International Journal of
Molecular Sciences

Special Issue Reprint

Neurodegenerative Disease

From Molecular Basis to Therapy, 3rd Edition

Edited by
Claudia Ricci

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Neurodegenerative Disease: From Molecular Basis to Therapy, 3rd Edition

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Guest Editor

Claudia Ricci

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This is a reprint of the Special Issue, published open access by the journal *International Journal of Molecular Sciences* (ISSN 1422-0067), freely accessible at: https://www.mdpi.com/journal/ijms/special_issues/2I33KXR45P.

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-7258-8965-5 (Hbk)
ISBN 978-3-7258-8966-2 (PDF)
https://doi.org/10.3390/books978-3-7258-8966-2

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About the Editor

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Claudia Ricci is a research scientist at the Neurogenetics laboratory in the Department of Medical, Surgical and Neurological Sciences at the University of Siena. Her expertise lies in the genetics of cerebral vascular malformations and motor neuron diseases, particularly amyotrophic lateral sclerosis (ALS), as well as genetic screening and biomarker research. She collaborates with the Italian Consortium for the Study of Genetics in ALS (ITALSGEN) and the Italy–USA Network, participating in large-scale studies on the genetic basis of ALS. These studies have led to the identification of several genes related to the disease, including C9orf72, TARDBP, and FUS. She has published approximately 70 papers in peer-reviewed journals (ORCID 0000-0002-2431-0308). Since 2021, she has been a member of the Scientific Committee of the University of Siena's Master's degree programmes in "Regulatory Medical Writing" and "Science Popularisation".

International Journal of
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Editorial

Neurodegenerative Disease: From Molecular Basis to Therapy, 3rd Edition

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Neurodegenerative diseases encompass a wide range of disorders linked to aging, predominantly impacting the central nervous system and leading to progressive neural deterioration. As the population continues to age, neurodegenerative diseases are becoming more prevalent, placing substantial financial pressure on healthcare systems. The majority of currently available treatments focus on managing symptoms rather than addressing the root causes, having little to no meaningful effect on slowing their progression.

The advancements in neurobiology and neurogenetics have enhanced our understanding of the pathogenesis of neurodegenerative diseases. These breakthroughs have enabled the development of molecularly targeted therapies designed to halt or slow the core pathological processes that cause neuronal damage and, consequently, cognitive and motor impairments. In some cases, these conditions are associated with genetic variants or disruptions in cellular pathways. Researchers have identified shared mechanisms among various neurodegenerative disorders, including the formation of misfolded protein aggregates, abnormal protein accumulation, neuroinflammation, and genetic and epigenetic influences. However, a precision medicine approach is becoming increasingly necessary to capture the variability between individuals and disease subtypes. A deeper understanding of these mechanisms is crucial for developing effective future treatments. Despite notable advancements in our understanding of the key processes involved in the development of these diseases, applying this knowledge to clinical practice remains in its early stages.

This Special Issue aims to provide a comprehensive update on the recent advancements in neurodegenerative disease research, from understanding their molecular basis to developing new therapies. This Special Issue includes a range of papers exploring the molecular mechanisms and pathways driving neurodegenerative conditions, alongside studies highlighting potential therapeutic targets and introducing novel drug candidates for specific diseases. Additionally, this Special Issue includes reviews focusing on the pathogenic processes and biomarkers associated with common neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease (PD).

Several papers describe new potential therapeutic treatments. Shimozono et al. [1] explored novel therapeutic approaches to spastic paraplegia 80 (SPG80), a pure form of juvenile-onset hereditary spastic paraplegia (HSP) caused by mutations in the ubiquitin-associated protein 1 (*UBAP1*) gene. They assessed the therapeutic potential of 4-phenylbutyric acid (4-PBA), a chemical chaperone and histone deacetylase inhibitor, as a treatment in a mouse model expressing a disease-associated truncated UBAP1 variant (Ubap1 knock-in (KI) mice). Administering 4-PBA significantly increased the motor performance of KI mice in the rotarod and beam walk tests. The maximal benefits were obtained when treatment was started in the pre- or early-symptomatic stages; however, partial efficacy was also observed when treatment began after symptom onset. In addition, 4-PBA reduced the activation of spinal microglia and partially restored their morphology, although astrocytic reactivity did

not change. These findings support 4-PBA as a treatment for SPG80 and show the potential of future therapeutic strategies that target proteostasis in HSP.

Ansari et al. investigated a potential treatment for Alzheimer's disease, focusing on amnesia, which involves the loss of short-term memory and the inability to retain facts, information, and experiences [2]. Amnesia is a primary symptom of dementia [3]. They explored the therapeutic potential of sobrerol (coded as NRM-331) in a scopolamine-induced amnesia mouse model, paying attention to its ability to improve memory deficits and enhance neuronal plasticity. Sobrerol is a synthetic mucolytic agent derived from terpenes with anti-inflammatory and antioxidant properties that has recently emerged as a potential treatment for multiple sclerosis [4]. Sobrerol may have additional, previously unreported pharmacological effects. The authors conducted multiple behavioral assessments, including the Y-maze, the passive avoidance, and the Morris water maze tests. They also biochemically analyzed serum samples (Aβ 1-40 and Aβ 1-42) and brain tissue (ACh and AChE), as well as histopathologically examined the brain using Nissl staining and tau-IHC. NRM-331 demonstrated an antiamnesic effect, likely due to its capacity to enhance cholinergic signaling in the hippocampus, combined with its antitau and anti-Aβ-synthesis actions. These findings indicate that NRM-331 shows potential as a therapeutic option for individuals with neurodegenerative conditions such as Alzheimer's disease. By targeting critical factors associated with memory loss and neuronal damage, NRM-331 could open avenues for developing multifunctional treatments incorporating NRM-331 to address Alzheimer's disease and other related cognitive disorders.

Liu et al. [5] describe a novel small molecule that enhances stable dopamine delivery to the brain in models of PD. PD is a progressive neurodegenerative disorder characterized by motor and nonmotor symptoms. PD is associated with the loss of dopamine in the neurons of the nigrostriatal pathway and the accumulation of misfolded alpha-synuclein. Although levodopa is the gold standard for managing PD symptoms, levodopa does not address the underlying progression caused by alpha-synuclein aggregation, pathological oxidative stress, brain inflammation, or the loss of dopamine neurons. Furthermore, motor fluctuations persist as a major challenge for patients, often arising from variability in levodopa absorption, plasma concentrations, and brain penetration. To address this critical medical issue, Liu et al. focused on a newly synthesized compound, named Pegasus [6], which demonstrated potential in addressing the multifactorial causes of PD. Pegasus is a small molecule with a molecular weight of 652 Da, formed by linking dopamine to a nonantibiotic derivative of doxycycline. Notably, the calculated physicochemical properties suggest that Pegasus is unlikely to cross the blood–brain barrier (BBB) via passive diffusion [7]. The authors obtained novel findings on the mechanisms through which dopamine is delivered to the brain. Following peripheral administration, Pegasus undergoes in vivo metabolism, breaking down into dopamine and tetracycline/doxycycline derivatives, which substantially enhance dopamine delivery. In mice harboring the human A53T mutation of alpha-synuclein, treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) caused motor symptoms resembling PD, whereas Pegasus was able to restore motor function and reduce anxiety-like behavior. Furthermore, Pegasus treatment reduced the levels of soluble and insoluble alpha-synuclein, protected dopamine-producing neurons, and reduced astrocytic activation in A53T mice. In vivo toxicity, pharmacology and toxicokinetic studies are still in the preliminary stages; however, these findings collectively suggest that Pegasus is a promising treatment for addressing the challenges associated with levodopa dosing.

Another key area of neurodegeneration research is identifying new drug targets. Ferrari et al. conducted a whole-transcriptome analysis of post mortem brain tissue samples from individuals with Alzheimer's disease, exploring the molecular mechanisms

https://doi.org/10.3390/ijms27114926

that drive the region-specific distribution of Alzheimer's disease pathology at various stages of progression [8]. Samples were obtained from the hippocampus (HI), temporal cortex (TC), parietal cortex (PC), cingulate gyrus (CG) and substantia nigra (SN). The samples were then compared those from individuals without dementia. The transcriptomic results revealed a higher abundance of differentially expressed genes (DEGs) in the TC and CG, and a lower abundance of DEGs in the HI, PC and SN. Notably, the shared DEGs primarily involved in synaptic transmission were significantly downregulated in the HI and TC, whereas genes primarily involved in protein folding and trafficking were significantly downregulated in the PC, CG, and SN. These findings imply that the AD progression follows a specific pattern related to time and severity, beginning with protein misfolding in the PC, CG, and SN and resulting in synaptic impairment in the HI and TC. The study suggests that a map outlining the molecular and biological processes involved in AD pathogenesis can be constructed. This map could facilitate the identification of novel biological targets for the development of effective and timely therapeutic approaches.

The patient must be characterized to identify the most suitable therapeutic strategy. Nowakowska et al. applied this approach to evaluate the risk of postoperative delirium in patients who had undergone cardiac surgery [9]. Delirium is commonly observed after major surgery, especially in older patients with physical and psychiatric illnesses or impaired cognition [10]. Postoperative delirium is associated with a poor prognosis, an increased risk of death, and impaired functioning, even after the syndrome has resolved [11]. Although the risk factors for delirium are well-understood, research into the pathophysiology of delirium has only recently begun. Authors investigated whether specific microRNAs (miRNAs) are associated with an increased risk of delirium They selected miRNAs related to brain function and development: miR-9-3p, miR-34c-5p, miR-96-5p, miR-183-5p and miR-374-3p. Serum samples that were obtained one day before and the day after surgery from 60 patients with delirium and 60 randomly selected individuals without delirium were analyzed. Univariate comparisons revealed that the preoperative levels of miR-96-5p and miR-183-5p, as well as postoperative levels of miR-34c-5p, miR-96-5p and miR-183-5p, were associated with an increased risk of postsurgery delirium. However, multivariate logistic regression showed that only the miR-183-5p level was an independent predictor of delirium. Decreased expression levels of miR-183-5p independently predisposed patients who underwent cardiac surgery to postoperative delirium. This association may be linked to increased oxidative stress and neuroinflammation in this patient group. This finding could inform subsequent actions based on a precision medicine strategy.

Research into neurodegeneration remains crucial, as a further understanding of the condition could lead to the development of new treatments and more effective approaches. Therefore, the majority of papers in this Special Issue logically address various aspects of the mechanisms underlying neurodegeneration. Three studies focused on this topic. Razak et al. studied diabetic peripheral neuropathy (DPN), which is one of the most prevalent and severe complications of diabetes [12]. DPN affects a large proportion of individuals with diabetes and can result in a range of debilitating outcomes, from early symptoms such as paresthesia to more severe complications such as limb loss and death [13]. DPN is characterized by Schwann cell dysfunction, demyelination, and impaired nerve regeneration. To deepen our understanding of the mechanisms responsible for Schwann cells modifications under hyperglycemic conditions, the authors studied the effects of high glucose treatment on the phenotype of Schwann cells, as well as assessing the involvement of the mTOR signaling pathway. The authors found that high-glucose conditions (50 mM) considerably reduce the expression of myelin basic protein (MBP), accompanied by the significant upregulation of the expression of total mammalian target of rapamycin (mTOR) and a concurrent reduction in the phosphorylation of mTOR at Ser2448. This finding

https://doi.org/10.3390/ijms27114926

suggests that hyperglycemic stress initiates Schwann cell dedifferentiation and dysregulates mTOR signaling. In addition, increased c-Jun and p75NTR expression levels, along with decreased Krox-20 levels, suggests a shift toward a repair-like phenotype, a process closely associated with demyelination and impaired nerve support. Taken together, these results show that impaired myelin protein expression, phenotypic changes associated with repair, and the altered activity of the mTOR pathway are key features of Schwann cell responses to hyperglycemia and contribute to the development of neuropathy.

Sánchez et al. explored the initial factors that contribute to Niemann–Pick disease type C (NPC), a genetic neurodegenerative disorder caused by mutations in the *NPC1* and/or *NPC2* genes [14]. This condition is marked by the selective vulnerability of specific cells, with cerebellar anterior Purkinje neurons being particularly affected. These neurons exhibit a specific degenerative pattern associated with the loss of NPC1 and/or NPC2 protein function, gradually progressing into the posterior regions of the cerebellum. Through the bioinformatics analysis of presymptomatic Npc1−/−Purkinje neurons in mice and in vitro testing on primary dermal fibroblasts from NPC patients, the researchers found that Purkinje neurons in the anterior cerebellum are particularly susceptible at an early stage due to oxidative stress and disruptions in iron homeostasis. The vulnerability to oxytosis/ferroptosis was higher in lobules I–III than in lobules VI and X, consistent with the sublethal threshold model of neurodegeneration in NPC. NPC-affected cells exhibited diminished antioxidant defenses, such as reduced levels of the enzyme GPx4, rendering the cells incapable of counteracting lipid peroxidation. The study suggests that antiferroptotic compounds can be used as innovative therapeutics to slow neurodegeneration, providing a new approach to treating NPC and other neurodegenerative disorders associated with oxytosis/ferroptosis.

The aggregation and accumulation of α-synuclein (α-Syn) are well-established contributors to the pathogenesis of PD. However, the involvement of the BBB in the mechanisms for clearing α-Syn remains poorly understood. Yokoya et al. focused on elucidating this involvement by examining the functional role of pericytes, a key cellular component of the BBB with the capacity to degrade various forms of α-Syn [15]. Utilizing primary cultures derived from rat pericytes, brain endothelial cells, and astrocytes, the authors found that, following α-Syn uptake by these cell types, the levels of intracellular α-Syn significantly decreased exclusively within pericytes. This pericyte-specific decrease in α-Syn was inhibited by treatment with the autophagy inhibitor bafilomycin A1 and the proteasome inhibitor MG132. Knocking down degradation enzymes or familial PD-associated genes such as cathepsin D, DJ-1 and LRRK2 did not affect α-Syn clearance. However, treatment with pharmacological inhibitors Akt, ERK and p38 MAPK inhibited the α-Syn degradation by pericytes. These findings suggest that α-Syn degradation by pericytes is mediated by the autophagy–lysosome and ubiquitin–proteasome systems via the α-Syn-activated Akt, ERK and p38 MAPK signaling pathways. From this perspective, these mechanisms represent potential therapeutic targets for developing disease-modifying strategies to halt the progression of α-Syn pathology in PD and other synucleinopathies.

Several reviews also offer in-depth analyses of the specific molecular mechanisms associated with neurodegeneration. Toader et al. conducted a comprehensive review describing the current state of research examining the molecular mechanisms through which neurodegeneration occurs and developing therapeutic strategies [16]. The authors examined the intricate network of molecular disruptions that underlie diseases such as Alzheimer's disease, PD, amyotrophic lateral sclerosis (ALS), and Huntington's (HD). Despite their unique clinical manifestations, these diseases share pathological features including protein misfolding and aggregation, neuroinflammation, the prolonged activation of brain immune cells, mitochondrial dysfunction and oxidative stress, as well as genetic

https://doi.org/10.3390/ijms27114926

and epigenetic factors. These mechanisms often act in intertwined pathways that drive the progression of these diseases. The review summarizes the most promising advances in identifying molecular biomarkers and explores innovative therapies emerging from cutting-edge research. Among these therapies are nanotechnology-driven drug delivery systems capable of crossing the BBB, CRISPR gene-editing tools developed to correct harmful genetic mutations, and stem cell techniques aimed at replacing damaged neurons and fostering neuroprotective conditions. In this review, attention is paid to precision medicine, where pharmacogenomics is transforming personalized treatment, enabling therapies tailored to individual genetic profiles. Molecular diagnostics and biomarkers are ushering in an era of early, precise disease detection. Additionally, emerging insights into the gut–brain axis are gaining attention, as evidence increasingly indicates that microbiome modulation could help mitigate the neuroinflammatory responses associated with the advancement of neurodegenerative conditions. In summary, this review emphasizes how molecular research is prompting a paradigm shift in the treatment of neurodegenerative diseases by providing novel therapeutic targets and facilitating more precise, multitargeted approaches. By addressing the web of interconnected mechanisms underlying these diseases, progress is being achieved toward treatments that exceed symptom management to intervene at critical stages of disease progression. This research is paving the way for ensuring neurodegenerative conditions are more manageable as well as potentially preventable in the future.

Ruiz-Sanchez et al. focused on a key component in neurodegeneration: nuclear receptor subfamily 4 group A member 2 (NR4A2) [17]. This transcription factor regulates the expression of genes involved in biological processes, such as cell proliferation, neuronal development, the immune response, cellular stress, apoptosis, DNA repair, and angiogenesis. *NR4A2* belongs to the class of genes known as immediate early genes, which are rapidly and transiently activated within minutes to an hour in response to cellular stimuli. Reduced *NR4A2* gene expression levels are associated with neurodegenerative and psychiatric disorders, including PD, Alzheimer's disease progression, schizophrenia, substance abuse (alcohol and amphetamines), neurodevelopmental disorders, and cognitive impairment. This review provides a comprehensive summary of the current understanding of the *NR4A2* gene, focusing on its structure and the molecular processes involved in regulating its expression. Special attention is paid to the primary epigenetic mechanisms influencing *NR4A2* expression, such as DNA methylation, histone deacetylation, and microRNA regulation. Additionally, the discussion highlights the gene's involvement in central nervous system disorders linked to disruptions in its expression. The authors conclude by exploring the potential of these regulatory mechanisms as biomarkers and therapeutic targets for addressing neurodegenerative diseases and psychiatric disorders.

Epigenetics is a cutting-edge field of neurodegenerative disease research. Among the various epigenetic mechanisms, histone acetylation has been identified as an epigenetic modification that directly affects gene transcription. Zhang et al. reviewed the role of histone lactylation in neurological disorders such as Alzheimer's disease, depression, neuroinflammation, and aging [18]. This review closes a gap, as previous reviews have summarized histone lactylation in cancer and immunology. The authors accurately describe the specific mechanisms and sites of histone lactylation, including lactylation and delactylation. The authors further examine the involvement of histone lactylation in a range of neurological disorders. In Alzheimer's disease, for example, histone lactylation activates the transcription of glycolysis genes, creating a positive feedback loop that intensifies the activation and dysfunction of microglial cells, thereby aggravating the pathological condition. This loop involves active glycolysis, H4K12la, and pyruvate kinase M2 (PKM2). However, inhibiting PKM2 disrupts the positive feedback loop in-

https://doi.org/10.3390/ijms27114926

volving glycolysis, H4K12la, and PKM2 in microglia. This reduces microglial activation and neuroinflammatory responses [19,20]. Moreover, astrocyte–neuron lactate transport modulates synaptic plasticity via BDNF lactylation, a process required for the formation of long-term memory [21]. Depression is characterized by the release of lactate by cortical astrocytes under fluoxetine/paroxetine treatment and by a reduction in astrocyte density in the prefrontal cortex due to chronic stress. These characteristics suggest that the dysregulation of astrocytic lactate is a core component of the etiology of depression [22,23]. These findings could affect future therapeutic approaches. However, the authors underline that the understanding of the role and regulation of histone lactylation in the brain is still developing, and further research is needed to completely understanding the therapeutic potential of this mechanism.

In their review, Elazar et al. focus on the importance of serine metabolism in the brain, specifically referencing the role of solute carrier family 1 member 4 (SLC1A4) in maintaining serine homeostasis during neurodevelopment and brain aging [24]. SLC1A4 is a neutral amino acid transporter, also known as alanine-serine-cysteine transporter 1 (ASCT1), which is encoded by the *SLC1A4* gene. In the brain, SLC1A4 transports the L- and D-isomers of serine. L-serine is involved in many cellular processes, including protein and sphingolipid synthesis. D-serine acts as a co-agonist essential for normal neurotransmission through N-methyl-D-aspartate receptors, which are fundamental for synaptic plasticity, learning, and memory. SLC1A4 plays a pivotal role in promoting and preserving brain health throughout life by facilitating the transport of L-serine across the BBB and regulating synaptic D-serine levels. The authors explore the role of SLC1A4 in neurodevelopment and neurodegeneration, evaluating the potential therapeutic benefits of serine supplementation in treating various conditions, including symptoms associated with SLC1A4 mutations in spastic tetraplegia, thin corpus callosum, and progressive microcephaly (SPATCCM), as well as neuropsychiatric disorders such as schizophrenia and depression, traumatic brain injury, and neurodegenerative diseases such as Alzheimer's disease and PD. The authors emphasize that, given the role of the SLC1A4 transporter in mediating the exchange of intracellular L-serine for extracellular D-serine, SLC1A4 may represent a key therapeutic target for maintaining serine homeostasis in the brain. Furthermore, when D-serine levels are reduced, the activity of SLC1A4 could be reduced using selective, high-affinity inhibitors that modulate the transporter's function. Moreover, identifying the pathways that regulate SLC1A4 levels and activity may reveal methods of increasing these levels to treat diseases associated with elevated levels of extrasynaptic D-serine. However, further studies are needed to assess potential side effects.

Finally, Athanasaki et al. [25] reviewed the molecular biomarkers used to diagnose Alzheimer's disease and determine eligibility for new disease-modifying treatments, such as the two antiamyloid antibodies currently approved by the FDA and EMA, lecanemab [26] and donanemab [27]. Rather than providing a comprehensive review, the authors highlight the ongoing discrepancies among the various existing guidelines. The authors consider various types of biomarkers—including amyloid (A) and tau (T) biomarkers in cerebrospinal fluid and plasma, biomarkers of neurodegeneration and neuroinflammation, and genetic biomarkers such as APOE—and note that hybrid ratios such as τP-181/Aβ42 and plasma τP-217/Aβ42 enhance diagnostic discrimination. A key point of contention is whether amyloid positivity (A+) alone is sufficient for diagnosing AD and accessing treatments or whether tau positivity (T+) is also required. The authors conclude that no satisfactory consensus currently exists, as conflicting diagnoses can arise in approximately 42% of patients when only one biomarker is abnormal. A diagnosis based solely on biomarkers is insufficient: comorbidities, clinical phenotype, and the predominantly European origin of the analyzed data—which limit generalizability—remain essential considerations. Over-

https://doi.org/10.3390/ijms27114926

coming these limitations will be key to more accurately identifying eligible patients and advancing a personalized approach to care.

Conclusions

The papers collected in this Special Issue reflect the breadth and dynamism of the current neurodegenerative disease research. Together, these studies illustrate the progression in multiple interconnected domains. These domains include the molecular analysis of pathogenic mechanisms and the identification of novel therapeutic targets, the development of promising drug candidates, and the refinement of diagnostic biomarker frameworks.

A recurrent theme is the complexity and heterogeneity of neurodegeneration. The evidence indicates the involvement of multiple pathways rather than a single cascade. This complexity underscores the necessity for targeted therapeutic strategies and a precision medicine approach that accounts for the variability among individuals and disease subtypes. Several papers highlight the translational potential of new molecular insights. Research using animal models and post mortem tissue is beginning to produce pharmacological candidates, including 4-PBA in hereditary spastic paraplegia, Pegasus in PD, and antiferroptotic compounds in Niemann–Pick disease. Additional research could result in these compounds being adopted in clinical settings. The increasing complexity of biomarker research, including the ongoing discussion on amyloid and tau profiles in Alzheimer's disease, shows the importance of correctly identifying patients before any treatment can be properly assessed. Additionally, novel regulatory mechanisms, such as histone lactylation, NR4A2 transcriptional control, and SLC1A4-mediated serine transport, have emerged. These provide insight into disease biology and could be exploited as therapeutic targets. These findings suggest that more opportunities exist for intervention than the classical pathological hallmarks suggest. Nevertheless, several challenges remain to be addressed. The process of translating laboratory findings into effective clinical treatments remains slow, and many of the biomarkers and targets discussed in this Special Issue require further validation, particularly in relation to diverse patient populations.

In conclusion, this Special Issue provides a timely and comprehensive overview of the current state of neurodegeneration research. The insights presented here will hopefully contribute to a deeper understanding of these devastating diseases and inspire future studies that bring us closer to treatments capable of not only alleviating symptoms but also fundamentally altering the course of neurodegeneration.

Conflicts of Interest: The author declares no conflicts of interest.

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International Journal of
Molecular Sciences

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Article

4-Phenylbutyric Acid Improves Gait Ability of UBAP1-Related Spastic Paraplegia Mouse Model: Therapeutic Potential for SPG80

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Abstract

Spastic paraplegia 80 (SPG80), caused by mutations in *ubiquitin-associated protein 1 (UBAP1)*, is a pure form of juvenile-onset hereditary spastic paraplegia (HSP) and leads to progressive motor dysfunction. Despite recent advances in the molecular analyses of HSP, disease-modifying therapy has not been established for HSP including SPG80. In the present study, we evaluated the therapeutic potential of 4-phenylbutyric acid (4-PBA), a chemical chaperone and histone deacetylase inhibitor, in *Ubap1* knock-in (KI) mice expressing a disease-associated truncated UBAP1 variant. We found that 4-PBA administration significantly improved the motor performance of KI mice in the rotarod and beam walk tests, with maximal benefits achieved when given during pre- or early-symptomatic stages. Partial efficacy was also observed when treatment began after symptom onset in KI mice. Furthermore, 4-PBA attenuated spinal microglial activation and partially restored microglial morphology, although astrocytic reactivity remained unchanged. These findings support 4-PBA as a candidate therapeutic compound for SPG80 and highlight the potential of proteostasis-targeted interventions in HSPs.

Keywords: hereditary spastic paraplegia (HSP); SPG80; *UBAP1*; 4-phenylbutyric acid (4-PBA); ER stress; microglia activation

1. Introduction

Hereditary spastic paraplegia (HSP) is a clinically and genetically diverse group of neurodegenerative disorders characterized by progressive spasticity and weakness of the

lower extremities [1]. Clinically, HSP is classified into a pure form, which is characterized by leg spasticity accompanied by bladder disturbance and disturbance of the vibration sense, and a complicated form, which is characterized by leg spasticity and many complications such as ataxia, a thin corpus callosum, chorioretinal dystrophy, intellectual disability, extrapyramidal signs, and peripheral neuropathy [1,2]. These disorders primarily result from the degeneration of corticospinal tracts, with over 90 associated genes or loci identified to date [1–3]. At the molecular level, many HSP-associated genes are implicated in essential cellular processes, including intracellular transport, organelle morphology, mitochondrial dynamics, and proteostasis, particularly affecting long motor axons [4].

In 2019, we and other investigators independently identified a juvenile-onset, pure form of autosomal dominant HSP caused by mutations in *ubiquitin-associated protein 1 (UBAP1)* (SPG80) [5–7]. UBAP1 is a core component of the endosomal sorting complex required for transport-I (ESCRT-1), which recognizes ubiquitinated cargos in early endosomes and facilitates their incorporation into multivesicular bodies (MVBs) for lysosomal degradation [8,9]. Dysfunction of UBAP1 or other ESCRT components impairs MVB formation and endolysosomal trafficking, resulting in the accumulation of misfolded or ubiquitinated proteins, a hallmark of many neurodegenerative diseases [10–12]. In SPG80, impaired UBAP1 function likely disrupts endosomal maturation and protein clearance, thereby contributing to axonal degeneration [13].

A very recent study of a murine model of SPG15, a complicated form of HSP, has shown that microglial activation precedes neuronal loss, implicating neuroinflammation as a contributing factor in disease progression [14]. This finding suggests that glial activation, alongside impaired proteostasis, may also play a role in the pathogenesis of HSP, including SPG80. Given that intracellular stress and protein misfolding are known to trigger glial responses [15,16], therapeutic strategies aimed at restoring proteostasis may help mitigate neuroinflammation and neuronal damage in HSP [17].

Chemical chaperones that restore intracellular proteostasis are attracting attention as potential therapeutics for neurodegenerative diseases [18,19]. Among them, 4-phenylbutyric acid (4-PBA), a low-molecular-weight fatty acid analog initially developed to treat urea cycle disorders [20], has shown promise. 4-PBA efficiently crosses the blood–brain barrier, mitigates endoplasmic reticulum (ER) stress, and promotes protein folding and degradation of misfolded proteins, thereby exerting cytoprotective effects [19–21]. In addition, its histone deacetylase (HDAC) inhibitory activity may affect gene expression and enhance cellular stress responses [22].

We previously demonstrated that a *Ubap1*$^{+/E176Efx23}$ knock-in (KI) mouse model exhibits progressive gait impairment due to upper motor neuron dysfunction, with no other detectable physical abnormalities. These features closely replicate human SPG80, validating the usefulness of the model for mechanistic and therapeutic studies [13]. In this paper, we evaluated the therapeutic efficacy of 4-PBA in our KI model. By varying the treatment dosage and timing, we evaluated the effects of 4-PBA on motor performance and glial pathology. The present findings offer novel insights into the potential use of chemical chaperones as a treatment strategy for SPG80.

2. Results

2.1. Therapeutic Effects of 4-PBA on Motor Function and Coordination in Ubap1 KI Mice

Motor performance was evaluated using the accelerating rotarod test (Figure 1A and Supplementary Video S1). Untreated KI mice exhibited significantly shorter latency to fall than wild-type (WT) controls, indicating motor deficits. Among the treatment groups, mice receiving 1000 mg/kg/day from 2.5 to 4.5 months showed the greatest improvement ($p < 0.05$). Importantly, even when treatment was initiated at 4.5 months (after the typical

onset of symptoms), partial functional recovery was observed. Our previous work indicated that most KI mice develop overt gait abnormalities by 4 months of age [13]. Thus, 4-PBA still exerts therapeutic potential even when administered after the onset of symptoms, highlighting its clinical relevance and flexible treatment windows.

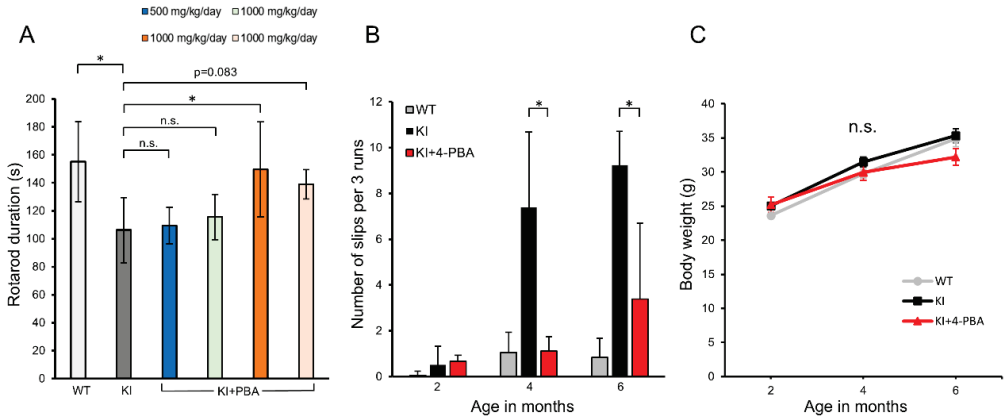


Figure 1. Effects of 4-PBA treatment on motor performance of KI mice. (**A**) Rotarod test results showing latency to fall (in seconds) for WT, KI, and KI+PBA-treated groups at approximately 7 months of age. Data are shown as mean ± SD. Sample sizes (*n*): WT = 23, KI = 9. The KI+PBA groups (blue: *n* = 4, light green: *n* = 3, orange: *n* = 8, light orange: *n* = 6) correspond to the treatment conditions described in Figure 4. (**B**) Number of slips across three trials in WT, KI, and KI+PBA groups across different ages. For the KI+PBA group, mice treated with 1000 mg/kg/day of 4-PBA from 2.5 months of age for 2 months (identified in panel B as the group with the best performance in the rotarod test) were used. Sample sizes (*n*): WT = 16, KI = 13, KI+4-PBA = 6. Data are shown as mean ± SD. (**C**) Changes in body weight over time in WT (*n* = 35), KI (*n* = 31), and KI+PBA (*n* = 6) groups. Data are shown as mean ± SEM. Data are presented as mean ± SD or SEM, as appropriate. Statistical analyses were performed as follows: (**A**) One-way ANOVA revealed a significant group difference ($F(5, 47) = 6.08$, $p = 0.0002$). Dunnett's post hoc test: WT vs. KI, $t = 4.63$, $p < 0.001$; KI vs. KI+PBA (blue), $t = 0.20$, $p = 0.9998$; KI vs. KI+PBA (light green), $t = 0.54$, $p = 0.9791$; KI vs. KI+PBA (orange), $t = 3.45$, $p = 0.0055$; KI vs. KI+PBA (light orange), $t = 2.40$, $p = 0.083$. (**B**) The Kruskal–Wallis test followed by Bonferroni-corrected pairwise Mann–Whitney *U* tests showed significantly fewer foot slips in KI+PBA vs. KI at 4 months 4 months ($U = 72$, $p = 0.0103$) and 6 months ($U = 69.5$, $p = 0.013$), indicating improved motor coordination. (**C**) Body weight was analyzed by one-way ANOVA at 2 months ($F(2, 65) = 2.853$, $p = 0.0649$) and by Kruskal–Wallis at 4 and 6 months ($\chi^2(2) = 3.41$, $p = 0.1819$; $\chi^2(2) = 1.45$, $p = 0.4834$), with no significant differences among WT, KI, and KI+PBA groups. Significance: $p < 0.05$ (*); n.s., not significant.

To further evaluate motor coordination, we conducted the beam walking test using the most effective regimen (1000 mg/kg/day from 2.5 to 4.5 months). Untreated KI mice exhibited significantly more foot slips across three trials than WT mice, indicating deficits in motor coordination (Figure 1B and Supplementary Videos S2 and S3). Notably however, 4-PBA treatment significantly reduced the number of foot slips ($p < 0.05$), confirming an improvement in coordination.

Longitudinal monitoring of body weight revealed no significant differences among WT, untreated KI, and 4-PBA-treated KI mice (Figure 1C). Additionally, no observable behavioral abnormalities or weight fluctuations were noted, indicating good tolerability to 4-PBA under the test conditions.

2.2. Glial Activation and Effects of 4-PBA on the Spinal Cord of Ubap1 KI Mice

To assess glial activation and the therapeutic effects of 4-PBA, we performed double immunofluorescence staining for ionized calcium binding adapter protein 1 (Iba1) and glial fibrillary acidic protein (GFAP)—established markers of microglia and astrocytes,

respectively—in the spinal cords of KI mice (Figure 2A). This approach enabled us to evaluate cell-type-specific neuroinflammatory responses in the principal site of motor dysfunction and to determine whether 4-PBA modulates glial reactivity in a region directly implicated in SPG80 pathology. Compared with WT controls, KI mice showed marked upregulation of both Iba1 and GFAP. We focused our analysis on the anterior horn and white matter—spinal regions essential for motor control (Figure 2B,E). Quantitative analysis revealed significant increases in Iba1 signal intensity in both regions, which were partially attenuated by 4-PBA treatment (Figure 2B,D). In contrast, the GFAP expression level remained elevated and was not significantly affected by 4-PBA (Figure 2C,E).

Morphological analysis further confirmed microglial activation in KI mice, as evidenced by enlarged soma and reduced process complexity. These changes were significantly ameliorated by 4-PBA: soma size was reduced (Figure 2G), and Sholl analysis demonstrated increased branching complexity (Figure 2H), indicating a partial restoration of microglial homeostasis.

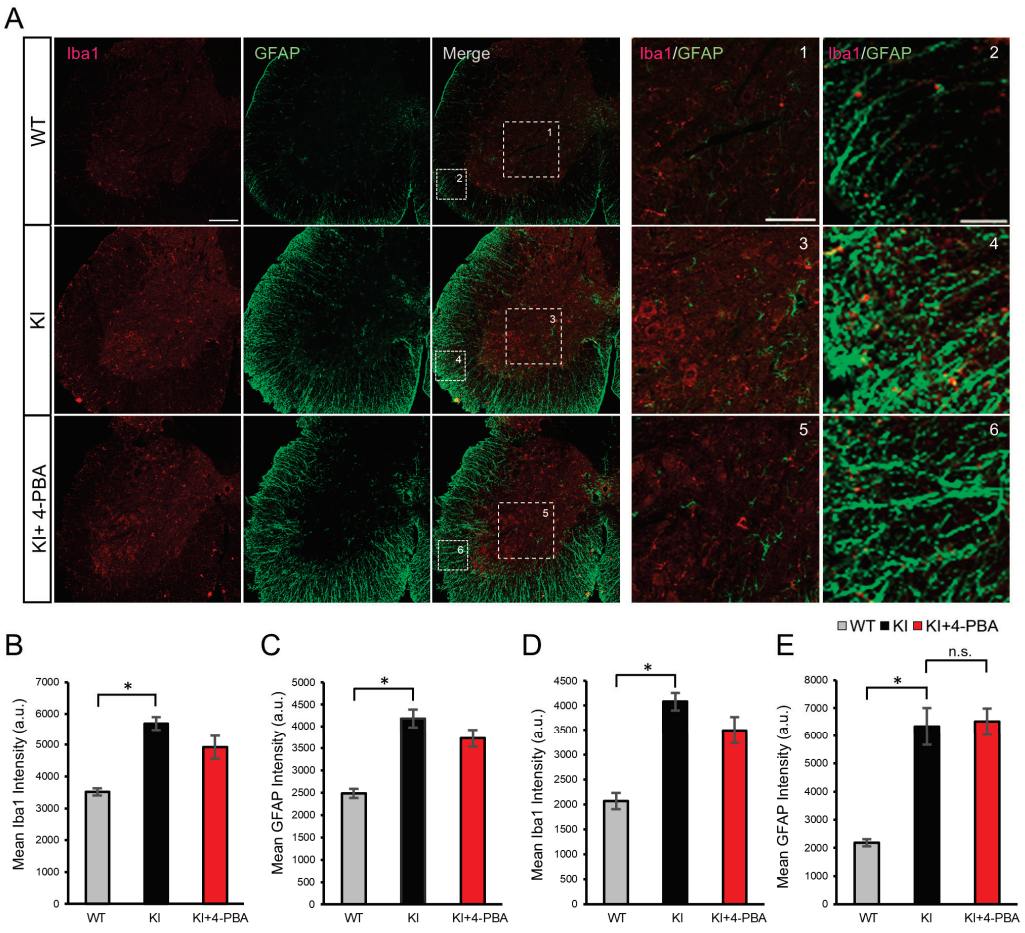


Figure 2. *Cont.*

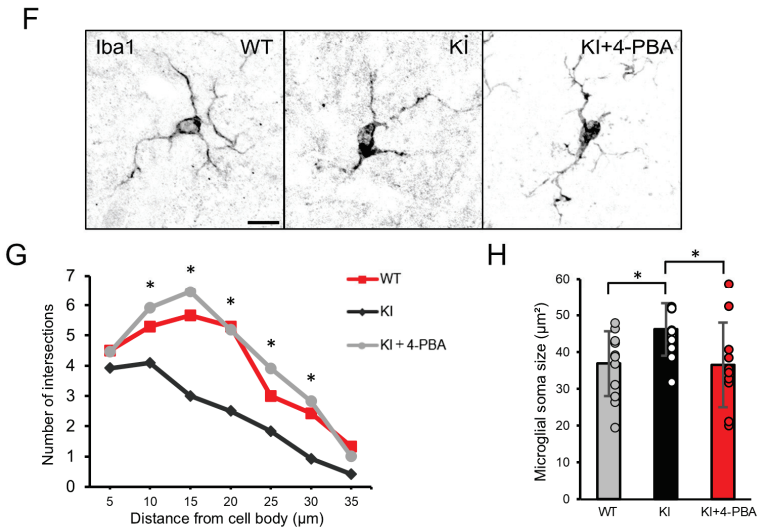


Figure 2. GFAP and Iba1 expression in the spinal cord of *Ubap1* mutant mice and the effect of 4-PBA treatment. (**A**) Representative immunofluorescence images of spinal cord sections from wild-type (WT), *Ubap1* knock-in (KI), and KI mice treated with 4-phenylbutyric acid (KI+4-PBA), stained with antibodies against Iba1 (red) and GFAP (green). Scale bars: 200 µm. Merged images are shown in the third column. White dashed boxes indicate regions shown at higher magnification on the right (boxes 1–6). Scale bars: 100 µm (left), 50 µm (right). (**B**–**E**) Quantification of mean fluorescence intensities of Iba1 and GFAP in the anterior horn (**B**,**C**) and white matter (**D**,**E**) of the spinal cord. Both Iba1 and GFAP expression levels significantly increased in KI mice. The Iba1 expression level clearly increased in KI mice and slightly decreased with 4-PBA treatment ($p = 0.13$). Sample sizes: WT ($N = 4$ mice, $n = 8$ sections), KI ($N = 4$ mice, $n = 8$ sections), KI+4-PBA ($N = 3$ mice, $n = 6$ sections). (**F**) Representative high-magnification images of Iba1-positive microglia in the anterior horn. Scale bar: 10 µm. (**G**) Sholl analysis showing the number of microglial process intersections as a function of distance from the cell body. KI+4-PBA mice showed increased ramification compared with KI mice ($p < 0.05$). Sample sizes: WT ($N = 3$ mice, $n = 7$ cells), KI ($N = 3$ mice, $n = 12$ cells), KI+4-PBA ($N = 3$ mice, $n = 11$ cells). (**H**) Quantification of microglial soma size. Sample sizes: WT ($N = 4$ mice, $n = 12$ cells), KI ($N = 3$ mice, $n = 14$ cells), KI+4-PBA ($N = 3$ mice, $n = 11$ cells). Sholl analysis showing the number of microglial process intersections as a function of distance from the cell body. KI+4-PBA mice showed enhanced ramification compared with KI mice ($p < 0.05$). Data are presented as mean ± SEM. Statistical analyses were performed as follows: (**B**) Kruskal–Wallis test: $\chi^2(2) = 13.445$, $p = 0.0012$; Bonferroni-corrected Mann–Whitney U test: WT vs. KI, $U = 64$, $p = 0.00046$; WT vs. KI+PBA, $U = 43$, $p = 0.03810$; KI vs. KI+PBA, $U = 35$, $p = 0.54300$. (**C**) One-way ANOVA: F(2, 19) = 28.16, $p = 2.08 \times 10^{-7}$; Bonferroni-corrected t-test: WT vs. KI, $p = 0.0000019$; WT vs. KI+PBA, $p = 0.00028$; KI vs. KI+PBA, $p = 0.24914$. (D) One-way ANOVA: F(2, 19) = 29.52, $p = 1.48 \times 10^{-6}$; Bonferroni-corrected t-test: WT vs. KI, $p = 0.000013$; WT vs. KI+PBA, $p = 0.00028$; KI vs. KI+PBA, $p = 0.17206$. (**E**) Kruskal–Wallis test: $\chi^2(2) = 14.616$, $p = 0.0006703$; Bonferroni-corrected Mann–Whitney U test: WT vs. KI, $U = 64$, $p = 0.00046$; WT vs. KI+PBA, $U = 48$, $p = 0.00200$; KI vs. KI+PBA, $U = 23$, $p = 1.00000$. (**G**) Kruskal–Wallis tests were performed at each radial distance from the soma, followed by Bonferroni-corrected Mann–Whitney U tests comparing KI and KI+PBA groups. The results were as follows: 10 µm, $\chi^2(2) = 6.51$, $p = 0.0386$; KI vs. KI+PBA, $U = 24.5$, $p = 0.03000$. 15 µm, $\chi^2(2) = 14.66$, $p = 0.000655$; KI vs. KI+PBA, $U = 12$, $p = 0.00250$. 20 µm, $\chi^2(2) = 12.49$, $p = 0.00195$; KI vs. KI+PBA, $U = 21$, $p = 0.01650$. 25 µm, $\chi^2(2) = 6.62$, $p = 0.0365$; KI vs. KI+PBA, $U = 28$, $p = 0.05000$. 30 µm, $\chi^2(2) = 8.94$, $p = 0.0114$; KI vs. KI+PBA, $U = 18$, $p = 0.00760$. No significant group differences were detected at 5 µm and 35 µm: 5 µm, $\chi^2(2) = 3.33$, $p = 0.1888$; 35 µm, $\chi^2(2) = 3.34$, $p = 0.1880$. (**H**) One-way ANOVA: F(2, 34) = 4.67, $p = 0.0161$; Tukey's HSD test: KI vs. KI+PBA, $p = 0.03326$; WT vs. KI, $p = 0.03771$; WT vs. KI+PBA, $p = 0.93207$. Significance: $p < 0.05$ (*); n.s., not significant.

2.3. Microglial Morphology and Therapeutic Effects of 4-PBA on the Cerebral Cortex

To determine whether the glial alterations observed in the spinal cord extended to other regions of the central nervous system, we next examined microglial morphology and the effects of 4-PBA on the cerebral cortex. Immunofluorescence staining for Iba1 and GFAP revealed no significant differences in the number or distribution of Iba1-positive microglia or GFAP-positive astrocytes between KI mice and wild-type (WT) controls (Figure 3A). Quantitative analysis confirmed that neither the density of Iba1-positive cells nor their regional coverage differed significantly between groups (Figure 3B,C), suggesting that microglial proliferation or recruitment is not markedly altered in the cortex.

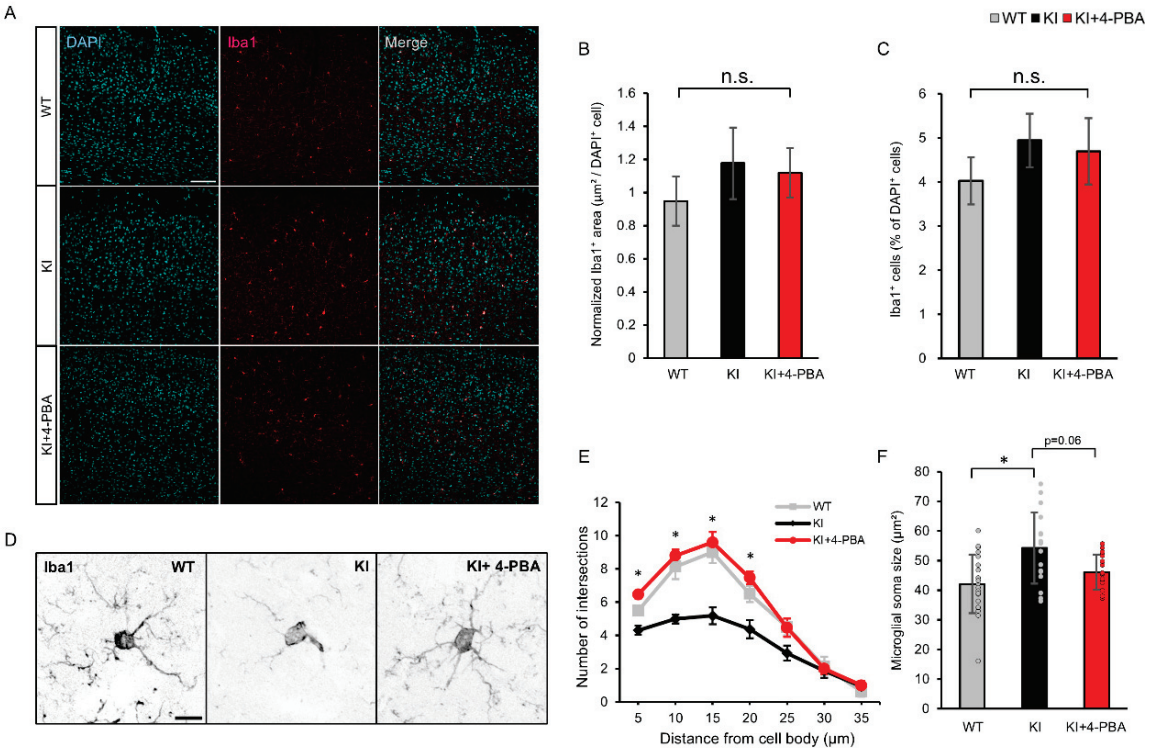


Figure 3. Microglial morphology in the cerebral cortex of *Ubap1* mutant mice and the effect of 4-PBA treatment. (**A**) Representative immunofluorescence images of cerebral cortex sections from wild-type (WT), *Ubap1* knock-in (KI) mice, and KI mice treated with 4-phenylbutyric acid (KI+4-PBA), stained with DAPI (cyan) and Iba1 (red). Merged images are shown. Scale bar: 100 μm. (**B**,**C**) Quantification of Iba1-positive area normalized to DAPI-positive cell number (**B**) and percentage of Iba1-positive cells among DAPI-positive cells (**C**). Sample sizes: WT (N = 4 mice, n = 10 sections), KI (N = 5 mice, n = 7 sections), KI+4-PBA (N = 4 mice, n = 7 sections). (**D**) Representative high-magnification images of Iba1-stained microglia in the cerebral cortex of each group. Scale bar: 10 μm. (**E**) Sholl analysis showing the number of microglial process intersections as a function of distance from the cell body. KI+4-PBA mice exhibited increased ramification compared with KI mice (* $p < 0.05$). Sample sizes: WT (N = 4 mice, n = 8 cells), KI (N = 4 mice, n = 16 cells), KI+4-PBA (N = 4 mice, n = 15 cells). (**F**) Quantification of microglial soma size. KI mice showed enlarged somata, which tended to decrease with 4-PBA treatment (p = 0.06). Data are presented as mean ± SEM. Sample sizes: WT (N = 4 mice, n = 21 cells), KI (N = 4 mice, n = 17 cells), KI+4-PBA (N = 4 mice, n = 17 cells). Data are presented as mean ± SEM (**B**,**C**,**E**) or mean ± SD (**F**). Statistical analyses were performed as follows: (**B**) One-way ANOVA: F(2, 21) = 0.357, p = 0.704; Tukey's HSD test: KI vs. KI+PBA, p = 0.87921; WT vs. KI, p = 0.94560; WT vs. KI+PBA, p = 0.67996. (**C**) Kruskal–Wallis test: $\chi^2(2) = 2.09$, p = 0.351; Bonferroni-corrected Mann–Whitney U test: WT vs. KI, U = 49, p = 0.58; WT vs. KI+PBA, U = 44, p = 1.00; KI vs. KI+PBA, U = 29, p = 1.00. (**E**) Kruskal–Wallis tests were performed at each radial distance from the soma, followed by Bonferroni-corrected Mann–Whitney U tests comparing KI and

KI+PBA groups. The results were as follows: 5 μm, $\chi^2(2) = 20.045$, $p = 0.0000444$; KI vs. KI+PBA, $U = 18$, $p = 0.00012$. 10 μm, $\chi^2(2) = 24.405$, $p = 0.00000502$; KI vs. KI+PBA, $U = 4.5$, $p = 0.000013$. 15 μm, $\chi^2(2) = 21.054$, $p = 0.0000268$; KI vs. KI+PBA, $U = 13.5$, $p = 0.000074$. 20 μm, $\chi^2(2) = 15.904$, $p = 0.000352$; KI vs. KI+PBA, $U = 29$, $p = 0.00088$. No significant group differences were detected beyond 25 μm: 25 μm, $\chi^2(2) = 5.202$, $p = 0.0742$; 30 μm, $\chi^2(2) = 0.0955$, $p = 0.953$; 35 μm, $\chi^2(2) = 0.882$, $p = 0.6435$. (**F**) Kruskal–Wallis test: $\chi^2(2) = 10.582$, $p = 0.00504$; Steel–Dwass test: WT vs. KI, $U = 280$, $p = 0.00811$; WT vs. KI+PBA, $U = 225$, $p = 0.35925$; KI vs. KI+PBA, $U = 210.5$, $p = 0.05958$. Significance: $p < 0.05$ (*); n.s., not significant.

Despite this, cortical microglia in KI mice exhibited distinct morphological changes, including somatic hypertrophy and reduced branching complexity (Figure 3D). Sholl analysis demonstrated a significant reduction in microglial arborization, which was partially reversed by 4-PBA treatment (Figure 3E). Similarly, 4-PBA tended to reduce soma size, although this effect did not reach statistical significance ($p = 0.06$) (Figure 3F). These findings indicate that microglial activation in the cortex occurs primarily at the morphological level rather than through increased cell number, and that 4-PBA can partially ameliorate these structural alterations.

3. Discussion

The present study demonstrates that 4-PBA improves motor function in the *Ubap1* KI mouse model, particularly when treatment begins at pre- or early-symptomatic stages. Even when administered after symptom onset, 4-PBA conferred measurable benefits. This finding is especially relevant to clinical settings, where diagnosis typically occurs post symptomatically. These findings support the hypothesis that impaired proteostasis accompanied by intracellular stress contributes to the progression of SPG80, and that 4-PBA acts by mitigating ER stress and promoting protein homeostasis [23,24]. The observation that motor deficits can be reversed, even partially, and post-symptomatically, highlights the potential of 4-PBA as a therapeutic candidate for human HSP.

In the spinal cord of KI mice, we observed microglial activation characterized by increased Iba1 expression levels, somatic hypertrophy, and reduced process complexity. These alterations were partially reversed by 4-PBA, while astrocyte activation—as indicated by the unchanged GFAP expression level—remained unaffected. Notably, although 4-PBA did not reduce the total number of Iba1-positive microglia, it partially restored their morphology, as evidenced by smaller cell bodies and more ramified processes. These findings suggest that 4-PBA attenuates microglia activation at the functional level, even though it did not normalize microglial numbers. Therefore, the improvement in motor performance may be attributed, at least in part, to modulation of microglial activity rather than to a complete rescue of microgliosis. This suggests a selective effect of 4-PBA on microglial regulation, likely through the reduction in proteotoxic stress and downstream inflammatory signaling [25,26].

The discrepancy between the robust behavioral improvements and the modest glial modulation suggests that 4-phenylbutyric acid (4-PBA) may exert additional protective effects on motoneurons. Although direct evidence for 4-PBA-mediated neuroprotection in motoneurons is currently limited, its established role in reducing endoplasmic reticulum stress and enhancing protein homeostasis provides a plausible mechanism by which it could support motoneuronal survival and function [27]. This may help explain the behavioral improvements observed in our study. To clarify the contribution of neuronal protection to the therapeutic effects of 4-PBA, future investigations should directly assess motoneuronal integrity and function using approaches such as immunostaining for choline acetyltransferase (ChAT) and NeuN, as well as electrophysiological recordings.

The differences in responses between microglia and astrocytes may reflect distinct activation mechanisms: microglia respond to intracellular proteotoxic stress, whereas astrocyte reactivity is more affected by extracellular signals and cytokines [28,29]. These observations align with a previous study demonstrating that 4-PBA enhances neuronal function but does not fully mitigate astrogliosis [27]. Consequently, targeting astrocyte activation may necessitate complementary strategies, such as anti-inflammatory or cytokine-directed therapies.

In contrast to the spinal cord, cortical glial responses were relatively mild. Despite morphological alterations in cortical microglia, there was no significant increase in glial marker expression level, consistent with milder cortical pathology in other HSP models such as SPG11 and SPG15 [30]. This regional specificity reflects the clinical phenotype of SPG80, which primarily affects lower limb function mediated by spinal motor pathways.

Our prior work showed that *UBAP1* mutations impair endosomal trafficking, leading to the accumulation of ubiquitinated proteins and axonal degeneration [13]. In the present study, 4-PBA treatment improved motor function and attenuated microglial activation in UBAP1-deficient mice, suggesting a beneficial effect on the spinal neuroimmune environment. These observations are consistent with a potential enhancement of proteostasis, although direct evidence for reduced ubiquitinated protein accumulation was not assessed. Given that activated microglia exacerbate neuronal stress and degeneration [14,31,32], the ability of 4-PBA to modulate microglial activity may be an important component of its therapeutic action.

While our study focused on UBAP1-related HSP, the beneficial effects of 4-PBA on proteostasis and motor function may also be relevant to other HSP subtypes, such as SPG11, SPG15, SPG4, SPG20, and SPG53, which involve microglial activation, lysosomal stress, or ESCRT dysfunction [14,33–37]. Given that these pathological features converge on impaired proteostasis, chemical chaperones like 4-PBA could offer broader therapeutic potential. However, direct experimental validation in each subtype will be necessary to confirm this possibility.

In summary, 4-PBA alleviates motor deficits and spinal microgliosis in a *UBAP1* mutant model of SPG80. Although it does not completely reverse the underlying cellular pathology, the observed improvements in motor performance and glial activation highlight its therapeutic potential. These findings support further investigation into the molecular basis of UBAP1-related diseases and the development of multimodal strategies, including gene therapy and proteostasis-targeting combination treatments.

Importantly, although 4-PBA significantly improved motor function in *Ubap1* mutant mice, caution is warranted when extrapolating these findings to humans. 4-PBA is already approved for clinical use in urea cycle disorders [38] and has also been considered for neurodegenerative diseases [39–41], supporting its potential repurposing for HSP. However, several key issues remain unresolved, including optimal dosing, CNS penetration, and long-term safety in patients. Further studies would be required to investigate dose–response relationships and assess potential toxicity to ensure safe clinical translation. Thus, while our results provide preclinical evidence supporting 4-PBA as a candidate therapy for HSP, well-designed clinical trials would ultimately be necessary to establish its therapeutic efficacy in patients.

4. Materials and Methods

4.1. Animal Experiments

All experiments were performed using C57BL/6J mice obtained from SLC (Hamamatsu, Japan). *Ubap1* KI mice were generated using the CRISPR/Cas9 system as previously reported [13]. Genotyping was performed by polymerase chain reaction using tail DNA.

Mice were housed under standard conditions (24 ± 1 °C, 12 h light/dark cycle, lights on from 8 a.m. to 8 p.m.) with ad libitum food and water. Only male mice were used to avoid estrous cycle variability. WT littermates from heterozygous crosses served as controls.

4.2. Drug Administration Timeline

To assess the therapeutic efficacy of 4-PBA, we employed four distinct treatment regimens in KI mice, varying in both dosage and duration. Mice received oral 4-PBA (sodium 4-phenylbutyrate; Cat. No. 11323, Cayman Chemical, Ann Arbor, MI, USA) at either 500 mg/kg/day or 1000 mg/kg/day, beginning at 2.5 or 4.5 months of age. Specifically, the regimens were as follows: 500 mg/kg/day for 1 month starting at 2.5 months; 1000 mg/kg/day for 1 month starting at 2.5 months; 1000 mg/kg/day for 2 months starting at 2.5 months; and 1000 mg/kg/day for 2 months starting at 4.5 months (Figure 4). These treatment windows were chosen to evaluate both early intervention and delayed administration after the emergence of motor symptoms. The doses were selected on the basis of previous studies demonstrating efficacy and tolerability in mouse models [19,21,27,42].

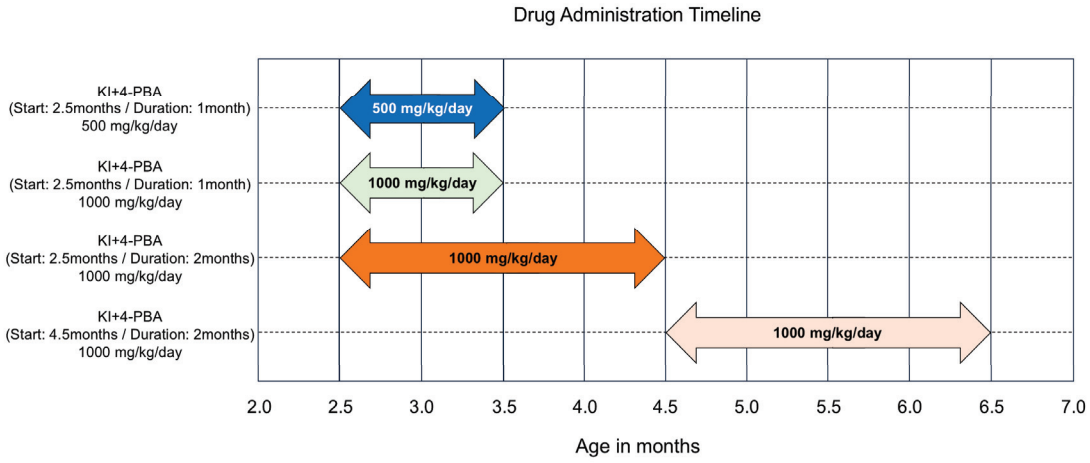


Figure 4. Drug administration timeline. Drug administration timeline. KI mice were treated with 4-PBA under the following conditions: 500 mg/kg/day from 2.5 months of age for 1 month (n = 4). 1000 mg/kg/day from 2.5 months of age for 1 month (n = 3). 1000 mg/kg/day from 2.5 months of age for 2 months (n = 8). 1000 mg/kg/day from 4.5 months of age for 2 months (n = 6).

4.3. Behavioral Tests

Motor function was assessed by rotarod and beam walk tests. In the beam walk test, 2- to 6-month-old male mice traversed a 1-cm diameter, 100-cm long stainless-steel bar elevated 70 cm above the ground. Foot slips were recorded over three trials, with a maximum score of 10 per trial.

In rotarod tests, mice were evaluated using an accelerated rotarod protocol (Muromachi, MK-610A, Tokyo, Japan). The rotation speed increased linearly from 4 to 40 rpm over 300 s, and the average latency to fall was calculated from three consecutive trials. Mice were trained prior to the test sessions. We employed the accelerated paradigm because it provides higher sensitivity to detect subtle motor impairments in HSP models compared with constant-speed protocols, as reported in the previous studies [43,44].

4.4. Tissue Preparation and Immunofluorescence Staining

Mice were anesthetized by the intraperitoneal injection of medetomidine, midazolam, and butorphanol, followed by transcardial perfusion with ice-cold phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and 4% paraformaldehyde (PFA) in PBS. Brains and spinal cords were post fixed in 4% PFA overnight at 4 °C, and then cryoprotected in a 30% w/v sucrose

solution for two days. Tissues were sectioned at 20 μm thickness using a cryostat (CM1520, Leica Biosystems, Nussloch, Germany). Sections were washed three times for 5 min each in PBS, blocked with 0.1% Triton X-100/10% NGS for 1 h at room temperature, and then incubated with primary antibodies in PBS at 4 °C for 24 h.

The primary antibodies used were rabbit anti-Iba1 (1:500; WAKO, Cat# 019-19741, Osaka, Japan) and mouse anti-GFAP (1:500; WAKO, Cat# MO389, Osaka, Japan). After the conjugation of the tissues with the primary antibodies, the sections were washed three times for 5 min each in PBS and then incubated with the corresponding secondary antibodies for 2 h at room temperature. The secondary antibodies were Alexa Fluor 546-conjugated goat anti-rabbit IgG (H+L) (1:1000; Thermo Fisher Scientific, Cat# A10035, Waltham, MA, USA) and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (1:1000; Thermo Fisher Scientific, Cat# A28175, Waltham, MA, USA).

For Sholl analysis and soma size measurements, microglial cells were selected from randomly chosen fields within the anterior horn of the spinal cord and the cerebral cortex. To minimize selection bias, only cells with clearly distinguishable and intact somata and processes, without overlap with neighboring cells, were included in the analysis.

4.5. Sholl Analysis

Dendritic complexity was quantified using the Sholl Analysis plugin in ImageJ (version 1.54p, NIH, Bethesda, MD, USA). Neurons were traced using the Simple Neurite Tracer plugin, with centric circles drawn at 10 μm intervals from the soma. The number of intersections at each radius was used to assess arbor complexity.

4.6. Statistical Analyses

Statistical analyses were performed using EZR (version 1.54), a graphical interface for R. Data normality was assessed by the Shapiro–Wilk test. ANOVA with Tukey's or Dunnett's post hoc tests was applied to normally distributed data. Non-parametric data were analyzed using Kruskal–Wallis followed by Bonferroni-corrected Mann–Whitney U tests. For Sholl analysis, group comparisons at each radial distance were conducted using Kruskal–Wallis tests followed by Bonferroni-corrected pairwise Mann–Whitney *U* tests.

5. Conclusions

Our study showed that 4-PBA partially restores motor function and attenuates microglial activation in KI mice with SPG80 expressing a truncated UBAP1 protein. Although not completely curative, 4-PBA shows promise as a component of future therapeutic strategies targeting proteostasis in HSP.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ijms26199779/s1.

Author Contributions: K.S. (Keisuke Shimozono), Y.-J.K., and Y.T. contributed to the study conception and design. K.S. (Keisuke Shimozono), T.H., H.N., K.S. (Kozo Saito), and Y.M. performed experiments and analyzed the data. Y.-J.K., Y.U., F.I., M.I., S.K., T.O., and Y.T. supervised the study. All authors contributed to interpreting the results. K.S. (Keisuke Shimozono) and Y.-J.K. wrote the initial draft and Y.M., Y.U., S.K., T.O., and Y.T. edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Grants-in-Aid from the Research Committee for Ataxic disease (YT), the Ministry of Health, Labor and Welfare, Japan, and JSPS KAKENHI Grant Numbers JP24K18681(KS) and JP21K07456 (YT) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

Institutional Review Board Statement: All experiments and animal housing adhered to the Guide for Animal Experimentation of the University of Yamanashi, and were approved by the Animal Care and Use Committee of the University of Yamanashi (Registration Number: A1-6 (13 September 2019) and A4-4 (20 June 2022)).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

HSP	hereditary spastic paraplegia
SPG80	spastic paraplegia 80
SPG15	spastic paraplegia 15
SPG11	spastic paraplegia 11
UBAP1	ubiquitin-associated protein 1
4-PBA	4-phenylbutyric acid
KI	knock-in
WT	wild type
MVB	multivesicular body
ESCRT-1	endosomal sorting complex required for transport-1
ER	endoplasmic reticulum
HDAC	histone deacetylase
Iba1	ionized calcium binding adapter protein 1
GFAP	glial fibrillary acidic protein
CRISPR/Cas 9	clustered regularly interspaced short palindromic repeats/CRISPR associated proteins
a.u.	arbitrary units
DNA	deoxyribonucleic acid
PFA	paraformaldehyde
PBS	phosphate-buffered saline
NGS	normal goat serum
ANOVA	analysis of variance
HSD	honest significant difference
Tukey's HSD test	Tukey's honestly significant difference test

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International Journal of
Molecular Sciences

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Sobrerol Improves Memory Impairment in the Scopolamine-Induced Amnesia Mouse Model

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Abstract: Memory impairment is a defining characteristic of Alzheimer's disease (AD), with amnesia often appearing as its earliest symptom. Given the multifactorial nature of AD pathogenesis, this study investigates the multi-target therapeutic potential of sobrerol (coded as NRM-331) in a scopolamine-induced amnesia mouse model, focusing specifically on its effects in ameliorating memory deficits and enhancing neuronal plasticity. Sixty male C57BL/6NCrljOri mice were divided into six groups (10 mice/group): vehicle control (CTL, saline), scopolamine (SPA, 10 mg/kg/day), Aricept (APT, 2 mg/kg/day), and three treatment groups receiving NRM-331 at doses of 40, 80, and 100 mg/kg/day. Several behavioral tests were conducted, including the Y-maze test, passive avoidance test, and Morris water maze test. Additionally, biochemical assays were performed in serum (to measure Aß 1-40 and Aß 1-42) and in the brain (to assess ACh and AChE levels), along with histopathological examination of the brain using Nissl staining and p-tau IHC. No significant change was observed in the Y-maze test or the acquisition trial of the passive avoidance test. However, improvements were noted in the retention trial of the passive avoidance test and the Morris water maze test (including escape latency, swim distance, and number of platform crossed) for the NRM-331 groups compared to the SPA group. Serum levels of Aß 1-40 and Aß 1-42 decreased in the NRM-331 groups compared to the SPA group. In the brain, levels of ACh significantly increased, while AChE levels significantly decreased compared to the SPA group. The number of neuronal cells improved in the CA1, CA3, and DG regions of the hippocampus, as indicated by Nissl staining. A significant reduction in p-tau accumulation was also observed in the NRM-331 groups. In conclusion, NRM-331 demonstrated an anti-amnesic effect by enhancing hippocampal cholinergic signaling, alongside exhibiting anti-tau and anti-Aβ synthesis properties. These therapeutic effects suggest that NRM-331 significantly mitigates memory impairment induced by SPA through a neuroprotective mechanism.

Keywords: amnesia; memory; scopolamine; acetylcholine; amyloid beta; phospho-tau

1. Introduction

Amnesia refers to the loss of short-term memory, particularly the inability to retain facts, information, and experiences, which is a primary symptom of dementia [1]. This memory loss is often the result of neuronal damage or dysfunction of the hippocampus, the brain region responsible for memory formation and retrieval [2]. The causes of amnesia include traumatic brain injury, stroke, inflammatory reactions from infections, oxygen deficiency, excessive alcohol consumption (such as in Korsakoff syndrome), brain tumors, use of sedatives like benzodiazepines in epilepsy, and neurodegenerative diseases like dementia [3]. Alzheimer's

disease (AD), the most common form of dementia, is characterized by the progressive loss of memory, learning, and other cognitive impairments due to the ongoing degeneration of neurons and synaptic dysfunction [4]. Amnesia is typically a temporary and reversible condition, while dementia is progressive and irreversible. Treatment options for amnesia can include non-specific muscarinic anticholinergic drugs, which may aid in recalling events that occurred before brain damage; however, individuals often struggle to remember events after the injury [5]. In contrast, neurodegenerative amnesia, as seen in AD, is characterized by the inability to recall past events or previously familiar information.

The fundamental processes for learning and memory formation, which rely on neural communication, are crucial for encoding, consolidating, and retrieving information. These processes depend on neuroplasticity and the activity of neurotransmitters [6]. Memory loss is a key symptom of AD, as is the accumulation of amyloid-beta (Aβ) plaques and tau tangles, central molecules involved in the progressive damage to neurons [7]. Aβ protein plaques accumulate between neurons and disrupt neuronal communication, while hyperphosphorylated tau protein tangles accumulate inside neurons, which impairs the intracellular transport of neurotransmitters. The accumulation of amyloid plaques and tau tangles exacerbates neuronal inflammation and damage, ultimately leading to neurodegeneration. This neurodegeneration contributes to cognitive decline and memory loss, which are hallmark features of AD-related dementia and amnesia [8]. Moreover, memory impairment is linked to dysfunction in the cholinergic system, which includes cholinergic neurons, neurotransmitters, and their receptors [9]. According to the cholinergic hypothesis of memory dysfunction, decreased integrity of cholinergic neurotransmission, particularly involving acetylcholine (ACh) and acetylcholinesterase (AChE), affects synaptic plasticity, attention, memory, and learning [10]. Disruption of synaptic plasticity is commonly observed in both amnesia and AD; however, it may be reversible through the action of chlorogenic acid, which exerts its effects via anti-AChE and anti-oxidative activities [11].

Alkaloids are pharmacologically characterized as causing sensory loss, which makes it difficult to recall memories. Scopolamine (SPA), a tropane alkaloid, is a potent psychoactive drug that affects cholinergic signaling, as actively investigated in the 1950s [12]. SPA induces both short-term and long-term memory loss by blocking cholinergic transmission through its antagonist action on muscarinic receptors in the brain, thereby impairing learning and memory [13]. Recent studies suggest that natural compounds like *Callicarpa dichotoma* (Lour.) K Koch, couarin, and ginsenosides (Rg5 and Rh3) have a neuroprotective effect against SPA-induced memory deficits [14–16]. Additionally, calcium homeostasis is dysregulated in AD, but this can be reversed by *Bacopa Monnier* (L) Wettst through the CREB pathway in SPA-induced amnesia [17].

There are currently no specific medications that can help alleviate symptoms or slow the disease's progression. These medications may have side effects, including nausea, diarrhea, insomnia, muscle cramps, and dizziness. Aricept (Donepezil), a commonly prescribed medication, works by inhibiting ACh breakdown, thereby enhancing its levels in the brain. This highlights the importance of cholinergic pathways in memory recovery and underscores the need to investigate underlying neurobiological markers, such as amyloid-β and p-tau; both are implicated in the neurodegenerative processes of AD. Sobrerol ($C_{10}H_{18}O_2$) is a synthetic mucolytic agent derived from terpenes, traditionally investigated as an oral mucolytic agent for lung and bronchitis complications [18–20]. Sobrerol consists of anti-inflammatory and antioxidant effects, a mechanism of breaking disulfide bonds in mucoproteins, and increased mucociliary transport [21,22]. In addition, it has recently emerged as a potential therapeutic candidate for multiple sclerosis, as demonstrated in contemporary studies [23]. Building upon these findings, we hypothesized that sobrerol (coded as NRM-331) may exert additional previously unreported pharmacological effects.

To explore this possibility, the present study aims to rigorously evaluate its potential impact on cognitive dysfunction, thereby expanding the understanding of its therapeutic scope. In this study, we aimed to investigate these factors and their interactions to better understand the SPA-induced amnesia mouse model and to evaluate the potential effects of NRM-331 in mitigating memory impairments and neuronal dysfunction. To achieve this, we conducted several behavioral tests (Y-maze test, passive avoidance test, and Morris water maze test), biochemical analysis in serum (Aß 1-40 and Aß 1-42), as well as in the brain (ACh and AChE) and brain histopathological analysis (Nissl staining and tau-IHC).

2. Result

2.1. Effect of NRM-331 Treatment on Animals During the Study

Six experimental groups (n = 10/group) were examined as follows: saline vehicle control (CTL); negative control with 10 mg/kg/day scopolamine (SPA); positive control with 2 mg/kg/day Aricept (ACT) and three groups treated with NRM-331 at doses of 40 mg/kg/day (N40), 80 mg/kg/day (N80), and 100 mg/kg/day (N100). The pathogen test report of the animal environment indicated that there were no pathogenic factors that could affect the experiment. Body weights were measured on days 1, 8, 15, 22, and 29. Throughout the study period, no significant change in body weight was observed in any of the NRM-331 administration groups (N40, N80, and N100) or the ACT group compared to the CTL group (Figure 1A). However, a significant weight loss was observed in the SPA group on day 29 compared to the CTL group ($p < 0.05$, Figure 1B). Additionally, throughout the study period, no abnormal symptoms or deaths were recorded in any of the NRM-331 administration groups (N40, N80, and N100), indicating no adverse effects from the administration of NRM-331.

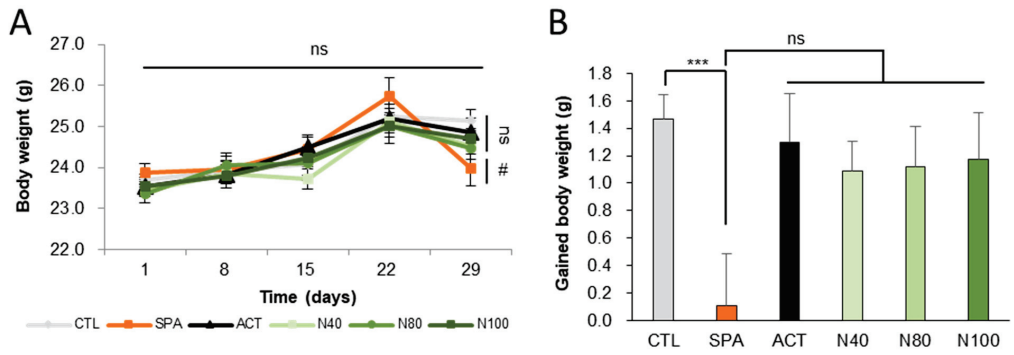


Figure 1. Body weights and gained body weight during the study. (**A**) Weekly body weight. (**B**) Gained body weight. Data were represented by mean ± S.E. The results were statistically analyzed by Welch's *t*-test and ONE-WAY ANOVA. The SPA group was compared to the CTL group, with significance indicated by *; the remaining groups were compared to the SPA group, with significance indicated by #. *** $p < 0.001$, # $p < 0.05$, ns: not significant. CTL (saline vehicle control); SPA (2 mg/kg/day scopolamine, negative control); ACT (2 mg/kg/day Aricept, positive control); N40 (40 mg/kg/day NRM-331); N80 (80 mg/kg/day NRM-331); N100 (100 mg/kg/day NRM-331).

2.2. Effect of NRM-331 in Behavioral Tests

The impact of NRM-331 on short-term memory was evaluated using the Y-maze test for behavior change on day 7. In the SPA group, a significant decrease in altered behavior was observed compared to the CTL group ($p < 0.05$). However, no significant changes were noted in the NRM-331 administration groups (N40, N80, and N100). Interestingly, the ACT group showed increased and altered behavior (31.77%) compared to the SPA group (Figure 2A). These findings suggest that NRM-331 administration did not significantly affect short-term memory performance in the Y-maze test compared to SPA administration.

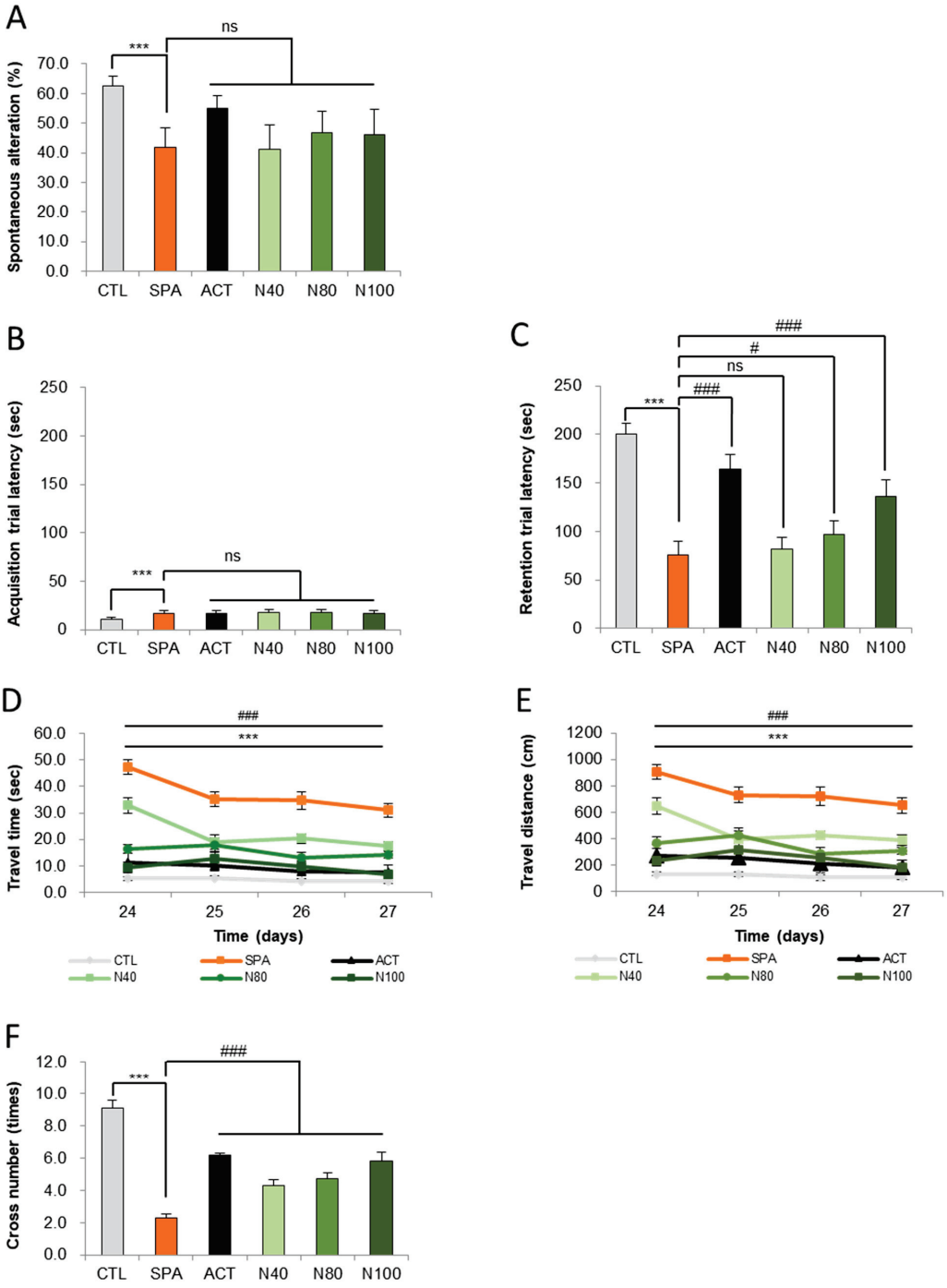


Figure 2. Behavioral tests of the Y maze, passive avoidance, and Morris water maze test. (**A**) Spontaneous alterations in the Y-maze test, (**B**) escape latencies in the passive avoidance test, (**C**) escape latencies in the Morris water maze test, (**D**) swim time in the Morris water maze test, (**E**) swim distances in the Morris water maze test, (**F**) number of platform crossing in the Morris water maze test. Data were represented by mean ± S.E. The results were statistically analyzed by ONE-WAY and TWO-WAY ANOVA. SPA group was compared to the CTL group, with significance indicated by *; the remaining groups were compared to SPA, with significance indicated by #. *** or ### $p < 0.001$, # $p < 0.05$, ns: not significant. CTL (saline vehicle control); SPA (2 mg/kg/day scopolamine, negative control); ACT (2 mg/kg/day Aricept, positive control); N40 (40 mg/kg/day NRM-331); N80 (80 mg/kg/day NRM-331); N100 (100 mg/kg/day NRM-331).

In addition to the Y-maze observations, we assessed short-term learning and memory through the passive avoidance test on days 14 and 15, focusing on the acquisition and

retention of trials. On day 14, the SPA group exhibited a significant increase in avoidance time (57.41%) compared to the CTL group. Conversely, no significant changes were observed in the NRM-331 administration groups (N40, N80, and N100), including the ACT group, compared to the SPL group (Figure 2B). On day 15, during the retention trial, a significant reduction in avoidance time was noted in the SPL group compared to the CTL group ($p < 0.01$). The N40 administration group did not show significant differences in avoidance time; however, the N80 administration group demonstrated a significant increase (26.97%), and the N100 administration group approximately showed a 60% increase in a dose-dependent manner when compared to the SPL group ($p < 0.05$ or $p < 0.01$). Additionally, the ACT group exhibited a significant increase in avoidance time compared to the SPL group ($p < 0.01$, Figure 2C). These results indicate that learning was successfully encoded in memory and could be recalled after a delay.

To assess long-term spatial learning and memory, the Morris water maze test was conducted on days 24, 25, 26, and 27. A significant increase in travel time to locate the platform was observed in the SPA group compared to the CTL group ($p < 0.01$) on all test days. In contrast, the NRM-331 administration groups (N40, N80, and N100), as well as the ACT group, showed a significant reduction in travel time on all test days ($p < 0.01$; Figure 2D). A similar trend was noted for travel distance, with the SPA group displaying a significant increase in distance traveled compared to the CTL group ($p < 0.01$). The N40, N80, and N100 administration groups showed a statistically significant decrease in travel distance compared to the SPA group, with the N100 group demonstrating a 15.07% decrease compared to the ACT group (Figure 2E). On day 28, the number of crossings over the platform location after its removal was assessed. The SPA group showed a significant decrease in the number of crossings compared to the CTL group ($p < 0.01$). Conversely, significant increases in the number of crossings were observed in the N40, N80, and N100 administration groups, including the ACT group, when compared to the SPA group ($p < 0.01$; Figure 2F). The representative track maps from the Morris water maze test on day 27 and the cross-number probe trial track map on day 28 illustrate that memory impairment appears complex in the SPA group, while the NRM-331 administration improved this impairment in a dose-dependent manner, as observed in the ACT group (Supplementary Figures S1 and S2).

2.3. Effect of NRM-331 on Biochemical Analysis

Amyloid-β peptides 1-40 and 1-42 were measured in serum using an ELISA kit. The results showed that amyloid-β 1-40 and 1-42 levels were significantly increased in the SPA group compared to the CTL group ($p < 0.01$). In the N40, N80, and N100 administration groups, significant reductions in either amyloid-β 1-40 or amyloid-β 1-42 were observed compared to the SPA group. Additionally, in the ACT group, there was a significant decrease in both amyloid-β 1-40 and 1-42 levels, compared to the SPA group ($p < 0.05$; Figure 3A,B). Acetylcholine (ACh) and acetylcholinesterase (AChE) levels were also measured in brain homogenate using ELISA. The results indicated a significant decrease in ACh levels and a significant increase in AChE levels in the SPA group compared to the CTL group ($p < 0.01$). In the N40, N80, and N100 administration groups, including the ACT group, ACh levels showed a significant increase in a dose-dependent manner compared to the SPA group ($p < 0.01$; Figure 3C). Conversely, AChE levels in the N40, N80, and N100 administration groups, including the ACT group, demonstrated a significant decrease compared to the SPA group, which was also in a dose-dependent manner ($p < 0.01$; Figure 3D).

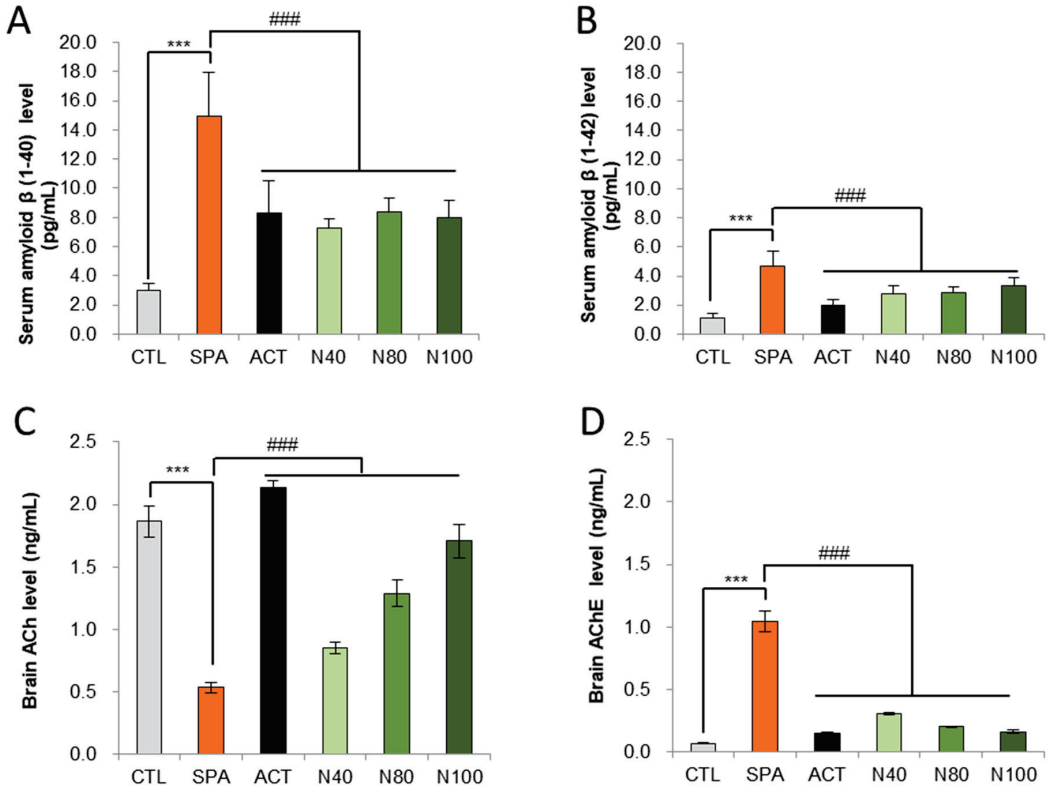


Figure 3. Biochemical analysis in serum for Aβ 1-40 and Aβ 1-42 levels and in brain tissue for ACh and AChE levels by ELISA. (**A**) Serum amyloid-β 1-40 level, (**B**) Serum amyloid- β 1-42 level, (**C**) Brain tissue ACh level, and (**D**) Brain tissue AChE level. The results were statistically analyzed by ONE-WAY ANOVA. The SPA group was compared to the CTL group, with significance indicated by *; the remaining groups were compared to SPA, with significance indicated by #. *** or ### $p < 0.001$, ns: not significant. CTL (saline vehicle control); SPA (2 mg/kg/day scopolamine, negative control); ACT (2 mg/kg/day Aricept, positive control); N40 (40 mg/kg/day NRM-331); N80 (80 mg/kg/day NRM-331); N100 (100 mg/kg/day NRM-331); Aβ: Amyloid beta; ACh: Acetylcholine; AChE: Acetylcholine esterase.

2.4. Effect of NRM-331 on Neuronal Plasticity

The histopathological analysis of neuron counts in the CA1, CA3, and DG regions of the hippocampus, using Nissl stain, showed no statistically significant difference in neuron numbers between the SPA group and the CTL group. However, a decrease in neuron counts was observed in the SPA group, with reductions of 16.69% in CA1, 15.34% in CA3, and 12.60% in DG. Furthermore, while there were no statistically significant changes in the neuron counts in the N40, N80, and N100 administration groups compared to the SPA group, increases in neuron counts were noted in the CA1 region for the N80 and N100 administration groups, showing increases of 11.95% and 10.44%, respectively. In the same groups, the DG region also showed increased neuron counts of 12.75%, 11.20%, and 11.42% compared to the ACT group (see Figure 4A,B).

Additionally, the quantitative analysis of p-tau accumulation in the CA1, CA3, and DG regions of the hippocampus was conducted through immunohistochemical staining, using the SPA group as a reference. A statistically significant increase in p-tau levels was observed in the SPA group, with increases of approximately 12.29% in both the CA1 and DG regions ($p < 0.01$). However, the increase in the CA3 region was not statistically significant compared to the CTL group. In both the N40 and N80 administration groups, a significant reduction in p-tau levels was noted in the CA3 region compared to the SPA group ($p < 0.05$ or $p < 0.01$). In the N100 administration group, a significant decrease in p-tau of around 11.16% was observed in the CA3 and DG regions ($p < 0.01$), although the decrease in the

CA1 region was not statistically significant compared to the SPA group. Furthermore, in the ACT group, significant reductions in p-tau levels were observed across all three sites (CA1, CA3, and DG) compared to the SPA group ($p < 0.01$; see Figure 4C,D).

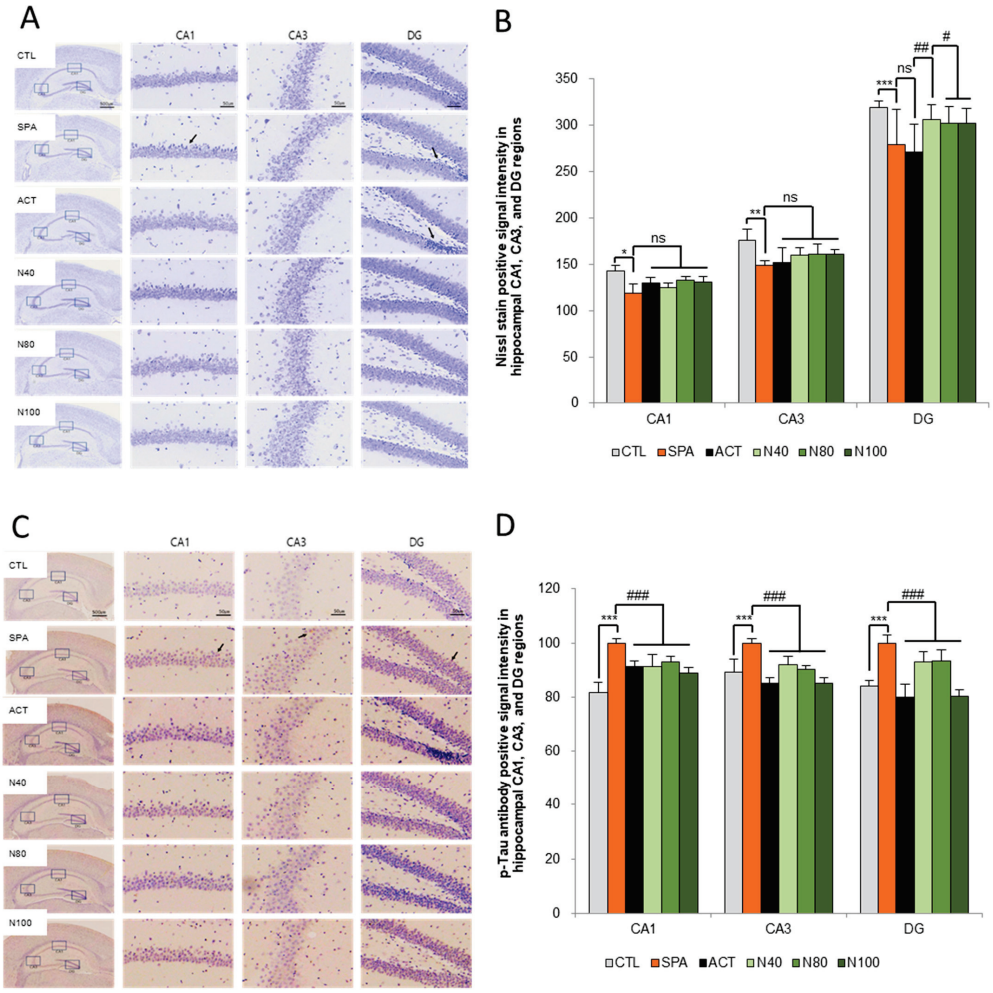


Figure 4. Histopathology of the brain for the Nissl stain and tau-accumulation by ELISA. (**A**) Representative images of Nissl staining neuronal cells in the hippocampus, (**B**) Quantification of Nissl stain, (**C**) Representative images of immunohistochemical staining for p-tau, and (**D**) Quantification of p-tau. The results were statistically analyzed by ONE-WAY and TWO-WAY ANOVA. SPA group was compared with the CTL group, and the remaining groups were compared with the SPA group. *** or $^{###}$: $p < 0.001$, ** or $^{##}$: $p < 0.01$, * or $^{#}$: $p < 0.05$, ns: not significant, Arrow: shrunken neuron or degenerated neuronal cell, or p-tau signal intensity. Scale bars: 500 μm or 50 μm. CTL (saline vehicle control); SPA (2 mg/kg/day scopolamine, negative control); ACT (2 mg/kg/day Aricept, positive control); N40 (40 mg/kg/day NRM-331); N80 (80 mg/kg/day NRM-331); N100 (100 mg/kg/day NRM-331).

3. Discussion

Memory loss is a hallmark symptom of AD, a multifactorial disorder characterized by a progressive decline of ACh levels, the accumulation of Aβ plaques between neurons, and the formation of tau tangles within neurons; these factors contribute to neurodegeneration and cognitive decline [9,24,25]. Due to the complex and multifunctional nature of AD, a multi-targeted therapeutic approach is preferred over a single-target drug to effectively address the intricate pathophysiological mechanisms underlying the disease. Current therapeutic approaches in the initial stages of AD often focus on enhancing cholinergic function in the brain, such as using Aricept (Donepezil), which inhibits the activity of AChE,

thereby increasing ACh levels. However, the underlying mechanisms of these treatments are not yet fully understood, and they can sometimes have adverse effects [26]. Recently, there has been growing interest in multi-target ligands and natural therapeutic agents as complementary and alternative treatments for neurodegenerative disorders [27]. In the present study, NRM-331 (sobrerol) approached the multifunctional properties of NRM-331 to evaluate its neuroprotective effect in a SPA-induced amnesia mouse model. Our results demonstrate that NRM-331 improves memory and cognitive impairment in this model. The improvements were validated by a reduction in serum Aβ concentrations, an increase in ACh levels in the brain, and a decrease in p-tau accumulation in the hippocampus, all of which reflect enhanced neuronal plasticity due to NRM-331.

The cholinergic antagonist properties of SPA provide insight into the mechanisms underlying memory impairment associated with neurodegenerative disorders and potential avenues for treating amnesia [28]. Previous studies have shown that SPA administration induces cognitive deficits in experimental models of learning and memory impairment [29,30]. To evaluate spatial learning and memory impairment, we conducted the Y-maze test, passive avoidance test, and Morris water maze test during NRM-331 treatment in SPA-induced amnestic mice. The Y-maze test, commonly used to study memory impairments and neurodegenerative diseases, evaluates short-term memory by observing rodents' instinctive behavior of exploring new environments [31]. Our findings from the Y-maze test indicate that administration of NRM-331 effectively inhibits the deterioration of short-term spatial memory compared to the SPA group. The passive avoidance test assesses short-term learning and memory by creating a conflict between fear and a natural preference for darkness, often induced by electric shock [32]. The passive avoidance tests are often employed to study memory retention, such as in neurodegenerative diseases [33]. In this test, we observed no significant difference in avoidance time at lower concentrations of NRM-331, while at higher concentrations, there was an increased avoidance time compared to the SPA group. This suggests that higher doses of the NRM-331 may enhance memory retention or reduce memory impairment, indicating potential cognitive-enhancing effects. The Morris water maze test assesses the acquisition of spatial memory, making it a valuable tool for studying hippocampal-dependent memory [34]. Over the trial time, the latency to find the platform reflects the animal's ability to learn and remember the location of the hidden platform, evaluating long-term spatial learning abilities. The results indicated a dose-dependent enhancement in cognitive function, as higher concentrations of NRM-331 (40, 80, and 100 mg/kg/day) significantly reduced travel time and distance, suggesting improved spatial memory and learning abilities compared to the SPA group. Overall, the findings from the Y-maze, passive avoidance, and Morris water maze tests align with previous studies that reported improvements in learning and memory following scopolamine-induced impairments [35,36]. Overall, the administration of NRM-331 significantly ameliorated learning and memory deficits induced by SPA.

Amnesia, often resulting from traumatic brain injury, is closely associated with the Aβ and tau protein aggregation as seen in AD [37]. The complex relationship between neurobiological factors and cognitive functioning is highlighted by the critical roles of Aβ, which serves as a key marker in the pathology of AD. A recent study indicated that the blood Aβ 1-42/1-40 ratio can serve as a predictive marker for the progression from mild cognitive impairment, leading to dementia [38]. Change in Aβ 1-40 and Aβ 1-42 levels can result in a decline in communication abilities and overall cognitive health, manifesting as amnesia and diminished learning capacity. In our study, we observed a decrease in serum Aβ 1-40 and Aβ 1-42 levels in the NRM-331 administration groups compared to the SPA group, indicating that NRM-331 has an effect in reducing these Aβ levels. Learning and memory processes are intricate and involve numerous neurotransmitters and receptors,

with cholinergic signaling being one of them. Cholinergic deficits are a significant mechanism in AD, contributing to the decline in cognitive functions, including memory loss [39]. Understanding the interactions between these biomarkers and cholinergic treatment strategies is crucial for developing effective therapies aimed at alleviating the cognitive deficits associated with SPA-induced amnesia [11]. To investigate the role of NRM-331 in neurotransmitter signaling, we examined ACh and AChE levels of cholinergic signaling by ELISA kits. Maintaining proper levels of ACh is essential for normal memory function; however, excessive activity of AChE can disrupt ACh availability in cholinergic synapses located in the hippocampus. In the context of SPA-induced amnesia, higher AChE activity is observed, which negatively impacts memory. Reducing or eliminating this excessive AChE activity may help restore functionality within the cholinergic neuronal system. Our results indicated increased ACh levels in the NRM-331 administration groups compared to the SPA group, along with a significant decrease in AChE levels in the same groups. Previous studies have shown that lower ACh levels can delay object recognition [40]. As mentioned, sobrerol consists of antioxidant properties, and oxidative stress and inflammation are key mechanisms involved in cognitive decline. In the current study, our primary objective was to investigate the effects of sobrerol on cholinergic markers (ACh, AChE), as well as neuropathological markers such as p-tau and Aβ, in relation to behavioral performance. Access to oxidative stress or inflammatory markers (e.g., ROS, IL-6, TNF-α, BDNF, CREB) would have provided a more comprehensive understanding of the mechanisms underlying sobrerol's cognitive protective effects. We did not assess oxidative stress or inflammatory markers, which is the limitation of this study.

The hippocampus is crucial for memory formation, and memory loss is a principal symptom of both AD and amnesia [41]. Damage to the hippocampus, particularly in the broader medial temporal lobe, leads to deficits in episodic memory, delayed recall, and recollective experience, associated with amnesia [42]. The impact of Aβ and p-tau protein on memory disorders such as AD cannot be overstated, as they contribute to the neurodegenerative processes underlying amnesia. Significant neurodegeneration has been linked to a decline in communication capabilities and overall cognitive health, leading to amnesia and diminished learning capacity. The hippocampus is recognized as central to memory formation and abnormal synaptic circuits and activity. The major input to the hippocampus comes from the CA1 to the subiculum through two main pathways: direct and indirect. In the indirect pathway, information reaches the CA1 region via the trisynaptic circuit. Axons project to the granule cells of the dentate gyrus (DG, first synapse), then continue through the mossy fibers to CA3 (second synapse), concluding the circuit with axons from CA1 (third synapse). Axons from CA1 project back to the entorhinal cortex (EC), completing the circuit. Any dysregulation in synaptic communication due to neurotransmitters (such as ACh) or neuroplasticity resulting from neurodegeneration (like Aβ or p-tau plaque accumulation) can impact the integrity of this trisynaptic circuit. In our study, following the administration of NRM-331, histopathological examination using Nissl stain revealed an increase in the number of neurons in the CA1, CA3, and DG sites of the hippocampus compared to the SPA group. Furthermore, immunohistochemical analysis for p-tau accumulation demonstrated a significant reduction in the NRM-331 administration group relative to the SPA group.

Conclusion: In conclusion, our findings provide evidence that NRM-331 exhibits an anti-amnesic effect, potentially attributable to its ability to enhance cholinergic signaling in the hippocampus, as well as its anti-tau and anti-Aβ synthesis activities. These properties suggest that NRM-331 could serve as a promising therapeutic agent for patients with neurodegenerative disorders, such as AD, by addressing key factors contributing to memory impairment and neuronal degeneration. This could pave the way for the

development of multifunctional treatments featuring NRM-331 for AD and other related cognitive disorders.

4. Materials and Methods

4.1. About NRM-331

Sobrerol (trans-p-Menth-6-ene-2,8-diol) is an aliphatic homomonocyclic compound (coded as NRM-331). One of the key mechanisms of action of Sobrerol is its antioxidant activity. Antioxidant activity is important for evaluating the capacity of compounds to reduce reactive oxygen species, thereby inhibiting oxidation and the production of free radicals. In this study, we assessed the neuroprotective effect of NRM-331 using the Scopolamine-induced amnesia mouse model.

4.2. Animal Study Design

Male C57BL/6NCrljOri mice (n = 91, eight weeks old) were purchased from Orient Bio Co., Ltd. (Seongnam-si, Republic of Korea). Upon arrival, the body weights of the animals were measured, ranging from 19.86 to 23.81 g. Five animals were housed per polycarbonate rearing cage (W 200 × L 260 × H 130 mm), with bedding consisting of sterilized wooden flakes (Saron Bio, Uiwang-si, Republic of Korea). Animals were acclimatized for seven days in a specific pathogen-free (SPF) environment, with free access to food and water. A solid rodent diet (Teklad global diets, 18% protein rodent diet, 2918C, ENVIGO, Indianapolis, IN, USA) was sourced from Coretech (Pyeongtaek-si, Republic of Korea), and ultraviolet sterilized water was provided in a polycarbonate drinking bottle with a microfilter. Water quality was tested by the Gyeonggi-do Institute of Health and Environment (Suwon-si, Republic of Korea). The animal room conditions were controlled at a temperature of 22 ± 3 °C, with relative humidity maintained at 55 ± 15% and a ventilation frequency of 10–20 times per hour. A 12-h light (08:00 a.m. to 08:00 p.m.) and 12-h dark (08:00 p.m. to 08:00 a.m.) cycle was implemented, with illuminance measured at 150–300 Lux. Throughout the study, temperature and humidity were recorded hourly using a computer system, while ventilation frequency and light levels were measured regularly. Bedding and water were checked daily, with bedding changed once a week. The animal room was routinely monitored for pathogen testing. Following the acclimatization period, a cued test was conducted to select healthy animals and exclude those deemed unhealthy, which is essential for reliable study outcomes. The pool was filled with water, with the platform submerged just below the surface. When placed in the pool, animals had 60 s to locate the platform; those that failed to find it were excluded from the experiment after two attempts. Following the cued test, 60 healthy animals were selected, weighed, marked, and randomly assigned to six experimental groups (n = 10 mice/group) to ensure uniform body weight distribution (ranging from 21.88 to 24.94 g). After grouping, similarly, five animals were housed per cage and cages were labeled with unique identifiers and color codes: G1 (M 10 1–10—10 Saline), G2 (M 10 11–20—10 Scopolamine), G3 (M 10 21–30—10 Aricept), G4 (M 10 31–40—10 NRM-331 at 40 mg/kg/day), G5 (M 10 41–50—10 NRM-331 at 80 mg/kg/day), and G6 (M 10 51–60—10 NRM-331 at 100 mg/kg/day). The experimental groups included a saline vehicle control (CTL), a negative control group receiving 10 mg/kg/day scopolamine (SPA), a positive control group receiving 2 mg/kg/day Aricept (ACT), and treatment groups receiving NRM-331 at varying dosages (40 mg/kg/day, 80 mg/kg/day, and 100 mg/kg/day).

4.3. Drug Preparation and Treatments

Scopolamine (SPA) was obtained from Sigma-Aldrich (cat. no. S1875; Burlington, MA, USA) and was prepared in physiological saline at a concentration of 1 mg/10 mL, then

injected intraperitoneally at a dose of 1 mg/kg. The SPA was administered 30 min after NRM-331 treatment and 30 min before behavioral tests (Y-maze test, passive avoidance test, and Morris water maze test) for all groups except the control group. Aricept (ACT), a donepezil product from Handok, Republic of Korea, was also prepared in physiological saline at a concentration of 1 mg/10 mL, injected intraperitoneally at a dose of 2 mg/kg, 30 min before the SPA injection, but only for the ACT group.

The NRM-331 (Sigma-Aldrich, cat. No. 247774, Burlington, MA, USA) was used for anti-amnesia treatment and prepared in physiological saline for administration by oral gavage, using a 1 mL syringe equipped with a zonda. NRM-331 was administered 30 min prior to SPA injection for all groups except the CTL group. The treatment duration was set for four weeks, administered daily (seven times a week) from day 1 to day 28, with mice being sacrificed on day 29. Body weights were recorded at the start of acclimatization (day −7), the time of grouping (day 0), and then weekly thereafter (day 1, 7, 14, 21, and 28), along with a final measurement at the time of sacrifice (day 29). Throughout the drug administration and study period, any notable general symptoms or fatalities were recorded.

4.4. Behavioral Tests

The Y-maze test was conducted on day 7 following the administration of NRM-331 using the Y-maze test setup (Smart, version 3.0.06, Panlab Harvard Apparatus, Holliston, MA, USA). The experimental apparatus consists of a Y-shaped maze made from transparent acrylic plates (dimensions: W 10 × L 41 × H 25 cm). Each arm of the maze was positioned at a constant angle of 120°. After designating the arms as areas A, B, and C, the experimental animal was carefully placed in one of the areas and allowed to move freely for 8 min. During this time, the animal's location was recorded by a camera mounted on the ceiling. An entry was counted when the animal's tail completely crossed into an area, and both the number and the order of entries into each section were measured to evaluate changes in behavioral patterns (spontaneous alternation percentage). A sequence of entries into three different areas was counted as one point (actual alternation, such as ABC, BCA, CAB, etc.). If the animal did not enter consecutively, it was not scored. The percentage of spontaneous alternation was calculated using the following formula:

$$\% \text{ Spontaneous alternation} = (\text{total number of alternations}/(\text{total number of entrances} - 2)) \times 100.$$

A passive avoidance test was performed on days 14 and 15 after the administration of NRM-331 using the passive avoidance test setup (cat no. RNV-253C, SG-716B, SG6080D, St. Albans, VT, USA). This setup comprised two compartments divided by a partition with a guillotine door in the middle. One compartment was illuminated, while the other remained dark. The floor of the compartments was gridded and capable of delivering an electric shock. The experiments were conducted at the same time over three consecutive days with 24-h intervals. Initially, on day 13 following NRM-331 administration, animals were allowed to stay in the shaded area for 2 min, after which they were returned to the illuminated area and then immediately taken back to the shaded area for adaptation training. After 24 h, on day 14, SPA was injected 30 min following the administration of NRM-331. Training occurred twice, with 2-min intervals after the SPA injection (acquisition trial). During the first training session, animals were placed in the passive avoidance test chamber for 60 s to adapt to the equipment. At this stage, the guillotine door was opened without illumination, allowing the animal to enter and exit freely. After this 60-s adaptation period, illumination was restored, and after an additional 120 s of free entry and exit, the guillotine door was closed while the animal received a 0.20 mA scrambled shock for 2 s. Finally, 24 h later on day 15 after NRM-331 administration, SPA was again injected 30 min

following NRM-331, and the time taken for the animal to move to the shaded area was measured (retention trial).

The Morris water maze test was conducted from day 22 to day 28 following the administration of NRM-331, using the Morris water maze test setup (Smart, version 3.0.06, Panlab Harvard Apparatus, Holliston, MA, USA). In this setup, a platform was submerged in a pool at one of four designated release points within the water tank (diameter: 1 m) and was intended to be located within 60 s. After locating the platform, the animal was allowed to rest on it for about 30 s. If the platform was not found within 60 s, the animal was placed on the platform and allowed to rest for 30 s. Following a trial involving five animals, the next trial commenced, with each trial conducted twice daily. The release points were randomly selected to avoid overlap. The time taken to find the platform was recorded over a period of 7 days (training: 2 days, behavioral experiment: 4 days, probe trial: 1 day). On the final day of the Morris water maze test (day 28), the platform was removed, and a probe trial was carried out for 60 s to measure the number of crosses at the previous platform location. As noted earlier, SPA was injected 30 min following the administration of NRM-331 for the Morris water maze test.

4.5. Biochemical Analysis: Serum and Brain

Blood samples were collected and transferred into 5 mL vacuum tubes containing a clot activator (Becton, Dickinson and Company, cat. no. 367955, Franklin Lakes, NJ, USA). The samples were left at room temperature for 15 to 20 min to allow clotting, followed by centrifugation at 3000 rpm for 10 min at 4 °C to separate the serum. The resulting serum was collected in sterilized pre-labeled EP tubes and stored in a −80 °C deep freezer for further molecular analysis. When needed, the serum samples were thawed on ice and used for the quantification of amyloid-β 1-40 (IBL Co. Ltd., cat. no. 27720, Fujioka-Shi, Japan) and amyloid-β 1-42 (IBL Co., Ltd., cat. no. 27721, Fujioka-hi, Japan) using ELISA kits, following the manufacturer's instructions.

Brain samples were also thawed and homogenized in ice-cold PBS (the ratio of brain weight to PBS volume, PBS molarity, and PBS pH to be specified). After homogenization, the samples were centrifuged at [rpm/min], and the supernatants were collected for acetylcholine (ACh) and acetylcholinesterase (AChE) analysis, which was conducted within 24 h. ACh (MyBiosource, cat. no. MBS733116, San Diego, CA, USA) and AChE (MyBiosource, cat. no. MBS721845, San Diego, CA, USA) levels were determined using ELISA kits, following the manufacturer's instructions.

4.6. Histopathology of the Brain

Prefixed brain samples (in 10% neutral buffered formalin) were prepared for Nissl staining and p-tau antibody analysis to evaluate neuronal plasticity and degeneration histopathologically. The examination focused on the areas of Cornu Ammonis 1 (CA1), Cornu Ammonis 3 (CA3), and the dentate gyrus (DG) in the hippocampus. The prefixed brain was trimmed, and the hippocampal region was processed for paraffin embedding to form paraffin blocks. Tissue blocks were sectioned to a thickness of 4 μm and placed on glass slides. The slides were stained with Nissl stain following the reference protocol and observed using an optical microscope (BX61, Olympus, Tokyo, Japan). Photographs were taken using a digital camera (DP80, Olympus, Tokyo, Japan) [13].

For quantification, the number of Nissl-stained cells in the CA1, CA3, and DG regions of the hippocampus was counted under a high-resolution microscope (at 400× magnification), and Image-Pro software (Media Cybernetics, version 10.0.15; Rockville, MD, USA) was employed for quantitative analysis of the stained cells compared to standard values after measuring positive signal intensity.

For p-tau immunohistochemical staining (IHC), the paraffin sections (4 μm thick) were mounted on SuperFrost glass slides (Thermo Fisher, cat. no. 22037246, Waltham, MA, USA). After deparaffinization with xylene and hydration using ethanol, slides were immersed in 0.01 M citric acid buffer for antigen retrieval and subjected to high temperature and pressure treatment for 10 min in a pressure cooker. Endogenous peroxidase activity was blocked using 3% H_2O_2 for 15 min. Following treatment with blocking agents (Vector Laboratories, cat. no. S-1000-20, Newark, CA, USA), anti-p-tau (Ser202, Thr205) antibody (Thermo Fisher, cat. no. MN1020, Waltham, MA, USA) was diluted 1:500 in 1x PBS buffer, incubated for 16 h, and washed three times with 1× PBS buffer. Detection was carried out using the Vectastain Elite ABC kit (Vector Laboratories, cat no. PK-6100, Newark, CA, USA) and 3,3′-Diaminobenzidine, followed by counterstaining with hematoxylin. Observations were made using an optical microscope (BX61, Olympus, Tokyo, Japan), and images were captured using a digital camera (DP80, Olympus, Tokyo, Japan).

For quantitative analysis of the immunohistochemical staining results, the captured images were analyzed for relative ratios based on standard values after measuring positive signal intensity using Image-Pro software (Media Cybernetics, version 10.0.15; Rockville, MD, USA).

4.7. Statistical Analysis

Statistical analyses were performed using SPSS (version 12.0K; SPSS Inc., Chicago, IL, USA,). Comparisons of the control (CTL) and study groups (SPA) involved parametric multiple comparison procedures to determine disease causation. The SPA and the APT were compared using a Student's *t*-test, with Welch's *t*-test applied when variances were unequal. A one-way ANOVA was utilized to compare the SPA across different administration groups (NRM-331, N40, N80, N100), followed by Duncan's test for equal variance and Dunnett's test for non-equal variance based on post-mortem analysis. Statistical significance was determined at $p < 0.05$.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ijms26104613/s1.

Author Contributions: Conceptualization, K.-H.J. and A.A.; methodology, K.-H.J. and A.A.; software, G.-S.P. and A.-R.G.; validation, K.-H.J. and S.-J.P.; investigation, K.-H.J.; data curation, A.A. and G.-S.P.; writing—original draft preparation, A.A.; writing—review and editing, K.-H.J.; visualization, A.A. and G.-S.P.; supervision, K.-H.J.; project administration, K.-H.J. and G.-S.P.; funding acquisition, K.-H.J. All authors have read and agreed to the published version of the manuscript.

Funding: This research study was conducted by NeurolMed Biopharma in collaboration with Corestem Chemon Co., Ltd. (study number: 22-ME-0967N, dated: 31 May 2023).

Institutional Review Board Statement: The experiment were carried out under the Laboratory Animal Steering Committee of Non-Clinical Laboratory Co., Ltd. (Deliberation No. 2022-1112) guidelines with the standard operating procedure by Corestem Chemon Co., Ltd. (240, Nampyeon-gro, Yangji-myeon, Cheoin-gu, Yongin-si, 17162, Republic of Korea) and Neurolmed Biopharma (Changryong-daero 256 beon-gil, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16229, Republic of Korea).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data is contained within the article with Supplementary Material.

Conflicts of Interest: All authors were employed by the company NeurolMed. The authors declare no conflict of interests related to this work.

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International Journal of
Molecular Sciences

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A Novel Small Molecule Enhances Stable Dopamine Delivery to the Brain in Models of Parkinson's Disease

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Abstract: Levodopa is the gold standard symptomatic treatment for Parkinson's disease. Disease progression due to alpha-synuclein accumulation, brain inflammation, and the loss of dopamine neurons, as well as motor fluctuations, due to variations in levodopa plasma levels, remain a significant problem for Parkinson's patients. Developing a therapeutic option that can simultaneously reduce the neuropathology associated with alpha-synuclein aggregation, attenuate oxidative stress and inflammation, and overcome variations in levodopa plasma levels is an unmet need to treat Parkinson's disease. We determined the pharmacokinetics and pharmacodynamics of a small molecule, dubbed Pegasus, that conjugates dopamine with a nonantibiotic doxycycline derivative via a molecular linker. Mice harboring the human A53T mutation of alpha-synuclein or treated with MPTP were injected once daily with 50 mg/kg Pegasus for 2 weeks and assessed for motor, behavioral, and cognitive effects, followed by biochemical and histochemical analysis. Pegasus is a poor brain penetrant but it was metabolized to stable dopamine and tetracycline derivatives, and abundant plasma and brain levels of these metabolites were detected. Pegasus reduced soluble and insoluble alpha-synuclein levels, protected dopamine-producing neurons, and reduced astrocytic activation in A53T mice. Mice treated with Pegasus exhibited motor improvement (6.5 h) and reduction in anxiety-like behavior. Rotarod and grip strength improved in MPTP-treated mice when mice were treated with Pegasus or levodopa. Pegasus may be a multi-modal therapeutic option that can deliver stable dopamine into the CNS and reduce misfolded alpha-synuclein, activate dopamine receptors, and attenuate variations in dopamine levels.

Keywords: Pegasus; tetracycline; dopamine; levodopa; Parkinson's disease

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder causing motor and non-motor symptoms, the loss of dopamine (DA) in neurons of the nigro-striatal pathway, and the accumulation of misfolded alpha-synuclein [1–3]. Levodopa is the gold standard treatment for PD symptoms, but progression due to alpha-synuclein accumulation, pathological oxidative stress, brain inflammation, and the loss of DA neurons are not abated by levodopa. Motor fluctuations, due to variations in levodopa absorption, levodopa plasma levels, and brain penetration remain significant problems for patients. Optimizing a therapeutic option that can simultaneously halt alpha-synuclein aggregation and overcome variations in levodopa plasma levels is an medical need to treat PD [4–6].

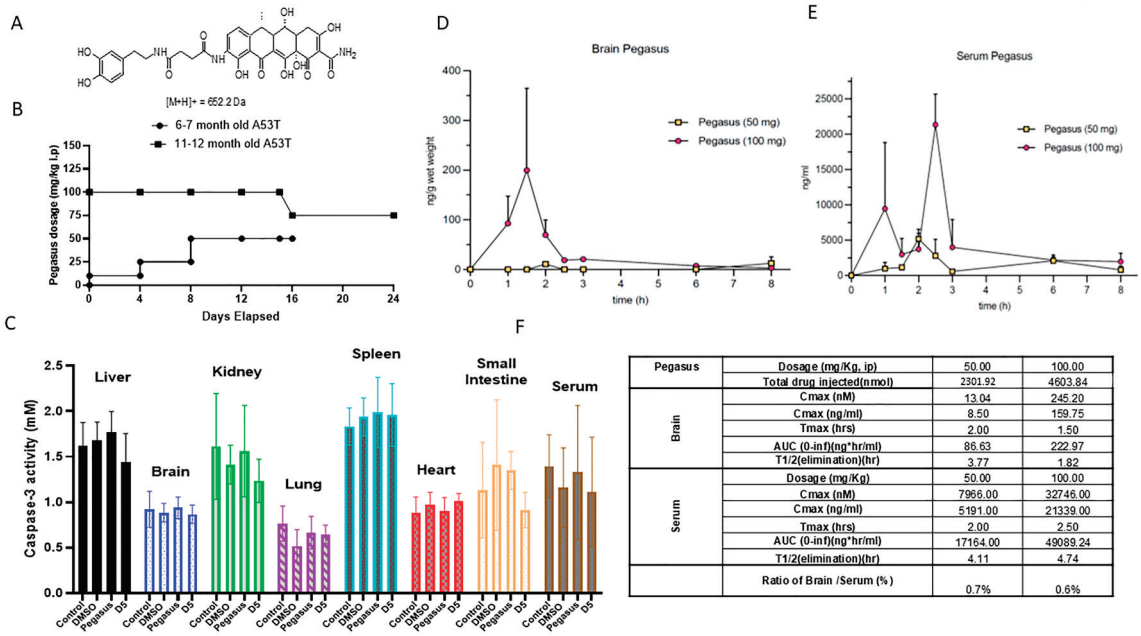
A novel compound dubbed Pegasus was synthesized [7] and shown to address the multifactorial cause of PD, including the aggregation of alpha-synuclein, oxidative stress,

and inflammation in vitro [7]. Pegasus is a small molecule (with a molecular weight of 652 Da) that links DA with a nonantibiotic doxycycline derivative [7], resulting in a molecule with calculated physicochemical properties not predicted to cross the blood–brain barrier (BBB) via passive permeability [8]. Here, we report novel mechanisms of DA delivery into the brain via the in vivo metabolism of Pegasus into DA and tetracycline/doxycycline derivatives to provide significantly enhanced DA delivery upon peripheral dosing. Treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes motor symptoms reminiscent of PD, while Pegasus can restore behavior like levodopa in vivo.

2. Results

2.1. Tolerated Dose and Tissue Toxicity

Pegasus synthesis and the D5 control derivative of tetracycline were previously described [7]. Pegasus is a 652.2 Da small molecule that consists of one DA conjugated to tetracycline via a molecular linker (Figure 1A). Extensive in vitro studies were previously performed and showed Pegasus's protective effects via the prevention of alpha-synuclein misfolding [7]. We determined a tolerated dose of Pegasus in young (6–7 months old) and old (11–12 months old) TgA53T mice (IP, once daily) treated with an ascending dose of Pegasus of 10 mg/kg, 50 mg/kg, and 75 mg/kg for 4 consecutive days (Figure 1B). Pegasus was tolerated up to 75 mg/kg without visible adverse effects. However, at 100 mg/kg, male and female (12–13 months old) mice showed some scruffiness and weakness at Day16, and one mouse showed gut toxicity at autopsy. We then reduced the dose to 75 mg/kg for another week. Autopsy was performed and no adverse effects were detected.



Pegasus	Dosage (mg/Kg, ip)	50.00	100.00
	Total drug injected(nmol)	2301.92	4603.84
Brain	Cmax (nM)	13.04	245.20
	Cmax (ng/ml)	8.50	159.75
	Tmax (hrs)	2.00	1.50
	AUC (0-inf)(ng*hr/ml)	86.63	222.97
	T1/2(elimination)(hr)	3.77	1.82
Serum	Dosage (mg/Kg)	50.00	100.00
	Cmax (nM)	7966.00	32746.00
	Cmax (ng/ml)	5191.00	21339.00
	Tmax (hrs)	2.00	2.50
	AUC (0-inf)(ng*hr/ml)	17164.00	49089.24
	T1/2(elimination)(hr)	4.11	4.74
	Ratio of Brain /Serum (%)	0.7%	0.6%

Figure 1. Toxicity and pharmacokinetic study of Pegasus. (**A**) Chemical structure of Pegasus. (**B**) Administration of Pegasus revealed maximum tolerable dose and in vivo toxicity verified by (**C**) caspase-3 activity. Pharmacokinetic analysis of Pegasus in (**D**) brain and (**E**) serum and (**F**) calculated pharmacokinetic parameters. One-way ANOVA with Dunnett's multiple comparisons test. N = 3–10. All values presented as mean ± SD.

We further verified cell death via caspase-3 activity assays of the liver, brain, kidney, lung, spleen, heart, serum, and small intestine in young TgA53T mice (6–7 months old). There were no significant changes in caspase-3 activity in all the tested tissues in Pegasus-

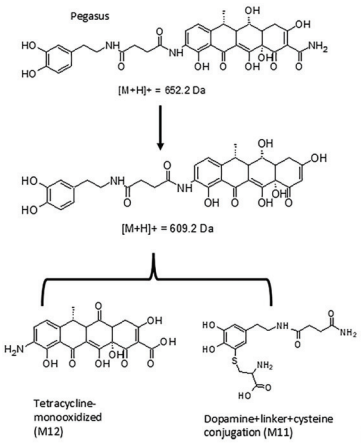
treated mice compared to naïve control (without any treatment), DMSO-, and D5-treated mice (Figure 1C).

2.2. Pharmacokinetics

PK analysis showed that Pegasus could be detected in both the serum and the brain after a single intraperitoneal (i.p.) injection (Figure 1D,E) in wild-type mice. Pegasus reached peak concentration at 2 h after an injection of 50 mg/kg in both the serum and the brain, and reached peak concentration at 1.5 h in the brain and 2.5 h along with an additional smaller peak at 1 h in the serum after an injection of 100 mg/kg i.p. $AUC_{0\text{-inf}}$ was 86.63 to 222.97 ng × h/mL in the brain and 17,164 to 49,089.24 ng × h/mL in the serum. The elimination half time $T_{1/2}$ of Pegasus was 4.11 to 4.74 h in the serum and 1.82 to 3.77 h in the brain. The percentage ratio of brain/serum was 0.6–0.7% (Figure 1F).

In vivo metabolism: We performed a metabolism study (Figure 2) in male Sprague Dawley rats (6–8 weeks old) after a single i.p. injection of 50 mg/kg Pegasus. Plasma and brain samples were serially collected and assessed for the presence of possible phase-I and phase-II metabolites. A total of thirteen metabolites were detected in the rat brain and plasma samples. They were identified as formed by the mono-oxidation -2H (M1), NH2 to OH + glucuronidation (M2), sulfation (M3), C-N bond cleavage (tetracycline) -2H (M4), C-N bond cleavage (tetracycline) + NH_2 to OH -2H (M5), C-N bond cleavage (tetracycline) + NH_2 to OH -2H + O (M6), C-N bond cleavage (tetracycline) + acetylation (M7), C-N bond cleavage (dopamine + linker) (M8), C-N bond cleavage (dopamine) + N-acetylcysteine conjugation (M9), NH_2 to OH (M10), C-N bond cleavage (dopamine + linker) + cysteine conjugation (M11), -$CONH_2$ (M12), and C-N bond cleavage (tetracycline) + both NH2 to OH + 2H (M13) of parent (M). The parent peak (Figure 2B) was very small in the rat plasma samples and was not detected in the brain samples. The possible metabolic pathways for Pegasus are presented in Figure 2A and Supplemental Figure S1. The [M + H] + (Q1) with retention time and product ions (MS/MS, fragmentation pattern) for the parent Pegasus and its putative metabolites, as well as the differences in the observation of the metabolites in the rat brain or plasma are shown in Figure 2B. The relative abundance of Pegasus and its metabolites in the brain and plasma was determined at the ionization parameters of the parent. The major metabolite was M6. The other relatively abundant metabolites in the brain followed the order M5, M13, M11, and M12. M6, M13, M11, and M12 were relatively more abundant in plasma (Figure 2B).

A



B

Possible Transformation	Metabolite Identification number	Retention Time (min)	MH+	Rat Brain	Rat Plasma	Relative brain abundance (%±SD)	Relative plasma abundance (%±SD)	Brain/plasma (%)
Parent	M	13.92	652.2	X	✓	0±0	0.55±0.62	0
Mono-oxidation -2H	M1	13.08	666.2	X	✓	0±0	0.2±0.2	0
NH2 to OH+ Glucuronidation	M2	13.31	829.2	X	✓	0.2±0.3	0.2±0.1	93
Sulfation	M3	14.01	732.2	X	✓	0±0	0.1±0.2	14
C-N bond cleavage (tetracycline)-2H	M4	12.13	415.2	✓	✓	4.6±3.9	8.4±2.2	54
C-N bond cleavage (tetracycline) + NH2 to OH -2H	M5	11.94	416.2	✓	✓	7.6±5.4	3.7±7.1	207
C-N bond cleavage (tetracycline) + NH2 to OH -2H + O	M6	12.17	432.2	✓	✓	40±6.2	27±7.1	148
C-N bond cleavage (tetracycline) + acetylation	M7	12.49	459.2	✓	✓	0.9±0.8	8.1±2.8	11
C-N bond cleavage (dopamine + linker)	M8	11.38	253.2	✓	✓	0.8±1.4	0.2±0.3	318
C-N bond cleavage (dopamine)+ N-acetylcysteine conjugation	M9	11.07	314.2	X	✓	0±0	1.5±2.6	0
NH2 to OH	M10	13.05	653.2	✓	✓	2.3±0.7	6.7±3	34
C-N bond cleavage (dopamine + linker) + cysteine conjugation	M11	11.53	372.1	✓	✓	16±6	12±5.7	131
-CONH2	M12	12.83	609.2	✓	✓	6.1±4.9	12±3	50
C-N bond cleavage (tetracycline) + both NH2 to OH +2H	M13	12.51	520.1	✓	✓	28±10	25±6.7	114

Figure 2. In vivo metabolism of Pegasus. (**A**) Possible metabolic pathways for Pegasus and matrix differences in metabolite formation and (**B**) relative abundance of metabolites of Pegasus in rat brain and plasma samples. All values presented as mean ± SD.

2.3. Pegasus Profoundly Alleviates Anxiety and Improves Motor Performance in TgA53T Mice

TgA53T mice (12–13 months old) were treated daily with Pegasus 50 mg/kg i.p. or DMSO for 3 weeks before performing behavioral analysis. A single day assessment (following pre-test training) showed that Pegasus significantly improves motor performance at 1–2 h post-injection (Figure 3A) and continues to improve motor behavior at 2–3 h (Figure 3B), 3–4 h (Figure 3C), and up to 5–6.5 h (Figure 3D) compared to DMSO, suggesting that Pegasus is a potential drug that provides long-lasting effects on motor symptoms. An elevated plus maze test was utilized to assess the anxiety-like behavior of TgA53T mice (12–13 months old). In Pegasus-treated mice, the total distance traveled (Figure 3E) and the total number of entries to arms (Figure 3F) were significantly decreased compared to DMSO. The percentage of time spent in open arms (Figure 3G) and the percentage of entries to open arms (Figure 3H) were also increased in Pegasus-treated mice, suggesting reduced anxiety.

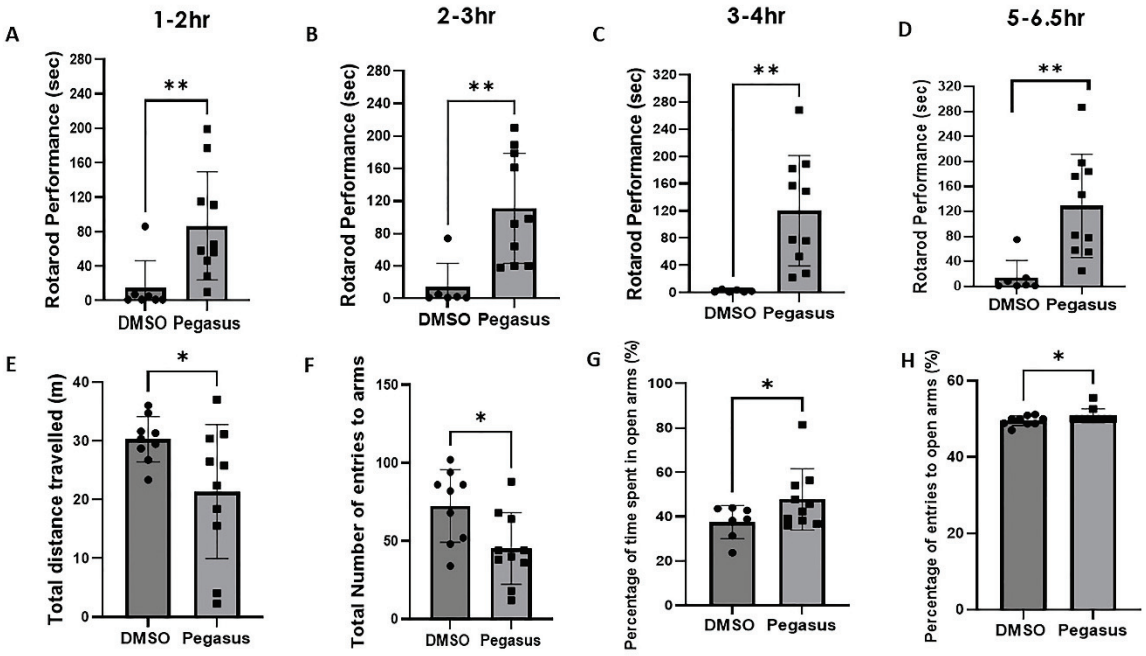


Figure 3. Pegasus profoundly improves anxiety and motor performance up to 6.5 h. Male and female 12–13-month-old TgA53T mice were treated with DMSO or Pegasus at a dosage of 50 mg/kg for 3 weeks. Behavioral assessment started from the 15th day of treatment. (**A**–**D**) The Rotarod test shows that Pegasus treatment improves animal motor performance up to 6.5 h. (**E**) The elevated plus maze test reveals that Pegasus treatment significantly decreases the total distance traveled and the total number of entries to arms (**F**), and significantly increases the percentage of time spent in open arms (**G**) and the percentage of entries to open arms (**H**). Actual *p*-value of the statistical analysis by a two-tailed t test. * $p < 0.05$, ** $p < 0.01$. N = 7–10. All values are presented as mean ± SD.

2.4. Pegasus Improves Behavioral Deficits of MPTP-Treated Mice

MPTP was injected to male C57BL/6 mice for 5 consecutive days (Figure 4A) to destroy DA neurons, and then the mice were treated with Pegasus alone or Pegasus + Benserazide compared to L-dopa alone or L-Dopa + Benserazide. Pegasus- and L-Dopa-treated mice had a significantly increased latency to fall, indicating improved motor deficiency induced by MPTP (Figure 4B). Forelimb muscular strength was significantly reversed back to normal levels in Pegasus- and L-Dopa-treated mice (Figure 4C), indicating that Pegasus is sufficient to alleviate the neuromuscular dysfunction induced by MPTP.

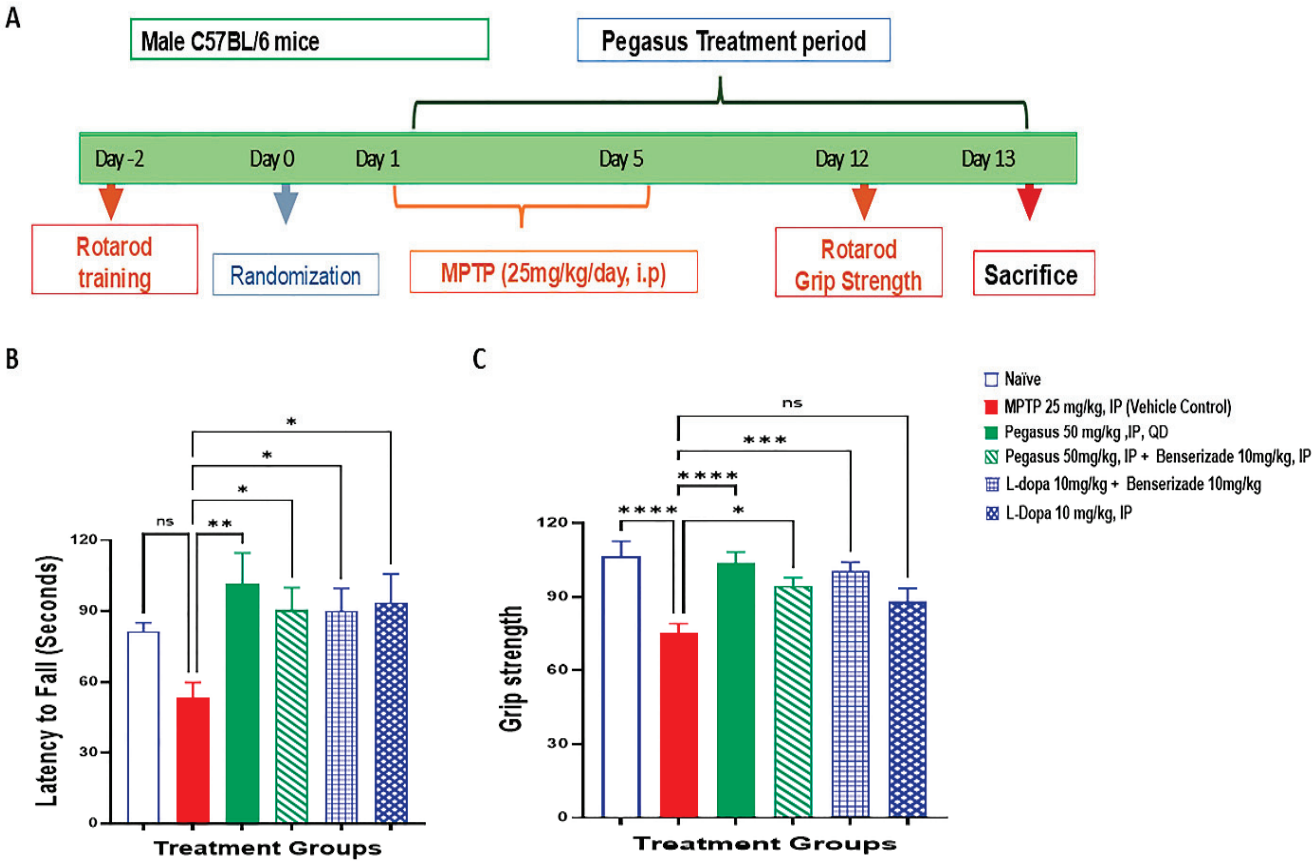


Figure 4. Pegasus improves behavioral deficits of MPTP-induced neuromuscular impairment. The study design (**A**) where C57BL/6 mice are treated with Pegasus and L-dopa alone or in combination with Benserazide significantly increases the latency to fall in the Rotarod test (**B**) and increases the grip strength in the grip strength test (**C**) compared with MPTP vehicle control. The asterisk indicates static significant difference. ns: not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to vehicle. One-way ANOVA; Dunnett's multiple comparisons test; N = 11–15. All values are presented as mean ± SD.

2.5. Pegasus Reduces Alpha-Synuclein Levels and Increases DA and Its Metabolite HVA Levels in the Brain of TgA53T Mice

Pegasus significantly increases the concentrations of DA in the brain (Figure 5A; $p = 0.0177$) and HVA (Figure 5B; $p = 0.0374$), and significantly increases the concentration of DA (Figure 5C; $p = 0.0057$) though not HVA in the serum (Figure 5D; $p = 0.4896$), compared to DMSO in 12–13-month-old mice, indicating that Pegasus may act as a dopamine carrier. The WB of brain samples harvested from young TgA53T mice (6–7 months old) revealed significant decreases in the levels of both monomeric (Figure 5E,G; $p = 0.0149$) and oligomeric alpha-synuclein (Figure 5F,H; $p = 0.0437$) after treatment with Pegasus compared with D5- and DMSO-treated mice. The concentration of CNS alpha-synuclein via ELISA was reduced in the brain lysates of old (11–12-month-old) TgA53T mice treated with Pegasus compared to DMSO-treated controls (Figure 5I).

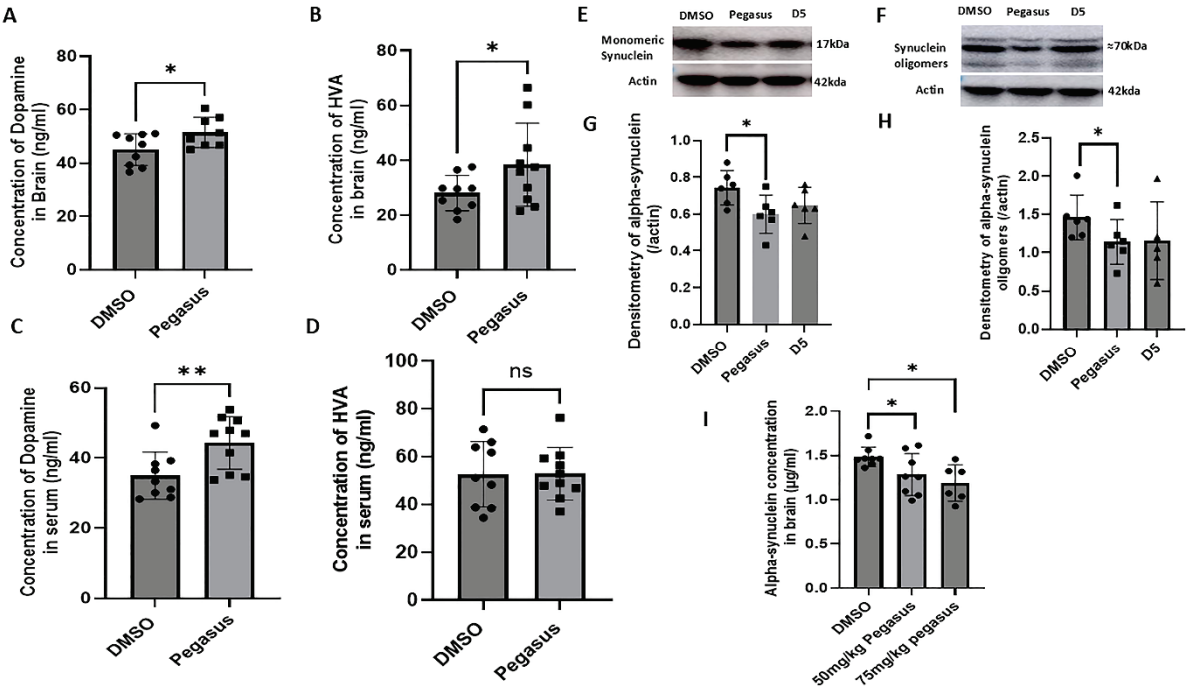


Figure 5. Pegasus reduces alpha-synuclein and increases DA/HVA levels in the brain of TgA53T mice. Old 12–13-month-old TgA53T mice were injected with Pegasus at the once daily dosage of 50 mg/kg or 75 mg/kg for 3 weeks. Pegasus 50 mg/kg significantly increased DA and its metabolite HVA levels in the brain (**A**,**B**) and DA levels in the serum (**C**) but not HVA levels in the serum (**D**). Younger 6–7-month-old TgA53T mice were injected with Pegasus or D5 once daily for 2 weeks. The WB of soluble brain extracts showed (**E**,**F**) monomeric alpha-synuclein and (**G**,**H**) oligomeric alpha-synuclein levels relative to actin in 4–12% Bis-tris Criterion XT precast gel. (**I**). The ELISA analysis of soluble brain extracts reveals alpha-synuclein levels. Actual *p*-value of the statistical analysis by a two-tailed t test. ns: not significant, * $p < 0.05$, ** $p < 0.01$. N = 5–10. All values are presented as mean ± SD.

2.6. Pegasus Significantly Increases the Number of DA-Producing Neurons in the SN and Striatum

To further examine whether Pegasus has any effects on DA-producing neurons, we performed immunohistochemistry to visualize tyrosine hydroxylase (TH+)-positive neurons in both the SN and striatum in Pegasus-treated versus untreated mice. TH is a rate-limiting enzyme in the biosynthesis of DA in the brain. We used DAB staining to highlight the TH+ neurons. Treatment with Pegasus dramatically increases the cell count of TH+ neurons in the SN (Figure 6A–I) and DA nerve terminals in the striatum (Figure 6J–R) in both young (6–7-month-old) and old (12–13-month-old) TgA53T mice compared to DMSO. This suggests that Pegasus is an effective agent for neuroprotection against DA deficiency.

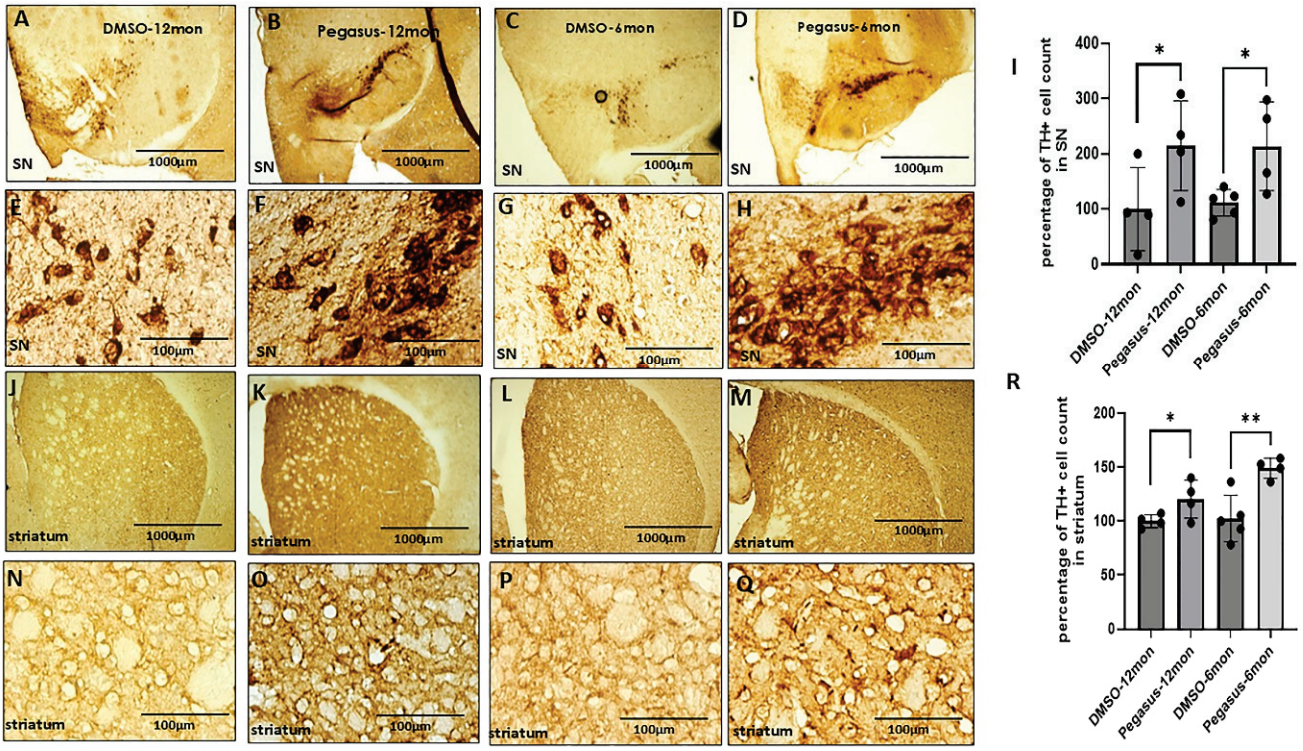


Figure 6. Pegasus significantly increases dopamine neurons in substantia nigra and striatum in TgA53T mice. Here, 12–13-month-old TgA53T mice were injected with Pegasus at the once daily dosage of 50 mg/kg for 3 weeks and 6–7-month-old TgA53T mice were injected with Pegasus at the once daily dosage of 10/25/50 mg/kg i.p. for a total of 2 weeks. The DAB staining of TH shows that Pegasus significantly increases the TH+ cell count in the substantia nigra and striatum in both 12–13-month-old TgA53T mice (**B**,**F**,**K**,**O**) and 6–7-month-old TgA53T mice (**D**,**H**,**M**,**Q**) compared with the corresponding DMSO-treated TgA53T mice ((**A**,**E**,**J**,**N**) and (**C**,**G**,**L**,**P**)). The results were verified by the quantification of the percentage of TH+ cell count in both the SN (**I**) and the striatum (**R**). Actual *p*-value of the statistical analysis by a two-tailed t test. * $p < 0.05$, ** $p < 0.01$. N = 4–5. All values are presented as mean ± SD.

2.7. Pegasus Reduces Astrocytic Staining in TgA53T Mice

Neuroinflammation is one of the most important pathogeneses in PD in addition to alpha-synuclein aggregation [9–12]. To examine the effects of Pegasus on glial cell activation, we performed immunohistochemistry of astrocyte and microglia from the brains of treated vs. untreated young (6–7-month-old) and old (12–13-month-old) TgA53T mice. The astrocyte staining with glial fibrillary acidic protein (GFAP) in the hippocampus of young TgA53T mice showed a significant reduction in astrocyte reactivity with Pegasus compared to DMSO in young (Figure 7A,B,E), but not old TgA53T mice (Figure 7C–E). GFAP staining was not appreciably different between treatments in other brain regions, including the SN and cortex. No differences in IBA1 staining were observed in the hippocampus of Pegasus-treated mice compared to DMSO control mice (Figure 7F–J).

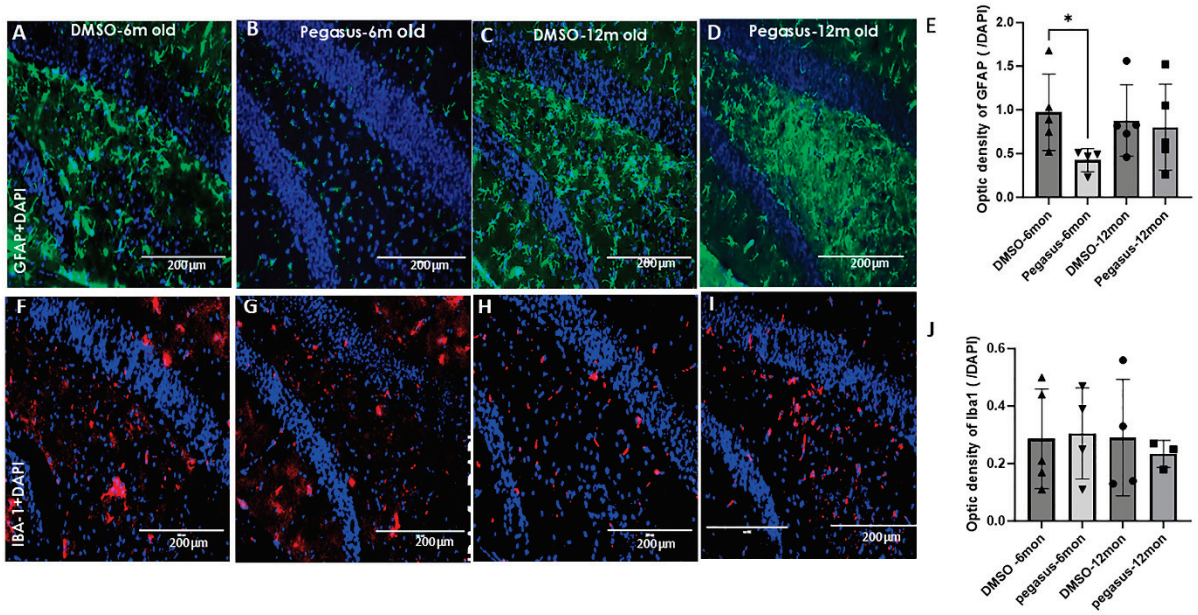


Figure 7. Pegasus reduces astrocytic reactivity in TgA53T mice. GFAP staining in the hippocampus reveals a decrease in the immunoreactivity of astrocytes in young (6–7-month-old) TgA53T mice treated with Pegasus 50 mg/kg i.p. (**B**,**E**) compared to mice treated with DMSO (**A**,**E**) but not in old (12–13-month-old) mice (**C**–**E**). IBA-1 staining in the hippocampus shows no immunoreactivity difference in microglia in young (6–7-month-old) (**F**,**G**,**J**) and old (12–13-month-old) (**H**–**J**) TgA53T mice. Actual *p*-value of the statistical analysis by a two-tailed t test. * $p < 0.05$. N = 4–5. All values are presented as mean ± SD.

3. Discussion

Pegasus is metabolized into a total of thirteen metabolites, which were observed in rat brain and plasma. Many of the metabolites were formed after the amide cleavage of the parent, Pegasus, into the tetracycline or the DA scaffold and further biotransformation of those scaffolds. In rat brain and plasma samples, the major metabolite was M6, which is a modified tetracycline consisting of C-N bond cleavage + NH_2 to OH-2H+O. The other relatively abundant metabolites in the brain followed the order M5, M13, M11, and M12. M11 is DA conjugated to cysteine (C N bond cleavage + DA + linker + cysteine) that was abundantly detected in both the plasma and brain, suggesting that the linker ± cysteine serve as stabilizers to protect DA from rapid plasma oxidation and breakdown and delivery into the brain at high relative brain–plasma abundance (130%). Pegasus enters the brain as the mouse studies showed (Figure 1D,E,F), albeit less than 1% is detected in the mouse and rat brain. However, further in-depth studies in rats showed that Pegasus is metabolized into 13 fragments (Figure 2B, table) and 2 of these fragments are DA + linker (M8) and DA + linker + cysteine (M11). Both M8 and M11 are abundantly detected in the brain and the blood in rats and the relative abundance of brain–plasma is 318 and 131%, respectively. These data suggest that Pegasus may either act as a "pro-drug" that is metabolized in the periphery (perhaps the liver) and yields DA that is stabilized via linker ± cysteine and these metabolites are in turn delivered to the brain, or intact Pegasus may directly enter the CNS where it is finally metabolized. Indeed, more data indicate that, under physiological conditions, 2–6% of all small molecules can cross the BBB intact [8,13]. Collectively, these findings suggest that the ability of Pegasus to yield neuropathological effects in PD models may be either due to its ability as an intact molecule to enter the brain where it can be metabolized, or the possibility of peripheral multiple liver passages and phases of metabolism that can increase the level of its metabolites. Furthermore, Pegasus is moderately bound to mouse, rat, dog, and human plasma (Avg. 91–98%) using the equilibrium dialysis method.

The abundance of the DA–cysteine metabolite in plasma and its detection in the brain is in stark contrast with the highly challenging delivery of peripheral levodopa. When levodopa is administered alone, only 1% of each oral dose can reach the brain with a short half-life ($T_{1/2}$ 0.77–1.08 h) [13]. However, various formulations of levodopa in combination with dopa-carboxylase enzyme inhibitor (DDCI) can enhance levodopa brain entry up to 10% with extended $T_{1/2}$ 1–1.5 h [14]. When levodopa is administered with DDCI and selective catechol-O-methyl transferase (COMT) inhibitors, it may reach up to 40% in the brain [15]. Our PK data indicate that the half-life of Pegasus is extended longer than 4 h ($T_{1/2}$ 4.11) in plasma, and despite its low brain/serum ratio (0.7%), the $T_{1/2}$ in the brain is also increased to 3.77 h. We observe a highly desirable total drug exposure over time as indicated by the area under the curve (AUC 17,164 ng $\times$ h/mL), suggesting that the metabolism of Pegasus via phase I and phase II provides extended-release time (or slower clearance) of the M11 metabolite, resulting in prolonged and improved performance on Rotarod in TgA53T mice. Importantly, the short plasma $T_{1/2}$ of existing levodopa formulations is manifest in the short duration of the antiparkinsonian response that parallels the plasma levodopa levels and their numerous side effects. PD patients may need multiple dosing (up to 10 a day) of levodopa and its various formulations [16] due to the shrinkage of the therapeutic window as the disease progresses [17,18]. Long-lasting drug effects or slower clearance may help reduce dosing frequency and improve patient adherence [6,19]. Pegasus may prolong the antiparkinsonian response due to the abundance of brain penetration of M11 and its plasma levels, which may result in the prolongation of the duration of its action, as seen in TgA53T mice that continued to perform well on Rotarod up to 6.5 h. Additionally, the prolongation of $t_{1/2}$ of dopaminergic therapy due to reduced dopamine breakdown (e.g., with monoamine oxidase inhibitors) may attenuate motor fluctuations and overcome the complications of long-term therapy with levodopa. Motor fluctuations could predictably improve with DA bioavailability. Long-term complications are attenuated by an early combination of levodopa with DA agonists [20], and the metabolism of Pegasus into DA, that may act as a DA receptor agonist [7], can potentiate the action of Pegasus and perhaps offer a wider therapeutic window.

Previous studies in vitro and in vivo demonstrated that a tetracycline derivative that crosses the BBB inhibited α-synuclein aggregation via the potential dismantling of α-synuclein fibrils into smaller fragments to prevent the seeding of alpha-synuclein and reduce inflammation [21–24]. These findings agree with our data that Pegasus reduces monomeric and oligomeric alpha-synuclein in TgA53T mice and suppresses astrocytic activity, suggesting an anti-inflammatory response, leading to the improvement of motor and behavioral outcomes in these mice. It is unlikely that Pegasus' effect on DA numbers is via neurogenic mechanisms; however, the increase in DA levels may be due to a neurotrophic effect due to the reduction in alpha-synuclein oligomerization and the possible scavenging of reactive oxygen species [21–24]. The relative high abundance of tetracycline metabolites of Pegasus (M6, M10, and M12) in the brain indicates that these molecules may be responsible for the reduction in brain alpha-synuclein levels [25]. It has been suggested that the aggregation of alpha-synuclein can induce glutamate release that can lead to excitotoxic effects, but DA may dampen glutamate excitotoxicity [24,26]. Our data indicate a simultaneous decrease in alpha-synuclein aggregation and DA increase in TgA53T mice in association with the attenuation of astrocytic activity that is responsible for the detoxification of glutamate. Tetracyclines are generally regarded as anti-oxidants and they represent a class of antibiotics in animal models and human disease [27,28]. Minocycline prevents striatal cell loss in MPTP models of PD [29], and our data show a reduction in cell death in TgA53T mice and the recovery of DA and its metabolite, HVA [30], both in plasma and the brain. Pegasus also counters the effects of the destruction of DA neurons

with the chronic administration of MPTP and leads to behavioral improvement. Taken together, these data suggest that Pegasus exerts a multifunctional role due to its ability to deliver DA into the brain, where it may act as a DA agonist [7] and reduce the seeding and oligomerization of alpha-synuclein and inflammation.

In conclusion, Pegasus provides a mechanistic strategy that concurrently lowers alpha-synuclein and neuroinflammation, replenishes DA loss, and acts as a DA receptor agonist. Pegasus may effectively target multiple phenomena associated with PD pathology. Although the capsase-3 studies showed no apparent toxicity in organs and 50 mg/kg was a tolerated dose in mice with no visible side effects, the fragmentation and metabolism of Pegasus into 13 metabolites, albeit beneficial for the release and delivery of stable DA to the brain, must be further investigated through in vivo toxicity, pharmacology, and toxicokinetic studies to determine the safety of these molecules and their bioavailability via different routes of administration. Furthermore, levodopa absorption is quite variable from individual to individual in PD, and the administration and optimization of peripheral levodopa to PD patients to reduce side effects and improve antiparkinsonian effects are very challenging. The enhanced bioavailability and exposure of Pegasus in plasma and its ability to deliver DA into the brain may help overcome the challenges of levodopa dosing, including the frequency of administration. Future human studies may help delineate the potential pro-drug effect of Pegasus on DA levels.

4. Materials and Methods

4.1. Pharmacokinetics

The formulation vehicle of Pegasus was 5% DMSO + 35% PEG400 + 60% normal saline. Pharmacokinetics (PK) was investigated after a single i.p. injection of 50 mg/kg or 100 mg/kg Pegasus to male and female 5–6-month-old wild-type C57BL/6 mice. The animals were euthanized with CO2 (3.7 L/min) in a mouse chamber ($6.5'' \times 12.5'' \times 5.5''$), equivalent to approximately 50% flow rate per minute, and death was verified by cervical dislocation. The brains were washed with normal saline for 1 min and then perfused with 4% paraformaldehyde (PFA) for 15 min. The blood was collected via cardiac puncture. The separation of the benzoyl derivatives was achieved on a column Luna Omega C18 PS, 150 × 2.1 mm, 1.6 μm (Phenomenex, Torrance, CA, USA). Pegasus concentrations in serum and brain were measured by UHPLC-MS/MS. Mobile phase A was water, 0.1% (*v*/*v*) formic acid (FA), and 6.7 mM ammonium formate. Mobile phase B was acetonitrile and 0.1% formic acid. Gradient elution (min—%A/%B): 0—98/2; 0.2—98/2; 6—30/70; 7.5—30/70; 7.6—98/2; 8.2—98/2; the flow rate was 400 μL/min.

4.2. TgA53T Mouse Experiment

Male and female (12–13 months) transgenic mice that harbor the arginine to threonine (A53T) mutation of human alpha-synuclein under the control of prion promoter [31] were treated with an i.p. injection of Pegasus 50 mg/Kg or 75 mg/Kg or DMSO daily for 3 weeks. The animals were sacrificed with CO_2. All experiments were conducted in full compliance with the recommendations of Georgetown University Animal Care and Use Committee.

4.3. Dose Escalation Study and Tissue Toxicity via Caspase-3 Activity

Tissue toxicity was assessed in young (6–7-month-old) and old (12–13-month-old) A53T mice. The mice received daily i.p. escalating doses of Pegasus or control D5 (tetracycline derivative with no DA) vs. DMSO of 10, 25, 50, and 100 mg/kg for 4 days with each dose up to 16 days, and tissues were analyzed via caspase-3 activity. The caspase-3 activity assay (EnzChek® Invitrogen, Waltham, MA, USA) was conducted according to manufacturer's protocols as we previously described [32,33].

4.4. In Vivo Metabolism

Metabolite profiling was performed in rat brain and plasma samples to detect phase-I and phase-II metabolites in male Sprague Dawley rats (6–8 weeks old, n = 6) injected (i.p.) with 50 mg/kg Pegasus. The formulation vehicle of Pegasus was 5% DMSO + 35% polyethylene glycol (PEG) 400 + 60% normal saline, and this formulation was used to measure the plasma and brain concentrations of Pegasus and all other metabolites. All samples obtained after Pegasus dosing in rats by the i.p. route of administration were used for metabolite profiling and compared to normal saline. Blood (n = 16 samples) was collected via retro-orbital plexus puncture 0.25, 1, 4, 6, 8, and 24 h pre-dose, and brain sampling was 1, 4, and 24 h post-dose (n = 6 per group). The brain samples were cleaned and perfused, then washed with PBS. The brains (approximately 1.5 g) were transferred to a 50 mL centrifuge tube and 36 mL of ice-cold PBS was added. The mixture was homogenized for about 1–2 min to obtain a uniform suspension. The precipitate was allowed to settle for 30 min at 4 °C, and 15 mL of the middle layer was collected. The plasma and brain samples were processed by the protein precipitation method. In total, 175 µL of acetonitrile was added to 25 µL of plasma samples. The samples were mixed, followed by centrifugation at 4000 rpm for 20 min. The supernatant from the samples was subjected to LC-MS/MS analysis.

The samples were analyzed by liquid chromatography tandem mass spectrometry (HPLC: LC series, Shimadzu, Chiyoda-ku, Tokyo, Japan; mass spectrometer: API4000 Triple Quad mass spectrometer, Applied Biosystems, Carlsbad, CA, USA). The samples (n = 6) were processed using protein precipitation and 1 mM stock solution, prepared in DMSO, spiked into acetonitrile to a final concentration of 4 µM, and processed using liquid chromatography tandem mass spectrometry, employing a linear gradient method using MH+/MH−(Q1) and product ion (MS/MS) scans. The evaluation of the Pegasus metabolites included demethylation, de-hydroxylation, amide cleavage, oxidation, de-hydrogenation, glucuronidation, sulfation, glutathione conjugation, and several other possibilities. The relative abundance of Pegasus and its metabolites in the brain and plasma was determined at the ionization parameters of the parent.

4.5. MPTP Experiment

During the experimental period, animals were housed in a single experimental room (5 per cage in standard cages) and weighed. On randomization day, the corresponding body weights were recorded, and intra-group variations did not exceed 15% of the mean body weight. The study was performed at an in-house facility registered by the Committee for the Control and Supervision of Experiments on Animals (CCSEA). The registration was in accordance with the protocol approved by the Institutional Animal Ethics Committee (IAEC) of Eurofins, India. All the ethical practices laid down in the CCSEA guidelines for animal care were followed during the conduct of the study. Furthermore, the procedures used in this study plan were designed to conform to the accepted practices and to minimize or avoid the risk of causing pain, distress, or discomfort to the animals. The number of animals that were selected for use in this study was considered the minimum requirement to meet rational scientific endpoints for this type of study.

4.6. Administration of MPTP

From day 1 to day 5, MPTP was administered to all the groups except Naive at a dose of 25 mg/kg, i.p., QD. Treatment with Pegasus alone (50 mg/kg, i.p., BID), a combination of Pegasus 50 mg/kg, BID + Benserazide 10 mg/kg, QD, or a combination of L-Dopa 10 mg/kg, QD + Benserazide 10 mg/kg, QD or standalone L-Dopa 10 mg/kg, QD, started on day 1 and continued until day 13. On day 13, after the first dose of Pegasus,

4 animals/time point from each group were sacrificed at 0.5, 1, 3, 6, and 12 h, and the blood and brains were collected.

4.7. Rotarod Test

The mice were placed on an accelerating Rotarod (Columbus Instruments, Columbus, OH, USA) equipped with individual timers for each mouse. The mice were trained to stay on the rod at a constant 5 rpm rotation for at least 5 min, and then the speed gradually increased by 0.2 rpm/min and the latency to fall was measured. The mice were trained for 3 consecutive days before the final measurement.

4.8. Elevated Plus Maze Test

The mice were placed individually in the center of an elevated, plus-shaped (+) apparatus with two open and two closed arms. The number of entries to open arms, the number of entries to closed arms, the time spent in open arms, the time spent in closed arms, the total distance traveled, and the total number of entries to both open and closed arms during a 10 min period were recorded by trained and blinded observers.

4.9. Grip Strength Test

The maximal muscle/grip strength of forelimbs was measured using a strength meter. Each mouse was made to hold the grid only by its forepaws, while gently being pulled back by its tail, and the maximal grip strength value displayed was recorded.

4.10. Immunohistochemistry

Animals were deeply anesthetized with a mixture of Xylazine and Ketamine (8:80 mg/kg), washed with normal saline for 1 min, and then perfused with 4% paraformaldehyde (PFA) for 15 min. The brains were dissected out and immediately stored in 4% PFA for 24 h at 4 °C, and then transferred to 30% sucrose at 4 °C for 48 h. The brains were cut using a cryostat microtome into 20 μm thick coronal sections and stored at −20 °C. Staining for tyrosine hydroxylase (TH) was probed with anti-tyrosine hydroxylase antibody (Cat No: AB152, Sigma-Aldrich, St. Louis, MO, USA). GFAP (glial fibrillary acidic protein) was stained with anti-GFAP monoclonal antibody (Cat No:MS1376P1, Fisher Scientific, Newington, NH, USA). Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) was performed according to the manufacturer's protocol (Life Technologies, Miami, FL, USA). TH^+ cells were counted in a stereology-like manner across multiple visual fields (typically 20 fields) in a single animal, and the same procedure was repeated on several animals to obtain the best approximation of TH staining.

4.11. Western Blot

To extract the soluble proteins, whole brain tissues, which excluded the olfactory bulb, cerebellum, and spinal cord, were isolated and homogenized in 1 × STEN buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 2 mM EDTA, 0.2% NP-40, 0.2% BSA, 20 mM PMSF, and protease cocktail inhibitor). The proteins were quantified and normalized for WB (and ELISA) on 4–12% Criterion™ XT Bis-Tris Protein Gel (Cat No: 3450125, Bio-rad, Hercules, CA, USA). Beta-actin (β-actin) was probed (1:3000) with monoclonal antibody (Cat No: MAB1501R, Emdmillipore, Danvers, MA, USA). Human alpha-synuclein was probed (1:4000) with monoclonal antibody (Cat No: AHB0261, Thermo Fisher, Waltham, MA, USA). Human oligomeric alpha-synuclein was probed (1:500) with anti-alpha-synuclein oligomer-specific syn33 antibody (Cat No: ABN2265, Sigma-Aldrich, St. Louis, MO, USA). WBs were quantified by densitometry using Quantity One 4.6.3 software (Bio Rad, Hercules, CA, USA) and Image J version 1.54m.

4.12. ELISA

The levels of DA and its metabolite HVA were measured using a commercially available ELISA (Cat No. MBS269234 and Cat# MBS1601074, MyBiosource, San Diego, CA, USA) on serum and brain tissue soluble extracts. Human alpha-synuclein was measured via a human-specific ELISA (Cat# 448607, BioLegend, San Diego, CA, USA) according to the manufacturer's protocol.

4.13. Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 10 (GraphPad software, Inc, San Diego, CA, USA). One-way analysis of variance (ANOVA) (and nonparametric or mixed) tests were used in the comparison of means of multiple groups. A two-tailed Student's t test (and nonparametric tests) was used in the comparison of means of two groups. The Shapiro–Wilk test was used for checking normality before performing statistic tests. Asterisks denote actual *p*-value significances (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$), and N is the number of animals or the number of independent experiments (cell cultures) per group. Unless otherwise indicated, data are expressed as mean ± SD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms26094251/s1.

Author Contributions: X.L. collected and analyzed data, curated the figures, performed statistical analysis, and co-wrote the manuscript. M.L.H. and M.S. assisted with animal treatment and behavior and provided input for experimental design. C.M. obtained funding, designed experiments, and co-wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: Funding for these studies was provided by SkyBio LLC to Charbel Moussa, Georgetown University Medical Center.

Institutional Review Board Statement: All animal work was approved by Georgetown University Animal Care and Use Committee (GUACUC protocol # 2016-1194). Animal work (MPTP treatment) conducted at Adgyl Sciences was internally approved by the animal welfare and ethics committee according to Indian local law and regulations. The study was performed at an in-house facility registered as a Test Facility by the Committee for the Control and Supervision of Experiments on Animals (CCSEA). The registration was in accordance with the protocol approved by the Institutional Animal Ethics Committee (IAEC) of Eurofins, India. All ethical practices laid down in the CCSEA guidelines for animal care were followed during the conduct of this study. Furthermore, the procedures used in this study plan were designed to conform to the accepted practices and to minimize or avoid the risk of causing pain, distress, or discomfort to the animals. The number of animals that were selected for use in this study was considered the minimum requirement to meet rational scientific endpoints for this type of study. The work presented in this manuscript conforms with all rigorous and ethical standards for research conduct, data collection and analysis, and data dissemination.

Informed Consent Statement: Not applicable.

Data Availability Statement: The final data generated and analyzed, materials, and all interpretations are available to the scientific and non-scientific community upon request (to C.M.). No datasets are deposited in a repository and there is no web link to any datasets.

Acknowledgments: The authors would like to acknowledge Pronexus Analytical AB, Bromma, Sweden, for the LC-MS/MS analysis of CSF HVA; DOPAC for performing the pharmacokinetics studies; Eurofins (India) for performing the in vivo metabolism studies; and Adgyl Life Sciences (India) for performing the MPTP studies.

Conflicts of Interest: Charbel Moussa is a paid consultant and shareholder and receives compensation from SkyBio LLC. All other authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

PD	Parkinson's disease
ELISA	Enzyme-linked immunosorbent assay
i.p.	Intraperitoneal
CNS	Central nervous system
PK	Pharmacokinetics
DA	Dopamine
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
SN	Substantia nigra
BBB	Blood–brain barrier
A53T	Alanine to threonine mutations
Tg	Transgenic
TH	Tyrosine hydroxylase
DMSO	Dimethylsulfide
HVA	Homovanillic acid

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International Journal of
Molecular Sciences

MDPI

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A Map of Transcriptomic Signatures of Different Brain Areas in Alzheimer's Disease

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Abstract: Alzheimer's disease (AD) is a neurodegenerative disorder that progressively involves brain regions with an often-predictable pattern. Damage to the brain appears to spread and worsen with time, but the molecular mechanisms underlying the region-specific distribution of AD pathology at different stages of the disease are still under-investigated. In this study, a whole-transcriptome analysis was carried out on brain samples from the hippocampus (HI), temporal and parietal cortices (TC and PC, respectively), cingulate cortex (CG), and substantia nigra (SN) of six subjects with a definite AD diagnosis and three healthy age-matched controls in duplicate. The transcriptomic results showed a greater number of differentially expressed genes (DEGs) in the TC (1571) and CG (1210) and a smaller number of DEGs in the HI (206), PC (109), and SN (60). Furthermore, the GSEA showed a difference between the group of brain areas affected early (HI and TC) and the group of areas that were subsequently involved (PC, CG, and SN). Notably, in the HI and TC, there was a significant downregulation of shared DEGs primarily involved in synaptic transmission, while in the PC, CG, and SN, there was a significant downregulation of genes primarily involved in protein folding and trafficking. The course of AD could follow a definite time- and severity-related pattern that arises from protein misfolding, as observed in the PC, CG, and SN, and leads to synaptic impairment, as observed in the HI and TC. Therefore, a map of the molecular and biological processes involved in AD pathogenesis may be traced. This could aid in the discovery of novel biological targets in order to develop effective and well-timed therapeutic approaches.

Keywords: Alzheimer's disease; transcriptomics; human brain samples; different brain regions; molecular map

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that is considered the main cause of dementia, and its prevalence is expected to double in Europe and triple worldwide by 2050 [1]. The clinical manifestations of AD are due to the progressive dysfunction of synapses and neuronal circuits, causing gradual impairment in all cognitive domains [2]. In the brain, the pathology of AD is represented by the extracellular aggregation of amyloid beta (Aβ) peptides in amyloid plaques and the intraneuronal aggregation of hyperphosphorylated Tau protein (pTau) in neurofibrillary tangles (NFTs) and threads. The multifocal coupling of Aβ plaques and NFTs in the cerebral cortex generates so-called neuritic plaques (NPs), which are the neuropathological hallmark of AD. The clinical evolution of AD reflects the propagation of synaptic failure, which is accompanied or followed by the propagation of the pathology across various brain areas; typically, AD pathology starts in the parahippocampal, entorhinal, and hippocampal zones and propagates to the temporo-parietal cortex and, in some cases, the whole neocortex [3–6]. However, it is important to state that a certain degree of AD pathology, as well as brain atrophy, is often observed in cognitive healthy elderly [7]. Considering that the newest anti-amyloid treatments resulted in no clinical changes that were perceived by patients and caregivers, it is becoming increasingly clear that the mechanisms that cause some subjects to develop AD and some to maintain cognitive function are quite complex and multifactorial, and they are not sufficiently explained by the amyloid cascade theory [8–10]. In this regard, different hypotheses about the etiology of AD have been proposed, ranging from the so-called "mitochondrial cascade hypothesis" [11] and neuroinflammation [12] to defective proteostasis, which is also strictly related to synapse failure [13]. Thus, it is becoming clear that AD's etiology is highly heterogeneous and requires new methodological approaches; genetic and transcriptomic investigations could be winning strategies for understanding the pathogenesis of AD. Transcriptomic analyses, in particular, are crucial for obtaining information about differentially expressed genes (DEGs) and alternative gene expression regulation mechanisms that were already observed to impact neurodegeneration [14]. Moreover, RNA-seq data could be used to build functional interaction networks and to obtain additional information about the active role of specific genes in a specific cellular model [15] or pathological context [16]. There are few transcriptomic studies focused on AD as a whole-brain pathology. Generally, analyses were performed on the most affected areas, such as the hippocampus and entorhinal cortex [14,17], considered specific aspects of the pathology [18,19], or mainly focused on a subtype of deregulated RNA, such as miRNA and lncRNA [20,21]. There are also studies focusing on less affected areas, such as the primary visual cortex, that aim to investigate AD without the influence of other collateral aspects of the pathology [22]. However, even if of great interest, no information about the differences between brain regions among the selected subjects has been reported. Finally, we did not find any other research aimed at tracing a molecular map of the spread of AD in the brain by simultaneously analyzing and comparing the transcriptomic profiles of different brain regions or, moreover, research comparing AD with CTRL subjects.

Considering the above-mentioned propagation pattern of AD, we performed a whole-transcriptome analysis on human post-mortem brain tissue samples derived from the hippocampus (HI), temporal cortex (TC), parietal cortex (PC), cingulate gyrus (CG), and substantia nigra (SN) of AD subjects and compared them with those of subjects without dementia. In order to increase the value of this study, we used post-mortem samples from a cohort of elderly subjects that we had monitored since 2010, when they were enrolled in the InveCe.Ab study [23], a population-based longitudinal study that is currently ongoing in Abbiategrasso. Therefore, our first goal was to define the "molecular signature" of AD in each brain region considered. To gain insight into the biological role of the observed deregulated genes (DEGs) and to build region-specific functional interaction networks, a gene set enrichment analysis (GSEA) was performed on the total number of DEGs and then only on the deregulated mRNAs. Our second objective was to switch from a region-specific molecular signature to a whole-brain molecular map of the spread of AD. To do

so, we compared the functional enrichment terms of every brain region, an analysis that resulted in the spontaneous clustering of specific brain regions based on two well-defined groups of functional enrichment networks. This clustering may have revealed a shared deregulation pattern, and it was hypothesized that it reflected the propagation pattern of AD. This would allow us to work backwards and identify specific coding DEGs whose deregulation could be related to the etiology of AD.

2. Results

The results are reported according to the two main research questions relative to the molecular signature of AD in each brain region and the molecular map of AD's spread in the brain.

2.1. AD Subjects Showed Different Transcriptomic Profiles from Those of the CTRL Subjects

2.1.1. Hippocampus—HI

In the HI of AD subjects, the total number of DEGs was 206, with the majority of them being of the protein-coding class (177) and downregulated (110). Their classification is reported in Table 1. A principal component analysis (PCA) division based on the expression levels of all deregulated mRNAs and lncRNAs in the AD and CTRL groups resulted in a consistent separation between the two groups of subjects and is represented in Figure 1A.

Table 1. Number of statistically significant differentially expressed genes in the HI, TC, PC, CG, and SN in terms of upregulated transcripts, downregulated transcripts, and total transcript count.

Brain Regions	Total Count	mRNA		lncRNA	
		Upregulated	Downregulated	Upregulated	Downregulated
HI	206	67	110	12	17
TC	1571	371	781	344	75
PC	109	54	38	10	7
CG	1210	617	110	436	47
SN	60	29	24	6	1

A GO term enrichment analysis was carried out for DEGs in brain tissue from the HI of AD subjects compared with that from CTRL subjects [24,25]. For the HI of AD subjects, the GO enrichment analysis resulted in a significant deregulation of processes mainly related to synaptic transmission, such as the "modulation of chemical synaptic transmission" and the "regulation of trans-synaptic signaling" (Figure 1C). Thus, alterations in gated and ion channels and transporters were observed, together with the involvement of several cellular components mainly related to synaptic functionality and, in general, neuron-to-neuron interactions (Figure 1D,E). A STRING analysis was performed on 96 out of 177 protein-coding DEGs with an adjusted p value of ≤ 0.05. In the HI, a total of four local network clusters were found, showing a functional correlation between DEGs that were exclusively involved in voltage-gated potassium and sodium- and calcium-activated potassium channel activity (Supplementary Table S3). A similar outcome was obtained when considering the top five GO terms in the STRING analysis for biological processes, molecular functions, and cellular components of the functional enrichment analysis. The deregulation of processes related to the synaptic vesicle cycle and synaptic plasticity was observed, with a strong involvement of the presynaptic active zone (AZ) (Supplementary Table S4), a result consistent with the GO analyses performed on all DEGs. Interestingly, the DEGs of the top five STRING GO terms of the functional enrichment analysis resulted in downregulation in the HI of AD subjects, except for *LTF*, which encodes for the protein Lactoferrin (Lf).

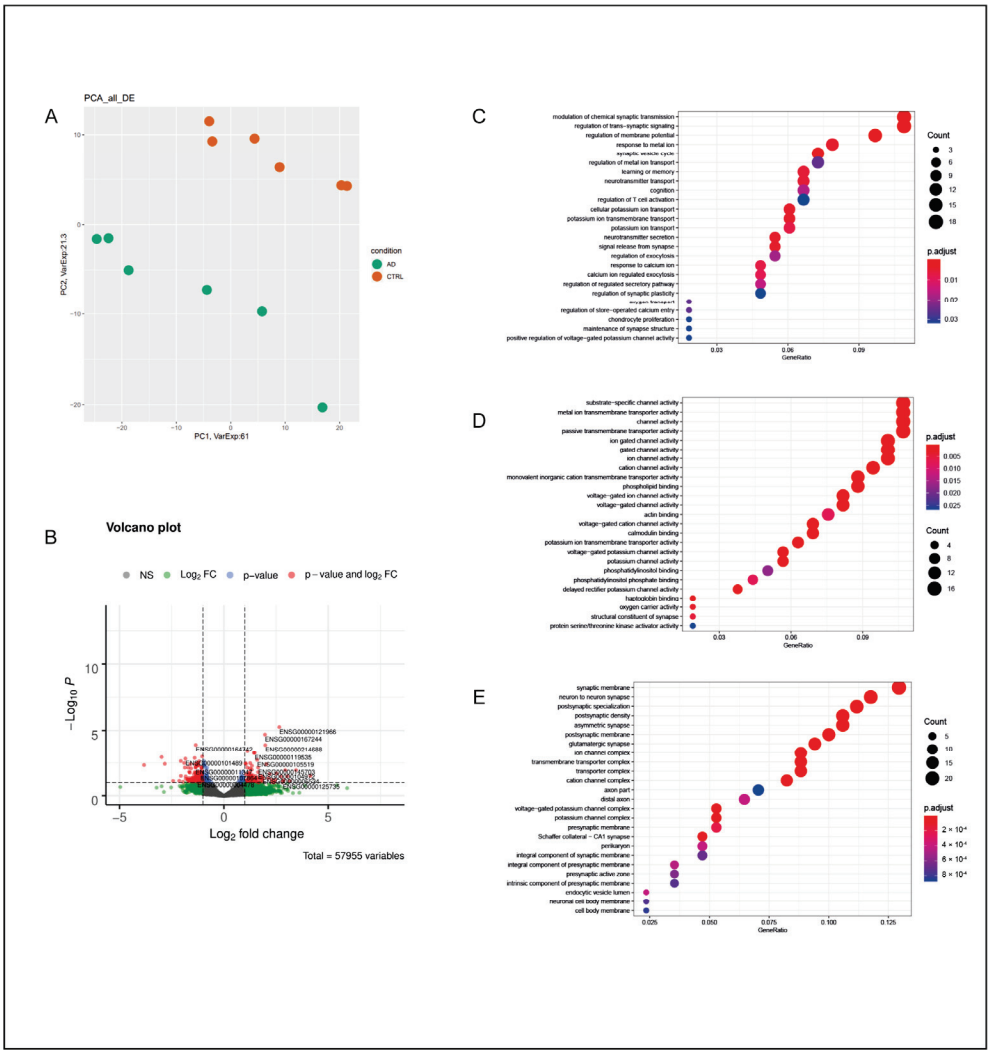


Figure 1. Hippocampus. (**A**) PCA of DEGs in the HI of AD subjects in comparison with those of the CTRL subjects; AD subjects and CTRL subjects were separated into two distinct groups, suggesting a substantial difference between the two groups from a statistical point of view. (**B**) Volcano plot of DEGs between AD and CTRL subjects. Genes were plotted in order to emphasize both statistical significance (*p*-value) and magnitude of change (Log_2 fold change). Genes whose deregulation was the most statistically significant and that had a large fold change are represented with red dots. (**C**) GO-enriched terms in the HI of AD vs. CTRL for biological processes, molecular functions (**D**), and cellular components (**E**). The dots in each category may vary in color and dimension; a color that tends toward red indicates a higher statistical significance, while large dots indicate a higher number of genes of that specific enrichment term that were deregulated in the analyzed sample in comparison with the original GO classification.

2.1.2. Temporal Cortex—TC

In the TC of AD subjects, the total number of DEGs was 1571, with the majority of them being protein-coding (1152) and downregulated (781). Their classification is reported in Table 1. The PCA division based on the expression levels of all deregulated mRNAs and lncRNAs in the TC of AD subjects and CTRL subjects resulted in a defined separation between the groups of subjects, and this is presented in Figure 2A. One AD sample is missing because it failed RNA sequencing, and no more samples were available.

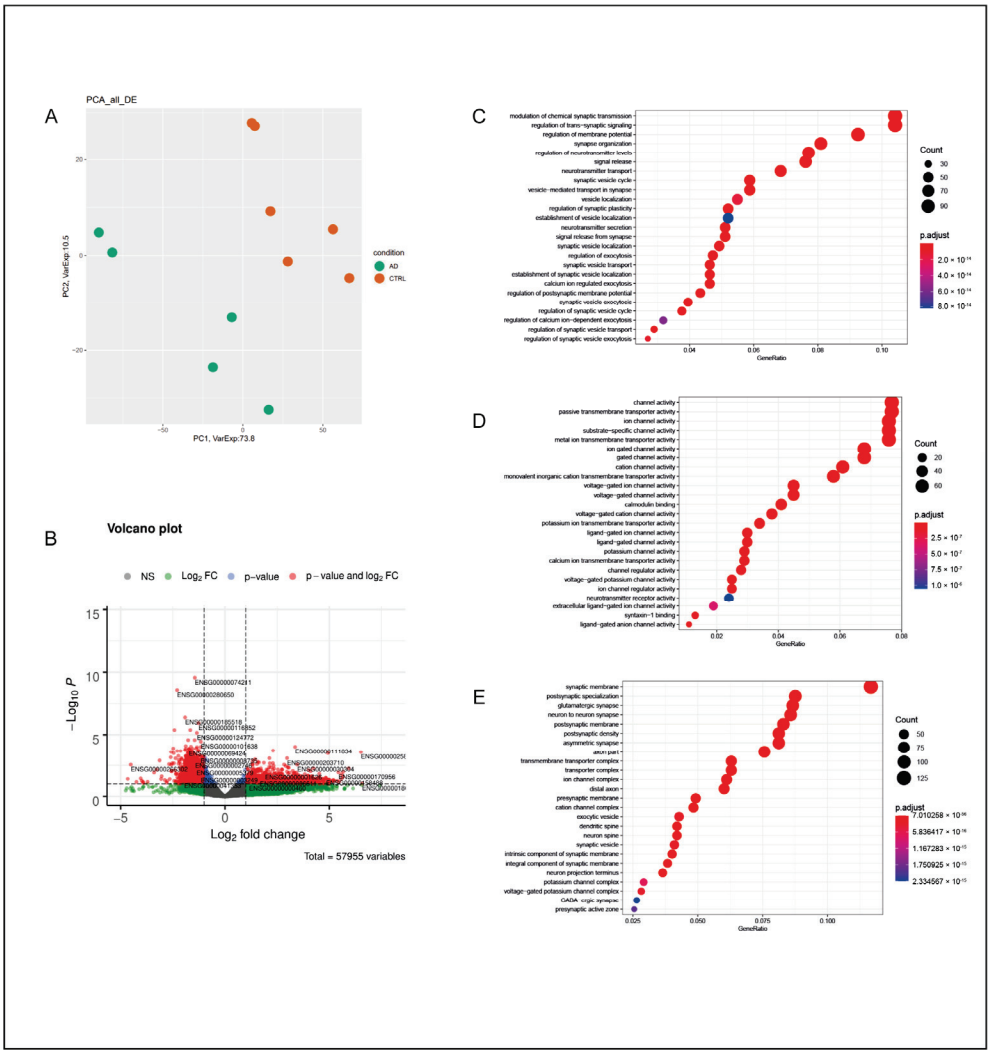


Figure 2. Temporal cortex. (**A**) PCA of DEGs in the TC of AD subjects in comparison with CTRL subjects. AD subjects and CTRL subjects were separated into two distinct groups, suggesting a substantial difference between the two groups from a statistical point of view. (**B**) Volcano plot of DEGs between AD and CTRL subjects. Genes were plotted in order to emphasize both statistical significance (*p*-value) and magnitude of change (Log_2 fold change). Genes whose deregulation was most statistically significant and that had a large fold change are represented by red dots; (**C**) GO-enriched terms in the TC of AD vs. CTRL for biological processes, molecular functions (**D**), and cellular components (**E**). The dots in each category may vary in color and dimension; a color that tends toward red indicates greater statistical significance, while large dots indicate a higher number of genes of that specific enrichment term that were deregulated in the analyzed sample in comparison with the original GO classification.

For the TC of AD subjects, the GO biological process analysis resulted in a significant deregulation of processes related to synaptic functionality and transmission (Figure 2C). Thus, a strong alteration in the activity of several families of ion channels was observed (Figure 2D), together with the involvement of cellular components (Figure 2E) related to pre- and post-synaptic membranes and neuronal components such as axons, neuron spines, and neurites. A STRING analysis was then performed on 727 out of 1152 protein-coding DEGs. In the TC, a total of 41 local network clusters were found. The top 10 network clusters showed a significant functional correlation among DEGs involved in synaptic functionality, and these were mainly related to the regulation of both glutamatergic and GABAergic synapses (Supplementary Table S5). A similar outcome was obtained in the functional enrichment analysis when considering the top five STRING GO terms for

biological processes, molecular functions, and cellular components (Supplementary Table S6), which was consistent with the GO analyses performed on all DEGs. Interestingly, the considered DEGs were downregulated in the TC of AD subjects, excluding *CFTR*, which was upregulated. *CFTR* encodes for a member of the ATP-binding cassette (ABC) transporter (ATP-Binding Cassette Sub-Family C, Member 7), whose expression, which is characteristic of epithelial tissues, was also observed in the human brain [26].

2.1.3. Parietal Cortex—PC

In the PC of AD subjects, the total number of DEGs was 109, with the majority of them being protein-coding (92) and upregulated (54). Their classification is reported in Table 1. The PCA division based on the expression levels of all deregulated mRNAs and lncRNAs in the PC of AD subjects and CTRL subjects is represented in Figure 3A, and this resulted in a consistent clustering of AD and CTRL subjects.

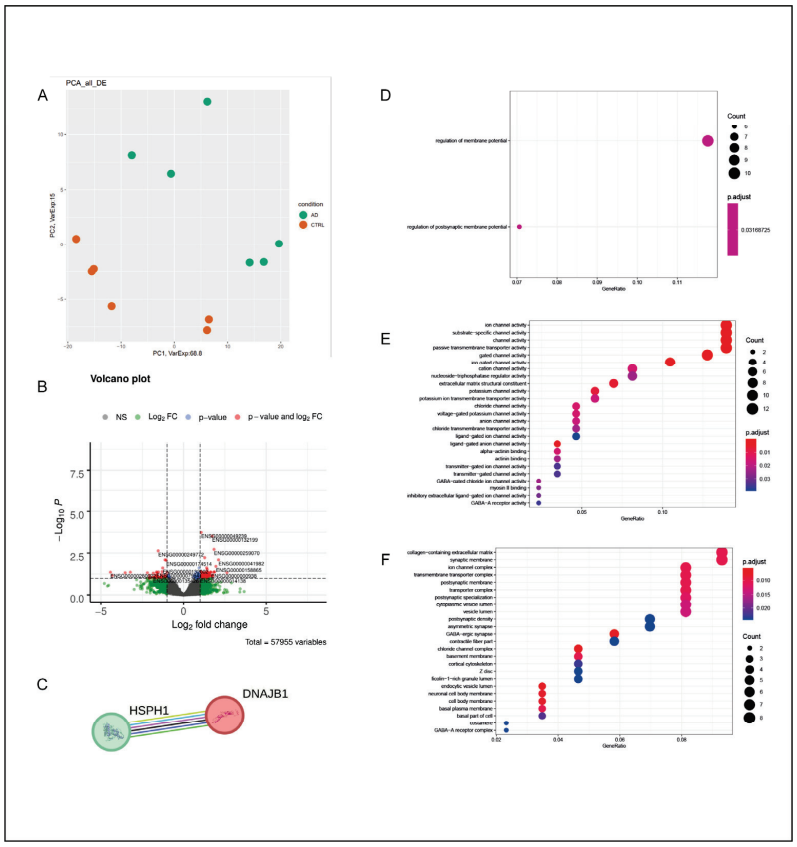


Figure 3. Parietal cortex. (**A**) PCA of DEGs in the PC of AD subjects in comparison with CTRL subjects. In this case, the separation between AD subjects and CTRL subjects was less defined, suggesting a minor difference between the two groups from a statistical point of view. (**B**) Volcano plot of DEGs between AD and CTRL subjects. Genes were plotted in order to emphasize both statistical significance (*p*-value) and the magnitude of change (Log_2 fold change). Genes whose deregulation was most statistically significant and those with a large fold change are represented by red dots. (**C**) Interaction network for the PC obtained through STRING. The two nodes are represented by *HSPH1* and *DNAJB1*. Interactions between the two nodes were determined using curated datasets and experimental determinations. (**D**) GO-enriched terms in the PC of AD vs. CTRL for biological processes, molecular functions (**E**), and cellular components (**F**). The dots in each category may vary in color and dimension; a color that tends toward red indicates a greater statistical significance, while large dots indicate a higher number of genes of that specific enrichment term that were deregulated in the analyzed sample in comparison with the original GO classification. In this case, the reduced number of DEGs observed in the PC resulted in a less defined enrichment analysis with a minor degree of statistical significance.

The GO biological process analysis resulted in the "regulation of membrane potential" and the "regulation of postsynaptic membrane potential" (Figure 3D), with the majority of deregulated biological activities (Figure 3E) being related to the functionality of ion and gated channels. Therefore, the involvement of cellular components (Figure 3F) that were primarily related to cellular membranes was observed. We then used the biological STRING database to obtain information about protein–protein interactions and perform a functional enrichment analysis in the PC. The number of considered protein-coding DEGs decreased from 92 to 27, and only one interaction network was found between *HSPH1* and *DNAJB1* (Figure 3C). Both *HSPH1* and *DNAJB1* encode for members of two different families of heat shock proteins (HSPs)—the Hsp70 protein family and the DnaJ or Hsp40 protein family [27,28]. Both *HSPH1* and *DNAJB1* were downregulated in the PC of our AD subjects compared with the CTRL subjects. On the other hand, no significant functional enrichment data were obtained with the above-mentioned DEG selection criteria.

2.1.4. Cingulate Gyrus—CG

In the CG of AD subjects, the total number of DEGs was 1210, with the majority of them being protein-coding (727) and upregulated (617). Their classification is reported in Table 1. The PCA division based on the expression levels of all deregulated mRNAs and lncRNAs in the CG of AD subjects and CTRL subjects is represented in Figure 4A.

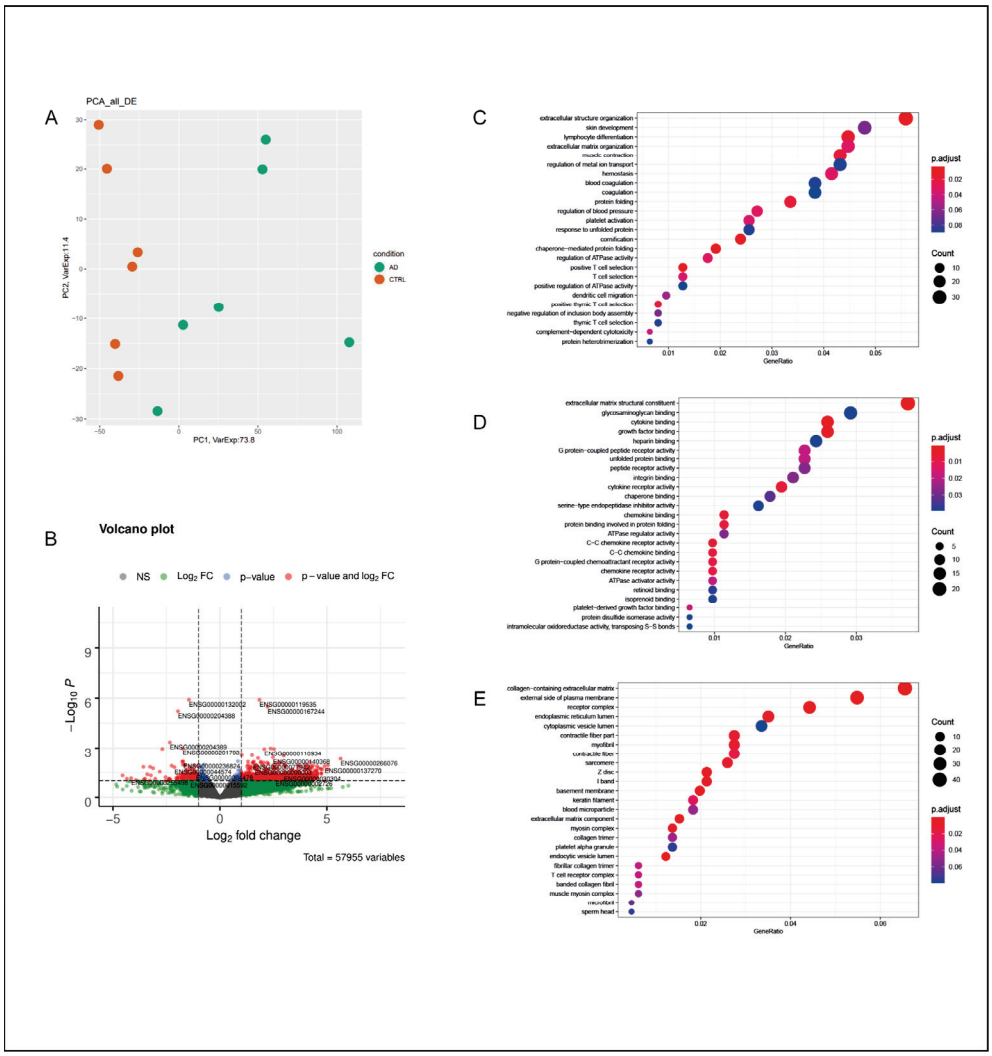


Figure 4. Cingulate gyrus. (**A**) PCA of DEGs in the CG of AD subjects compared with the CTRL subjects. In this case, the separation between AD subjects and CTRL subjects was less defined, suggesting

a minor difference between the two groups from a statistical point of view. (**B**) Volcano plot of DEGs between the AD and CTRL subjects. Genes were plotted in order to emphasize both statistical significance (*p*-value) and the magnitude of change (Log_2 fold change). Genes whose deregulation was most statistically significant and those that had a large fold change are represented by red dots. (**C**) GO-enriched terms in the CG of AD vs. CTRL for biological processes, molecular functions (**D**), and cellular components (**E**). Dots in each category may vary in color and dimension; a color that tends toward red indicates a higher statistical significance, while large dots indicate a higher number of genes of that specific enrichment term that were deregulated in the analyzed sample in comparison with the original GO classification.

For the CG of AD subjects, the GO biological process analysis resulted in a significant deregulation of processes related to protein folding, such as "protein folding", "response to unfolded protein", and "chaperone-mediated protein folding" (Figure 4C). Thus, a deregulation of the response to extracellular stimuli was observed, with an alteration in unfolded protein binding and chaperone binding. Moreover, an alteration in cell-signaling-related functions was also observed, and there was involvement of cytokine and chemokine receptor activity (GO molecular function analysis) (Figure 4D), the external side of plasma membranes, and extracellular matrix components (GO cellular component analysis) (Figure 4E). A STRING analysis was performed on 202 out of 727 protein-coding DEGs. In the CG, a total of eight local network clusters were found. This analysis showed a functional correlation between the DEGs involved in several protein-folding-related processes, which were mainly chaperone-related, and in extracellular matrix organization (Supplementary Table S7). A similar outcome was obtained in the functional enrichment analysis when considering the top five STRING GO terms for biological processes, molecular functions, and cellular components (Supplementary Table S8), and the results were consistent with those of the GO analyses performed on all DEGs. The number of DEGs of the top five STRING GO terms in the functional enrichment analysis was 50. Moreover, 16 out of the 50 DEGs were downregulated, while 34 were upregulated. Considering the 16 downregulated genes, 8 of them (*DNAJB1*, *HSPA1B*, *HSPA1A*, *HSPA5*, *DNAJB4*, *HSP90B1*, *HSP90AA1*, and *HSP90AB1*) encode for heat shock protein family members [29], while five of them (*FKBP4*, *PDIA4*, *PDIA2*, *CHORDC1*, and *STIP1*) encode for proteins related to protein folding and trafficking [30–33]. Interestingly, the remaining three downregulated genes (*CACNA2D1*, *CRELD1*, and *HBB*) are not related to protein folding and trafficking processes.

2.1.5. Substantia Nigra—SN

In the SN of the AD subjects, the total number of DEGs was 60, with the majority of them being protein-coding (53) and upregulated (29). Their classification is reported in Table 1. The PCA division based on the expression levels of all deregulated mRNAs and lncRNAs in the AD and NOLD is represented in Figure 5A.

For the SN of AD subjects, the GO biological process analysis resulted in a significant deregulation of processes related to protein folding and chaperone-mediated protein folding. Thus, a strong deregulation of heat shock proteins and chaperone functions was observed (GO molecular function). On the other hand, the GO cellular component analysis revealed a deregulation of genes related to hematic components (Figure 5C–E). A STRING analysis was performed on 30 out of 53 protein-coding DEGs. In the SN, a total of five local network clusters were found, showing a functional correlation between DEGs involved in denatured protein binding and folding and those involved in the haptoglobin–hemoglobin complex (Supplementary Table S9). This result was consistent with the top five STRING GO terms for biological processes, molecular functions, and cellular components in the functional enrichment analysis, which was statistically significant even when considering the small number of selected DEGs (Supplementary Table S10). The DEGs of the top five STRING GO terms in the functional enrichment analysis were all downregulated in the SN of AD subjects. Interestingly, the most downregulated genes (log2(AD subjects/CTRL subjects) ≤ -4) were *HBA2* and *HBB*.

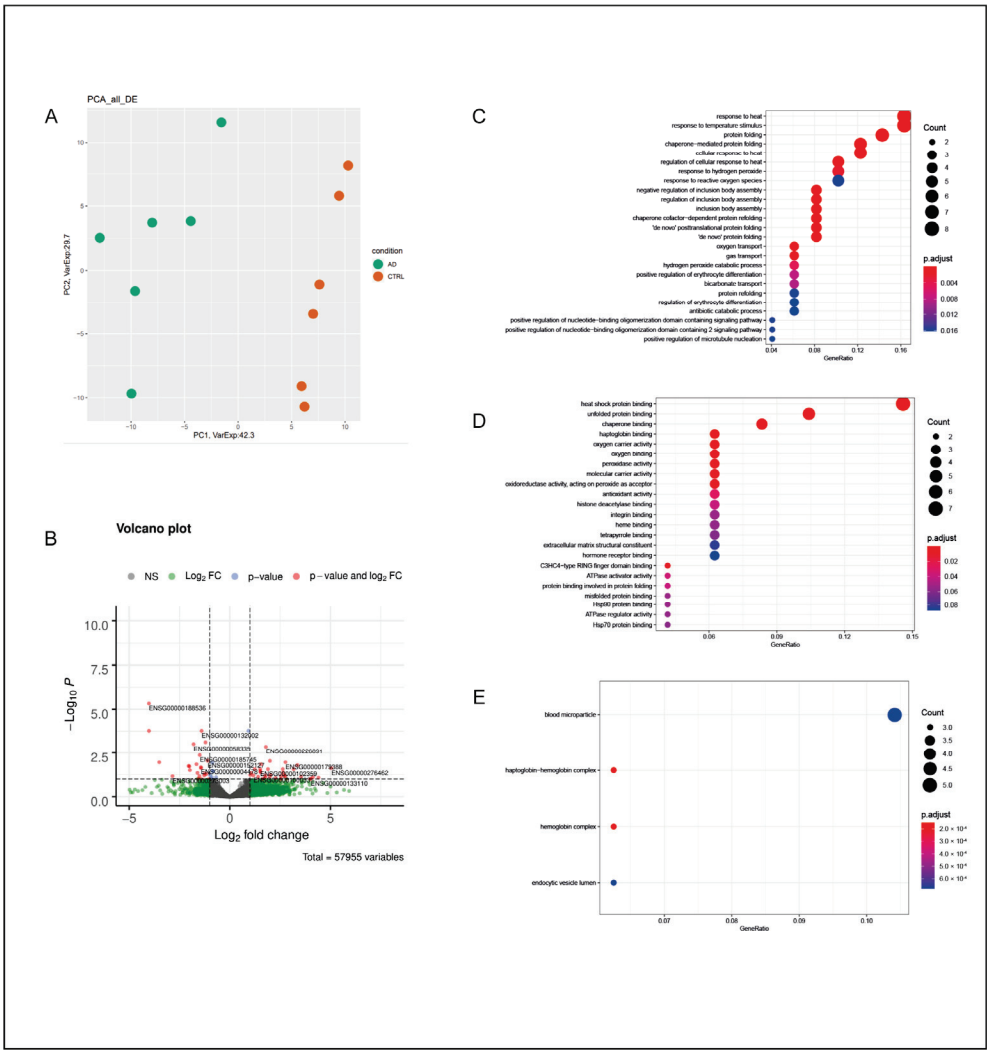


Figure 5. Substantia nigra. (**A**) PCA of DEGs in the SN of AD subjects in comparison with CTRL subjects. AD subjects and CTRL subjects were separated into two distinct groups, suggesting a substantial difference between the two groups from a statistical point of view. (**B**) Volcano plot of DEGs between the AD and CTRL subjects. Genes were plotted in order to emphasize both statistical significance (*p*-value) and the magnitude of change (Log_2 fold change). Genes whose deregulation was most statistically significant and those that had a large fold change are represented by red dots. (**C**) GO-enriched terms in the SN of AD vs. CTRL for biological processes, molecular functions (**D**), and cellular components (**E**). Dots in each category may vary in color and dimension; a color that tends toward red indicates a higher statistical significance, while large dots indicate a higher number of genes of that specific enrichment term that were deregulated in the analyzed sample in comparison with the original GO classification.

2.2. Brain Areas in the AD Cluster According to the Deregulation of the Same Class of Enrichment Terms

All of the investigated brain areas of AD subjects shared the deregulation of two specific genes, *HBB* and *IGF2*. As seen in Figure 6A, in our study, these were both downregulated in all of the analyzed areas. The downregulation of *HBB* in all of the selected areas was validated through qPCR (Supplementary Figure S2).

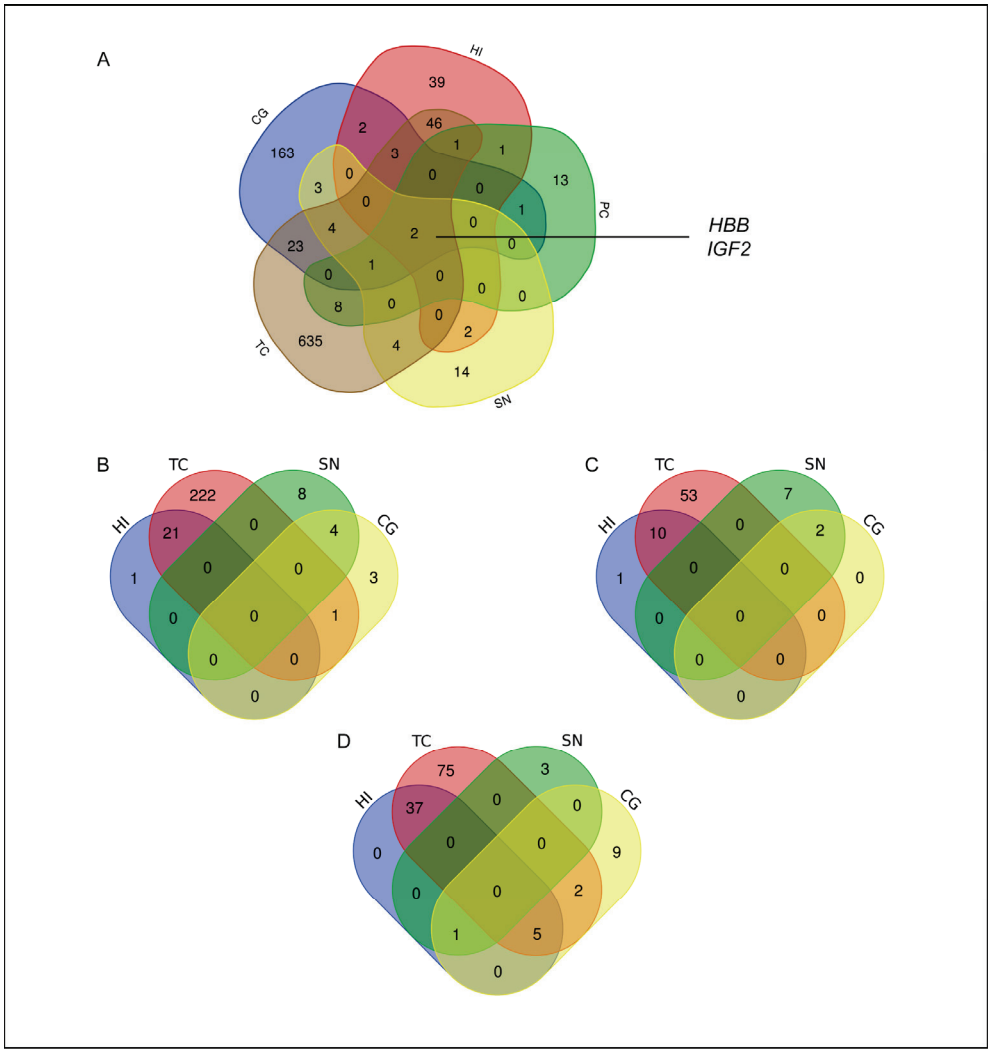


Figure 6. Brain areas of AD subjects were clustered according to the deregulation of the same class of enrichment terms. (**A**) Venn diagram of DEGs across the analyzed brain areas in AD subjects. The selection of DEGs was made by considering protein-coding genes with an adjusted p value of $\leq$0.05. The Venn diagrams referring to the overlapping of GO enrichment terms resulted from the STRING functional enrichment analysis: (**B**) GO biological process terms; (**C**) GO molecular function terms; and (**D**) GO cellular component terms.

In addition to the simple overlapping in gene expression profiles, it was possible to observe a similarity in the STRING functional enrichment analysis results between specific brain areas. Interestingly, the GO enrichment terms for biological processes and molecular functions were spontaneously clustered in two different groups of brain areas: the HI and TC, with 21 shared GO terms for biological processes and 10 shared GO terms for molecular functions, as well as the SN and CG, with four shared GO terms for biological processes and two for molecular functions. On the other hand, the grouping of GO enrichment terms for cellular components was less defined. Once again, there was an almost exclusive overlapping between the HI and TC but not for the SN and CG, with an overlapping of deregulated GO cellular component terms that affected the CG, HI, TC, and SN (Figure 6B–D), which was a sign that even if there was a marked difference between areas in terms of biological functionality, the involved cellular components remained almost unaltered.

Therefore, there was a common specialization between the HI and TC and between the CG and SN. In particular, the 21 GO terms for biological processes shared by the HI and TC were related to synaptic functionality; thus, the 10 GO terms for molecular functions

were mainly related to the activity of potassium channels and voltage-gated channels (Supplementary Table S11). On the other hand, the four GO terms for biological processes and the two GO terms for molecular functions shared by the SN and CG were related to protein folding and chaperone activity (Supplementary Table S12). No data were reported for the PC due to the absence of significant STRING functional enrichment analysis results. However, it should be noted that the only interaction network found in the PC comprised *HSPH1* and *DNAJB1*, two genes encoding for proteins involved in protein-folding-related processes, as previously reported. Moreover, considering the top five shared GO terms over the two groups of areas, it was possible to observe that the involved genes were all downregulated in AD subjects in comparison with the CTRL subjects.

2.2.1. The HI and TC in AD Are Subject to a Ca^{2+}-Related Synaptic Failure with Major Involvement of AZs

The top five GO terms in the STRING functional enrichment analysis for biological processes and molecular functions shared by the HI and TC are reported in Table 2.

The GO:0098693 term includes *BSN*, *CDK5R1*, and *PRKAR1B*. *BSN* encodes for the Bassoon protein, a fundamental zinc-finger protein of AZs, which are the release sites of chemical neurotransmitters [34,35]. Bassoon activity is strictly related to the activity of Piccolo, another zinc-finger protein of AZs that has a link domain for Ca^{2+} and is involved in synaptic vesicle clustering [36]. Furthermore, *PCLO*, the gene encoding for Piccolo, was downregulated in the HI of our AD subjects, together with *ERC2*, a gene encoding for another AZ protein that forms a protein complex with Piccolo and Bassoon and regulates, in particular, the Ca^{2+}-dependent release of neurotransmitters [37]. *ERC2* was also downregulated in the TC; *CDK5R1* encodes for p35, the main activator of Cdk5 (cyclin-dependent kinase-5), a key regulator of cytoskeletal remodeling and synaptic activity in the brain parenchyma. Cdk5 is able to inhibit Rac1 signaling through the phosphorylation of RasGRF2, which, in turn, reduces its exchange activity, leading to Rac1 inhibition and the downregulation of the axonal growth process [38]. Rac1 is a Rho family GTPase that regulates actin dynamics to induce axonal growth [39]. Moreover, a downregulation of *RASGRF2*, the gene encoding for RasGRF2, was observed only in the HI of our AD subjects; *PRKAR1B* encodes for a cyclic AMP (cAMP)-dependent protein kinase A (PKA) subunit. Since we observed a downregulation of *PRKAR1B*, it is important to also consider the role of PKA in the neural context. In fact, it was reported that PKA plays a neuroprotective role by modulating several cAMP-dependent processes related to survival mechanisms and synaptic plasticity [40]. *BSN*, *CDK5R1*, and *PRKAR1B* were downregulated in both the HI and TC of our AD subjects. Moreover, we also observed the downregulation of *ADCY1* (GO:0050804, GO:0048167), a gene encoding for the adenylate cyclase 1, which catalyzes the synthesis of cAMP itself in the brain. Notably, ADCY1, as well as the other components of group I of ADCYs (ADCY3 and ADCY8), is activated by Ca^{2+}/calmodulin and is five-fold more sensitive to Ca^{2+} than ADCY3 and ADCY8 [41]. Moreover, loss of ADCY1 was associated with impaired synaptic plasticity and deficits in spatial learning [42]. Furthermore, we also observed the shared downregulation of *SLC8A2* and *SYT7* (GO:0050804, GO:0048167). *SLC8A2* encodes for a Na^+/Ca^{2+} exchanger whose reduced expression was observed in hippocampal CA1 of APP23 and APP-KI mouse models of familial AD, and it was associated with deficits in hippocampus-dependent learning and memory processes [43]. On the other hand, *SYT7* encodes for the presynaptic Ca^{2+} sensor synaptotagmin-7. Synaptotagmin plays a pivotal role in Ca^{2+}-dependent synaptic activity, and its expression was also found to be markedly decreased by the combined action of mutated presenilin (PS) and γ-secretase proteolytic activity, which requires amyloid precursor protein (APP) as a substrate, suggesting a role for mutated PS in presynaptic mechanisms [44]. Another Ca^{2+}-related protein whose gene expression was downregulated in both the HI and TC of our AD subjects was *NRGN* (GO:0050804, GO:0048167). *NRGN* encodes for the protein neurogranin, which is localized at the post-synaptic terminal and binds calmodulin in the absence of Ca^{2+}. Lower *NRGN* expression was associated with higher Aβ and

tau pathology in the perirhinal cortex. Moreover, this negative correlation may indicate that the Aβ and tau proteins may contribute to synaptic degeneration in AD through the downregulation of *NRGN* itself [45].

Other genes encoding for synaptic proteins that were downregulated in both the HI and TC were *NPTX1* (Neuronal pentraxin 1, GO:0050804) and *LRRC4* (Leucine-Rich Repeat Containing 4; GO:0050804). The remaining shared DEGs were related to the activity of voltage-gated K^+ and Na^+ channels, K^+-Cl^- cotransporter 2, and RNA-binding proteins. Among these genes, the downregulation of *KCNC1* was validated through qPCR in both the HI and LT (Supplementary Figure S3).

Table 2. Classification of the GO terms for biological processes and molecular functions, with the related DEGs, shared by the group of brain areas consisting of the HI and TC and the group of brain areas consisting of the CG and SN.

Brain Regions	GO Aspects	GO Unique Identifier	GO Term Name	Shared DEGs
HI, TC	Biological processes	GO:0098693	Regulation of synaptic vesicle cycle	*BSN*, *CDK5R1*, *PRKAR1B*
		GO:0001508	Action potential	*SCN2B*, *KCNB1*, *KCNIP2*, *KCNC2*, *KCNA2*
		GO:0048167	Regulation of synaptic plasticity	*SLC8A2*, *CPEB3*, *NRGN*, *ADCY1*, *KCNB1*, *PRKAR1B*, *SYT7*
		GO:0050804	Modulation of chemical synaptic transmission	*SLC8A2*, *LRRC4*, *CPEB3*, *NRGN*, *ADCY1*, *NPTX1*, *KCNB1*, *PRKAR1B*, *CELF4*, *SYT7*
		GO:0010038	Response to metal ions	*KCNC1*, *ADCY1*, *NPTX1*, *KCNB1*, *KCNIP2*, *DMTN*, *SYT7*, *KCNC2*
	Molecular functions	GO:0005251	Delayed rectifier potassium channel activity	*KCNC1*, *KCNB1*, *KCNC2*, *KCNA2*
		GO:0005249	Voltage-gated potassium channel activity	*KCNC1*, *SCN2B*, *KCNB1*, *KCNV1*, *KCNC2*, *KCNA2*
		GO:0005267	Potassium channel activity	*KCNC1*, *SCN2B*, *KCNB1*, *KCNIP2*, *KCNV1*, *KCNC2*, *KCNA2*
		GO:0015079	Potassium ion transmembrane transporter activity	*KCNC1*, *SCN2B*, *KCNB1*, *SLC12A5*, *KCNIP2*, *KCNV1*, *KCNC2*, *KCNA2*
		GO: 0022843	Voltage-gated cation channel activity	*KCNC1*, *SCN2B*, *CACNA1E*, *KCNB1*, *KCNV1*, *KCNC2*, *KCNA2*
CG, SN	Biological processes	GO:0051085	Chaperone cofactor-dependent protein refolding	*DNAJB1*, *HSPA1B*, *HSPA1A*
		GO:0061077	Chaperone-mediated protein folding	*FKBP4*, *DNAJB1*, *CHORDC1*, *HSPA1B*, *HSPA1A*
		GO:0006457	Protein folding	*FKBP4*, *DNAJB1*, *CHORDC1*, *STIP1*, *HSPA1B*, *HSPA1A*
		GO:0090084	Negative regulation of inclusion body assembly	*DNAJB1*, *HSPA1B*, *HSPA1A*
	Molecular functions	GO:0044183	Protein folding chaperone	*DNAJB1*, *HSPA1B*, *HSPA1A*
		GO:0051082	Unfolded protein binding	*DNAJB1*, *HSPA1B*, *HSPA1A*

2.2.2. Molecular Chaperone Activity Impairment Is a Common Aspect of AD Pathology in the CG and SN

The top five GO terms shared by the CG and SN in the STRING functional enrichment analysis for biological processes and molecular functions are reported in Table 2.

All of the involved DEGs in both the CG and SN of AD subjects were downregulated. The shared DEGs involved in the two GO terms for molecular functions were *DNAJB1*, *HSPA1A*, and *HSPA1B*, and they were also part of all of the GO terms for biological processes. *DNAJB1*, *HSPA1A*, and *HSPA1B* are part of different families of heat shock proteins. Notably, *HSPA1A* and *HSPA1B* encode for two members of the Hsp70 family. Even if Hsp70 family members' expression decreases with age, induced overexpression of these proteins was associated with a reduction in Aβ peptides in microglial cells and a reduction in tau protein in cell lines and animal models [46,47].

The other DEGs involved in the GO terms for biological processes were *FKBP4*, *CHORDC1*, and *STIP1*. They are part of both the GO:0061077 and the GO:0006457 terms and encode for proteins related to protein folding and trafficking that, however, are not properly molecular chaperones. In particular, *FKBP4* encodes for FKBP Prolyl Isomerase 4, an enzyme that also displays chaperone activity. Its expression was reduced in AD subjects, and it was found to strongly correlate with the aggregation of pathological tau [48]. *STIP1* encodes for the Stress-Induced Phosphoprotein 1, a co-chaperone that modulates the activity of Hsp70 and Hsp90 families' proteins and is considered as a neuroprotective factor in the context of neurodegeneration. Moreover, in plasma samples from Parkinson's disease subjects, STIP1-specific autoantibodies were found, suggesting an autoimmune component of the pathology [49]. On the other hand, *CHORDC1* (Cysteine- and Histidine-Rich Domain Containing 1) encodes for a protein involved in centrosome duplication and chaperone-mediated protein folding. Interestingly, a specific methylation profile of *CHORDC1* was found either in the hippocampus or blood of epileptic subjects [50]. Despite this, *CHORDC1* has not been related to AD so far. *HSPA1A* downregulation was validated through qPCR in both the CG and SN (Supplementary Figure S4).

3. Discussion

The first objective of this study was to define the "molecular signature" of AD pathology in the brain by comparing the transcriptomic profiles of five different brain regions of AD subjects with those of CTRL subjects. Our second objective was to define a "molecular map" of the spread of AD by overlapping these transcriptomic profiles to highlight common pathways underlying neuropathological changes that are the consequence but not the cause of the disease. This is an original exploratory approach to AD. Indeed, despite using more recent technology with a deeper resolution (single-cell or spatial transcriptomics), previous studies mainly focused on animal models or on a single brain region [51,52]. A recent meta-analysis aimed to explore the differences between transcriptomic signatures across different brain regions in several neurodegenerative diseases, but, again, no information on single regions was reported, and fewer brain regions were analyzed [53].

Brain Areas Involved in Early and Late Alzheimer's Disease Show Different Molecular Signatures; Transcriptomic Analysis Highlights Possible Pathogenetic Mechanisms

In all areas, the classification of DEGs suggested a generalized impairment of the related biological processes. In the HI, the deregulation of processes that were mainly related to the synaptic vesicle cycle and synaptic plasticity, together with the involvement of the presynaptic active zone, was observed. We observed a downregulation of all genes except for *LTF*, the gene encoding for Lf. As reported in the reference literature, an increase in the protein level of Lf was observed in AD subjects in comparison with the CTRL subjects [54]. Moreover, a multivariate non-parametric analysis identified Lf as one of the most powerful novel targets in classifying AD subjects from normal old (NOLD) subjects. This, added to the evidence of a strong correlation between Lf secretion from activated microglia and Aβ production, led to the consideration of the inhibition of the interaction

between Lf and APP as a potential therapeutic target for AD treatment [55]. In the context of AD pathology, the HI is also known to be prone to early atrophy, an aspect that, together with general cortical gray matter atrophy, is related to the global cognitive impairment that is characteristic of AD subjects [56]. Therefore, it is not surprising that we observe a broader deregulation of GO terms related to synaptic function in the HI of our AD subjects, which was a result that was also in line with previous work in which, in the HI of late-onset AD (LOAD) subjects, deregulation of genes enriched in synapse function, learning, and memory was observed [14].

In the TC, the main outcome was, again, the deregulation of genes related to synaptic functionality; they were all downregulated except for *CFTR*, whose expression, which is characteristic of epithelial tissues, was also observed in the human brain [26]. In the central nervous system (CNS), several ABC transporters mediate the removal of metabolites from the brain interstitial fluid and cerebrospinal fluid (CSF) into peripheral blood. On the other hand, they regulate the passage of potentially toxic circulating compounds into the CNS [57]. In the context of AD, ABC transporters seem to be involved in protecting the CNS against Aβ neurotoxicity through the export of Aβ to the peripheral blood [58]. On the other hand, downregulation of *CFTR* expression was observed in the hypothalamus of AD subjects, suggesting a relationship between *CFTR* downregulation and the reduction in body fat observed in AD pathology [59]. Although the exact role of *CFTR* in physiology and pathology in the CNS remains unclear, it appears to be widely expressed in brain tissue [26].

Concerning the PC, the reduced number of DEGs resulted in only one functional interaction network that was found between *HSPH1* and *DNAJB1*, as both were downregulated in the PC of our AD subjects. The Hsp70 and DnaJ families' members form oligomers that are involved in protein refolding/degradation. Furthermore, it has been shown that tau fibrils derived from brain tissue can be successfully disaggregated when mixed with DnaJB1 and Hsp70 [29]. Nonetheless, it is important to state once again that, due to the reduced number of DEGs observed in the PC, no significant functional enrichment data were obtained.

In the CG, on the other hand, we observed a deregulation of genes encoding for heat shock protein family members or related to protein folding and trafficking, except for *CACNA2D1*, *CRELD1*, and *HBB*. *CACNA2D1* (Calcium Voltage-Gated Channel Auxiliary Subunit alpha-2/delta-1) encodes for the $\alpha_2\delta$ preprotein, which is post-translationally cleaved to form two polypeptides that are involved in the trafficking and function of voltage-gated calcium Ca_V1 and Ca_V2 channels [60]. CaV1 and CaV2 channel family members play a pivotal role in integrating synaptic inputs in neurons and in initiating synaptic transmission at fast synapses, respectively [61]. CRELD1 (Cysteine Rich With EGF-Like Domains 1) encodes for a protein involved in heart development that was recently associated with immune system homeostasis regulation [62]. In particular, low *CRELD1* expression is associated with a low $CD4^+$ T cell number in peripheral blood, which is a sign of immunosenescence [63]. Moreover, *CRELD1* acts as an upstream modulator of Wnt signaling, and its upregulation can restore blood–brain barrier dysfunctions due to the disruption of brain endothelial cells that is characteristic of AD [64]. *HBB* (Hemoglobin Subunit Beta) encodes for the adult hemoglobin subunit beta chain, and its expression was also observed in mouse and human neurons [65,66]. As previously reported, the upregulated genes observed in the CG were also involved in the deregulation of several biological processes related to protein folding, but, surprisingly, the most upregulated genes (log2(AD subjects/CTRL subjects) $\geq$ 4) were *KRT1*, *TYR*, and *ANXA8*. High expression of *KRT1* (Keratin 1) was observed to be protective against oxidative stress in the context of cerebral cavernous malformations [67]. *TYR* encodes for the protein Tyrosinase (Tyr), which is one of the key enzymes in mammalian melanin synthesis, and its expression was also observed in murine brains [68]. Moreover, PS deficiency leads to the aberrant accumulation of Tyr-containing post-Golgi vesicles, a phenomenon that was related to the PS1M146V familial AD mutation; subjects affected by this exhibited a partial loss of

function in melanin synthesis and, by extension, Tyr trafficking [69]. *ANXA8* encodes for Annexin A8, a Ca^{2+}-regulated phospholipid-binding protein [70] that was found to be involved in long-term potentiation in mice [71]. Other Annexin family members, such as Annexin A1 and Annexin A5, were related to AD pathology. In particular, Annexin 1 was found to be overexpressed in microglia in proximity to Aβ plaques in post-mortem AD brain tissue, suggesting an adaptive response of microglia during inflammation at early stages of the disease [72]. On the other hand, Annexin A5 was found to play a role in reducing neurotoxicity by interacting with amyloidogenic proteins in the context of neurodegenerative diseases and type II diabetes mellitus [73]. These upregulations could express attempts to cope for damage related to neurodegeneration, including oxidative stress, aberrant protein deposition, and inflammation.

Finally, in the SN, the DEG analysis and the functional enrichment analysis resulted in the deregulation of processes related to denatured protein binding and folding and to the haptoglobin–hemoglobin complex. In particular, the most downregulated genes were *HBA2* and *HBB*. *HBA2* encodes for the hemoglobin subunit alpha-2 chain, whose expression was found to be reduced in the context of several neurodegenerative diseases [74]. A similar role is played by HBB, as previously reported. Our second objective was to switch from a region-specific "molecular signature" to a whole-brain "molecular map" of the spread of AD. When overlapping the DEG profiles between the selected regions, the first outcome was the shared deregulation of *HBB* and *IGF2*. As stated above, HBB encodes for the beta polypeptide chain of adult hemoglobin. The decrease or even the absence of hemoglobin expression in neurons and the downregulation of the *HBB* gene are associated with several pathological conditions, including AD [75,76]. In particular, brain hemoglobin is involved in O2 brain homeostasis and the balance of the redox system. In the context of AD pathology, Aβ was observed to induce a molecular response pathway that led to a strong downregulation of the expression of the HBB gene [76]. On the other hand, *IGF2* encodes for insulin-like growth factor 2, a member of the insulin family of polypeptide growth factors that seems to play a pivotal role in brain plasticity, learning, and memory [77]. In addition, its administration in mice subjected to several mnemonic tasks led to memory enhancement [78]. Then, we looked at the shared STRING functional enrichment analysis terms, and we found that there was a spontaneous clustering of brain regions.

The results obtained for the HI and TC underline the importance of Ca^{2+} homeostasis in the HI and TC in the context of AD and in relation to proper synaptic transmission. In AD, neuronal loss is prominent in the cortex and hippocampus [79], in which one of the main signs observed in both in vivo and post-mortem conditions is synaptic loss, an aspect that is more tightly correlated with cognitive impairment than either Aβ deposition or tangle counts [80,81]. All of these aspects also highlighted the possibility of searching for synaptic biomarkers in the CSF of AD patients, a research line that led to encouraging results, such as the identification of higher levels of SNAP-25 and SYT1 in the CSF of AD and prodromal AD cases [82,83].

The transcriptomic signatures of the PC, CG, and SN, on the other hand, could be explained by the fact that loss of proteostasis is also a hallmark of aging [84]. Proteostasis involves mechanisms for the stabilization of correctly folded proteins, which are mainly dependent on the activity of Hsp family proteins, as well as mechanisms for protein degradation [85,86]. Thus, in this pathological context, a positive modulation of these mechanisms could have a neuroprotective effect.

Interestingly, even though the accumulation of Aβ aggregates spread throughout the brain in combination with AD progression, this modulation was absent in the SN of AD subjects, where the expression of molecular chaperones was observed to be downregulated, suggesting that the neuroprotective mechanism against protein misfolding either was not requested or was already compromised in the SN [87]. Notably, we observed a similar pattern in both the SN and CG, in which a low or absent AD pathology was found. This fact suggested that areas with a similar pathological stage may share similar deregulated pathways.

Considering all of these findings, we could make some assumptions in order to explain the pattern of the spread of AD that we observed. 1. We investigated the variations in the transcriptomic profiles of different brain regions in AD using post-mortem brain tissue samples from elderly subjects. 2. The spread of AD starts from the hippocampus and temporal lobe and leads, in its final stage, to the involvement of the whole brain [7]. 3. We observed that there was a clear and "spontaneous" clustering of deregulated GO terms for biological processes and molecular functions between different areas. In particular, the downregulation of DEGs related to the loss of proteostasis was only localized in the CG and SN of our AD subjects. Therefore, we can assume that the loss of proteostasis could be an early aspect of AD pathology that occurs when a specific brain region starts to be affected; this result is also in line with another study highlighting that there are significant differences in gene expression over the course of AD progression, at least for TC, ultimately leading to cognitive impairment [88]. At an early stage, then, the HI and TC also could have shown a similar molecular signature related to the loss of proteostasis, which eventually switched to the deregulation of biological processes related to synaptic transmission. Considering that cognitive impairment is not significantly related to Aβ deposition and, instead, correlates with pTau pathology and synaptic transmission impairment [7,89], we can also assume that the switch from the loss of proteostasis to synaptic impairment occurs when a significant number of unfolded or misfolded proteins are reached, regardless of Aβ deposition, and this could be a significant hallmark of the beginning of pathology.

Limitations: We worked on a small number of subjects. Nonetheless, using post-mortem samples of human brain tissue remains the gold standard for studying brain disorders [90]. We should also consider that the selected subjects were not free from comorbidities, as shown in Table 3. This could also explain the sub-clustering of the subjects in the reported PCAs. On the other hand, this awareness granted us the opportunity to perform the case selection as carefully as possible. Finally, we obtained no information about the roles of specific cell populations and tissue alterations in transcriptomes since no single-cell sequencing analyses or spatial transcriptomic analyses were performed.

Indeed, our results, while consistent with the reference literature, provide new observations that are useful for further research that investigates the relationships between brain areas in brains with increasing neuropathological AD severity.

Table 3. Characteristics of the selected subjects. Legend: CDR = Clinical Dementia Rating scale; AD = Alzheimer's disease; F = female; M = male; NOLD = normal old; NCD = neurocognitive disorder; DM2 = diabetes mellitus type 2; CVD = cardiovascular disease; AHT = arterial hypertension; SVD = small-vessel disease; CAA = cerebral amyloid angiopathy: 1 mild; 2 moderate; 3 severe; P = parenchymal; M = meningeal; cap = capillary involvement; LATE = limbic-predominant age-related TDP-43 encephalopathy (TDP-43—TAR DNA-binding protein 43); PMI = post-mortem interval.

Samples	Gender	Comorbidity	ApoE	Cognitive State	MMSE	CDR	Age at Death	Death Cause	Thal Phase	Braak Stage	AD Scoring (Montine)	CAA (Love)	Additive Pathologies	Vascular Pathology (Skrobot)	PMI	Groups
1	M	Cirrhosis, CVD	2//3	NOLD	28	0.5	81	cachexia	0	0	no	no	no	low	19h30′	CTRL
2	F	Lung cancer	3//3	NOLD	30	0	71	cachexia	0	I	no	no	no	low	16h	CTRL
3	M	CVD; DM2	3//3	NOLD	26	0	79	cachexia	1	I	low	no	no	low	3h15′	CTRL
4	F	AHT; CVD; DM2	3//4	Major-NCD (AD; SVD)	21	2	82	cachexia	4	III	int	2M	no	moderate	11h	AD
5	F	CVD; DM2	2//3	Major-NCD (AD)	0	5	78	arrhythmia	4	IV	high	no	no	low	8h	AD
6	M	AHT	3//3	Major NCD (AD)	0	4	80	cachexia	4	V	high	3P; 3M	no	low	15h30′	AD
7	F	CVD; CVD	3//3	Major-NCD (AD; SVD)	2	4	85	arrhythmia	5	V	high	2Pcap; 3M	LATE	moderate	15h30′	AD
8	F	AHT; CVD	3//4	Major-NCD (AD; SVD)	0	5	89	cachexia	5	V	high	3Pcap; 3M	LATE	moderate	15h20′	AD
9	M	no	3//3	Major-NCD (AD)	4	4	80	arrhythmia	5	VI	high	2M	LATE	no	15h	AD

4. Materials and Methods

4.1. Clinical and Neuropathological Assessments

All clinical assessments were performed by the same team (neurologists, geriatricians, and neuropsychologists) according to the Abbiategrasso Brain Bank (ABB) protocol [91]. The ABB autopsy and sampling protocol was approved by the Ethics Committee of the University of Pavia on 6 October 2009 (committee report 3/2009) in the context of the InveCeAb study (A Longitudinal Multidimensional Population Study on Brain Aging (InveCeAb), ClinicalTrials.gov, NCT01345110). This study's procedures were in accordance with the principles outlined in the Declaration of Helsinki of 1964 and the following amendments. All subjects were asked to sign an exhaustive consent form, and the signature of a guardian or the next of kin was requested in the case of non-compos mentis subjects. This study was also supervised by "Associazione Alzheimer Italia". During the longitudinal observation, brain donors were subjected to a neuromotor and neurocognitive evaluation that included global cognition and a complete neuropsychological assessment of specific cognitive domains (memory, attention, executive functions, language, and visuospatial abilities) according to the DSM-V. The Clinical Dementia Rating (CDR) was used for a synthetic definition of dementia severity [92].

After death, a brain-harvesting procedure was performed within 24 h, and the brain was processed according to the above-mentioned protocol [91]. Briefly, both hemispheres were cut fresh into slices that were alternately frozen or fixed in 10% phosphate-buffered formalin solution. Then, the samples were processed for paraffin inclusion and cut into 8 µm thick serial sections. All neocortical areas and the basal ganglia (BG), hippocampus (HI), cerebellum, and brainstem (SN, pons, and medulla oblongata) were analyzed to carry out a detailed neuropathological characterization with the following stainings and immunohistochemical reactions: hematoxylin and eosin, Cresyl Violet, Luxol Fast Blue, Gallyas, antibodies against amyloids (4G8; BioLegend (San Diego, CA, USA) Cat# 800722, RRID:AB_2861076), phosphoTau (pTau—AT8; Thermo Fisher Scientific (Waltham, MA, USA) Cat# MN1020, RRID:AB_223647), alpha-synuclein (α-SYN—KM51; Leica Biosystems (Nussloch, Germany) Cat# NCL-L-ASYN, RRID:AB_442103), and phospho-TDP-43 (pS409/410-2; Cosmo Bio (Tokyo, Japan) Cat# CAC-TIP-PTD-P02, RRID:AB_1961898) (Supplementary Table S1). Representative pathological pictures of the HI of 3 different subjects with different AD scores (no AD; intermediate AD; high AD) are shown in Supplementary Figure S1.

For this study, six subjects with a neuropathological diagnosis of AD (AD scoring: intermediate AD, high AD) and three CTRL subjects in duplicate (no dementia) were selected. Sample 1 was classified as CTRL despite CDR = 0.5, since the patient's minimal cognitive disorder was presumably due to the presence of some degree of small-vessel disease in the absolute absence of neurodegenerative burden. The population age ranged from 71 to 89 years old. The particular characteristics of the selected subjects are reported in Table 3.

4.2. Transcriptome Profiling

4.2.1. Tissue Sampling and Total RNA Extraction

For this study, 5 brain areas were selected to perform a whole-transcriptome analysis: the temporal cortex (TC), parietal cortex (PC), cingulate gyrus (CG), hippocampus (HI), and substantia nigra (SN). First, 15 mg of cryopreserved brain tissue sample was obtained and homogenized using the CK14 Precellys Lysing Kit (Bertin Technologies, Aix-en-Provence, France). Then, total RNA was extracted using TRIzol Reagent (Sigma Aldrich, Milan, Italy) according to the manufacturer's instructions. Quantification of the extracted RNA was performed using a NanoDrop™ One/OneC Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA, RRID:SCR_023005), and the quality and integrity of the extracted RNA were assessed using a 2200 TapeStation Instrument (RRID:SCR_014994) and an RNA ScreenTape Kit (Agilent, Waldbronn, Germany).

4.2.2. Preparation of Libraries for RNA-Seq

Libraries for RNA-seq from the selected brain areas were prepared with the CORALL Total RNA-seq Library Prep Kit (Lexogen, Wien, Austria) using 500 ng of total RNA according to the manufacturer's instructions. The quality of the libraries was then analyzed using a Tape Station system (D1000 High-Sensitivity Kit, Agilent, Waldbronn, Germany) and quantified using a Qubit™ dsDNA HS Assay Kit (Invitrogen, Waltham, MA, USA). The sequencing was performed with a NextSeq 500/550 High Output v2.5 kit (150 cycles, Illumina, San Diego, CA, USA; Illumina NextSeq 550 System, RRID:SCR_016381).

4.2.3. Quantitative PCR (qPCR)

Using human gene sequences available from the NCBI (www.ncbi.nlm.nih.gov/nucleotide, accessed on 15 January 2023), PCR oligonucleotides for gene pairs were selected using Primer3plus online (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi, accessed on 20 January 2023) (Supplementary Table S2). Then, 500 ng of RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad, Segrate, Italy). Quantitative PCR amplification was performed using SYBR Green Master Mix (BioRad, Segrate, Italy) and 25 ng of cDNA. The reaction was performed using the CFX Connect™ Real-Time PCR Detection System (BioRad, Segrate, Italy, RRID:SCR_018064). Cycle threshold (Ct) values were automatically recorded for each replicate qPCR reaction, and the mean Ct values were normalized against those determined for GAPDH. Fold-expression differences were determined using the 2-ΔΔCt method.

4.2.4. Bioinformatic Data Analysis

FastQ files were generated via Illumina bcl2fastq2, starting from raw sequencing reads produced by an Illumina NextSeq sequencer (Version 2.17.1.14, http://support.illumina.com, accessed on 4 October 2022). Gene and transcript intensities were computed using the STAR/RSEM software with GRCh38 (STAR 2.7.10b: RSEM v 1.3.3 Gencode Release 27) as a reference while using the "stranded" option. Differential expression analysis was performed using the R package DESeq.2. DEGs, which were grouped as coding and non-coding genes, were considered differentially expressed and retained for further analysis with $|\log2(\text{AD subjects}/\text{CTRL subjects})| \geq 1$ and a false discovery rate (FDR) of ≤ 0.1, considering the CTRL subjects as a reference. qPCR was conducted for the technical validation of RNA-seq.

4.2.5. Gene Set Enrichment Analysis (GSEA) and Statistical Analysis

GSEA was performed on the coding genes. Pathway analysis was performed using KEGG pathway analysis (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.ad.jp/KEGG, accessed on 29 November 2022), WikiPathways analysis, and Gene Ontology (GO) for the biological processes, cellular components, and molecular functions of differentially expressed genes via the enrichR web tool [93]. R software (R software (Version 2023.09.1+494. pro2)) was used to generate Dotplot graphs (using the ggplot2 library). For the functional enrichment analysis, the STRING database (https://string-db.org/, accessed on 10 October 2023) was used, and only protein-coding DEGs with an adjusted p value of ≤ 0.05 were selected. To improve statistical significance and increase the stringency of the selection, a minimum required interaction score with a high level of confidence (0.700) was set. The statistical significance of local network clusters and GO terms was defined with the "strength" and "false discovery rate" values. Venn diagrams were obtained using a specific web tool (https://bioinformatics.psb.ugent.be/webtools/Venn/, accessed on 20 October 2023). For the qPCR results, figures and comparisons of fold-expression differences (2-ΔΔCt method) between brain areas were obtained with RStudio Desktop (Version 2023.09.1+494.pro2, Posit Software, Boston, MA, USA). For comparisons between two areas, the Mann–Whitney test was performed; for comparisons between more than two areas, the Kruskal–Wallis test with Dunn's post hoc test and Benjamini–Hochberg correction was performed. Data with p values of ≤ 0.05 were considered statistically significant.

5. Conclusions

With this work, we were able to observe the transcriptomic signature of AD pathology in different areas of the post-mortem human brain. We then had the opportunity to explore the molecular basis of AD and speculate on the etiology of the disease, reporting that different brain areas have different gene expression profiles. In particular, we observed that the HI and TC are affected by synaptic transmission impairment, while the CG and SN, as well as the PC in a non-statistically significant way, are characterized by a loss of proteostasis. This led us to assume that the spread of AD may follow a time- and severity-related pattern that arises from the age-dependent loss of proteostasis and leads to synaptic impairment.

All things considered, our step in this direction highlights the transcriptomic differences between AD brain areas and the identification of novel biological targets that could be useful for the development of therapeutic approaches. However, further experiments are required in order to confirm this hypothesis and potentially investigate what happens in relation to the severity of AD pathology.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms252011117/s1.

Author Contributions: Conceptualization, R.R.F., T.E.P. and A.G.; methodology, R.R.F., V.F. and M.G.; validation, R.R.F., R.D.G., F.D., E.S. and B.R.; formal analysis, R.R.F. and M.R.; investigation, R.R.F. and V.F.; resources, M.G., R.D.G., F.D., B.R., X.P. and C.C.; writing—original draft, R.R.F.; writing—review and editing, R.R.F., A.G., T.E.P., A.D., S.G. and M.C.; visualization, R.R.F.; supervision, A.D., S.G. and T.E.P.; project administration, T.E.P. and A.G.; funding acquisition, A.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Serpero Foundation, 20129, Milano, Italy, and the Golgi Cenci Foundation, 20081, Abbiategrasso, Italy, in equal parts. The Serpero Foundation and Golgi Cenci Foundation provided financial support for this research; in particular, they financed the data collection, analysis, interpretation of data, and the result publication.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and all human subjects provided informed consent for autopsy, sampling protocols, and further analysis in the context of the InveCeAb study (A Longitudinal Multidimensional Population Study on Brain Aging (In-veCeAb), ClinicalTrials.gov, NCT01345110), approved by the Ethics Committee of the University of Pavia on 6 October 2009 (committee report 3/2009).

Informed Consent Statement: All human subjects provided informed consent for autopsy and sampling protocols in the context of the InveCeAb study (A Longitudinal Multidimensional Population Study on Brain Aging (InveCeAb), ClinicalTrials.gov, NCT01345110), and this was approved by the Ethics Committee of the University of Pavia on 6 October 2009 (committee report 3/2009).

Data Availability Statement: The datasets generated and/or analyzed during the current study are available in the GEO repository.

Acknowledgments: We would like to thank all donors and patients who participated in this study, without whom this work would not have been possible.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of this study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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International Journal of
Molecular Sciences

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Circulating Brain-Related miRNAs as Predictors of Postoperative Delirium in Cardiac Surgery Patients †

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† This article is a revised and expanded version of a conference poster, "Decreased preoperative miR 183-5p expression and an episode of depression are the independent predictors of delirium after cardiac surgery", which was presented at the 35th ECNP Congress, 15–18 October 2022, Vienna, Austria.

Abstract

Delirium is a neuropsychiatric syndrome that is pathophysiologically related to both mental (dementia, depression) and physical illness. Its occurrence results in a poor prognosis. This study investigates whether specific miRNAs (miR-9-3p, miR-34c-5p, miR-96-5p, miR-183-5p, and miR-374-3p) related to brain function are associated with an increased risk of postoperative delirium. A total of 224 adult individuals scheduled for elective cardiac surgery were eligible to participate in the study. Delirium diagnosis was established with the use of the Confusion Assessment Method. Following miRNA expression profiling, cDNA synthesis was conducted on the samples obtained one day before and the day after surgery, from 60 delirium patients and 60 randomly selected non-delirium individuals. Univariate comparisons revealed that preoperative miR-96-5p (p = 0.05) and miR-183-5p (p = 0.001), along with postoperative miR-34c-5p (p = 0.009), miR-96-5p (p = 0.07), and miR-183-5p (p = 0.05), were associated with the risk of post-surgery delirium. However, after conducting multivariate logistic regression analysis, only miR-183-5p was found to be independently associated with the risk of delirium development. Other predictors of delirium included an ongoing episode of depression, peripheral vascular disease, female gender, active smoking, and increased postoperative pCO2 concentration. The current study revealed that preoperatively decreased expression of miR-183-5p predicts delirium development after cardiac surgery.

Keywords: miR-183-5p; delirium; oxidative stress; neuroinflammation; antioxidant activity

1. Introduction

Delirium is a neuropsychiatric syndrome that is commonly observed after major surgery, particularly among elderly patients with physical and psychiatric comorbidities, as well as those with impaired cognitive status [1,2]. It is characterized by sudden onset, fluctuations in attention and cognitive deficits, reduced awareness, and altered psychomotor activity. The frequency of delirium in elderly hospitalized individuals is exceptionally high, with rates of up to 70% [3]. Similarly, patients hospitalized in intensive care units due to COVID-19 infection have comparable rates of delirium at 73% [4]. Unfortunately, delirium is associated with poor prognosis, increased mortality, and impaired functioning even after the syndrome resolves [5].

The risk factors for delirium are well-known and described. However, investigations into its pathophysiology have only recently begun. The processes underlying delirium include an imbalance in the hypothalamic–pituitary–adrenal (HPA) axis, increased oxidative stress, and inflammation [6,7]. Despite significant progress, the role of miRNA in the development of delirium remains unclear.

MicroRNAs (miRNAs) are small, single-stranded RNA molecules that play a crucial role in post-transcriptional gene expression. Their post-transcriptional activity includes both the inducement of mRNA degradation and the stimulation of gene expression [8,9]. Specific miRNAs are recognized as regulators of three basic areas: central nervous system (CNS) development, neuroinflammation, and neurodegeneration.

The role of miRNAs in neural development was first demonstrated through conditional knockout mice experiments involving enzymes responsible for miRNA biogenesis [9]. For instance, miR-9 is highly expressed in neural precursors and regulates the number of neural stem cells [10,11]. Its overexpression controls proliferation and promotes neural differentiation by suppressing the orphan receptor [12].

Fu et al. (2020) conducted a study that demonstrated that miR-34c-5p is significantly reduced in individuals with drug-resistant epilepsy as compared to the control group [13]. The findings of the study suggest that the decrease in miR-34c-5p levels in drug-resistant epilepsy is responsible for inducing neuroinflammation, leading to the loss of hippocampal neurons and worsening of the disease. Additionally, Tu and Hu's (2021) research found that the lowered expression of miR-34c-5p is associated with cerebral ischemia/reperfusion injury in experimental artery occlusion ischemic models and reperfusion-induced models in animal subjects [14]. The authors suggest that inflammatory and apoptotic signaling pathways may mediate the above process.

A recent study revealed that the upregulation of miR-96-5p increases the level of the glutamate transport-associated protein (GTRAP3-18), leading to a decrease in the concentration of the excitatory amino acid transporter (EAAC1) [15]. EAAC1 regulates the concentration of neuronal glutathione (GSH), an important neuroprotective antioxidant. When the level of EAAC1 decreases, GSH activity also diminishes, resulting in oxidative stress, neuronal damage, and the onset of neurodegenerative processes [15].

Although some miRNAs have been identified as regulators of the CNS and mediators of neuroinflammation, only a few are critical for regulating synaptic plasticity and cognitive status. One of these miRNAs is miR-9-3p, which is highly expressed in developing and mature brains [16]. Both miR-9-3p and miR-9-5p are produced from the miR-9 precursor and are involved in developing and losing neurons, synapses, and microglia activation [16, 17]. The level of miR-9-3p is decreased in neurodegenerative diseases such as Huntington's and Alzheimer's disease (AD) [18,19]. It has been described as a biomarker differentiating between Parkinson's disease (PD) and Multiple System Atrophy (MSA) [20].

Das Gupta et al. (2021) found that after experimental traumatic brain injury (TBI), plasma miR-9-3p levels increase [21]. The study showed that in patients with severe TBI,

plasma miR-9-3p levels were 6.5- and 9.2-fold higher compared to those with mild TBI and the control group, respectively. These findings illuminate miRNA's role in neuronal loss and brain tissue repair. In the study conducted by Wang et al. (2020), it was found that miRNA-183-5p expression in the mouse brain decreased after experimental intracerebral hemorrhage (ICH) [22]. The authors investigated the effect of miRNA-183-5p on the injury and repair of brain tissue after ICH by injecting miRNA-183-5p agomir or miRNA-183-5p antagomir into the lateral ventricles of mice with experimentally induced ICH. After several days of treatment, mice treated with exogenous miRNA-183-5p showed less brain edema, neurobehavioral defects, inflammation, oxidative stress, and ferrous deposition than antagomir-treated mice. Two studies have identified the specific miRNA involved in the development of postoperative delirium [23,24]. The first study found a correlation between microRNA-320 and postoperative delirium in patients undergoing tibial fracture internal fixation surgery [23]. The second study revealed that preoperative miR-210 could potentially predict the occurrence of postoperative delirium in elderly gastric cancer patients undergoing curative resection [24]. Recent research has shown that certain miRNAs can target genes that regulate the permeability of the blood–brain barrier (BBB) in both animal and human in vitro models. This discovery suggests that miRNAs have a role in modifying the integrity of the BBB. For instance, Coxsackievirus A16 can penetrate the BBB and decrease the expression of miR-1303 [25]. As a result, junctional complexes are disrupted by directly regulating matrix metallopeptidase 9 (MMP9), ultimately leading to pathological changes in the CNS [25]. The purpose of the present study was to investigate whether specific miRNAs (miR-9-3p, miR-34c-5p, miR-96-5p, miR-183-5p, and miR-374-3p) related to brain function are associated with an increased risk of delirium among patients who have undergone cardiac surgery.

2. Results

Elective cardiac surgery was performed in 294 patients during the study period; of these, 58 subjects did not meet the inclusion criteria since they underwent something different than CABG/CABG plus CVR surgery (isolated CVR surgery without CABG, including minimally invasive mitral valve repair [n = 58]), and 12 individuals did not sign an informed consent form. Of the 224 patients who signed their informed consent and were enrolled, 4 patients were lost to follow-up since they died before the observational period was completed, and 43 individuals had incomplete study data (these patients were not included in the analysis due to failure during sample collection or inappropriate sample collection (coagulation), n = 28, and due to incomplete postoperative delirium evaluation, n = 15). The incidence of delirium among the 177 remaining patients was 34% (61 delirium cases). After the miRNA expression profiling, cDNA synthesis was performed in 60 delirium patients and 60 randomly selected non-delirium individuals (simple randomization using the computer random number generation program). Figure 1 outlines the selection process of eligible participants, including accurate inclusion and exclusion criteria. miRNA dqPCR analysis was performed in the serum samples obtained one day before and the day after surgery (see Section 4.4).

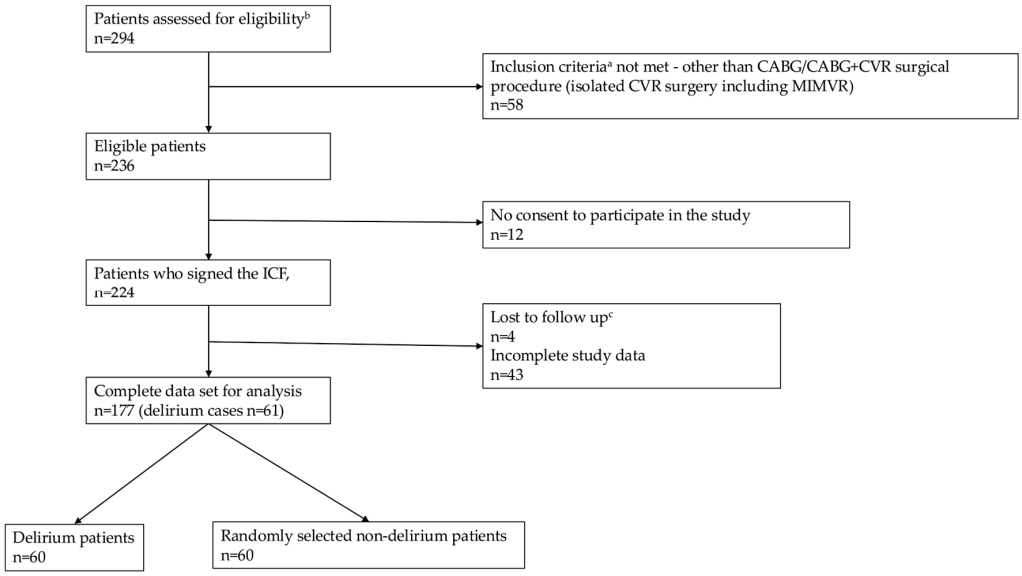


Figure 1. The selection process of eligible participants. CABG: coronary-artery bypass graft; CVR: cardiac valve replacement; MIMVR: Minimally Invasive Mitral Valve Repair. [a] Inclusion criteria: patients scheduled for cardiac surgery CABG/CABG with valve surgery, age $\geq$ 18 years. [b] Exclusion criteria: unstable general condition of the patient, diagnosis of dementia before surgery, delirium diagnosed in the week preceding the procedure, surgery other than CABG or CABG with CVR, chronic inflammatory or autoimmune diseases, use of corticosteroids, cytokine/anticytokine treatment 6 months before surgery, active alcohol or other substance addiction (abstinence period shorter than 3 months), severely impaired hearing or vision, illiteracy. [c] Death before the observation period was completed.

2.1. Patients' Characteristics, Univariate and Multivariate Comparisons

Study population characteristics are presented in Table 1.

Table 1. Demography, comorbidities, and perioperative characteristics of the study participants.

Characteristic	Mean or N	SD or %
Age (years)	68.13	6.80
Gender (Male)	92	76.67%
Weight (kg)	81.69	12.38
Height (cm)	170.22	8.20
Education (years)	11.74	3.75
CDT test	5.77	2.44
MMSE Score	27.44	2.22
Ejection fraction (%)	51.68	10.26
CCS score	2.47	0.77
NYHA grade	2.18	0.72
Urea concentration (mmol/L)	7.58	8.76
Creatinine concentration (μmol/L)	90.59	35.70
Presence of anxiety disorders	7	5.83%
Presence of depression	25	20.83%
Diabetes	45	37.50%
Arterial hypertension	102	85%
Peripheral vascular disease	22	18.33%
Vascular diseases of the CNS	12	10%
Epilepsy	3	2.50%
Head injuries	1	0.83%
Asthma	4	3.33%
Chronic obstructive pulmonary disease	7	5.83%
Smoking tobacco	53	44.17%
Anemia (Hb 10 mg/dL for female; 12 mg/dL for male)	25	20.83%
Creatinine > 1.2 mg/dL	18	15.00%

Table 1. *Cont.*

Characteristic	Mean or N	SD or %
Atrial fibrillation	18	15.00%
Surgery duration (h)	3.93	0.85
ECC	91	75.83%
Intraoperative resuscitation	3	2.50%
Intraoperative steroid use	2	1.67%
Postoperative $pCO_2 \geq 45$ mmHg	25	20.83%
Postoperative $pO_2 \leq 60$ mmHg	16	13.33%
Postoperative hyperthermia	15	12.50%
Massive postoperative blood transfusion (>4 units)	6	5.00%
Plasma transfusion (≥1 unit)	15	12.50%
Length of stay in the ICU (days)	3.71	2.24
Aortic cross-clamping time (min)	36.26	28.54

CCS: Canadian Cardiovascular Society degree; CDT: Clock Drawing Test; MMSE: Mini-Mental State Examination; NYHA: New York Heart Association grade; CNS: central nervous system; pCO_2: partial pressure of carbon dioxide; pO_2: partial pressure of oxygen; ICU: intensive care unit; ECC: extracorporeal circulation; Hb: hemoglobin concentration.

The median preoperative and postoperative miRNA expression levels and oxidative stress biomarker concentrations in the whole population are presented in Table 2.

Table 2. The median miRNA expression and biomarker concentrations in the whole study population.

miRNA	Pre-Operative Level Copies/mL [a]	Post-Operative Level Copies/mL [a]
miR-9-3p	79 (13.0–172.1)	101.4 (20.7–191.9)
miR-34c-5p	12.5 (0.0–45.4)	25.7 (0–108.1)
miR-96-5p	311.6 (110.2–696.8)	156.7 (68.7–325.7)
miR-183-5p	78.9 (14.3–179.2)	73.4 (10.4–168.2)
miR-374-3p	2.8 (0.0–30.04)	0 (0.0–16.3)
Superoxidase dismutase (ng/mL)	2.68 (2.06–3.53)	2.13 (1.62–3.01)
Antioxidant activity (μmol/L)	2.1 (1.3–2.9)	1.8 (1.2–2.6)

[a] For continuous variables, the medians and interquartile ranges (IQRs) are given.

The findings of the univariate analysis of demographics, comorbidities, and factors related to anesthesia and surgical procedures are shown in Tables 3 and 4.

Table 3. The expression of preoperative and postoperative miRNA in univariate comparisons.

Variable	Non-Delirious [a]	Delirious [a]	Effect Size [b]	*p* Value [c]
Preoperative miR-9-3p	87.4 (34.7–187.2)	53.8 (5.1–156.6)	0.32	0.39
Preoperative miR-34c-5p	20.5 (0.0–63.1)	8.0 (0.0–42.5)	0.19	0.28
Preoperative miR-96-5p	368.2 (169.7–832.5)	165.7 (55.0–507.9)	0.49	**0.05**
Preoperative miR-183-5p	210.5 (67.7–347.6)	53.02 (9.6–173.2)	0.77	**0.0005**
Preoperative miR-374-3p	9.3 (0.0–39.7)	0 (0.0–11.2)	0.34	0.26
Postoperative miR-9-3p	118.5 (45.04–185.9)	57.13 (0–201.9)	0.32	0.39
Postoperative miR-34c-5p	51.4 (15.7–121.4)	7.6 (0–69.6)	0.56	**0.009**
Postoperative miR-96-5p	187.2 (96.5–364.2)	119.2 (33–278.6)	0.47	0.07
Postoperative miR-183-5p	89.1 (14.5–247)	39 (0–98.1)	0.49	**0.05**
Postoperative miR-374-3p	0 (0–18.0)	0 (0–15.4)	0.09	0.99

[a] For continuous variables, the medians and interquartile ranges (IQRs) are given. [b] For continuous variables, Cohen's d coefficient was calculated; for categorical variables, Cramer's V coefficient was presented. [c] *p* value with Sidak correction for multiple comparisons was calculated. Significant values are in **bold**.

Table 4. Perioperative characteristics in univariate comparisons.

Variable	Non-Delirious [a]	Delirious [a]	Effect Size [b]	*p* Value [c]
CABG plus valve surgery	2 (3.3%)	8 (23.3%)	0.18	**0.04**
Duration of surgery (h)	4 (3–4.5)	4 (3.4–4.4)	0.15	0.41
Extracorporeal circulation	40 (66.7%)	51 (85%)	0.21	**0.02**
Intraoperative circulatory support	16 (26.7%)	17 (28.3%)	0.24	0.16
Post-op. hyperthermia > 38 °C	6 (10%)	9 (15%)	0.08	0.40
Post-op. $pO_2 \leq 60$ mmHg	5 (8.3%)	11(18.3%)	0.15	0.10
Post-op. $pCO_2 \geq 45$ mmHg	7 (11.7%)	18 (30%)	0.40	**0.01**
Plasma transfusion > 1 unit	6 (10%)	9 (15%)	0.08	0.40
Blood transfusion > 4 units	1 (6.7%)	5 (8.3%)	0.15	0.21

CABG: Coronary Artery Bypass Graft Surgery; pO_2: partial pressure of oxygen; pCO_2: partial pressure of carbon dioxide. [a] For continuous variables, the medians and interquartile ranges (IQRs) are given; for categorical variables, the number of observations (n) and fraction (%) were calculated. [b] For continuous variables, Cohen's d coefficient was calculated; for categorical variables, Cramer's V coefficient was presented. [c] *p* value with Sidak correction for multiple comparisons was calculated. Significant values are in **bold**.

Univariate comparisons revealed that preoperative miR-96-5p ($p = 0.05$) and miR-183-5p ($p = 0.001$) and postoperative miR-34c-5p ($p = 0.009$), miR-96-5p ($p = 0.07$), and miR-183-5p ($p = 0.05$) were associated with the risk of post-surgery delirium. However, according to the results of multivariate logistic regression analysis, only preoperative miR-183-5p was independently associated with the risk of postoperative delirium development (Table 5). Other predictors of post-surgery delirium included an ongoing episode of depression, peripheral vascular disease, female gender, active smoking, and an increased postoperative pCO2 concentration. There were no statistical differences between men and women with regard to miR-183-5-p expression. The median level of preoperative miR-183-5p among men and women in the whole study population was 164.5 (QR: 24.9–279.5) and 63.1 (QR: 14.5–174.8), respectively ($p = 0.11$, Cohen's d coefficient = 0.29). The median level of postoperative miR-183-5p among men and women in the whole study population was 57.7 (QR: 5.5–181.0) and 47.7 (QR: 8.5–113.6), respectively ($p = 0.53$, Cohen's d coefficient = 0.11). The median level of preoperative miR-183-5p among men and women in the delirium group was 51.5 (QR: 5.5–178.5) and 53.0 (QR: 17.9–166.3), respectively ($p = 0.89$, Cohen's d coefficient = 0.03). The median level of postoperative miR-183-5p among men and women in the delirium group was 25.5 (QR: 0–93.2) and 55.3 (QR: 8.5–94.4), respectively ($p = 0.36$, Cohen's d coefficient = 0.23).

Table 5. Factors independently associated with delirium after cardiac surgery revealed in a multivariate stepwise logistic regression analysis.

Variable	Coefficient	Standard Error	OR (95% CI)	*p* Value
Depression	2.53	0.79	12.6 (2.7–59.2)	<0.001
Preoperative miR-183-5p	−0.002	0.001	0.99 (0.995–0.999)	0.005
Postoperative pCO_2 >= 45	1.32	0.62	3.7 (1.1–12.6)	0.03
Cigarette smoking	0.91	0.46	2.4 (1.007–6.15)	0.05
Female Gender	1.26	0.56	3.5 (1.2–10.5)	0.02
Peripheral vascular disease	1.38	0.68	3.9 (1.04–15.2)	0.04
Constant	−1.005	0.40	-	0.01

pCO_2: partial pressure of carbon dioxide. The regression model is statistically significant: $\chi 2 = 49.641$, df = 6, $p < 000.1$; Hosmer–Lemeshow test: $\chi 2 = 11.203$, $p = 0.190$; Nagelkerke $R^2 = 0.451$.

2.2. Optimal miRNA Thresholds and Correlations with Other Analyzed Variables

According to the ROC analysis, the most optimal cutoff value of preoperative miR-183-5p that predicts the development of delirium was ≤193 copies/mL, with a sensitivity of 82%, specificity of 57%, positive predictive value of 0.65 and negative predictive value of 0.75 (area under the curve = 0.7; standard error = 0.05; 95% CI: 0.61 to 0.80; $p < 0.001$).

In the group of patients who did not experience delirium, there was a trend towards a significant correlation between preoperative miR-183-5p expression and preoperative superoxidase dismutase (SOD) activity (Spearman's rank correlation 0.264; $p = 0.09$). However, a significant positive correlation was found between preoperative SOD activity and the preoperative levels of miR-96-5p ($p < 0.05$). Additionally, there was a significant positive correlation between postoperative miR-96-5p and preoperative plasma antioxidant activity, which is a marker that reflects the total activity of all plasma antioxidative agents (Spearman's rank correlation 0.199; $p = 0.04$).

3. Discussion

The current study revealed that preoperatively decreased expression of miR-183-5p predicts delirium development after cardiac surgery.

In previous studies, upregulation of miR-183-5p has been reported to alleviate liver and brain injury induced by ischemia–reperfusion (I/R) [26]. Moreover, exosomal miR-183-5p was revealed to protect against myocardial I/R injury by targeting forkhead box protein O1 (FOXO1), reducing apoptosis and oxidative stress in I/R cardiomyocytes and improving cardiac function [27]. Zhu et al.'s study (2020) evaluated the effects of miR-183-5p on ischemia injury using ischemic models of mouse brains exposed to transient middle cerebral artery occlusion and Neuro-2A (N2A) neuroblastoma cells exposed to oxygen–glucose-deprivation (OGD) [26]. Their study investigated ischemia, miR-183-5p expression, N2A cell viability, and apoptosis-associated proteins' expression. The results revealed that miR-183-5p expression was decreased, and brain damage was increased in ischemic mice compared with the sham group. Furthermore, N2A cells exposed to ischemia were characterized by lower miR-183-5p expression levels and increased apoptosis compared with the control group. Following the administration of agomiR-183-5p, cerebral ischemic injury and apoptosis were reduced in the stroke model and OGD-induced N2A cells.

Also, Wang et al. (2020) conducted a study to investigate the effect of miR-183-5p on brain injury in animal models [22]. Their analysis revealed that the expression of miR-183-5p decreased in the mouse brain after experimental intracerebral hemorrhage (ICH). However, mice treated with exogenous miRNA-183-5p showed improved brain edema, neurobehavioral defects, inflammation, oxidative stress, and ferrous deposition three days after ICH onset compared to control non-treated mice. As one of the miR-183-5p targets is heme oxygenase-1 (HO-1) (a molecule reported to exacerbate ICH brain injury), the authors investigated the association between agomir-183-5p and antagomir-183-5p injections, HO-1 levels, and cerebral injury. In the agomir group, the expression of HO-1 decreased significantly ($p < 0.05$), whereas there was no difference in its expression between the antagomir group and the ICH group. The study revealed that the positive impact of miR-183-5p on brain injury was related to inflammation and oxidative damage reduction via HO-1 inhibition.

Roser et al. investigated the role of microRNAs in Parkinson's disease, hypothesizing that glial cell line-derived neurotrophic factor (GDNF) may enhance the survival of dopaminergic (DA) neurons in Parkinson's disease (PD) models [28]. They demonstrated that transfecting synthetic miR-182-5p and miR-183-5p resulted in increased neurite outgrowth and provided neuroprotection for DA neurons both in vitro and in vivo, similar to the effects of GDNF. This effect was associated with decreased expression of the transcription factors FOXO3 and FOXO1 and enhanced PI3K-Akt signaling. Both of these transcription factors are known to promote neuronal apoptosis in response to oxidative stress and affect neurite growth [29].

In another study of animal models, extracellular vesicles derived from bone marrow mesenchymal stem cells carrying miR-183-5p were used to assess the impact on a diabetic

intracerebral hemorrhage. The study showed that miR-183-5p alleviated neuroinflammation and oxidative stress via the PDCD4/NLRP3 pathway [30].

Interestingly, Zhou et al. revealed that extracellular vesicle-encapsulated miR-183-5p has a protective effect against the methamphetamine-induced dependence model in mouse brains by targeting neuregulin 1 [31]. Furthermore, in the prospective cohort study conducted among concussed children with and without persistent post-concussive symptoms (PPCS), expression of miR-183-5p was evaluated over time post-concussion. The results indicated statistically significant miRNA 183-5p overexpression after concussion in children with PPCS compared to children without PPCS [32].

The present study is the first to reveal the role of miR-183-5p in the development of neurocognitive disorders. Specifically, the analysis showed that decreased miR-183-5p expression independently predisposes to postoperative delirium development among CVD patients. Available studies suggest that the mechanisms responsible for the impact of miR-183-5p on brain injury involve neuroinflammation and oxidative stress. As cardiac surgery burdens the brain's functioning, lower expression of miR-183-5p may contribute to postoperative delirium via less efficient inhibition of neuroinflammatory and oxidative stress processes. Current evidence suggests that miRNAs are involved in pathways that regulate redox biology [33]. In their experimental study, Li et al. propose that miR-183-5p specifically serves as a protective factor in neuronal survival. MiR-183-5p reduces neuronal death by regulating two distinct cell death pathways: PDCD4 (a key protein in cell apoptosis) and RIPK3 (a regulator of necroptosis) [33]. Furthermore, their study indicates that miR-183-5p is stress-inducible, meaning it acts as an immediate response factor to neuronal stress. Therefore, miR-183-5p regulates stress sensing and response in neurons and is crucial for neuron survival under stress conditions. This is a potential pathogenic pathway in the current study, where patients with lower miR-183-5p expression are more vulnerable to oxidative stress, neuronal death and delirium [Figure 2].

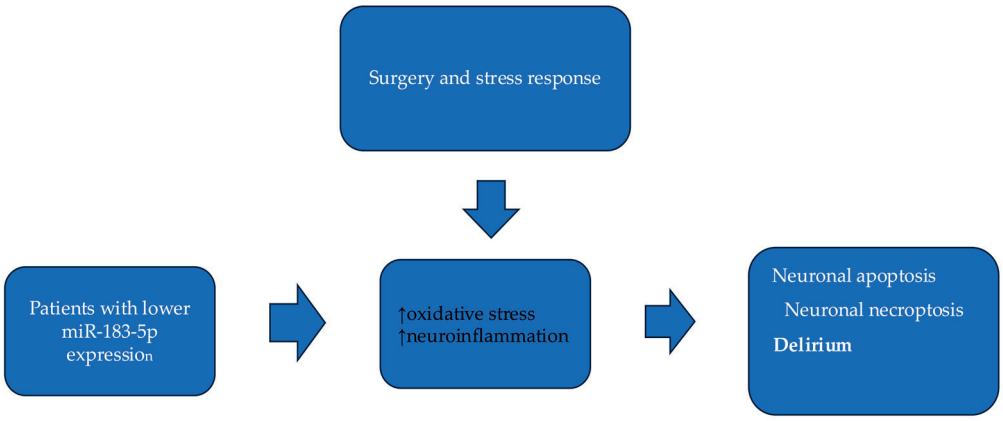


Figure 2. Proposed mechanism of miR-183-5p contributing to postoperative delirium. ↑: increase of process.

Our previous studies revealed that CABG patients with decreased preoperative antioxidant activity (less efficient antioxidative mechanisms) and those with depressive episodes complicated with lower postoperative antioxidant activity are at significantly higher risk of delirium after cardiac surgery [6]. As pre- and postoperative antioxidant capacity levels were negatively correlated with postoperative soluble receptor for advanced glycation end products (sRAGE) concentration, we concluded that sRAGE overexpression may be a protective mechanism against increased oxidative stress and subsequent cell damage. In another study, we found that individuals with less efficient baseline antioxidative mechanisms have a higher postoperative peak of myeloperoxidase (MPO), a lysosomal enzyme known for its strong pro-oxidative and pro-inflammatory properties [2]. Consequently,

these patients were more susceptible to experiencing delirium after surgery. Additionally, a higher preoperative plasma concentration of monocyte chemoattractant protein-1 (MCP-1)—a key chemokine involved in neuroinflammation and myelin degradation—has been identified as a predictor of postoperative delirium development [7]. Other studies among non-cardiac surgery patients also revealed the role of inflammation in postoperative delirium development [34,35].

The reason for decreased preoperative miR-183-5p expression in the studied population is unknown. However, the present analysis revealed that individuals with higher preoperative superoxide dismutase (SOD) concentration who did not develop delirium were characterized by increased miR-183-5p levels (a trend toward significance; $p < 0.1$). The same positive correlation was observed between preoperative SOD activity and miR-96-5p levels ($p < 0.05$). Additionally, a significant positive correlation was revealed between postoperative miR-96-5p and preoperative plasma antioxidant capacity ($p < 0.05$).

SOD is the first line of antioxidative defense, and its increased activity was associated with protective effects against mortality from cancer [36] and with lower all-cause mortality in older women [37]. Antioxidant capacity (AC) reflects the cumulative action of all antioxidants present in plasma, and its lower measures may indicate higher vulnerability to diseases related to oxidative stress.

Interestingly, in previous studies, increased miR-183-5p expression was significantly upregulated post-concussion in children with persistent post-concussive symptoms (PPCS) as compared to those without PPCS. The different distributions of the identified miRNAs in children with vs. without PPCS may signal a differential physiological response to the concussive injury or its subsequent repair [32].

Based on the mentioned findings, it can be hypothesized that specific miRNAs may have protective mechanisms against excessive oxidative stress and neuronal damage. The presence of miR-183-5p may indicate a curative role, while reduced levels of this miRNA could increase the risk of neuronal damage following cardiac surgery and other traumas. Notably, in univariate analysis, we identified additional associations, specifically between miR-34c-5p, miR-96-5p, and delirium. In Hamada et al.'s study, miR-34c-5p was significantly downregulated after ischemia, which was correlated with cognitive decline [38]. Also in our study, univariate analysis revealed lower postoperative expression of miR-34c-5p among patients with delirium. Based on animal studies, downregulation of miR34c-5p may contribute to delirium through hippocampal neuronal loss and increased neuroinflammation. A similar observation was made for miR-96-5p. Overexpression of miR-96-5p suppresses pyroptosis and reduces brain damage during the acute phase of ischemic stroke in an animal model, with the opposite effect observed when miR-96-5p expression decreases [39].

Limitations

This well-designed and innovative study has some limitations. MiRNA was measured only once before the operation and once after; there were no additional tests to evaluate how miRNA expression changed over time postoperatively. However, in the current study, we aimed to assess the predictive power of miRNA in the development of delirium. Therefore, the postoperative sampling was carried out preoperatively and on the first postoperative day, before the onset of postoperative delirium. To our knowledge, there is only one study on a group of 30 people, of whom only 12 had delirium, in which miR was assessed at three time points (start of surgery, end of surgery, and 24 h after surgery) [40].

Additionally, the entire study group consisted of patients with advanced cardiovascular disease, and there was no control group without CVD for comparison. Recent studies have shown that patients with carotid atherosclerosis exhibit higher serum levels of miR-

183-5p compared to healthy individuals [41,42]. Increased levels of circulating miR-183-5p were also detected in patients with acute coronary syndrome (ACS) and non-ST-segment elevation myocardial infarction (NSTEMI) [43,44], with the observation that serum miR-183-5p levels positively correlated with the Gensini score and hs-CRP [36]. Additionally, further investigations revealed a positive correlation between circulating miR-183-5p levels and coronary artery disease (CAD) compared to individuals without CAD [45]. According to the studies previously mentioned, miR-183-5p has a protective effect not only on neurons but also on cardiomyocytes. Our patient group consisted of individuals with CVD, making it challenging to determine whether the higher expression of miR-183-5p in those with a lower risk of delirium was linked to a cardioprotective or neuroprotective mechanism or a general effect against oxidative stress. This raises the question of whether the direct examination of miR-183-5p overexpression in neurons and cardiomyocytes should be pursued. On the other hand, our analysis revealed that only preoperative miR-183-5p was independently associated with postoperative delirium. It may suggest that the postoperative increase in miR-183-5p, related to the systemic stress response or change in CAD severity, has a lesser impact on the risk of delirium development. Furthermore, as described in the laboratory section, we initially assessed the expression levels of 754 human miRNA genes in serum samples from four patient groups: samples obtained before and after surgery from patients who developed delirium, and samples obtained before and after surgery from patients who did not develop delirium. Of note, only miRNAs with Cq < 35 were considered detected, and miRNAs with altered expression profiles were selected for further investigations. Furthermore, the severity of CAD and heart failure, as well as other perioperative factors, were included in our multivariate analysis to ensure that the expression of miR independently distinguishes between delirium and non-delirium patients. Although the study included a large number of participants, only 120 (60 with delirium and 60 without delirium) were assessed in detail, which may be a limitation. However, we entered into analysis a number of variables associated with demography, mental and physical condition, and surgery. Therefore, the impact of different potential risk factors was analyzed in multivariate comparisons.

4. Materials and Methods

The study received approval from the Ethics Committee of the Medical University of Lodz, Poland, under Approval Number RNN/95/17/KE. The procedures followed ethical standards in accordance with the Declaration of Helsinki. Adult individuals scheduled for elective cardiac surgery between April 2017 and November 2019 were eligible to participate. Patients were included if they provided informed consent and were set to undergo isolated coronary artery bypass grafting (CABG) surgery or CABG with cardiac valve replacement (CVR). Participants experienced either on-pump (with cardiopulmonary bypass (CPB)) or off-pump surgery; however, the effects of CPB on the risk of postoperative delirium were controlled for in the statistical analysis. The exclusion criteria included concomitant surgeries other than CABG or CABG with CVR; preoperative delirium; active alcohol or other substance addiction (with an abstinence period shorter than three months); illiteracy; and significant hearing or visual impairment. Participants were recruited consecutively.

4.1. Neuropsychiatric Assessment

Patients' cognitive status was assessed using the Mini-Mental State Examination (MMSE) the day before their scheduled operation [46]. Diagnostic criteria from the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) were used to diagnose Major Depressive Disorder (MDD) and anxiety disorders [47]. Each patient was screened for delirium once daily using the Confusion Assessment Method for the Intensive Care Unit

(CAM for ICU) and the Memorial Delirium Assessment Scale (MDAS), both of which evaluate the past 24 h [48,49]. After surgery, all patients were monitored in an intensive care unit, where they received specialized medical care and enhanced monitoring. If there were doubts or discrepancies in the CAM and MDAS assessments, the final consensus on delirium diagnosis was determined by the study team of physicians, who reviewed all available information. Nurses and doctors were interviewed, and clinical notes were examined for mentions of delirium or its symptoms.

4.2. Anesthesia

For the induction of anesthesia, fentanyl (5–10 mcg/kg), propofol (1–2.5 mg/kg), and rocuronium (0.6–1.0 mg/kg) were administered. During the maintenance phase, fentanyl was delivered via continuous intravenous infusion at doses of 2–10 mcg/kg/h, with propofol at 3–10 mg/kg/h and intermittent doses of rocuronium. Ventilation was provided with a breathing mixture of FiO_2 0.5 and air to maintain end-tidal CO_2 levels between 35 and 45 mmHg. From the surgical incision to connection with cardiopulmonary bypass, sevoflurane (0.5–2 vol%) was used. After the surgical intervention, patients were transferred to the ICU for continued mechanical ventilation. Until extubation, morphine was administered via continuous infusion at a rate of 1–2 mg/h, alongside propofol perfusion at 1–2 mg/kg/h for sedation. The criteria for extubation included arterial blood gases and oxygen saturation greater than 92%, along with stabilization of hemodynamic parameters. Patients were included if they signed informed consent and presented for isolated CABG surgery or CABG surgery with cardiac valve replacement (CVR). Patients with CABG underwent both on-pump (without cardiopulmonary bypass (CPB)) and off-pump (with CPB) surgery; however, the impact of CPB on the risk of postoperative delirium was controlled in the statistical analysis. The exclusion criteria were as follows: concomitant surgery other than CABG or CABG with CVR; preoperative delirium; active alcohol or other substance addiction (abstinence period shorter than three months); illiteracy; pronounced hearing and/or visual impairment. The participants were recruited consecutively.

4.3. Surgery

Patients who underwent coronary artery bypass grafting (CABG) or CABG with concurrent valve surgery were operated on using median sternotomy and placed on cardiopulmonary bypass (CPB) under normothermic conditions. All patients received anterograde Del Nido cardioplegia during the procedure. In some cases, CABG was performed off-pump, meaning the surgery was conducted on a beating heart. This approach was carried out either through median sternotomy or through a left-sided mini-thoracotomy.

4.4. Laboratory Measurements

Venous blood samples were collected on two occasions during the study period: once the day before the surgery (baseline measurement) and again on the first postoperative day, between 7:00 and 9:00 a.m. The blood samples were centrifuged at 7000 rpm for 10 min and then frozen at -80 °C until the biochemical parameters were analyzed.

4.5. Exosome Isolation

According to the manufacturer's instructions, total exosomes were extracted using the miRCURY Exosome Serum/Plasma Kit (Cat No. 76603; Qiagen, Hilden, Germany). In brief, plasma was thawed on ice and centrifuged at 3000× g for 10 min at 4 °C. Then, 800 µL of supernatant was transferred to a new tube, and 8 µL of thrombin (stock concentration of 500 U/mL) was added and incubated for 5 min at room temperature. The plasma was centrifuged at 10,000× g for 5 min, and 700 µL of the supernatant was transferred to a new tube. Then 280 µL Precipitation Buffer A was added, incubated for 60 min at

4 °C, and centrifuged twice at 500× *g* for 5 min to remove the supernatant. The pellet was resuspended in 240 µL of Resuspension Buffer for RNA extraction.

4.6. Isolation and Purification of miRNA

Total RNA, including miRNA, was extracted using the miRNeasy Mini Kit (Cat No. 217004; Qiagen, Germany) according to the manufacturer's protocol with one minor variation. After the sample was mixed with 700 µL of QIAzol Lysis Reagent, 5 µL of 5 nM cel-miR-39-3p (Cat No. 4464066, Ambion, Austin, TX, USA) was added as an exogenous spike-in control. RNA was eluted from spin columns in 30 µL of RNase-free water and stored at −80 °C until use.

4.7. Expression Profile of miRNA Genes

Broad miRNA profiling was carried out using TaqMan® Human MicroRNA Array A and B (Cat No. 4444913, Thermo Scientific, Waltham, MA, USA) as described by the manufacturer. Briefly, 3 µL of RNA extracts was reverse-transcribed using MegaplexTM RT Primers Human Pool A and B (Cat No. 4444745, Thermo Scientific) in a final volume of 7.5 µL. cDNA targets were then pre-amplified using 2.5 µL of the RT product in a 25 µL pre-amplification reaction. The PCR mixture for each array contained 450 µL of TaqMan Universal PCR Master Mix, 9 µL of diluted PreAmp product, and 441 µL of nuclease-free water. The plates were inoculated with 100 µL of the mixture into each port and sealed. Amplifications were performed using the 7900HT Fast Real-Time PCR System (Thermo Scientific) under the following conditions: 2 min at 50 °C, 10 min at 94.5 °C, and 40 cycles each for 30 s at 97 °C and 1 min at 57 °C. The expression levels of 754 human miRNA genes were assessed in serum samples of 4 patient groups: samples obtained before surgery from patients who developed delirium (n = 10), samples obtained after surgery from patients who developed delirium (n = 10), samples obtained before surgery from non-delirious patients (n = 10), and samples obtained after surgery from non-delirious patients (n = 10). Only miRNAs with Cq < 35 were considered as detected. miRNAs with altered expression profiles were chosen for further investigations.

4.8. cDNA Synthesis and Digital Quantitative PCR

According to the manufacturer's instructions, the purified total RNA was reversely transcribed into cDNA using the TaqManTM advanced miRNA cDNA Synthesis Kit (A28007, Thermo Scientific, Waltham, MA, USA). The cDNA synthesis protocol consisted of a poly(A) tailing reaction, a ligation reaction adding an adaptor sequence, and a reverse transcription reaction; 5 µL of RT product was pre-amplified to increase the amount of cDNA for all miRNAs (miR-Amp reaction) uniformly and stored at −20 °C. Selected miRNAs were profiled using digital quantitative PCR with a QX200 droplet digital PCR system (Bio-Rad, Warsaw, Poland) according to the manufacturer's instructions. The ddPCR mixture was composed of 11 µL of 2 × ddPCR Supermix for Probes (No dUTP) (Bio-Rad), 1.1 µL of appropriate TaqMan Advanced miRNA Assay (hsa-miR-96-5p (Assay ID: 478215_mir), hsa-miR-34c-5p (Assay ID: 478052_mir), hsa-miR-9-3p (Assay ID: 4782151_mir), hsa-miR-183-5p (Assay ID: 477937_mir) or hsa-miR-374b-3p (Assay ID: 479421_mir)), 8.9 µL DNase/RNase free MilliQ water and 1 µL of 10 time-diluted cDNA, in a final reaction volume of 22 µL. A Bio-Rad QX200 droplet generator was used to partition each PCR reaction into up to 20,000 nano-sized droplets by loading 20 µL of the reaction mixture and 70 µL of droplet generation oil for probes (Bio-Rad) onto matched wells of a DG8 cartridge (Bio-Rad). Then, 40 µL of the droplet/oil mixture was transferred to a 96-well plate (Bio-Rad). The plate was then heat-sealed using a PX1 PCR plate sealer (Bio-Rad) set to run at 180 °C for 5 s. The PCR was performed in a T100 Thermal Cycler (Bio-Rad). The PCR reaction setups and

thermal cycling conditions used for individual miRNA quantification are summarized in Appendix A, Table A1.

The fluorescence signals were measured by the QX200 Droplet Reader (Bio-Rad). The positive droplets containing amplified products were distinguished from negative droplets by applying a threshold above the negative droplets. Reactions with more than 10,000 accepted droplets per well were analyzed using QuantaSoft™ Analysis Pro software version 1.0.596 (Bio-Rad). Subsequently, the results were converted into copies/1 mL of the input material concerning the input plasma volume and dilutions at the RT and PCR reaction levels.

4.9. Statistical Analysis

Quantitative variables are expressed as medians and interquartile ranges (IQRs). For categorical variables, the number of observations (n) and fraction (%) were calculated. Normality was tested using Shapiro–Wilk's test for normality. Differences between two independent samples for continuous data were analyzed using the Mann–Whitney U test (since the distributions of variables were different from normal). The effect size for continuous variables was calculated with Cohen's d. For categorical variables, statistical analysis was based on the chi-squared test or Fisher's exact test. Cramer's V coefficient was calculated to assess the effect size for categorical variables. Spearman's rank correlation coefficients were calculated to determine the correlation between two quantitative variables. The minimum study sample size was calculated using the power analysis, estimating the expected effects from our previous studies and assuming an alpha level of 0.10 and a power of 80% (the minimum sample size for each group is 37 patients). To evaluate the discriminant power of miRNAs, receiver operating characteristic (ROC) curves were drawn (Area Under Curve with Standard Error was given), and optimal decision thresholds (based on Youden's index value) were found. The sensitivity, specificity, and positive and negative predictive values were calculated. Odds ratios with 95% confidence intervals were also presented. All investigated miRNAs and other factors significant in univariate comparisons ($p < 0.10$) were included in a forward stepwise logistic regression model to identify independent risk factors for delirium. The results were considered significant for $p < 0.05$. The calculations were performed using STATISTICA (version 13.3, 2017; StatSoft, Inc., Tulsa, OK, USA) and the R project (the rcompanion package, Version 2.4.15).

5. Conclusions

The current study indicates that patients with reduced preoperative expression of miR-183-5p face a greater risk of experiencing postoperative delirium. This association may be related to increased oxidative stress and neuroinflammation in this patient group.

The potential pathogenic pathway of this association may be related to increased oxidative stress and neuronal death, mediated by PDCD4 and RIPK3 regulators.

Author Contributions: Conceptualization, J.K. and K.N.; methodology J.K.; validation, E.S.-M., D.K.-W. and M.K.; formal analysis, E.S.-M. and D.K.-W.; investigation, K.N., E.F., P.M., A.P., K.W. and J.K.; resources, G.W.; data curation, G.W.; writing—original draft preparation, K.N.; writing—review and editing, K.N., J.K., M.W. and E.S.-M.; visualization, K.N.; supervision, J.K. and M.W.; project administration, J.K.; funding acquisition, J.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research has received funding from the European Union's Horizon 2020 research and innovation program TO_AITION under grant agreement No 848146 and the Polish National Science Centre under Grant No. OPUS-1, 2016/21/B/NZ5/00495.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Medical University of Lodz, Poland (RNN/95/17/KE. Approval date: 14 March 2017).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare that they have no conflicts of interest. The funders had no role in the design of the study, in the collection, analysis, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Appendix A

Table A1. The PCR thermal cycling conditions (T100 Thermal Cycler, BioRad).

	Cycling Step	Temperature, °C	Time	Ramp Rate	No of Cycles
miR_96-5p	Enzyme activation	95	10 min	1 °C/s	1
	Enzyme activation	94	30 s		50
	Annealing/extension	62.5	1 min		50
	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1
miR_34c-5p	Enzyme activation	95	10 min	2.5 °C/s	1
	Denaturation	94	30 s		40
	Annealing/extension	60	1 min		40
	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1
miR_9-3p	Enzyme activation	95	10 min	1 °C/s	1
	Denaturation	94	30 s		50
	Annealing/extension	62.5	1 min		50
	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1
miR_183-5p	Enzyme activation	95	10 min	1 °C/s	1
	Denaturation	94	30 s		50
	Annealing/extension	62	1 min		50
	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1
miR_374-3p	Enzyme activation	95	10 min	2.5 °C/s	1
	Denaturation	94	30 s		40
	Annealing/extension	60	1 min		40
	Enzyme deactivation	98	10 min		1
	Hold	4	Infinite		1

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International Journal of
Molecular Sciences

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Article

Hyperglycemia Modulates mTOR Signaling and Myelin Protein Expression in Schwann Cells

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Abstract

Diabetic peripheral neuropathy (DPN) is a common complication of diabetes, marked by Schwann cell dysfunction, demyelination, and impaired nerve regeneration. Although Schwann cells undergo phenotypic changes under hyperglycemic conditions, the underlying molecular mechanisms remain unclear. This study aimed to examine the effects of high glucose on Schwann cell phenotype and assess the involvement of the mTOR signaling pathway. Primary Schwann cells were isolated from rat sciatic nerves and cultured in media containing 5 mM (control), 25 mM, or 50 mM glucose for five days. Immunofluorescence staining and corrected total cell fluorescence (CTCF) analysis were used to evaluate expression of key markers: c-Jun, Krox-20, $p75^{NTR}$, MBP, mTOR, phosphorylated mTOR (Ser2448), and AKR1B1. Among these, significant changes were observed in MBP ($p = 0.002$), total mTOR ($p = 0.001$), and phosphorylated mTOR (Ser2448) ($p = 0.0179$), indicating impaired mTOR activation and loss of myelin protein expression. Non-significant changes in the other markers are discussed as preliminary observations. These findings highlight mTOR dysregulation and impaired myelin protein expression as central features of Schwann cell responses to hyperglycemia, which may contribute to the development of DPN.

Keywords: diabetic peripheral neuropathy; Schwann cell reprogramming; hyperglycemia; plasticity; mTOR signalling

1. Introduction

Diabetic peripheral neuropathy (DPN) is one of the most prevalent and severe complications of diabetes, affecting a large proportion of individuals living with the disease [1]. It can result in a range of debilitating outcomes, from early symptoms like paresthesia to more severe complications such as limb loss and even death. Research indicates that between 10% and 20% of diabetic patients are diagnosed with DPN at the time of their diabetes diagnosis, and an estimated 50% to 66% of patients with diabetes will develop DPN over their lifetime [2,3]. High blood glucose levels have been identified as a major contributing factor, driving oxidative stress, chronic inflammation, and cellular damage, which all play a role in the development and progression of DPN [4]. Although DPN is widespread, its

exact cause remains elusive [5]. The pathophysiology of DPN involves multiple interconnected processes, including axonal atrophy, Schwann cell demyelination, and a loss of nerve regenerative capacity. Schwann cells form the myelin sheath around axons, allowing efficient nerve signal transmission [6]. However, in diabetic patients, Schwann cells show signs of dedifferentiation and demyelination, impairing their function and hindering the ability to repair damaged nerves [7,8]. This leads to the progressive loss of nerve function and exacerbates the clinical symptoms of DPN, such as sensory loss, muscle weakness, and discomfort [9]. Several hypotheses have been proposed to explain the molecular and cellular mechanisms underlying DPN, with metabolic dysfunction [10], microvascular damage [11], and inflammatory dysregulation [12] being some of the leading theories.

Meanwhile, high blood glucose levels contribute to a toxic environment in peripheral nerves by inducing oxidative stress [13]. This oxidative stress disrupts critical cellular signaling pathways, thereby impairing Schwann cell function and diminishing the regenerative potential of peripheral nerves [14]. Among these disrupted pathways, the polyol and poly(ADP-ribose) polymerase (PARP) pathways have been extensively studied in the context of diabetic neuropathy. In contrast, pathways such as Wnt, mitogen-activated protein kinase (MAPK), and the mechanistic target of rapamycin (mTOR) have more recently emerged as important contributors to Schwann cell dysfunction and nerve degeneration under hyperglycemic conditions [15]. Under normal conditions, mTOR supports Schwann cell function by controlling cellular processes such as growth, differentiation, and myelin formation. However, in hyperglycemia, dysregulated mTOR activation contributes to Schwann cell plasticity, mitochondrial dysfunction, and dedifferentiation [16]. In Schwann cell injury response, c-Jun expression was induced by the temporary activation and upregulation of the mTORC1 signaling pathway [17], suggesting a shift toward a repair phenotype [18]. Meanwhile, Krox-20 expression, which is essential for myelination, downregulated in a repair phenotype that favors dedifferentiation and remyelination inhibition [19]. Aldose reductase, Aldo-keto reductase family 1 member B1 (AKR1B1), is also a highly expressed protein in Schwann cells that catalyzes the conversion of glucose to sorbitol and ultimately to fructose by sorbitol dehydrogenase [20]. Their activation and accumulation of sorbitol in Schwann cells under hyperglycemia play a key role in the pathogenesis and progression of DPN [20,21].

Our previous study demonstrated that in vitro cultured Schwann cells do not dedifferentiate into an immature phenotype, as traditionally thought, but rather reprogram into a distinct repair phenotype characterized by elevated c-Jun and $p75^{NTR}$ expression and reduced Krox-20 and MBP levels [7]. Building on these findings, the current study aims to further explore the molecular mechanisms driving Schwann cell plasticity under diabetic conditions by focusing on the regulatory roles of mTOR activation, c-Jun, Krox-20, and AKR1B1 in Schwann cell reprogramming during hyperglycemia.

2. Results

2.1. Immunofluorescence Analysis on the Expression of c-Jun and Krox-20, Transcription Factors That Regulate Schwann Cell Phenotypes

Figure 1A shows an in vitro culture of Schwann cells with positive expression of S100β. (Figure 1B–D) Schwann cell culture expressed an increased trend of c-Jun expression with no significant differences between the three glucose concentration groups. c-Jun expression was the highest in high glucose media (50 mM) with a mean of 2,881,296 ± 121,760 compared to 5 mM (1,867,655 ± 413,012), $p = 0.1124$, and 25 mM (1,984,390 ± 277,093), $p = 0.9582$. Meanwhile, Krox-20 expression in the 50 mM media was the lowest, with no significant differences detected as shown in Figure 1D. The mean value of Krox-20 expression in 50 mM media was (625,218 ± 54,618) compared to 5 mM (776,103 ± 112,833), $p = 0.3763$

and 25 mM (913,606 ± 22,652), $p = 0.4348$. High glucose exposure showed a non-significant trend toward increased c-Jun and decreased Krox-20 expression, changes that may suggest early features of a repair-like phenotype but did not reach statistical significance.

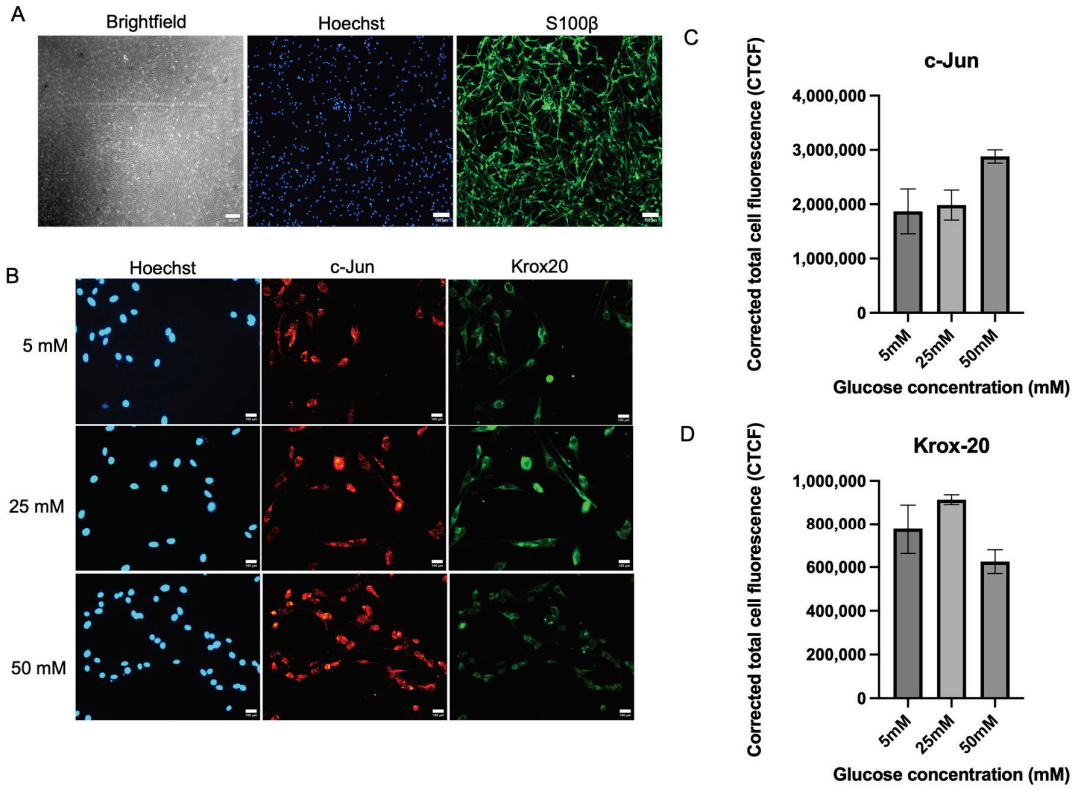


Figure 1. Expression of c-Jun and Krox20 in Schwann cells. (**A**) Brightfield picture of Schwann cell culture and its positive expression of S100β. (**B**) Representative immunofluorescence micrographs of Schwann cells expressing c-Jun and Krox20 under 40× magnification. Schwann cells have been cultured in 5 mM, 25 mM, and 50 mM of glucose for 5 days and were stained with Hoechst, c-Jun, and Krox-20. Scale bar = 100 μM. (**C**) Measurement of corrected total cell fluorescence for repair transcription factor, c-Jun expression, (**D**) Measurement of corrected total cell fluorescence for myelinating transcription factor Krox-20 expression. The mean CTCF value was presented as mean ± SD. ($n = 3$, three independent experiments).

2.2. Immunofluorescence Analysis on the Expression of p75^{NTR} and MBP in In Vitro Cultured Schwann Cells

Figure 2A shows the expression of p75^{NTR} and MBP of Schwann cells in hyperglycemic conditions. For p75^{NTR}, there were no significant differences between the culture media with three different glucose concentrations. The expression level of p75^{NTR} was expressed highest by Schwann cells in 50 mM culture media (2,564,124 ± 529,524) when compared to 25 mM (2,149,244 ± 258,103), $p = 0.7306$ by 19.35% and 5 mM (1,967,167 ± 287,152), $p = 0.5399$ by 30.35%, but no significant difference was detected. However, Schwann cells in the 5 mM media showed the highest MBP expression (801,545 ± 391,903) compared to 25 mM media (419,577 ± 14,520), with a significant difference ($p = 0.0041$) and an 81.6% increase, and 50 mM media (310,960 ± 18,573), with a significant difference ($p = 0.0020$) and a 157.8% increase.

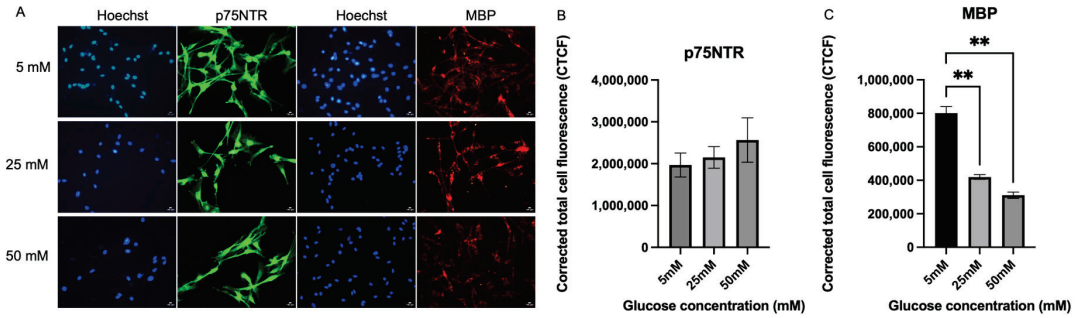


Figure 2. Expression of $p75^{NTR}$ and MBP. (**A**) Representative immunofluorescence micrographs of Schwann cells expressing $p75^{NTR}$ and MBP under 40× magnification. Scale bar = 50 µm. Schwann cells have been cultured in 5 mM, 25 mM, and 50 mM of glucose for 5 days and stained for $p75^{NTR}$ and MBP. (**B**) Measurement of corrected total cell fluorescence for $p75^{NTR}$ in three different culture media (5 mM, 25 mM, 50 mM). (**C**) Measurement of corrected total cell fluorescence for MBP in three different culture media (5 mM, 25 mM, 50 mM). The mean CTCF value was presented as mean ± SD, ** $p < 0.001$. ($n = 3$, three independent experiments).

2.3. Immunofluorescence Analysis on the Expression of Aldose Reductase in In Vitro Cultured Schwann Cells

Figure 3A shows the representative fluorescence micrograph images of Schwann cells expressing aldose reductase (akr1b1), which is the key enzyme for the polyol pathway across all three different glucose concentration media (5 mM, 25 mM, and 50 mM). The aldose reductase expressions were detected within the nucleus of the cells. There is no significant difference in akrb1 expression across the tested hyperglycemia conditions. Figure 3B shows that there were no significant differences between the expression of aldose reductase (akr1b1) in Schwann cells cultured in three different glucose media. However, expression of aldose reductase was seen as highest in the 50 mM glucose media (164,315 ± 27,988) when compared to 25 mM media (132,633 ± 29,987), $p = 0.8288$, and 5 mM glucose media (156,192 ± 26,158), $p = 0.9773$. Expression of aldose reductase (akr1b1) by Schwann cells cultured in 50 mM glucose media was 23.89% higher when compared to the Schwann cells cultured in 25 mM glucose media. A slight but non-significant increase in AKR1B1 expression was observed in Schwann cells exposed to high glucose, which may indicate a potential trend toward polyol pathway involvement.

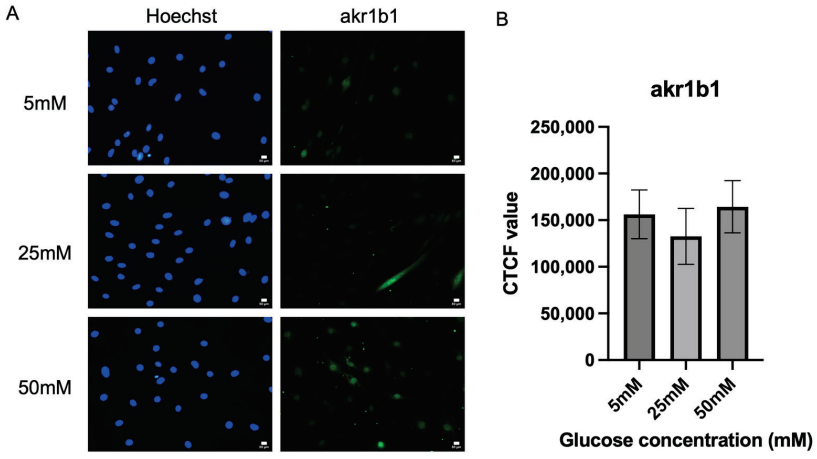


Figure 3. Expression of akr1b1. (**A**) Representative immunofluorescence micrographs of Schwann cells expressing akr1b1 under 40× magnification. Scale bar = 50 µm. Schwann cells have been cultured in 5 mM, 25 mM, and 50 mM of glucose for 5 days and stained for akr1b1. (**B**) Measurement of corrected total cell fluorescence for aldose reductase in three different culture media (5 mM, 25 mM, 50 mM). The mean CTCF value was presented as mean ± SD. ($n = 3$, three independent experiments).

2.4. Immunofluorescence Analysis on the Expression of mTOR In Vitro Cultured Schwann Cells

Following the investigation of the activation of the polyol pathway, this study also examined the expression level of mTOR. mTOR was also quantified by measuring the fluorescence intensity, as shown in Figure 4. A significant increase in mTOR expression was detected in the Schwann cells cultured in 50 mM glucose concentration media (450,901 ± 47,517), $p = 0.0015$ when compared to Schwann cells cultured in 5 mM (146,390 ± 25,282) and 25 mM glucose concentration media (159,830 ± 20,053). Schwann cells cultured in 50 mM glucose media expressed mTOR by 182.113%, significantly higher than 25 mM glucose media.

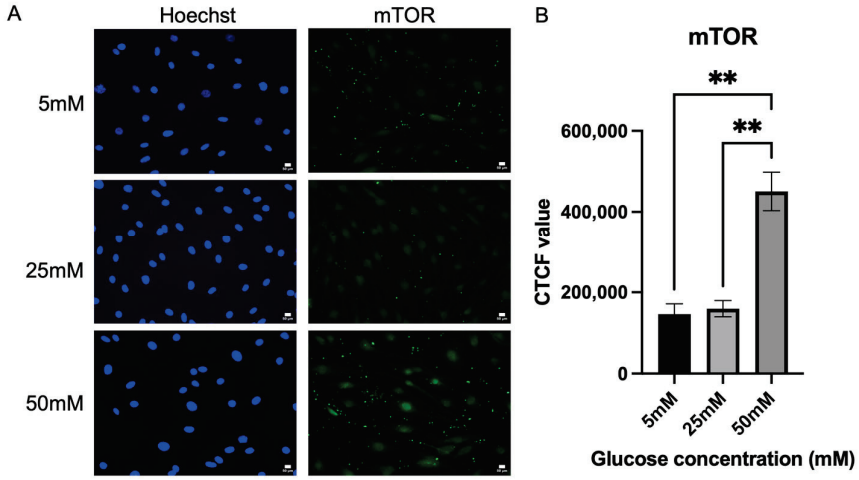


Figure 4. Expression of mTOR. (**A**) Representative immunofluorescence micrographs of Schwann cells expressing mTOR under 40× magnification. Scale bar = 50 μm. Schwann cells have been cultured in 5 mM, 25 mM, and 50 mM of glucose for 5 days and stained for mTOR. (**B**) Corrected total cell fluorescence for mTOR in three different culture media (5 mM, 25 mM, 50 mM). The fluorescence intensity of mTOR was quantified to observe the effect of hyperglycemia on the mTOR activity. The mean CTCF value was presented as mean ± SD, ** $p < 0.001$. ($n = 3$, three independent experiments).

2.5. Immunofluorescence Analysis on the Expression of Phosphorylated mTOR in In Vitro Cultured Schwann Cells

Following mTOR expression, phosphorylated mTOR was also evaluated in Schwann cells in the hyperglycemic condition. Figure 5A shows the fluorescence intensity of expression of p-mTOR by Schwann cells in the three different glucose concentration media. Figure 5B shows that there was no significant difference in the p-mTOR expression level between the three different glucose media. However, a slight increase in the p-mTOR expression level was observed in the 50 mM glucose media, which is the hyperglycemic condition with a mean of (156,208 ± 22,361), when compared to 25 mM glucose concentration (141,144 ± 20,658), an increase of 10.67%, $p = 0.8764$, and 5 mM glucose concentration (141,558 ± 21,547), $p = 0.8826$. Figure 5C shows the ratio of pmTOR/mTOR; the mean values for the 5 mm, 25 mm, and 50 mm groups were 1.273 ± 0.2402, 0.8960 ± 0.1666, and 0.3510 ± 0.0883, respectively. A statistically significant difference was observed between the 5 mm and 50 mm groups ($p = 0.0179$), indicating that the mTOR activation is inhibited as glucose concentration increases.

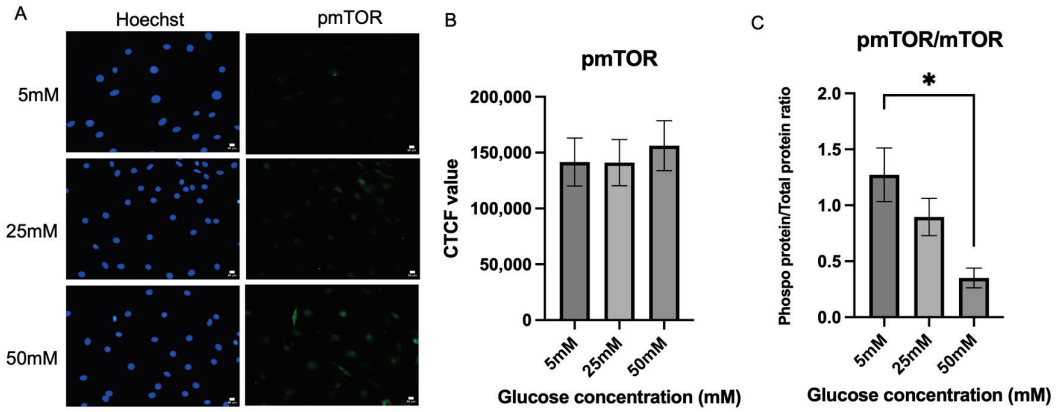


Figure 5. Expression of phosphorylated mTOR. (**A**) Representative immunofluorescence micrographs of Schwann cells expressing phosphorylated mTOR under 40× magnification. Scale bar = 50 µm. Schwann cells have been cultured in 5 mM, 25 mM, and 50 mM of glucose for 5 days and stained for phosphorylated mTOR. (**B**) Corrected total cell fluorescence for phosphorylated mTOR in three different culture media (5 mM, 25 mM, 50 mM). The fluorescence intensity of phosphorylated mTOR was quantified to observe the effect of hyperglycemia on the phosphorylated mTOR activity. (**C**) Quantification of pmTOR/mTOR ratio. The mean CTCF value was presented as mean ± SD. * $p < 0.01$, ($n = 3$, three independent experiments).

3. Discussion

Diabetic peripheral neuropathy (DPN) is a complex disorder where Schwann cell dysfunction, or Schwannopathy, plays a pivotal role in the progression of the disease [22]. Emerging evidence suggests that Schwann cell dysfunction may occur independently of axonal damage, contributing significantly to the pathology of DPN [23]. Research suggests that hyperglycemia affects Schwann cells, and in severe cases, it can cause demyelination, leading to central and peripheral sensitization in diabetic patients [24]. In line with that, we studied the plasticity of Schwann cells under high glucose conditions. This study is the first to investigate the regulation of the mTOR pathway and its interaction with key transcription factors such as c-Jun, Krox-20, $p75^{NTR}$, MBP, and AKR1B1 in Schwann cells under hyperglycemic conditions. Our study hypothesized that hyperglycemia might influence Schwann cell responses through the regulation of the mTOR pathway.

In the context of peripheral nerve injury, Schwann cells reprogram into a repair phenotype to support axonal regeneration during Wallerian degeneration, with c-Jun being selectively upregulated [18]. In our study, c-Jun expression showed a non-significant upward trend at 50 mM glucose compared to 5 mM glucose. These observations are consistent with previous studies, which reported an increase in c-Jun expression following ascorbic acid-induced peripheral nerve injury [25]. Similarly, an in vitro model of Wallerian degeneration also showed elevated c-Jun expression, indicating the reprogramming of nerves into a repair phenotype [26]. Moreover, the upregulation of c-Jun can also occur without physical nerve injury, as seen in hereditary demyelinating neuropathies, where it helps prevent the excessive secondary loss of sensory axons [27]. The absence of statistical significance in our data indicates that this finding should be regarded as a preliminary observation. Further studies with larger sample sizes are needed to confirm whether c-Jun is truly involved in Schwann cell adaptation under hyperglycemia.

On the other hand, Krox-20 expression showed a downward trend at 50 mM glucose, although this change was not statistically significant. During nerve injury, Krox-20 is downregulated to facilitate Schwann cell dedifferentiation and repair [28], and the microenvironment can further inhibit Krox-20 expression, preventing satellite glial cells from adopting a Schwann cell-like phenotype [29]. In our study, the non-significant decrease in Krox-20 may suggest a potential similarity to these injury-related changes.

Notably, clinical studies in diabetic patients support this speculation, as sural nerve biopsies have revealed Schwann cell dedifferentiation with reduced myelin proteins and increased $p75^{NTR}$ expression, along with ultrastructural changes such as segmental demyelination and axonal degeneration, closely resembling the Wallerian-like response observed after peripheral nerve injury [30,31]. Regarding $p75^{NTR}$, it is well established that this receptor is expressed during Schwann cell development and in response to peripheral nerve injury [32,33]. In our study, $p75^{NTR}$ expression showed an upward but non-significant trend under high-glucose conditions. While this aligns with reports linking $p75^{NTR}$ to an immature/repair phenotype and protective roles in neuropathy, the lack of statistical significance means our finding should be considered preliminary. The elevated c-Jun expression in hyperglycemic environments may explain the concurrent rise in $p75^{NTR}$ levels, as activation of c-Jun has been shown to regulate the expression of neurotrophin receptors, including $p75^{NTR}$, during peripheral nerve injury [34]. Further, $p75^{NTR}$ expression in Schwann cells is often associated with an immature/repair phenotype and may serve a protective role, as deficiency of this receptor has been shown to exacerbate diabetic neuropathy through immune pathway overactivation and lysosomal stress, with related miRNAs further linking $p75^{NTR}$ signaling to autophagy and mTOR regulation [35].

Conversely, MBP expression significantly declined with increasing glucose levels, indicating a reduction in the myelinating phenotype under hyperglycemic stress. These results are consistent with a previous study that reported a reduction in MBP, MAG, and P0, alongside the upregulation of $p75^{NTR}$, which has been linked to Schwann cell dedifferentiation in both in vivo and in vitro models, potentially due to sorbitol accumulation from polyol pathway hyperactivation, although the exact mechanism remains unclear [36]. Likely, a significant reduction in MBP was also reported in the oligodendrocytes that were exposed to high glucose [37]. This reduction in expression plays a crucial role in how cells respond to nerve injury, enabling Schwann cells to aid in axonal repair and regeneration [7,28]. Taken together, our significant findings highlight that hyperglycemia induces myelin protein loss through MBP downregulation, while other observed changes (such as $p75^{NTR}$ increase) remain non-significant trends requiring further validation. Collectively, these changes indicate that hyperglycemia may promote a shift in Schwann cell differentiation status. The upward trends in c-Jun and $p75^{NTR}$, both established markers of the repair phenotype, together with the significant reduction in MBP and the downward trend in Krox-20, markers of the myelinating phenotype, suggest that Schwann cells under high-glucose stress begin to reprogram away from a myelinating state toward features of a repair-like phenotype. This pattern resembles the dedifferentiation process observed during Wallerian degeneration and peripheral nerve injury, where Schwann cells adopt an immature/repair phenotype to facilitate axonal survival and regeneration. Although some changes did not reach statistical significance, the overall expression profile supports the interpretation that hyperglycemia primes Schwann cells for a reparative response.

This study also examined the effects of hyperglycemia on the expression of aldose reductase (AR), also known as AKR1B1, which serves as a key indicator of polyol pathway activation. Overactivation of AR has been linked to the accumulation of sorbitol and fructose, promoting osmotic stress, oxidative imbalance, and pro-inflammatory responses in neural tissues [38,39]. More recently, modulation of AR has been shown to attenuate microglial metabolic reprogramming and neuropathic pain in diabetic models, highlighting its functional relevance across neural and glial contexts [40]. In our study, AR expression showed only a slight, non-significant increase in Schwann cells cultured under hyperglycemic conditions, consistent with previous reports that primary Schwann cell cultures are not substantially affected by moderate hyperglycemia (20–30 mM) but respond more strongly to severe hyperosmotic stress ($\geq$100 mM [41]. The AR is not substantially

affected by moderate hyperglycemic condition (20–30 mM), but the response to severe hyperosmotic stress (≥100 mM) is due to the differences in the Schwann cell line used. The isolated primary Schwann cell culture did not increase the level of aldose reductase compared to control (5.6 mM) in the period of incubation to high glucose for 7 days, and no sorbitol accumulation was observed. However, the treatment with an AR inhibitor such as SNK-860 (fidarestat), reduced the sorbitol intracellular level in high glucose, showing that glucose is converted to sorbitol by aldose reductase. When the hyperosmotic stress elicited by the addition of sodium chloride (NaCl) or raffinose to the medium was applied, the AR activity in isolated primary Schwann cells increased [41]. This is due to the hyperosmotic conditions that can induce AR mRNA and increase AR activity in cultured cells of various origins [42–45]. In contrast, in the IMS32 cell, which is the spontaneously immortalized cell established from adult ICR mice, a significant increase in AR mRNA/protein expression was observed under exposure to high glucose (≥30 mM) conditions. However, the reason behind the increase of the polyol pathway in IMS32, but not in primary cultured Schwann cells cultured in high glucose concentration (20–30 mM), has not yet been discovered [20].

We recognize that while 72 h exposure to 25–50 mM glucose was sufficient to induce Schwann cell reprogramming markers (such as c-Jun upregulation and MBP downregulation) and to reveal alterations in mTOR phosphorylation, more prolonged exposure or more severe hyperglycemic stress may be required to fully activate apoptotic cascades or elicit stronger effects on downstream mTORC1 targets. For example, Zhu et al. (2018) reported that 50 mM glucose for 72 h inhibited the mTORC1/S6K1 pathway and induced apoptosis in Schwann cells [46], suggesting that higher doses or longer exposure may amplify these effects. Future studies will therefore examine extended incubation periods and higher glucose concentrations to determine whether the paradoxical mTOR profile observed here reflects an early adaptive response or progresses toward sustained pathway inhibition.

Our observation of increased total mTOR with a concomitant reduction in p-mTOR suggests a feedback-inhibited state in Schwann cells rather than simple pathway activation. Previous work has demonstrated that high glucose can modulate other signaling cascades in Schwann cells, such as the ERK pathway, which was shown to be activated and to influence cell viability and differentiation [47]. In addition, hyperglycemia-induced apoptosis of Schwann cells has been linked to inhibition of mTORC1/S6K1 signaling, supporting the concept that glucose stress suppresses anabolic pathways despite changes in protein abundance [46]. Interestingly, Nunes et al. (2021) reported that mitochondrial dysfunction in Schwann cells activates mTORC1 and c-Jun, as evidenced by increased phosphorylation of downstream targets S6 and 4E-BP1, despite no significant change in mTOR Ser2448 phosphorylation [16]. In contrast, our findings under hyperglycemic stress reveal increased total mTOR but reduced Ser2448 phosphorylation, suggesting a distinct feedback-inhibited state. This divergence highlights how different cellular stressors, metabolic versus mitochondrial, can differentially modulate the mTOR pathway in Schwann cells. Furthermore, oxidative and ER stress are known to induce REDD1 and activate AMPK, which in turn repress Rheb–mTORC1 signaling via TSC2, providing another mechanism for the uncoupling between mTOR expression and phosphorylation [48]. Taken together, these studies suggest that the "mTOR paradox" observed here reflects a convergence of stress-driven negative feedback and impaired Akt signaling, rather than uniform pathway activation. Future studies will therefore assess Akt phosphorylation (Thr308, Ser473), downstream mTORC1 readouts (p-S6K1 (Thr389), p-4EBP1 (Thr37/46), ULK1 (Ser757)), and regulators such as REDD1 and sestrin2 in Schwann cells exposed to high glucose, with pharmacological interventions (rapamycin, Torin-1, SC79, NAC/apocynin) to clarify the mechanistic basis of this paradox in diabetic neuropathy.

Similarly, Hei et al. demonstrated that hyperglycemia can exacerbate neuronal injury by activating components of the mTOR pathway under both normoxic and ischemic conditions [49]. Chronic mTOR activation by sustained high glucose may trigger compensatory negative feedback mechanisms that reduce its phosphorylation over time, even when total mTOR protein levels remain elevated. This could involve increased oxidative stress, depletion of upstream activators, or upregulation of endogenous mTOR inhibitors [50]. Moreover, mTOR is reported to be overactivated in obesity and insulin resistance models, which elevated serine phosphorylation of IRS-1, leading to impaired insulin signaling to Akt [51]. These findings are further supported by studies showing that mitochondrial damage in Schwann cells under diabetic conditions can lead to upregulation of both c-Jun and mTOR components without necessarily altering their phosphorylation status [16]. We propose that these apparent discrepancies reflect the tissue- and context-specific roles of mTOR signaling. In systemic metabolic disorders such as obesity and insulin resistance, chronic nutrient overload results in persistent mTORC1 overactivation, driving IRS-1 serine phosphorylation and impairing Akt signaling. By contrast, in Schwann cells, hyperglycemia and mitochondrial stress appear to induce a feedback-inhibited state, in which total mTOR and c-Jun levels are elevated but phosphorylation is not, possibly as an adaptive mechanism to limit excessive anabolic signaling under stress [16,52].

Additionally, our data showed a slight but non-significant upward trend in aldose reductase (AR) expression under high glucose conditions. While this may point to potential polyol pathway involvement, the finding does not reach statistical significance and should be considered preliminary. The polyol pathway has been reported to stimulate NADPH oxidase (Nox), a protein kinase C-dependent enzyme. Notably, hyperglycemia has been shown to increase Nox1 expression, which is typically low under physiological conditions, and Nox1 has been implicated in mTOR activation and peripheral nervous system injury in diabetic models. Hyperactivation of the polyol pathway consumes NADPH and generates excess NADH, shifting the cellular redox balance toward oxidative stress. This can enhance Nox enzyme activity, particularly Nox1 and Nox4, leading to superoxide production and contributing to Schwann cell dysfunction in diabetes [53]. In our study, however, the lack of statistical significance for AR expression indicates that its role remains inconclusive, and further studies with larger sample sizes are necessary to confirm whether polyol pathway–Nox interactions contribute to Schwann cell stress under hyperglycemia.

4. Limitations and Future Perspectives

This study has several limitations that should be acknowledged. First, the experimental work was conducted exclusively in vitro using primary Schwann cells, which may not fully replicate peripheral nerves' complex in vivo environment under diabetic conditions. This controlled setting did not account for factors such as immune cell interactions, vascular changes, and systemic metabolic influences. Future studies should therefore incorporate in vivo validation using diabetic animal models, such as streptozotocin-induced diabetic mice, to confirm whether Schwann cell reprogramming and the associated changes in c-Jun, Krox-20, MBP, $p75^{NTR}$, mTOR, and AKR1B1 expression also occur in the context of diabetic neuropathy. Protein expression can then be evaluated directly from Schwann cells isolated from diabetic nerves, providing stronger translational relevance to the findings.

Second, while the study identified changes in the expression levels of key markers such as c-Jun, Krox-20, MBP, $p75^{NTR}$, and mTOR, it did not investigate the upstream signaling pathways or downstream functional outcomes of these molecular changes. It will be essential in future work to examine whether the observed molecular reprogramming translates into measurable changes in nerve function, for example, by assessing nerve conduction velocity, axonal regeneration capacity, or behavioral outcomes in diabetic

neuropathy models. This functional validation would help establish whether the molecular signatures identified here are directly linked to impaired or adaptive nerve repair processes. Third, the study was limited by the relatively small sample size, which may have reduced the statistical power to detect more subtle but biologically relevant changes. Future studies with larger sample numbers will be needed to confirm whether these trends reflect true biological differences. Fourth, the ability of Schwann cells to sustain a repair phenotype can also be reduced by the loss of axonal contact. While axonal deprivation initially induces c-Jun upregulation and repair cell reprogramming, prolonged absence of regenerating axons results in a decline of c-Jun expression within 8–10 weeks, thereby limiting Schwann cell repair capacity [54–56]. In diabetic neuropathy, additional factors such as chronic hyperglycemia, oxidative stress, vascular insufficiency, and inflammation may further compromise Schwann cell regenerative potential, highlighting the importance of in vivo models that capture these influences. Fifth, this study did not assess gene expression profiles, which could provide deeper insights into the transcriptional regulation underlying Schwann cell reprogramming. Future studies will explore these aspects, including gene expression analysis, in vivo validation, and functional assays, to better understand how Schwann cells respond to high glucose and how this affects nerve repair.

5. Materials and Methods

5.1. Cell Isolation and Culture

Isolation of primary Schwann cells was conducted following ethical guidelines approved by the Animal Ethics Committee of the Institute of Medical Science Technology, Universiti Kuala Lumpur, under approval number AEC/MESTECH-UNIKL/2018/001. Wistar rats, aged 9 to 12 weeks, were euthanized, sterilized with 70% alcohol, and handled by ethical standards. Under sterile conditions, the sciatic nerves were carefully dissected, with the surrounding tissues and epineurium removed. The nerves were then cut into 1 mm segments and digested enzymatically with 0.05% collagenase-A for 90 min at 37 °C. After digestion, the resulting material was filtered through a sterile 40 μm cell strainer, and the cell pellet was collected and resuspended in Schwann cell isolation media. DMEM D-valine (Sigma Aldrich, Saint Louis, MO, USA Cat. No. D9443) was supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Thornton, NSW, Australia, Cat. No. A4766801), 1% GlutaMAX (Sigma Aldrich, Saint Louis, MO, USA, Cat. No. G7513), 1% Penicillin-Streptomycin (Sigma Aldrich, Saint Louis, MO, USA, Cat. No. P0781), 0.5% Amphotericin B (Gibco, Waltham, MA, USA, Cat. No. 15290026), 1% N-2 Supplement, 100× (Gibco, Waltham, MA, USA 02451, Cat. No. 17502048), 20 μg/mL Bovine Pituitary Extract (BPE; Sigma Aldrich, Saint Louis, MO, USA, Cat. No. P1476), and 5 μM Forskolin (Sigma Aldrich, Saint Louis, MO, USA, Cat. No. F6886). Schwann cells were seeded onto plates precoated with Poly-L-lysine (Sigma Aldrich, Saint Louis, MO, USA, Cat. No. P4707) and Laminin (Sigma Aldrich, Saint Louis, MO, USA, Cat. No. L4544) and cultured for 12 weeks at 37 °C with 5% CO_2 until reaching ~80% confluency.

5.2. Immunocytochemical Labeling

Cells were fixed with 4% paraformaldehyde (in PBS) and permeabilized with 0.1% Triton X-100. Then, cells were blocked with 3% bovine serum albumin (BSA) in PBS for 60 min at room temperature. Then, the samples were incubated at 4 °C overnight with primary antibodies and washed three times with PBS before incubating with fluorescent-tagged secondary antibodies for 90 min at room temperature. The primary antibodies used were anti-Krox20 rabbit (1:1000, Invitrogen, Carlsbad, CA, USA, Cat. No. PA5-112409), polyclonal anti-c-Jun mouse (1:200, Santa Cruz, Dallas, TX, USA, Cat.No.sc-166540), phospho-mTOR-S2448 Rabbit (1:200, ABclonal, Cat. No. AP0094), mTOR Rabbit (1:100, ABclonal,

Woburn, MA, USA, Cat. No. A11354), AKR1B1 Rabbit (1:200, ABclonal, Woburn, MA, USA, Cat. No. A1684), anti-MBP mouse monoclonal (Santa Cruz, Dallas, TX, USA, Cat. No. sc-271524) (1:200), and anti-$p75^{NTR}$ (Promega, Madison, WI, USA, Cat. No. G3231) (1:200). The secondary antibodies used were DyLight 549-conjugated anti-mouse IgG (1:100, Vector Laboratories, Newark, CA, USA, Cat. No. DI-2549) and goat anti-rabbit IgG fluorescein (1:100, Vector Laboratories, Newark, CA, USA, Cat. No. F1-1000). The primary antibodies were diluted in 1% BSA solution, while the secondary antibodies were diluted in 1% BSA solution containing 1% goat and 1% horse serum. Lastly, Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA, Cat. No. 62249) (1:200) was added to stain for nuclei for 30 min at room temperature and washed three times with PBS before imaging. Cells were imaged using a Nikon Eclipse Ni (Nikon Corporation, Tokyo, Japan) fluorescence microscope equipped with Lumenera's Infinity 3 digital microscopy camera (Lumenera Corporation, Ottawa, ON, Canada).

5.3. Quantitative Analysis on the Expression of the Biomarkers Using Corrected Total Cell Fluorescence (CTCF) Measurement

Quantitative analysis of biomarker expression was performed by measuring Corrected Total Cell Fluorescence (CTCF) using ImageJ software (ImageJ 1.54g), as previously described [57]. For each image, individual cells were outlined as regions of interest (ROIs), and the integrated density, area, and mean background fluorescence intensity were obtained. Background intensity was determined from three randomly selected areas lacking fluorescence in each image to normalize against autofluorescence. The CTCF was calculated using the following formula: CTCF = Integrated Density − (Area of selected cell × Mean Background Intensity). A total of three images were analyzed per group, with 30–40 cells measured, and the resulting values were exported to Microsoft Excel (Microsoft 365 Apps for Enterprise) for quantification of fluorescence intensity.

5.4. Statistical Analysis

GraphPad Prism (Version 9.0.0) was used for all statistical analyses. The Shapiro–Wilk test was preferred for determining normality due to the small sample size. For data that were not normally distributed, log transformation was applied to achieve normality, allowing for parametric tests to be conducted. All data were expressed as mean ± S.D from three independent experiments. Statistical comparisons were performed by using one-way ANOVA, with statistical significance set at $p < 0.05$.

6. Conclusions

This study provides new insights into the molecular mechanisms by which hyperglycemia affects Schwann cells in the context of diabetic peripheral neuropathy (DPN). Our findings demonstrate that high glucose conditions (50 mM) lead to a significant reduction in MBP expression, indicating a marked loss of the myelinating phenotype and initiation of Schwann cell dedifferentiation under hyperglycemic stress. This change was accompanied by significant upregulation of total mTOR expression and a concurrent reduction in phosphorylated mTOR (Ser2448), suggesting dysregulated mTOR signaling. In addition, trends of increased c-Jun and $p75^{NTR}$ expression alongside decreased Krox-20 point to a reprogramming toward a repair-like phenotype, a process inherently linked to demyelination and impaired nerve support. Taken together, these results highlight impaired myelin protein expression, repair-associated phenotypic changes, and altered mTOR pathway activity as central features of Schwann cell responses to hyperglycemia that may contribute to the development of neuropathy.

Author Contributions: Conceptualization, M.F.D. and I.M.I.; methodology, M.F.D., N.H.A.R., and U.V.; software, N.H.A.R. and U.V.; validation, N.H.A.R., U.V., J.I., and N.H.H.; formal analysis, N.H.A.R. and U.V.; investigation, M.F.D. and N.H.A.R.; resources, M.F.D., I.M.I., F.Z., and N.M.; data curation, M.F.D., N.H.A.R., U.V., and N.M.; writing—original draft preparation, N.H.A.R. and U.V.; writing—review and editing, M.F.D., N.H.A.R., U.V., and J.I.; visualization, N.H.A.R. and U.V.; supervision, M.F.D., I.M.I., F.Z., and N.M.; project administration, M.F.D. and I.M.I.; funding acquisition, M.F.D. All authors have read and agreed to the published version of the manuscript.

Funding: We acknowledge the financial support of the Fundamental Research Grant Scheme. (FRGS/1/2019/SKK08/UNIKL/02/3) from the Ministry of Higher Education (MOHE).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Institute of Medical Science Technology, Universiti Kuala Lumpur, under approval number AEC/MESTECH-UNIKL/2020/009/SEP-2019-AUG-2022., approval date: 1 September 2019.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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International Journal of
Molecular Sciences

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Evidence of Oxytosis/Ferroptosis in Niemann–Pick Disease Type C

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Abstract: Niemann–Pick Disease Type C (NPC) is a hereditary neurodegenerative disease characterized by selective cell vulnerability, particularly affecting cerebellar anterior Purkinje neurons. These neurons exhibit a distinctive pattern of degeneration due to the loss of NPC1 and/or NPC2 protein function, progressively extending towards posterior cerebellar regions. Our study aimed to explore the early factors influencing this selective vulnerability of anterior Purkinje neurons in NPC. Oxytosis/ferroptosis, a novel form of regulated cell death, has been implicated in neurodegenerative diseases, with its inhibition showing promising therapeutic potential. Our laboratory has previously identified parallels between NPC cellular pathology and ferroptotic markers, including elevated levels of lipid peroxidation and iron, mitochondrial dysfunction, and Ca^{2+} dyshomeostasis. However, whether oxytosis/ferroptosis underlies NPC cellular pathology remains unexplored. We hypothesize that loss of NPC1 function increases vulnerability to ferroptosis and that anti-ferroptotic compounds will reverse NPC cellular pathology. Through bioinformatic analyses of pre-symptomatic *Npc1*$^{-/-}$ Purkinje neurons and in vitro studies using primary dermal fibroblasts derived from NPC patients, we provide evidence suggesting that oxytosis/ferroptosis may play a pathogenic role in NPC. These findings highlight the potential of anti-ferroptotic compounds as a promising therapeutic strategy to mitigate neurodegeneration in NPC and potentially other related disorders.

Keywords: Niemann–Pick disease; ferroptosis; cerebellum; neurodegeneration; J147

1. Introduction

Niemann–Pick Disease Type C (NPC) is a rare neurodegenerative disorder caused by mutations in the genes *NPC1* (Chr.18q11.2) and/or *NPC2* (Chr.14q24.3), with *NPC1* mutations accounting for 95% of cases. While the precise roles of the NPC1 and NPC2 proteins are still under investigation, it is suggested that they serve as lipid transporters from the late endosome/lysosome to various cellular compartments [1].

The initial clinical presentation of NPC is widely heterogeneous; however, most cases result in progressive demyelination and neuronal atrophy [2]. Cell death occurs

more prominently in the anterior cerebellar lobe where Purkinje neurons, the sole output of the GABAergic neuronal pathway, exhibit high vulnerability to the loss of NPC1 [3,4]. Consequently, individuals experience motor, neurological, and cognitive deficiencies, which diminish quality of life and eventually result in premature death [5].

The anterior-to-posterior patterns of cerebellar Purkinje cell death observed in NPC are not unique to this condition. Instead, similar gradients of vulnerability are evident in multiple cerebellar degenerative disorders, with lobule X exhibiting a natural resistance [6]. These pathological similarities suggest potential commonalities among these diseases. If a shared mechanism does underlie these patterns, elucidating the pathways involved in NPC cell death could also provide valuable insights into the molecular basis of other cerebellar diseases and shed light on the complex biology of Purkinje neurons.

The prevailing hypothesis that NPC neurodegeneration is primarily driven by impaired cholesterol transport has been a focal point for research; however, cholesterol-lowering therapies have not yielded significant benefits in slowing neurodegeneration [4]. Additionally, mouse studies have shown that while cholesterol levels are uniformly elevated across all *Npc1*$^{-/-}$ cerebellar lobes, cell viability does not follow this pattern [7], indicating that other factors likely play a critical role in neurodegeneration.

Recent evidence points to a complex cellular pathology in NPC, characterized by lipid peroxidation, oxidative stress, elevated iron, and Ca^{2+} dyshomeostasis [8–11]. These conditions are reminiscent of a novel non-apoptotic regulated cell death pathway known as oxytosis/ferroptosis. Initially termed oxytosis [12], the process was renamed as ferroptosis to incorporate its iron-dependent nature [7]. Oxytosis/ferroptosis is traditionally characterized by a disruption in the cystine/glutamate transporter (system X_c^-) or the glutathione (GSH)/ glutathione peroxidase 4 (GPx4) pathway. This leads to the accumulation of toxic lipid peroxides [9] due to the inability of GPx4, often due to a loss of its essential substrate GSH, to convert these peroxides into safe lipid alcohols [13]. Additionally, the Fenton reaction, where excessive ferrous iron (Fe^{2+}) and reactive oxygen species (ROS) interact, can initiate oxytosis/ferroptosis by generating highly reactive hydroxyl radicals that can oxidize lipids [14].

Despite these mechanisms being well documented in other contexts, an analysis of their role in NPC brain pathology is lacking. Here, we propose that oxytosis/ferroptosis may be an early critical contributor to neurodegeneration in NPC.

A study by Ko et al. (2005) revealed a notable disparity in cell death among Purkinje neurons in cerebellar lobules I–III versus lobule VI of *Npc1*$^{-/-}$ mice [7]. Building on this observation, we propose the concept of a chronic, sublethal level of oxytosis/ferroptosis to explain the differential vulnerability to neurodegeneration in the NPC cerebellum. As illustrated in Figure 1, we hypothesize that loss of NPC1 function leads to reduced antioxidant defenses (i.e., reduced GSH levels) and a subsequent increase in intracellular oxidative stress in Purkinje neurons. This imbalance begins before symptom onset and accumulates over time, causing chronic cellular dysfunction that remains at sublethal levels. However, as patients age, the oxidative stress imbalance reaches a critical, irreversible point, leading to ferroptotic cell death. If that scenario is accurate, and oxytosis/ferroptosis is an early pathogenic factor in NPC, it would logically follow that Purkinje neurons in anterior lobules (I–III) are closer to this lethal threshold than those in more posterior lobules, consistent with the observed pattern of neurodegeneration.

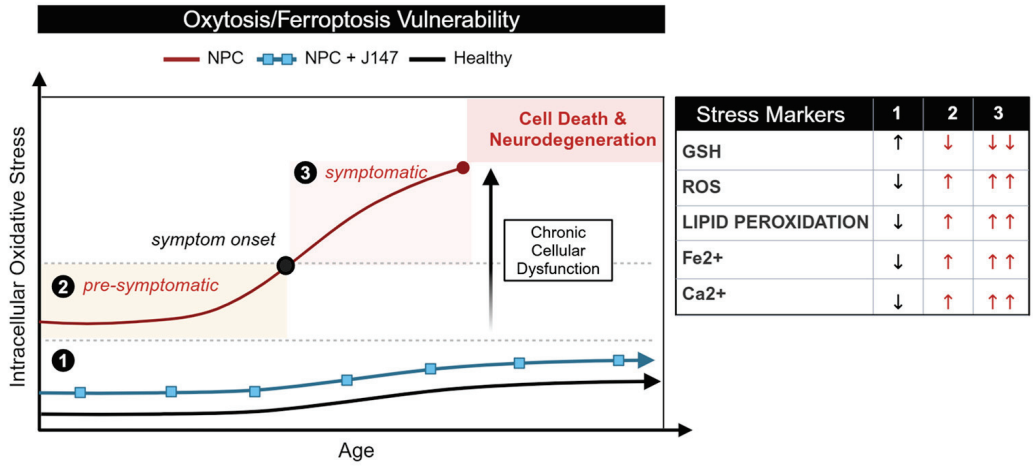


Figure 1. Proposed vulnerability of Niemann–Pick and healthy cells to oxytosis/ferroptosis. In NPC cells, a compromised antioxidant defense system (i.e., GSH reduction) leads to elevated baseline levels of lipid peroxides and reactive oxygen species (ROS). This imbalance predisposes NPC cells to oxytosis/ferroptosis more readily than healthy cells when exposed to intrinsic stress over time. In this scenario, the addition of anti-oxytotic/ferroptotic compounds, such as J147, has the potential to reduce the baseline level of oxytotic/ferroptotic stress close to that present in healthy, age-matched cells. ↑ indicates increased levels. ↓ indicates decreased levels. ↑↑ and ↓↓ indicate significantly increased or significantly decreased levels, respectfully.

As a corollary, confirmation of oxytosis/ferroptosis activation in the more vulnerable anterior lobules would conceivably open the possibility for anti-oxytotic/ferroptotic therapeutic strategies to delay pathogenesis by mitigating sublethal levels of oxytotic/ferroptotic stress to those closer to the levels in the posterior lobes. In that regard, the compound J147, generated in the laboratories of Dr. David Schubert and Pamela Maher, is effective in reversing oxytotic/ferroptotic pathology in animal models of Alzheimer's Disease [13,14]. J147 is an inhibitor of α-F1-ATP Synthase 5 (ATP5a) which reduces free fatty acids [15] and oxidative stress, maintains mitochondrial homeostasis [16], and stabilizes Ca^{2+} flux against glutamate and Ras-Selective Lethal 3 (RSL3), an inducer of oxytosis/ferroptosis that inhibits GPx4 [17]. Because those cellular pathological hallmarks are also present in NPC, compounds such as J147 have the potential for therapeutic value in NPC.

In this study, we carried out in silico analysis of pre-symptomatic Purkinje neurons isolated from anterior and posterior regions of the *Npc1*$^{-/-}$ cerebellum and provide evidence for higher vulnerability to oxytosis/ferroptosis in lobules I–III when compared to lobules VI and X, consistent with our sublethal threshold model of neurodegeneration in NPC (Figure 1). In addition, using dermal fibroblasts derived from NPC patients, we provide mechanistic evidence showing that NPC cells exhibit a constitutively higher pro-oxytotic/ferroptotic environment than age-matched healthy controls, also consistent with our model.

2. Results

2.1. Oxytosis/Ferroptosis Is Upregulated in NPC Purkinje Cells of Cerebellar Lobule III

Clinically, the NPC anterior cerebellum is the first region to exhibit cellular injury [15]. We hypothesize that this selective phenomenon reflects a differential vulnerability to oxytosis/ferroptosis in Purkinje neurons across individual cerebellar lobules.

To investigate this further, we retrieved a subset of transcriptomic data isolated from cerebellar lobules III, VI, and X of *Npc1*$^{-/-}$ (BALB/cNctr-Npc1miN/J) and control mice generated by Martin et al. (2020) [17]. In NPC, as observed in other cerebellar diseases, the anterior lobules (I–III) exhibit greater vulnerability, whereas lobule X has a higher degree

of resistance [16]. We performed a secondary analysis of these *Npc1*$^{-/-}$ cerebellar lobule datasets [17] to evaluate the presence of differential oxytotic/ferroptotic pathway activation that might correlate with neurodegeneration vulnerability.

Using Ingenuity Pathway Analysis (IPA), we discovered that the *Npc1*$^{-/-}$ cerebellar lobule III contains significant upregulation of oxytosis/ferroptosis genes (p-value < 0.05, z-score = 2.00) when compared to wildtype cerebellar lobule III (Figure 2b). In contrast, *Npc1*$^{-/-}$ cerebellar lobules VI (z-score = 0; p-value > 0.05) and X (z-score = 0; p-value > 0.05) showed no significant differences within the oxytosis/ferroptosis pathway when compared to their respective healthy controls (Figure 2b). These findings are consistent with our working model proposing the presence of sublethal levels of pro-oxytotic/ferroptotic stress (Figure 1) in the most vulnerable lobules of the NPC cerebellum. It is also noteworthy that the mice evaluated in this study were pre-symptomatic, suggesting that oxytosis/ferroptosis may be an early, and therefore therapeutically valuable, mechanism contributing to neurodegeneration in the NPC brain.

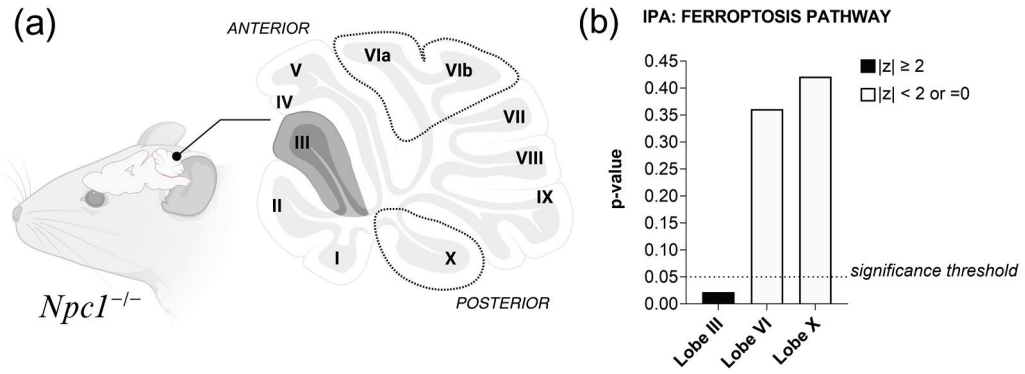


Figure 2. IPA Core Analysis of pre-symptomatic *Npc1*$^{-/-}$ cerebellar lobules. (**a**) Anatomical illustration of the *Npc1*$^{-/-}$ mouse cerebellum, depicting regions of interest potentially affected by oxytosis/ferroptosis. (**b**) Results of the IPA Ferroptosis Pathway show significant upregulation in lobule III of the pre-symptomatic *Npc1*$^{-/-}$ cerebellum (z-score= 2.00; p-value 2.23×10^{-2}) when compared to the control cerebellum lobule III. *Npc1*$^{-/-}$ cerebellar lobules VI (z-score= 0; p-value 3.61×10^{-1}) and X (z-score= 0; p-value 4.21×10^{-1}) are not significant compared to their control counterparts. Significance is determined by a z-score of ≥ 2.00 and p-value < 0.05.

To gain a more detailed understanding of the genes involved in initiating oxytosis/ferroptosis, we further investigated the oxytosis/ferroptosis pathway within NPC cerebellar lobules III, VI, and X. (Figure 3). Of the genes significantly upregulated in cerebellar lobule III, four areas were implicated in oxytosis/ferroptosis activation: the extracellular space, lysosome, endosome, and nucleus (Figure 3a). Of the 136 genes associated with the oxytosis/ferroptosis pathway, *Npc1*$^{-/-}$ cerebellar lobule III had 5 significantly upregulated genes: Ftl, Steap3, Hmox1, Cybb (Nox2), and Emp1. Three of these differentially expressed genes are significantly linked to iron regulation. Steap3 metalloreductase (Steap3) and heme oxygenase 1 (Hmox1) can contribute to the production of intracellular ferrous ions (Fe^{2+}), and increases in ferritin light chain levels (Ftl) can be a reflection of increases in intracellular iron. The increased iron can contribute to the production of ROS and lipid peroxides, as depicted in Figure 3a. Two DEGs additionally contribute to the production of ROS. Cybb, cytochrome b-245 (Nox2), is a subunit of the NADPH oxidase complex and serves as a producer of superoxide [18,19]. Emp1 regulates the expression of Nox4 that generates H_2O_2. By contrast, both lobules VI and X exhibit upregulation of a single gene, Cybb (Figure 3b,c). While Cybb may be responsible for ROS generation, this alone may not be sufficient to induce oxytosis/ferroptosis. Instead, ROS levels may remain below the sublethal threshold described in our working model (Figure 1).

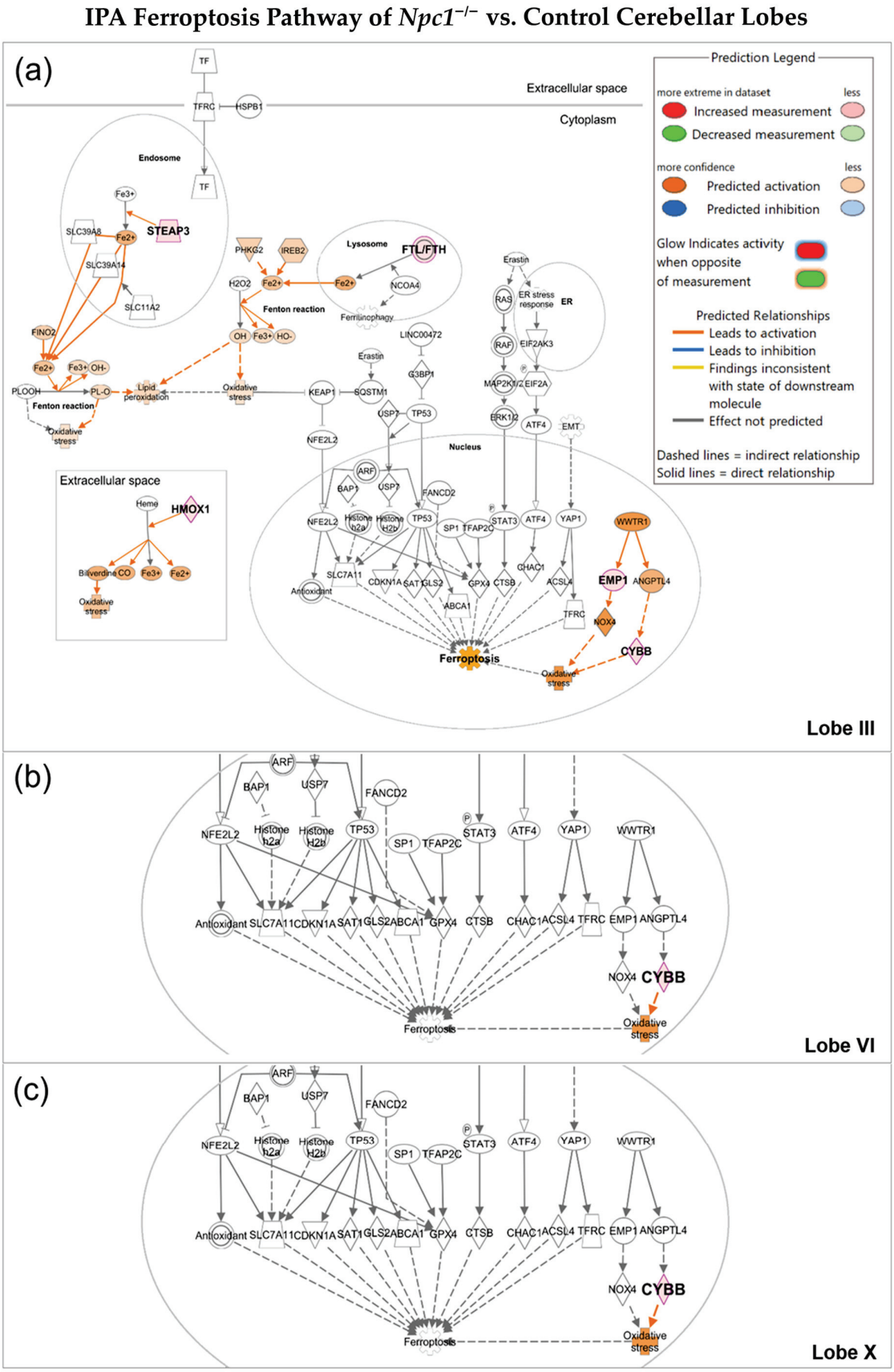


Figure 3. Overview of IPA Oxytosis/Ferroptosis Pathway. (**a**) *Npc1*$^{-/-}$ lobule III shows significant upregulation of genes reflecting iron dyshomeostasis and elevated oxidative stress when compared to wildtype control. Cybb (Nox2) is upregulated in lobules VI (**b**) and X (**c**), and both show no evidence of iron imbalance or upregulation of Nox4.

Additional analysis highlighting the differences between lobule III and lobules VI and X is presented in Supplemental Figures S2–S4. In contrast to *Npc1*$^{-/-}$ cerebellar lobule III, which exhibits dysregulated iron homeostasis (Supplemental Figure S2), lobules VI and X do not show evidence of altered iron handling or other cellular pathways associated with oxytosis/ferroptosis in these lobules (Supplemental Figures S3 and S4).

2.2. NPC Cells Are More Vulnerable to Ferroptosis than Age-Matched Controls

Despite their physiological differences, dermal fibroblasts have been widely recognized as a valuable model for studying cellular mechanisms relevant to brain cells, including lipid peroxidation, Ca^{2+} dyshomeostasis, mitochondrial dysfunction, and immune responses [8–11]. Leveraging this established model, we utilized dermal fibroblasts from NPC patients and age-matched controls to test our hypothesis that a heightened baseline of oxytotic/ferroptotic stress in NPC cells contributes to increased susceptibility to oxytotic/ferroptotic death as patients age (Figure 1).

In the context of our model, we reasoned that additional exposure to oxytotic/ferroptotic stress would reveal a higher number of NPC cells dying by oxytosis/ferroptosis than age-matched control cells, because of the higher baseline of oxytotic/ferroptotic stress in NPC (Figure 1). To begin to test this hypothesis, we treated dermal fibroblasts from NPC patients and controls with Ras-selective Lethal 3 (RSL3), a ferroptosis inducer that inhibits GPx4 activity, thereby impairing antioxidant defenses against lipid peroxidation [20,21] (Figure 4a). If NPC cells are indeed closer to an irreversible threshold of oxytotic/ferroptotic death (Figure 1), the additional oxytotic/ferroptotic stress generated by RSL3 treatment would be expected to push a greater number of NPC cells beyond that threshold. To align with our previous studies investigating the role of RSL3 in oxytosis/ferroptosis in cultured cells [22,23], we treated cells with 100 nM RSL3 at various timepoints up to 24 h and quantified cell death using the MTT assay. Figure 4b shows the effect of RSL3 on cell survival in NPC cells (6 months old; #GM17923) and healthy controls (1 year old; #GM05659). Differences in vulnerability to RSL3 are measurable at all timepoints. Figure 4c provides a visualization of the morphological changes in cells exposed to RSL3 at the corresponding time. Notably, co-treatment with the anti-oxytotic/ferroptotic compound J147 completely reversed cell death induced by RSL3 (Figure 4b,c), confirming that oxytosis/ferroptosis is the primary mechanism of cell death in this experimental context. Supplemental Figure S1 provides a comparable analysis of NPC and control cells from different age-matched individuals, further highlighting the differential response to RSL3, with NPC cells displaying increased vulnerability.

These results collectively demonstrate that NPC cells exhibit heightened vulnerability to oxytosis/ferroptosis compared to healthy controls, aligning with our working model that posits an elevated baseline of intracellular oxidative stress and, consequently, oxytotic/ferroptotic stress in NPC cells (Figure 1).

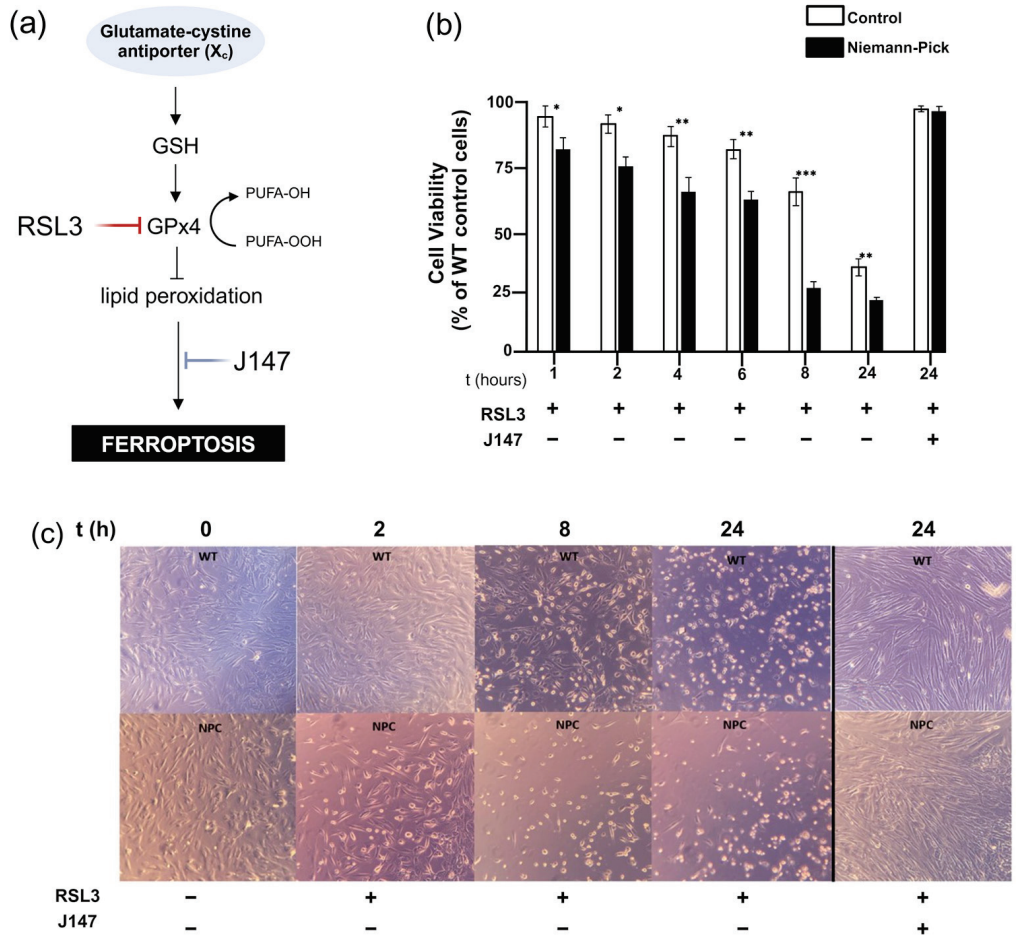


Figure 4. NPC cells show increased vulnerability to ferroptosis. (**a**) Overview of drug targets used in this study and their effects on the oxytosis/ferroptosis pathway. (**b**) MTT assay showing NPC fibroblasts died at a higher rate at all timepoints, with the most significant difference at 8 h. Error bars display SEM. Student's *t*-test was used to calculate statistical differences between NPC 6-month and WT 1-year fibroblasts (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Data are from 3 independent experiments performed in triplicate. (**c**) Representative image of NPC and healthy cells treated with 100 nM RSL3 and/or 1 µM J147.

2.3. Glutathione Peroxidase 4 (GPx4) Is Lower in NPC Compared to Age-Matched Controls

GPxs are a group of antioxidant enzymes that protect against oxidative stress, namely the reduction of free hydrogen peroxide to water and lipid peroxides into alcohol [22]. Among the eight isoforms that exist, GPx4 is the only enzyme capable of reducing esterified oxidized fatty acids and cholesterol hydroperoxides into non-toxic lipid alcohols, which has established it as a key player in the oxytosis/ferroptosis pathway [24]. To assess GPx4 expression as a possible contributor to oxytosis/ferroptosis sensitivity in NPC, Western blot analyses were conducted using dermal fibroblasts from NPC patients and controls following treatment with 100 nM RSL3 at 2, 4, and 8 h timepoints. Timepoints for 12 and 24 h were excluded from this analysis due to the high levels of cell death. In untreated (vehicle only) NPC cells, there was consistently lower expression of GPx4 compared to controls (Figure 5). Wildtype control cells exhibited a decrease in GPx4 levels at 2 and 4 h following treatment with RSL3, consistent with previously reported responses to RSL3-induced ferroptosis [25]. By 8 h, GPx4 levels in wildtype cells increased, potentially reflecting the activation of a protective mechanism to counteract ferroptotic stress. In contrast, NPC cells failed to show any increase in GPx4 levels after RSL3 treatment. This

inability to upregulate GPx4 suggests that NPC cells, possibly due to heightened sublethal oxytotic/ferroptotic stress, are unable to activate an effective protective response.

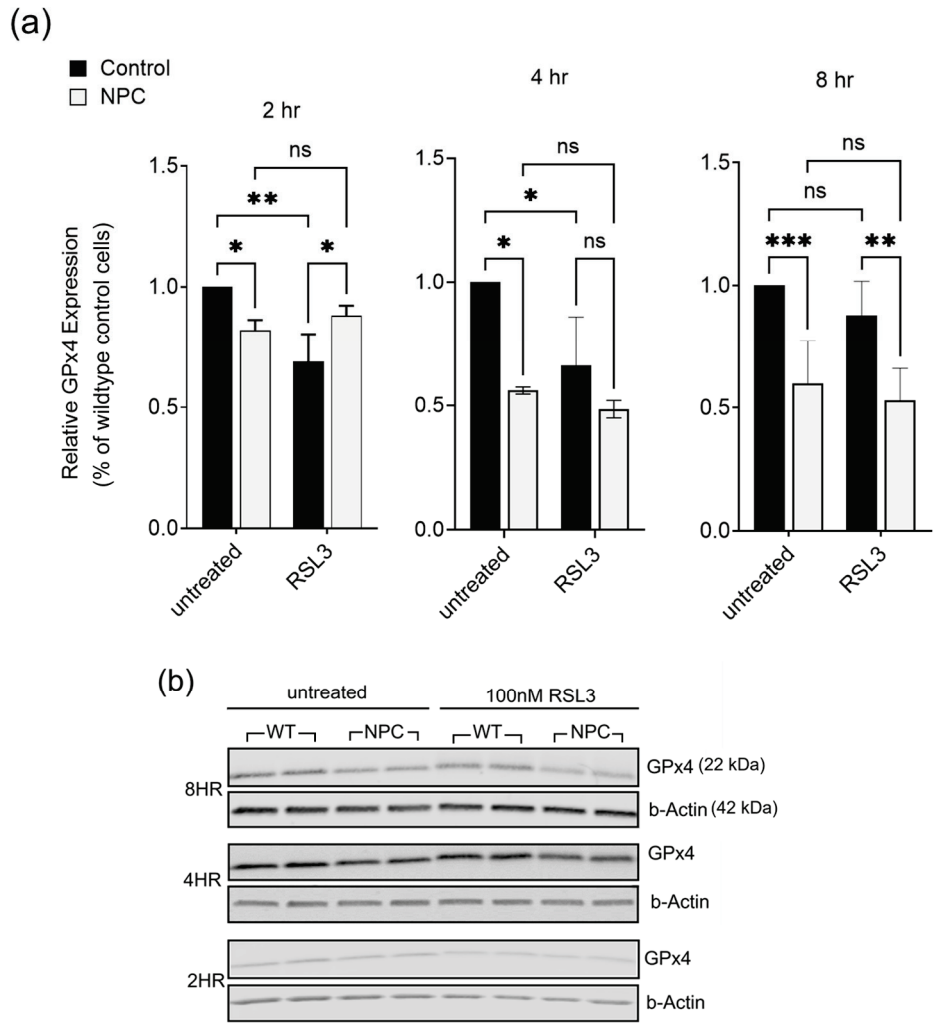


Figure 5. NPC cells express lower Glutathione Peroxidase 4 (GPx4) levels. (**a**) Relative levels of GPx4 expression across 2, 4, and 8 h following 100 nM RSL3 treatment. Experiments were run in duplicates (n = 3 per timepoint). Data were normalized to β-actin and two-way ANOVA was performed (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). ns: non-significant (**b**) Representative images of Western blots for 6-month-old NPC (#GM17923) and 1-year-old wildtype control (#GM05659).

3. Discussion

Effective treatments for NPC remain a significant challenge. While cholesterol buildup has long been proposed as the primary pathogenic trigger, therapies targeting cholesterol have largely failed to improve neurological symptoms, despite reducing systemic cholesterol levels [25,26].

Among the therapies under investigation, 2-hydroxypropyl-β-cyclodextrin (HP-β-CD), a widely used carrier molecule for lipophilic drugs, has shown limited success in clinical trials, with insufficient data on its long-term efficacy [25,26]. Recent advances include the FDA approval of Arimoclomol [27], a heat shock response activator, for use in combination with Miglustat, a glycolipid synthesis inhibitor. While Miglustat alone has only shown modest improvements in NPC [28], this combination therapy restores lysosomal function [29]. However, this approach has only been tested in patients with mild neurological symptoms [28].

In September 2024, N-acetyl-L-leucine (NALL) received approval for use in the United States. NALL offers a novel therapeutic approach by enhancing lysosomal and mitochondrial function, modulating ATP production, restoring neuronal membrane potential, and reducing neuroinflammation [30,31].

While these recent therapeutic developments mark a promising step forward in NPC symptom management [29,30], they remain palliative, addressing consequences rather than root causes of the disease, and understanding the fundamental molecular and cellular mechanisms driving NPC neurodegeneration remains a key objective for the development of evidence-based therapies. Here, we propose the hypothesis that NPC pathology may be driven, at least in part, by the early activation of the regulated cell death pathway known as oxytosis/ferroptosis. Our hypothesis is based on the shared characteristics of NPC and oxytosis/ferroptosis, including increased lipid peroxidation, elevated levels of iron, mitochondrial dysfunction, and Ca^{2+} dyshomeostasis [8–11]. Furthermore, we hypothesize that the activation of cell death by oxytosis/ferroptosis in NPC is not a binary event, but rather the culmination of iron-driven oxidative stress that contributes to the differential cell vulnerability as described in our sublethal threshold model (Figure 1). At early stages prior to reaching that threshold, this heightened vulnerability is potentially reversible, as our own data (Figure 4) and multiple studies in other neurodegenerative conditions [32,33] using the anti-oxytotic/ferroptotic compound J147 suggest. However, as oxidative stress continues to increase with age and the antioxidant systems become unable to counteract it, there is a transition to cell death (Figure 1).

Given the distinct spatiotemporal neurodegeneration in the cerebellum in NPC, which begins in the anterior lobules I–III and progresses to more posterior lobules, it is plausible that this pattern reflects varying levels of sublethal oxytotic/ferroptotic stress, as hypothesized in Figure 1. Higher oxytotic/ferroptotic stress in the anterior lobules, such as increased iron, lipid peroxidation, and ROS, may lead to earlier activation of cell death. Our secondary in silico analysis of pre-symptomatic *Npc1*$^{-/-}$ cerebellar data generated by Martin et al. (2020) [17] supports this model. Specifically, IPA analysis of *Npc1*$^{-/-}$ Purkinje neurons of anterior lobule III reveals significant activation of oxytosis/ferroptosis, whereas lobules VI and X are not affected (Figure 3). Among the genes upregulated in *Npc1*$^{-/-}$ lobule III, Steap3, Hmox1, and Flt are key regulators of iron homeostasis, while Emp1 and Cybb are involved in ROS generation. The dysregulation of iron homeostasis coupled with increased ROS production could thereby promote conditions favorable to an increase in lipid peroxidation [14]. In contrast to *Npc1*$^{-/-}$ anterior lobule III, cerebellar lobules VI (Figure 3b) and X (Figure 3c) both share a single upregulated gene in the oxytosis/ferroptosis pathway, Cybb, that alone is not predicted to increase oxytotic/ferroptotic cell death. Aligning with our hypothesis, we conclude that upregulation of genes involved in Fe^{2+} regualtion and ROS production supports the idea that the pre-symptomatic anterior lobule III is in an early state of sublethal oxytotic/ferroptotic stress while posterior lobules VI and X have yet to be affected in pre-symptomatic *Npc1*$^{-/-}$ mice.

Additional evidence of a link between oxytosis/ferroptosis and NPC was identified in hepatocytes [34] and HEI-OC1 cells [35]. While these findings were not reported in the brain, they represent additional evidence that NPC mutations can lead to ferroptosis. In addition, IP-10/CXCL10 impairs neuronal function in a ferroptosis-dependent manner and induces lipid peroxidation in neurons; these phenotypes are exacerbated by ferroptotic agents and reversed by ferroptosis inhibitors [36]. These findings are relevant because we have reported IP-10/CXCL10 in the *Npc1*$^{-/-}$ cerebellum as the only upregulated cytokine at 3 weeks of age, prior to symptom onset, therefore providing additional evidence for the role of ferroptosis early in NPC pathogenesis [9].

In summary, we demonstrate that NPC cells exist in a heightened oxytotic/ferroptotic environment, making them more vulnerable to cell death via this pathway. We propose that this vulnerability may help explain the selective neurodegeneration observed in anterior Purkinje neurons. Future research should prioritize validating these results in vivo and investigating the efficacy of anti-ferroptotic therapies, which could open new avenues for treating NPC and other neurodegenerative disorders associated with oxytosis/ferroptosis.

4. Materials and Methods

4.1. Cell Lines

Patient-derived NPC dermal fibroblasts (6 months old, #GM17923; 4 years old, #GM17921) and wildtype healthy controls (1 year old, #GM05659; 5 years old, #GM05381) were purchased from Coriell Institute for Medical Research (Camden, NJ, USA). Cell lines were cultured in Dulbecco's Modified Eagle Medium (11965092;Thermo Fisher Scientific, Pittsburgh, PA, USA) supplemented with 5% fetal bovine serum (R&D Systems, Minneapolis, MN, USA) and 1% PenStrep (15140-122; Thermo Fisher Scientific). Cells were maintained in a humidified chamber at 37 °C with 5% CO_2. For subsequent experiments, cell populations were seeded at ~30% confluency and treated at ~80% confluency.

4.2. Ingenuity Pathway Analysis

Transcriptomic data for cerebellar Purkinje neurons in lobules III, VI, and X of *Npc1*$^{-/-}$ (BALB/c$^{Nctr\text{-}Npc1miN/J}$) and *Npc1*$^{+/+}$ mice were sourced from Martin et al. (2020) [17]. Differentially expressed genes (DEGs) from the wt-mut_III-III, wt-mut_VI-VI, and wt-mut_X-X datasets were selected based on an adjusted p-value < 0.05 and log2 fold change greater than 0.58 (>1.5-fold increase) or less than −0.58 (< 1.5-fold change), as described by Martin et al. (2020) [17]. DEGs from each dataset were imported into Ingenuity Pathway Analysis Version 24.0.2 (IPA, Qiagen, Redwood City, CA, USA) to look at Canonical Pathways and Mechanistic Maps. Significant activation or inhibition of a Canonical Pathway was determined by a z-score of $|Z| \geq 2$ and p-value < 0.05.

4.3. MTT Cytotoxicity Assay

Cell viability was determined by the MTT Assay Kit (ab211091; Abcam, Cambridge, UK) according to the manufacturer's instructions. NPC dermal fibroblasts and controls were harvested at approximately 80% confluency and seeded onto 96-well plates. Plates were incubated in a humidified chamber at 37 °C with 5% CO_2 for 24 h. After incubation, the cell culture medium was discarded and treated with various doses of RSL3 (0.1, 0.25, 0.5 μM, or 1 μM) or vehicle alone. Following treatment for the indicated times, the cells were incubated with MTT at 37 °C for 3 h and then MTT solvent for 15 min. Absorbance was measured with a Spectra Max i3X and read with Soft Max Pro (version 6.5.1) at 590 nm. Percent cytotoxicity was calculated using (100 × (control-sample)/control).

4.4. Western Blot Analysis

Cells were lysed using 1x RIPA buffer (50 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, 1% Sodium Deoxycholate, 0.1% SDS) and supplemented with Phosphatase Single-Use Inhibitor Cocktail 1: 10 (RC232651; Thermo Fisher Scientific, Pittsburgh, PA, USA). Cells were collected and sonicated 1× for 3 s. Samples were centrifuged at 13,000× g for 20 min at 4 °C. The supernatant was collected, and protein concentrations were determined using the Pierce BCA protein assay kit, following the manufacturer's instructions. Cell protein lysates were separated on a 4–20% Novex Tris-Glycine Plus WedgeWell Gel using Invitrogen MiniBlot for 1 h at 120V. Proteins were transferred to a 0.45 μM nitrocellulose membrane (1212602; GVS North America, Sanford, ME, USA) for 45 min at 120V. Membranes were

blocked with 3% milk in Tris-buffered saline with 0.05% Tween® 20 Detergent (TBS-T) at room temperature for 1 h. Following 0.5% TBS-T washes (3×), membranes were probed overnight at 4 °C with GPx4 at 1:1000 (Abcam, 125066) with β-actin (MA1-140; Invitrogen, Waltham, MA, USA). The molecular weight of the protein bands was determined using a Color-coded prestained protein marker (74124S; Cell Signaling Technology, Danvers, MA, USA).

4.5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 10.2.0 (Graphpad Software, La Jolla, CA, USA). Data are represented as the mean ± SEM. Analyses were performed using an unpaired, two-tailed Student *t*-test in two-group comparisons or two-way ANOVA for multiple-group comparisons. Values of $p < 0.05$ were considered statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ijms26072915/s1.

Author Contributions: Conceptualization, S.S. and K.L.S.; methodology, S.S., K.L.S., and J.K.; software, K.L.S.; validation, K.L.S.; formal analysis, K.L.S.; resources, S.S.; data curation, K.L.S.; writing—original draft preparation, K.L.S. and S.S.; writing—review and editing, S.S., K.L.S., J.K., J.B.W., A.T., N.P.R., D.W.A., A.N.S., S.D.S., A.C., D.S.-C. and P.M.; supervision, S.S.; project administration, S.S.; funding acquisition, S.S. All authors have read and agreed to the published version of the manuscript.

Funding: K.L.S. was funded by the National Institutes of Health, grant number R25GM060507. A.C. was funded by the National Institutes of Health, grant number R01AG067331. P.M. was funded by NIH grants AG069206 and AG067331. D.S.-C. was funded by the Bundy Foundation. The LLUSM Advanced Imaging & Microscopy Core facility was funded by the National Institutes of Health, grant number S10OD034427-01.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

NPC	Niemann–Pick Disease Type C1
AD	Alzheimer's Disease

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International Journal of
Molecular Sciences

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α-Synuclein Degradation in Brain Pericytes Is Mediated via Akt, ERK, and p38 MAPK Signaling Pathways

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Abstract: Parkinson's disease (PD) is characterized by widespread distribution of Lewy bodies, which are composed of phosphorylated and aggregated forms of α-Synuclein (α-Syn), in the brain. Although the accumulation and propagation of α-Syn contribute to the development of PD, the involvement of the blood–brain barrier (BBB) in these processes remains unknown. Pericytes, one of the cell types that constitute the BBB, degrade various forms of α-Syn. However, the detailed mechanisms involved in α-Syn degradation by pericytes remain poorly understood. Therefore, in this study, we aimed to determine the ability of the BBB-constituting cells, particularly primary cultures of rat pericytes, brain endothelial cells, and astrocytes, to degrade α-Syn. After α-Syn uptake by the cells, intracellular α-Syn decreased only in pericytes. This pericyte-specific α-Syn decrease was inhibited by an autophagy inhibitor, bafilomycin A1, and a proteasome inhibitor, MG132. siRNA-mediated knockdown of degradation enzymes or familial PD-associated genes, including cathepsin D, DJ-1, and LRRK2, did not affect α-Syn clearance in pericytes. However, pharmacological inhibitors of Akt, ERK, and p38 MAPK inhibited α-Syn degradation by pericytes. In conclusion, our results suggest that α-Syn degradation by pericytes is mediated by an autophagy–lysosome system and a ubiquitin–proteasome system via α-Syn-activated Akt, ERK, and p38 MAPK signaling pathways.

Keywords: alpha-Synuclein; pericyte; Parkinson's disease; blood–brain barrier; autophagy; ubiquitin–proteasome system; Akt; ERK; p38 MAPK

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by the aggregation and accumulation of α-Synuclein (α-Syn), a protein involved in synaptic function and neuronal survival, in the brain [1]. The neuropathological hallmark of PD includes the progressive loss of substantia nigra dopaminergic neurons and presence of Lewy bodies [2,3]. The loss of dopaminergic neurons leads to dopamine depletion, which is a major cause of PD motor symptoms (bradykinesia, tremor, rigidity, and postural instability). The primary therapy for PD is dopamine replacement therapy, with levodopa serving as the gold standard for PD treatment. However, owing to the incomplete understanding of PD pathogenesis, there is currently no treatment that can stop or hinder PD progression.

Aggregation of α-Syn, a small protein (14 kDa) that predominantly exists as a soluble monomer [2,4], is associated with PD. Genetic and environmental factors modulate monomeric α-Syn aggregation [5,6]. These factors contribute to the formation of insoluble and highly toxic α-Syn aggregates, such as oligomers, fibrils, and ribbons, leading to the formation of Lewy bodies [7,8]. The progressive accumulation of α-Syn aggregates leads to neurodegeneration and neuronal cell death [9,10]. In addition to α-Syn aggregation, several postmortem studies have shown that tau is co-localized with α-Syn in Lewy bodies in PD brains, suggesting that tauopathies are associated with the pathogenesis of PD [11]. Neuroinflammation contributes to the pathophysiology of PD and other Parkinsonian syndromes, including multiple system atrophy (MSA) and progressive supranuclear palsy (PSP) [12–14]. Differences in inflammatory factors have been revealed in various Parkinsonian syndromes [13,15]. Microglial activation has been observed in the brains of patients with these synucleinopathies and tauopathies. Neurodegeneration and cell death in these diseases are partly caused by inflammatory responses due to exposure to pathogenic proteins, such as aggregated α-Syn and tau. These pathogenic proteins also induce microglial activation to release various inflammatory cytokines [12,16]. In turn, activated microglia facilitate α-Syn aggregation, oligodendrocyte apoptosis in MSA, and tau deposition in PSP [12–14]. In addition, a recent study revealed that α-Syn-mediated neurotoxicity is induced by the interaction of tissue plasminogen activator with N-methyl-D-aspartate receptor 1 [17]. Thus, the removal of α-Syn aggregates and the prevention of their formation are considered promising disease-modifying strategies for synucleinopathies, such as PD.

α-Syn is a neuronal protein that predominantly localizes to presynaptic nerve terminals [18,19]. α-Syn is propagated from neuron to neuron through tunneling nanotubes or extracellular release [20–22] as well as between neuron and glial cells, such as astrocytes and microglia [23–25]. Normally, these cells degrade intracellular α-Syn via the autophagy–lysosome pathway (ALP) and the ubiquitin–proteasome system (UPS) [26–31]. However, lysosome and proteasome activity tend to decline with age, leading to α-Syn accumulation in the brain [32–35]. Dysfunction of these degradation systems and α-Syn inclusions in neurons and glial cells have been observed in the brain of patients with PD [36–38], suggesting that disruption of the α-Syn clearance system in the brain is involved in the pathogenesis of PD.

In addition to neurons and glial cells, pericytes, which are located at the wall of cerebral capillaries, take up and degrade α-Syn aggregates [39,40]. In our previous in vitro study, we showed that pericytes take up α-Syn and then release various inflammatory mediators to induce blood–brain barrier (BBB) dysfunction [41]. The location of pericytes enables these cells to receive α-Syn through two primary pathways: (1) direct transfer from neurons and glial cells through tunneling nanotubes and extracellular release [42,43] and (2) transport from circulating blood across the BBB [44]. BBB disruption may contribute to blood-borne α-Syn penetration into the brain [45]. Furthermore, in PD brains, α-Syn aggregates are present in pericytes, microglia, and astrocytes [46], indicating that α-Syn degradation in pericytes is impaired in PD.

Although it is clear that pericytes possess α-Syn phagocytic or degradative abilities, the detailed molecular mechanisms remain unclear. Therefore, in this study, we aimed to determine the pericyte-driven mechanism of α-Syn clearance and elucidate the involvement of pericytes in PD pathogenesis. Furthermore, we aimed to identify the molecules involved in α-Syn clearance by pericytes.

2. Results

2.1. Time-Course of Oligomeric and Monomeric α-Syn Uptake by Pericytes, Brain Endothelial Cells, and Astrocytes

Western blot analysis (Figure 1A) showed that recombinant human α-Syn had a 17 kDa band, indicating that recombinant α-Syn contained mainly monomeric α-Syn (17 kDa). Several lots of HiLyte488-conjugated human recombinant α-Synuclein (HL-α-Syn, AnaSpec Inc., Fremont, CA, USA) exhibited a broad band, containing both monomeric (17 kDa) and oligomeric α-Syn (>17 kDa; Figure 1A). Therefore, in this study, we employed HL-α-Syn to evaluate the monomeric and oligomeric α-Syn uptake ability of BBB-constituting cell types (pericytes, rat brain endothelial cells (RBECs), and astrocytes) using Western blot.

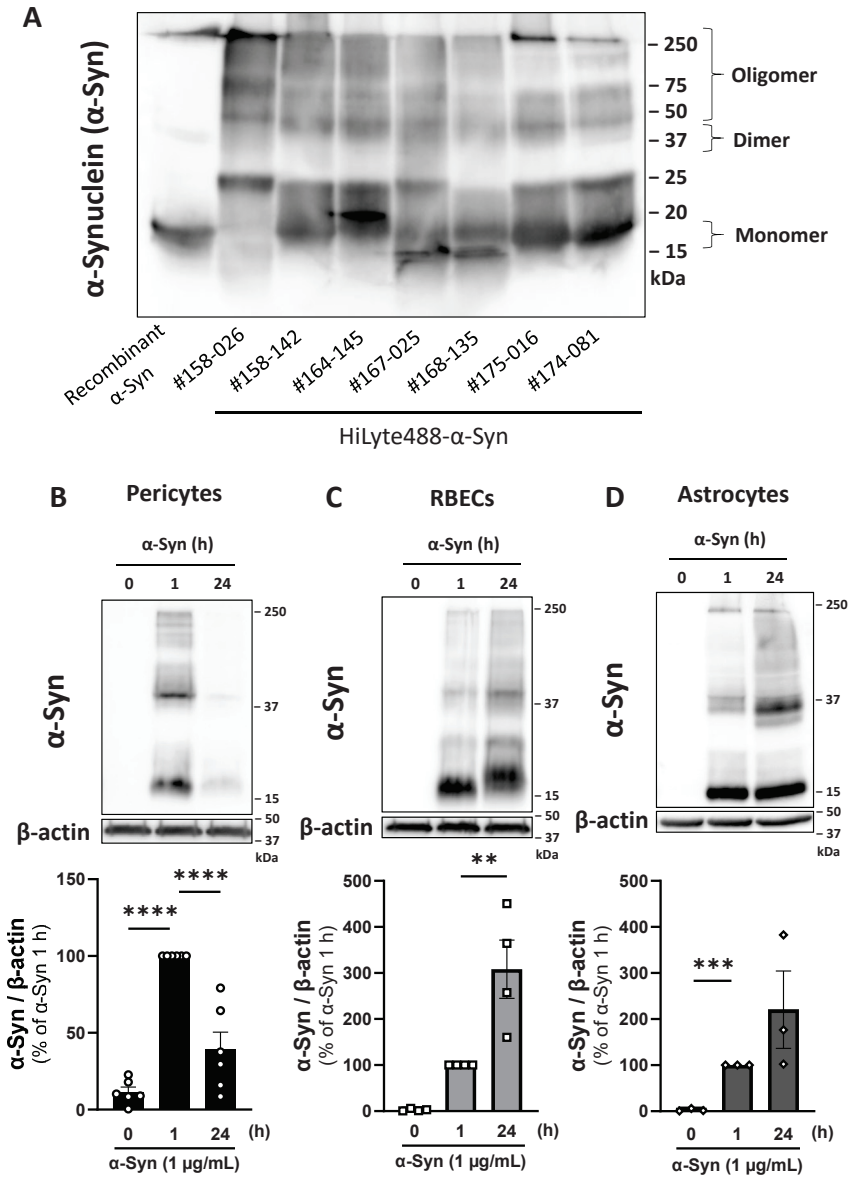


Figure 1. Time-course of α-Syn uptake by pericytes, RBECs, and astrocytes. (**A**) Representative Western blot images showing the molecular weight of α-Syn included in recombinant human α-Syn and each lot of HiLyte488-α-Syn (HL-α-Syn; AnaSpec Inc.). α-Syn (1 μg/lane) was loaded onSDS-PAGE, and α-Syn immunoreactivities were detected with an anti-α-synuclein antibody (MJFR1). (**B**–**D**) Intracellular α-Syn in pericytes (**B**), RBECs (**C**), and astrocytes (**D**) at 0, 1, and 24 h after HL-α-Syn (1 μg/mL) treatment. The top of each panel shows representative Western blots of α-Syn

(~17 kDa) in cell lysates. Cell lysates were loaded on SDS-PAGE, and α-Syn was detected with an anti-α-synuclein antibody (MJFR1). β-actin was used as a loading control. The lower panel shows quantitative analysis of Western blots using densitometry. The results are expressed as the percentage of each corresponding α-Syn level at 1 h. Values are the means ± SEM (n = 3–6). Statistical analysis was performed using Dunnett's multiple comparisons test. (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

The uptake of α-Syn by pericytes, RBECs, and astrocytes was evaluated over time. The cells were HL-α-Syn (1 μg/mL) treated for 24 h. Pericytes took up oligomeric and monomeric α-Syn within 1 h. Following HL-α-Syn exposure, intracellular levels of oligomeric and monomeric α-Syn in pericytes at 24 h were significantly lower (60%) than those at 1 h (Figure 1B). Intracellular α-Syn levels increased in RBECs and astrocytes with increasing time. Following HL-α-Syn exposure, α-Syn levels in RBECs and astrocytes at 24 h were four- and two-fold higher than those at 1 h (Figure 1C,D).

2.2. Degradation of α-Syn in Pericytes Is Mediated Through the Autophagy–Lysosome Pathway and Ubiquitin–Proteasome System

To determine whether the decrease in α-Syn in pericytes at 24 h after exposure was due to α-Syn degradation via autophagy and the ubiquitin–proteasome pathway, pericytes were treated with HL-α-Syn in the presence of bafilomycin A1 (lysosome–autophagy inhibitor) or MG132 (ubiquitin–proteasome inhibitor) for 24 h. Bafilomycin A1 (50 and 100 nM) significantly increased both oligomeric and monomeric α-Syn in pericytes (241.9 and 285.3% of vehicle, respectively; Figure 2A). The autophagy markers p62 and LC3 significantly increased in pericytes treated with 10 to 100 nM bafilomycin A1 (p62; 430.7, 445.4, and 357.8%, LC3; 534.2, 478.8, and 383.6% of vehicle; Figure 2B,C). To further investigate the involvement of autophagy in α-Syn degradation by pericytes, pericytes were treated with HL-α-Syn and an autophagy promoter, rapamycin (10–100 nM), for 24 h. Rapamycin (100 nM) significantly decreased α-Syn in pericytes (55.01% of vehicle; Figure 2D). Furthermore, pericytes were treated with the ubiquitin–proteasome inhibitor MG132 (50 and 100 nM), resulting in a significant increase in monomeric and oligomeric α-Syn in pericytes during 24 h exposure to HL-α-Syn (164.3 and 133.8% of vehicle; Figure 2E). These results suggest that the degradation of monomeric and oligomeric α-Syn by pericytes is mediated by autophagy and the ubiquitin–proteasome system.

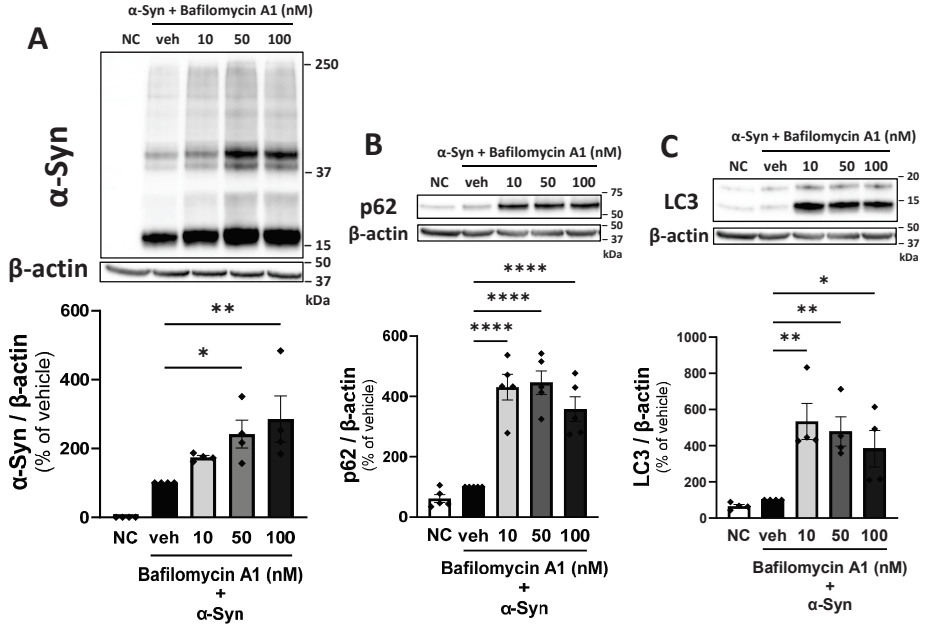


Figure 2. *Cont.*

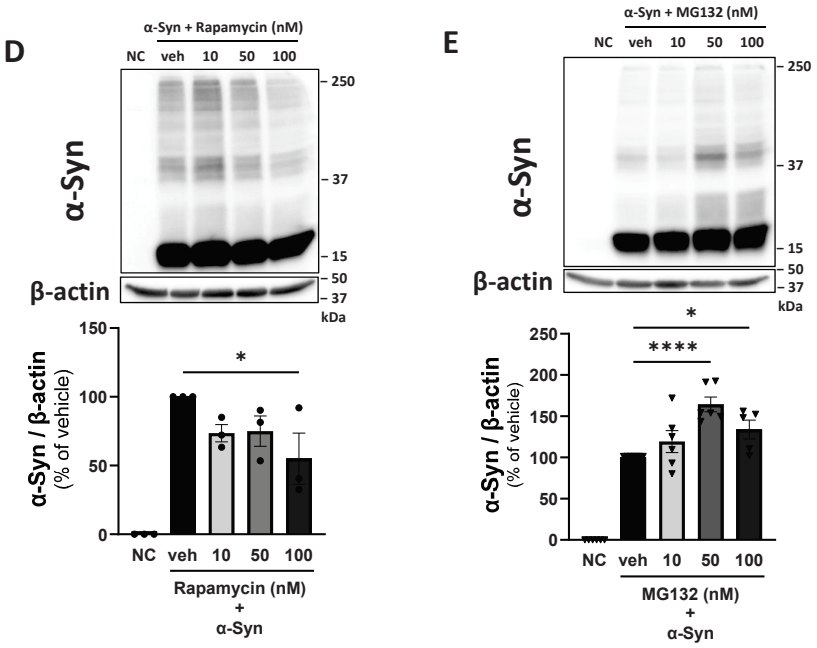


Figure 2. α-Syn degraded in pericytes through the autophagy–lysosome pathway and ubiquitin–proteasome system. (**A**–**C**) Effect of bafilomycin A1 on intracellular α-Syn (**A**) and expression of p62 (**B**) and LC3 (**C**) in pericytes. (**D**) Effect of rapamycin on intracellular α-Syn in pericytes. (**E**) Effect of MG132 on intracellular α-Syn in pericytes. Pericytes were treated with HL-α-Syn (1 μg/mL) in the presence of bafilomycin A1 (autophagy–lysosome inhibitor; 10–100 nM) (**A**–**C**), rapamycin (autophagy inducer; 10–100 nM) (**D**), and MG132 (ubiquitin–proteasome inhibitor; 10–100 nM) (**E**) for 24 h. Each panel shows representative Western blot images and quantitative analysis. β-actin was used as a loading control. The results are expressed as the percentage of the corresponding vehicle-treated pericytes, which were incubated with HL-α-Syn alone for 24 h. NC indicates non-treated pericytes as a negative control. Values are the means ± SEM (n = 3–6). Statistical analysis was performed using Dunnett's multiple comparisons test. (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

2.3. Pericytes Decrease Internalized α-Syn Intracellularly via Degradation

To assess whether pericytes secreted intracellular α-Syn into the extracellular space, we measured α-Syn levels in both cell lysates and culture supernatants up to 6 h following 1 h incubation with HL-α-Syn (Figure 3A). One hour after HL-α-Syn exposure, intracellular α-Syn in pericytes decreased significantly in a time-dependent manner (1 h: 45.31%, 3 h: 29.90%, 6 h: 12.79% of 0 h; Figure 3B). However, α-Syn was not detected in pericyte-derived supernatants, regardless of exposure time (Figure 3C). Intracellular levels of α-Syn in RBECs and astrocytes did not decrease (Figure 3D,E). These findings suggest that α-Syn uptake by pericytes and subsequent α-Syn clearing occur via the protein degradation system rather than through extracellular secretion.

To confirm whether the decrease in intracellular α-Syn following α-Syn uptake over 6 h is inhibited by bafilomycin A1 and MG132, pericytes were treated with bafilomycin A1 (10–100 nM) and MG132 (10–100 nM) for 6 h, as illustrated in the schematic diagram (Figure 4A). Oligomeric and monomeric α-Syn in pericytes increased significantly in the presence of 50 nM bafilomycin A1 (210.4% of vehicle; Figure 4B). Additionally, the autophagy markers p62 and LC3 led to a significant increase in α-Syn in pericytes (p62; 167.5%, LC3; 270.4% of vehicle; Figure 4C). However, MG132 did not increase α-Syn in pericytes (Figure 4D).

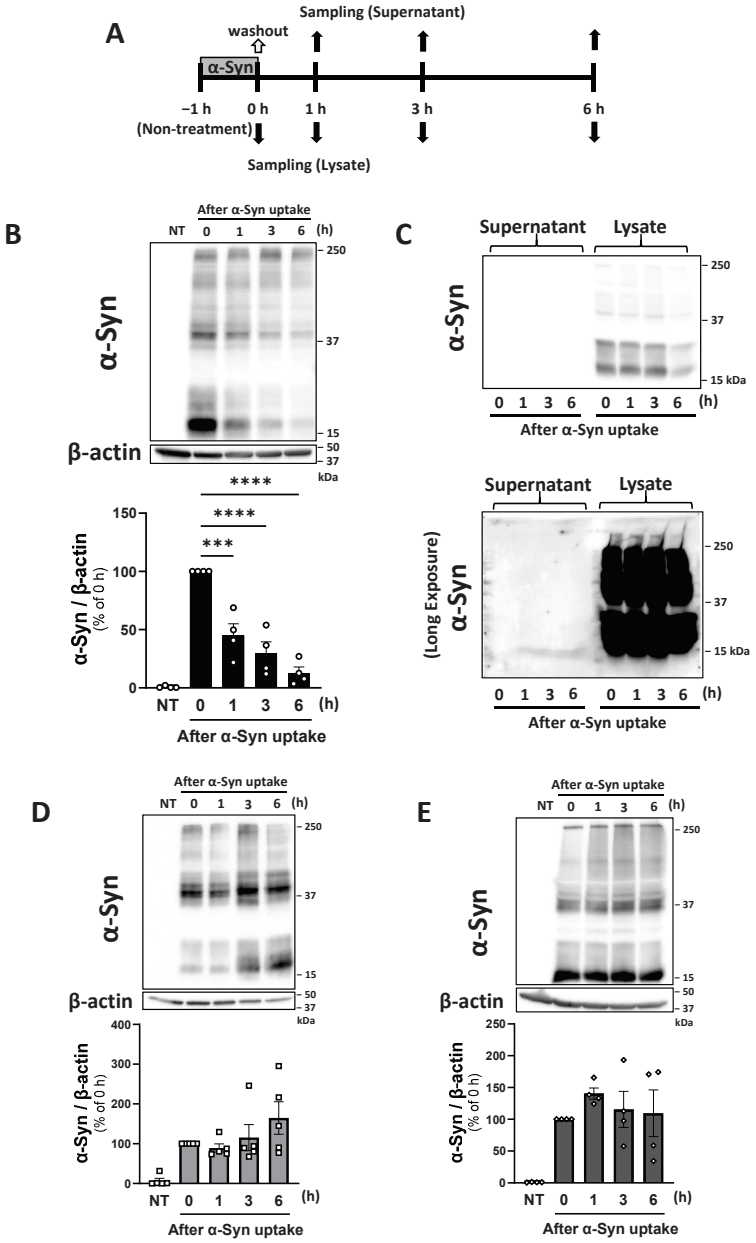


Figure 3. Internalized α-Syn in pericytes decreased in a time-dependent manner. (**A**) Experimental procedures for evaluating degradation and extracellular release of α-Syn. Cells were incubated with HL-α-Syn (1 μg/mL) for 1 h and subsequently washed twice with fresh physiological buffer. Cells were then incubated in fresh physiological buffer for the indicated time up to 6 h. Culture supernatants were collected to measure extracellular release. (**B**–**E**) α-Syn levels in cell lysates obtained from pericytes (**B**), RBECs (**D**), and astrocytes (**E**). Each panel shows representative Western blot images and quantitative analysis. β-actin was used as a loading control. NT indicates non-treated cells as a negative control. The results are expressed as the percentage of each corresponding control (0 h), which are cells after a 1 h HL-α-Syn uptake. Values are the means ± SEM (n = 4–5). Statistical analysis was performed using Dunnett's multiple comparisons test (*** $p < 0.001$, **** $p < 0.0001$). (**C**) Representative Western blot of α-Syn levels in culture supernatants obtained from pericytes. Aliquots of supernatants (350 μL out of 1 mL) were concentrated to approximately 25 μL using an Amicon Ultra centrifugal filter (3 kDa cut-off). Aliquots of lysates (35 μL of 100 μL) obtained from pericytes were loaded as a positive control. The bottom in the panel shows representative Western blot images with an extended exposure to detect immunoreactive bands for α-Syn. No immunoreactive bands were detected in culture supernatants.

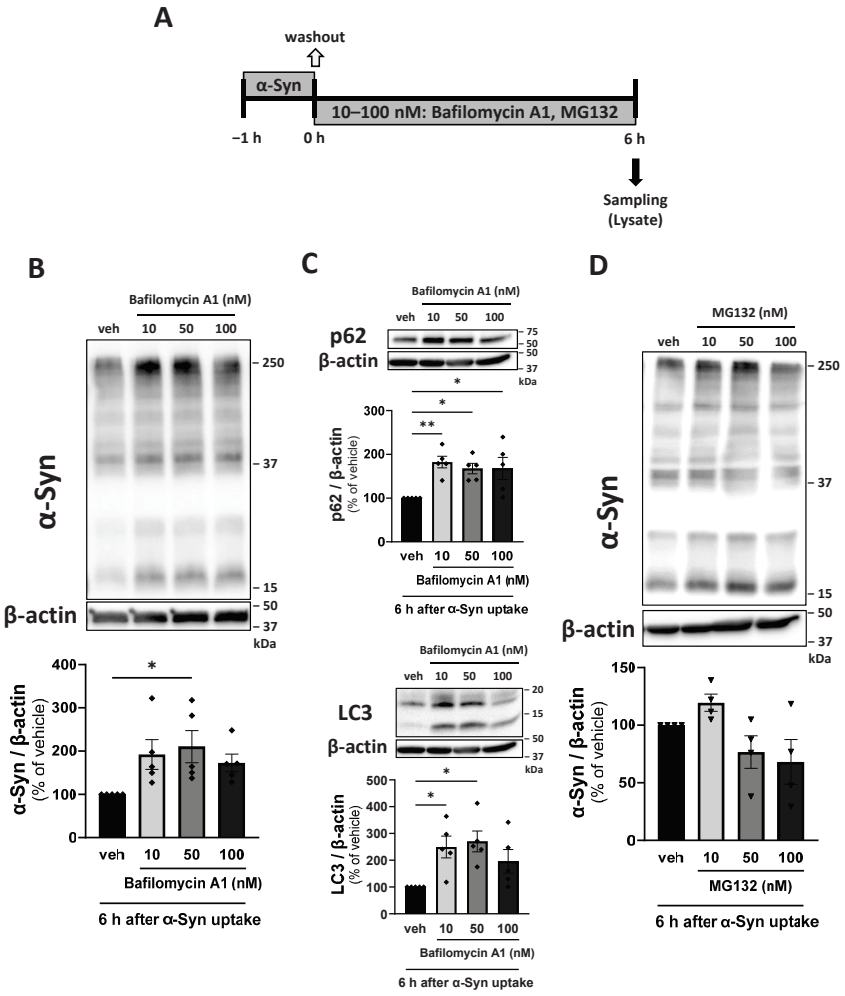


Figure 4. Effect of bafilomycin A1 and MG132 on the rapid decrease in internalized α-Syn in pericytes 0–6 h after α-Syn uptake. (**A**) Experimental procedures for investigating the degradation system of α-Syn in pericytes 0–6 h after α-Syn uptake. Pericytes were incubated with HL-α-Syn (1 μg/mL) for 1 h and subsequently washed twice with fresh physiological buffer. Pericytes were then incubated in fresh physiological buffer containing bafilomycin A1 (10–100 nM) (**B**,**C**) and MG132 (10–100 nM) (**D**) for 6 h. (**B**,**C**) Intracellular α-Syn (**B**), p62 (**C**), and LC3 (**C**) levels in pericytes treated with bafilomycin A1 for 6 h after α-Syn uptake. (**D**) Intracellular α-Syn levels in pericytes treated with MG132 for 6 h after α-Syn uptake. Each panel shows representative Western blot images and quantitative analysis. β-actin was used as a loading control. The results are expressed as the percentage of vehicle-treated pericytes. Values are the means ± SEM (n = 4–5). Statistical analysis was performed using Dunnett's multiple comparisons test. (* $p < 0.05$, ** $p < 0.01$).

2.4. Cathepsin D, DJ-1, and Lrrk2 Are Not Involved in α-Syn Degradation by Pericytes

To explore candidate molecules involved in α-Syn degradation in pericytes, we focused on cathepsin D (*Ctsd*), the major lysosomal protease involved in α-Syn degradation [47], and the familial PD-related genes DJ-1 (*Park7*) and Leucine-rich repeat kinase2 (*Lrrk2*). mRNA expression of Ctsd, DJ-1, and Lrrk2 was observed in BBB-constituting cell types (Figure 5A,D,G). Treatment of pericytes with siRNAs for Ctsd, DJ-1, and Lrrk2 significantly reduced the expression of each corresponding molecule (Figure 5B,E,H). However, Ctsd, DJ-1, and Lrrk2 knockdown did not alter α-Syn degradation activity in pericytes (Figure 5C,F,I).

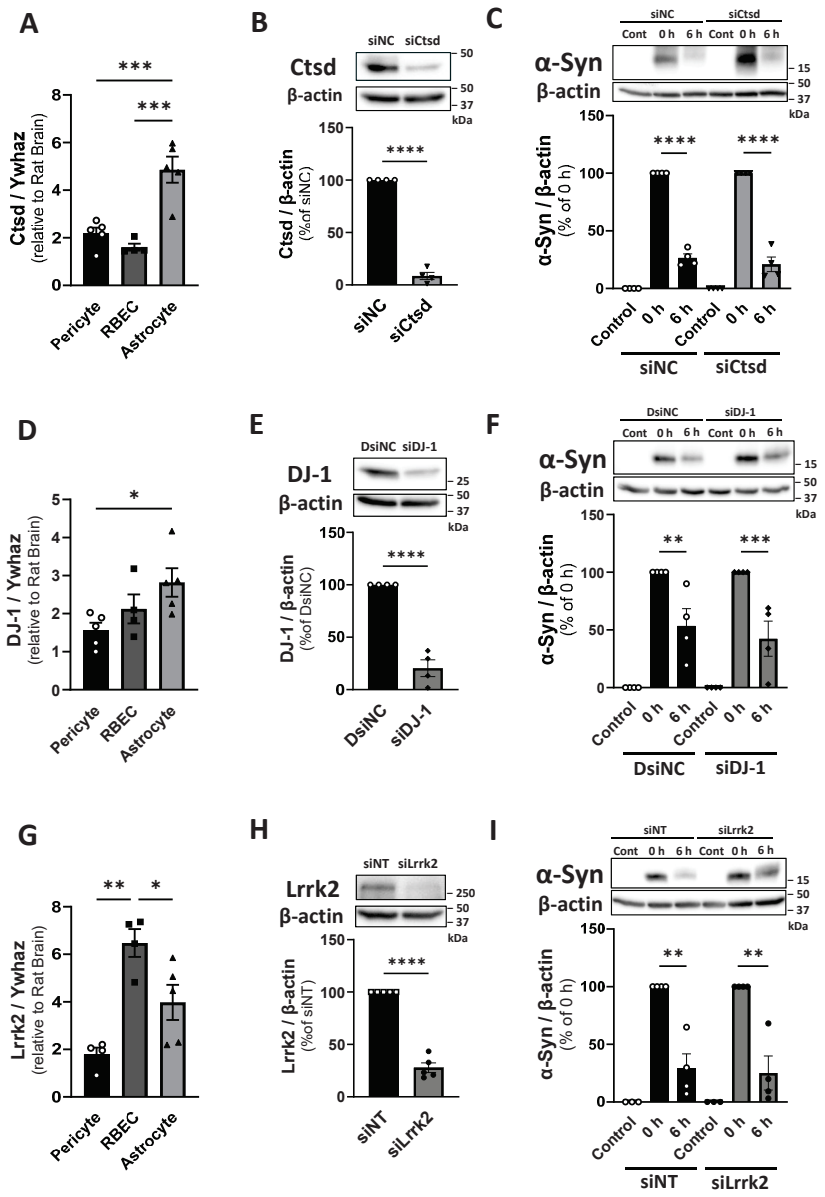


Figure 5. Cathepsin D, DJ-1, and Lrrk2 do not mediate α-Syn degradation in pericytes. (**A**,**D**,**G**) mRNA expression levels of Ctsd (**A**), DJ-1 (**D**), and Lrrk2 (**G**) in pericytes, RBECs, and astrocytes. mRNA levels measured by RT-qPCR using the *Ywhaz* gene as the reference gene for normalization. The results are expressed as fold changes relative to a calibrator (total RNA derived from rat brain). (**B**,**E**,**H**) siRNA-mediated knockdown of cathepsin D (**B**), DJ-1 (**E**), and Lrrk2 (**H**) in pericytes. Pericytes were transfected with negative control siRNA (siNC, DsiNC, and siNT) or siRNA targeting cathepsin D (siCtsd), DJ-1 (siDJ-1), and Lrrk2 (siLrrk2) for 72 h. The results are expressed as the percentage of negative control siRNA-transfected pericytes. (**C**,**F**,**I**) Intracellular monomeric α-Syn in pericytes transfected with negative control siRNA or siRNA targeting cathepsin D (**C**), DJ-1 (**F**), and Lrrk2 (**I**) after α-Syn uptake. After 72 h of siRNA transfection, pericytes were incubated with α-Syn (1 μg/mL) for 1 h and subsequently washed twice with fresh physiological buffer. Pericytes were then incubated in fresh physiological buffer for 6 h. β-actin was used as a loading control. Each panel shows representative Western blot images and quantitative analysis. The results are expressed as the percentage of the corresponding siRNA-transfected pericytes after 1 h α-Syn uptake (0 h). Control indicates pericytes without α-Syn incubation as a negative control. Values are the means ± SEM (n = 3–5). Statistical analysis was performed using Student's *t*-test or one-way ANOVA, followed by the Tukey–Kramer test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

2.5. Pericytes Degrade α-Syn Through Akt, ERK, and p38 MAPK Pathway Activation

We determined which pathways were rapidly activated by α-Syn in pericytes to determine intracellular signaling pathways leading to α-Syn degradation in pericytes. Phosphorylation levels of Akt, ERK, and p38 MAPK in pericytes increased significantly 1 h after α-Syn exposure (Figure 6B–D). Next, we determined whether the inhibition of these pathways, using pharmacological inhibitors (LY294002, U0126, and SB203580), attenuated α-Syn degradation in pericytes. Pericytes were treated with these inhibitors during the degradation phase after α-Syn uptake (1 h) to exclude the possibility that these inhibitors affect α-Syn uptake by pericytes. Intracellular α-Syn levels in vehicle-treated pericytes decreased 3 h after 1 h α-Syn uptake. Intracellular α-Syn in LY294002-, U0126-, and SB203580-treated pericytes was significantly higher than that in vehicle-treated pericytes (237.8, 263.7, and 239.1% of vehicle, respectively; Figure 6E).

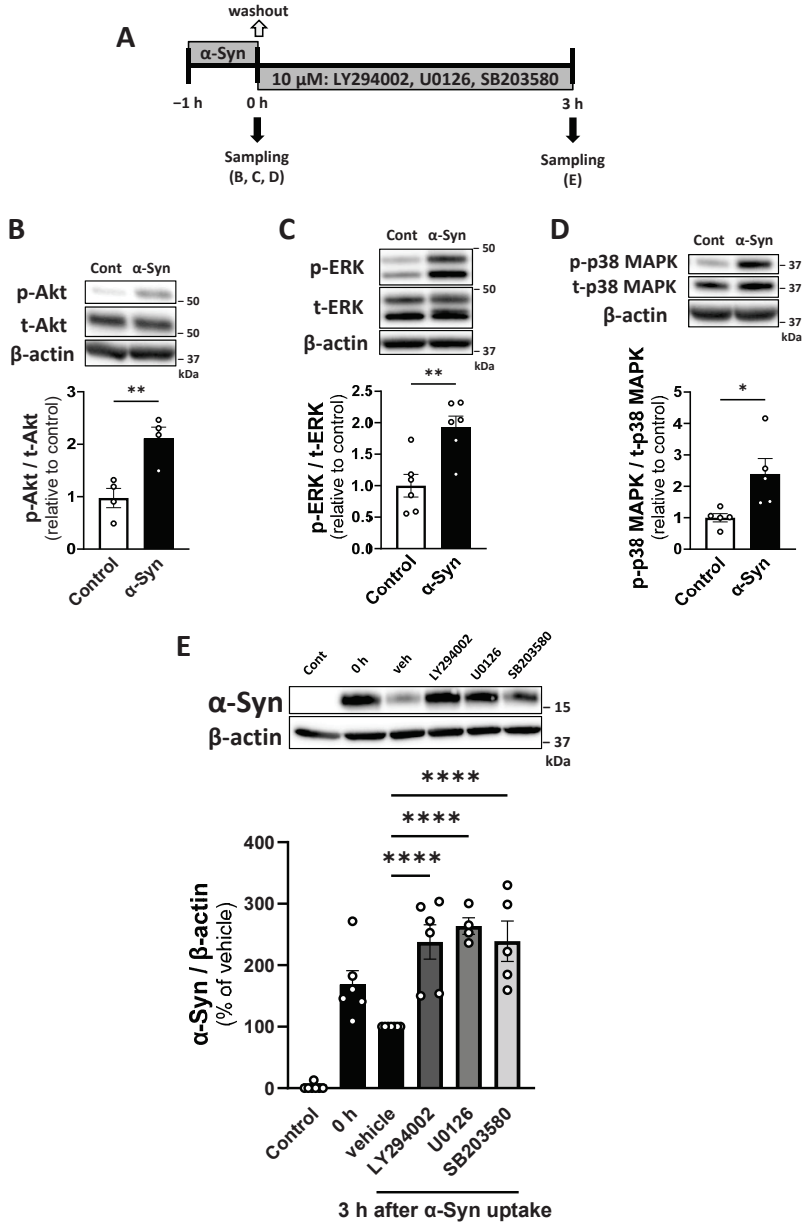


Figure 6. α-Syn degradation in pericytes is mediated by Akt, ERK, and p38 MAPK signaling pathways. (**A**) Experimental procedures for investigating the involvement of signaling pathways in degradation of α-Syn by pericytes 3 h after α-Syn uptake. Pericytes were incubated with recombinant human α-Syn

(1 μg/mL) for 1 h and subsequently washed twice with fresh physiological buffer. Pericytes were then incubated in fresh physiological buffer containing LY294002 (10 μM), U0126 (10 μM), and SB203580 (10 μM) for 3 h. Pericytes with or without 1 h of α-Syn incubation were used as a positive (defined as "0 h") and negative control (defined as "control"), respectively. (**B**–**D**) Phosphorylation levels of Akt (**B**), ERK (**C**), and p38 MAPK (**D**) in pericytes treated with α-Syn. Pericytes were incubated with recombinant human α-Syn (1 μg/mL) for 1 h. Each panel shows representative Western blot images of total and phosphorylated protein and quantitative analysis of the relative ratio of phosphorylated protein to total protein. β-actin was used as a loading control. (**E**) Representative Western blot images of intracellular α-Syn levels in pericytes in the absence or presence of LY294002, U0126, and SB203580 3 h after 1 h α-Syn uptake. β-actin was used as a loading control. The bar graph in the bottom shows quantitative analysis. The results are expressed as the percentage of vehicle-treated pericytes 3 h after 1 h α-Syn uptake. Values are the means ± SEM (n = 4–7). Statistical analysis was performed using Student's *t*-test or one-way ANOVA, followed by Dunnett's multiple comparisons test. (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$).

3. Discussion

To date, no disease-modifying therapies or preventive interventions targeting the elimination of the pathogenic protein α-Syn have been established. Although α-Syn interacts with the BBB and spreads between cells during the progression of PD, there is limited evidence on α-Syn uptake and degradation processes at the BBB, particularly as a potential α-Syn clearance mechanism. This study provides novel insights, demonstrating that α-Syn clearance is pericyte-specific at the BBB, by comparing α-Syn clearance across different BBB-constituting cell types. We demonstrated that pericytes degrade α-Syn without releasing it into the extracellular space. Furthermore, we identified the intracellular signaling pathways involved in the α-Syn degradation system within pericytes.

We found that commercially available HL-α-Syn contains α-Syn of various molecular weights. Therefore, we employed HL-α-Syn as monomeric and oligomeric α-Syn to evaluate the α-Syn uptake ability of BBB-constituting cell types (pericytes, brain endothelial cells, and astrocytes). Intracellular levels of oligomeric and monomeric α-Syn in all cells increased 1 h after exposure, indicating that all cells possessed the ability to take up α-Syn. Twenty-four hours after α-Syn exposure, unlike RBECs and astrocytes, pericytes exhibited decreased intracellular α-Syn levels. These findings suggest that, among the BBB-constituting cells, pericytes possess the unique ability to clear α-Syn. In contrast to previously reported findings [48], we were unable to confirm α-Syn clearance by astrocytes during 24 h. This study is the first to demonstrate that the α-Syn internalization profile varies among different BBB-constituting cells. Our findings suggest that pericytes clear α-Syn more rapidly than astrocytes and brain endothelial cells.

Our findings are consistent with recent studies showing that pericytes take up and degrade α-Syn aggregates [39]. However, there is little experimental evidence regarding the mechanisms underlying α-Syn clearance by pericytes. To elucidate these mechanisms, we first focused on the autophagy–lysosome pathway (ALP) and ubiquitin–proteasome system (UPS), which are major protein degradation pathways. This focus was based on previous findings that microglia, a key cell type in the brain [31,49,50], degrade α-Syn received from neurons via the ALP. We found that both oligomeric and monomeric α-Syn and the autophagy markers p62 and LC3 increased significantly when pericytes were treated with bafilomycin A1, an autophagy inhibitor, for 24 h. This suggests that bafilomycin A1 effectively inhibited autophagy activity in pericytes, leading to the increase in intracellular α-Syn. Moreover, these results were supported by rapamycin, an autophagy inducer, causing a significant decrease in monomeric and oligomeric α-Syn levels in pericytes. In addition, when UPS, a protein degradation pathway, was inhibited using MG132, intracellular α-Syn levels in pericytes increased after 24 h. These results suggest that the

decrease in α-Syn levels in pericytes observed during 24 h α-Syn incubation was due to degradation through the ALP and UPS.

These results, showing a decrease in α-Syn in pericytes, were obtained by continuously treating cells with α-Syn for 24 h. Therefore, the constant influx of α-Syn into the cells likely masked the extracellular release of α-Syn, as the observed decrease in α-Syn in pericytes could be attributed to both degradation and extracellular release. Thus, to investigate the role of extracellular release, a potential additional clearance pathway, we separately evaluated intracellular degradation and extracellular release of α-Syn. Intracellular levels of α-Syn in pericytes decreased in a time-dependent manner after α-Syn uptake. However, despite extending the exposure time for imaging, α-Syn was not detected in pericyte-derived supernatants. Therefore, we concluded that extracellular release of α-Syn could be ruled out as a mechanism for the observed decrease in α-Syn in pericytes. These findings suggest that pericytes internalize and clear α-Syn primarily through protein degradation systems rather than by extracellular secretion. Consistently, our results indicated that RBECs and astrocytes exerted insufficient α-Syn clearance ability.

To investigate the observed reduction in α-Syn in pericytes, we investigated the mechanism by which pericytes degrade α-Syn up to 6 h after α-Syn uptake. When the ALP in pericytes was inhibited by bafilomycin A1, oligomeric and monomeric α-Syn levels increased significantly. Unlike in the 24 h incubation of α-Syn with MG132, inhibition of the UPS in pericytes by MG132 did not lead to an increase in α-Syn levels. These results suggest that the ALP is predominantly involved in the degradation of both oligomeric and monomeric α-Syn during the early phase following α-Syn uptake by pericytes. This is likely due to differences in substrate specificity between the UPS and ALP. The UPS is a highly selective pathway that requires the identification and ubiquitination of target proteins via specific recognition sites, followed by the recognition and degradation of ubiquitinated proteins by the proteasome. It primarily degrades relatively low molecular weight proteins, such as IκB and p53, and plays a crucial role in maintaining cellular homeostasis, stress response, and cell cycle regulation [51]. In contrast, the ALP does not require substrate recognition sites and non-specifically degrades organelles and high molecular weight proteins [52,53]. Thus, our findings suggest that the UPS may not be as efficient in degrading high molecular weight aggregated proteins such as α-Syn in the early stages, as ubiquitination and proteasomal degradation require more time. Although both the UPS and ALP can recognize α-Syn as a substrate, the latter pathway likely acts more rapidly on the protein. Previous studies have shown that α-Syn clearance in human pericytes is mediated only by the ALP [39]. Our study is the first to demonstrate that both the ALP and UPS are involved in α-Syn clearance in healthy pericytes, despite differences in species of cells (human vs. rat) compared to previous research [39].

Based on the presented results, both monomeric and oligomeric α-Syn were cleared in pericytes via similar mechanisms. Therefore, in the following experiments, we evaluated monomeric α-Syn using recombinant human α-Syn. Next, we focused on identifying the molecules involved in α-Syn degradation by pericytes, specifically evaluating enzyme (cathepsin D) and familial PD-related molecules (DJ-1 and LRRK2). Cathepsin D (CTSD) is the principal lysosomal aspartate protease involved in α-Syn degradation in neurons [47,54]. DJ-1 is a multifunctional protein encoded by the gene *PARK7* (peroxiredoxin-like peroxidase), with mutations identified in familial PD [55]. DJ-1 primarily suppresses α-Syn aggregation through the activation of chaperone-mediated autophagy and provides a neuroprotective effect via its antioxidant activity [56]. *LRRK2* (Leucine-rich repeat kinase 2) is a commonly mutated gene that emerges in both familial and sporadic PD [57]. There is limited experimental evidence demonstrating a direct relationship between *LRRK2* and α-Syn; however, it has been suggested that *LRRK2* may indirectly

influence α-Syn through protein degradation pathways, such as the ALP [58–60]. We found that Ctsd, DJ-1, and Lrrk2 are expressed in BBB-constituting cell types. Pericytes expressed these molecules; however, knockdown of these molecules did not confer any significant changes in α-Syn clearance in pericytes. Therefore, these results suggested that these molecules were not involved in α-Syn clearance by pericytes. Further investigations are needed to clarify the role of these molecules at the BBB in the pathogenesis of PD.

The ALP and UPS are regulated by transcriptional factors, which serve as downstream targets for several intracellular signaling pathways [61–63]. These regulatory mechanisms are crucial for maintaining intracellular protein quality control, ensuring complete degradation of misfolded or damaged proteins. Further, as an additional approach to identify molecular targets involved in α-Syn degradation in pericytes, we investigated signaling pathways that initiate α-Syn degradation in pericytes. We demonstrated that α-Syn activated the Akt, ERK, and p38 MAPK signaling pathways in pericytes. To determine whether these signaling pathways are involved in α-Syn degradation in pericytes, we examined the effects of specific inhibitors targeting each signaling pathway. We found that LY294002, U0126, and SB203580 significantly inhibited degradation of intracellular α-Syn. Therefore, our findings suggest that α-Syn degradation in pericytes is mediated through the Akt, ERK, and p38 MAPK signaling pathways. Studies of microglia found that α-Syn activated the Akt and p38 MAPK signaling pathways to affect autophagy [31,64]. However, these signaling pathways were unlikely to mediate α-Syn degradation in microglia. Other studies reported that the activation of these signaling pathways induces autophagy under pathological conditions, such as starvation and oxidative stress [65–70]. Thus, although the precise signaling pathways involved in autophagy-mediated α-Syn degradation in pericytes require further detailed investigation, our results suggest that pharmacological activation of the Akt, ERK, and p38 MAPK pathways could enhance α-Syn degradation by pericytes. This presents a potential novel therapeutic strategy targeting α-Syn clearance.

The BBB disruption observed in PD patients can participate in the progression of pathogenesis. Several in vitro studies have demonstrated the ability of α-Syn to disrupt the BBB. Preformed fibril α-Syn contributes to the impairment of barrier integrity of the BBB through directly activating brain microvascular endothelial cells (BMECs) to downregulate tight junction-associated proteins [71,72]. BMECs took up preformed fibril α-Syn by endocytosis, which resulted in cell death and reduced expression of tight junction-associated proteins [73]. We previously showed that monomeric α-Syn was taken up by BMECs without any changes in permeability. However, monomeric α-Syn activated pericytes to induce impaired barrier integrity of BMECs [41]. In addition, intravenous monomeric and aggregated α-Syn can cross the healthy BBB without disruption [74], although the exact α-Syn transport mechanism remains unknown. An immunohistochemical study of α-Syn in healthy human brains showed α-Syn immunoreactivity in brain endothelial cells but not in capillary walls, such as pericytes [75], whereas perivascular α-Syn immunoreactivity was detected in PD brains [71]. These findings suggest that pericytes in the healthy brain cleared α-Syn. Considering these findings, our results suggest that pericytes may clear α-Syn that penetrates BMECs to prevent α-Syn deposition in the brain parenchyma.

The α-Syn clearance ability of pericytes derived from PD patients is likely to be impaired, as suggested by a previous study [39]. Therefore, our findings that the activation of the Akt, ERK, and p38 MAPK signaling pathways mediates α-Syn degradation in healthy pericytes could partly clarify the mechanisms underlying impaired α-Syn clearance. These insights may also provide therapeutic targets for restoring α-Syn clearance ability in pericytes in patients with PD. In this context, exploring therapeutic drugs that can cross the BBB for enhancing the α-Syn clearance ability in pericytes would be a potential therapeutic

strategy. Moreover, supplementation of pericytes with α-Syn clearance capability to the brain may be an effective approach.

This study has some limitations. First, we investigated the mechanism of monomeric and oligomeric α-Syn clearance in pericytes using rat-derived healthy cells. To better understand the role of pericytes in the pathogenesis of PD, future studies should address species differences in α-Syn clearance mechanisms using human-derived pericytes. Additionally, evaluating α-Syn clearance by pericytes under disease conditions—using animal models of PD or pericytes derived from PD patients or animals—will provide more insights. Furthermore, it is essential to assess the clearance capacity of pericytes for higher molecular weight and more toxic species of α-Syn, such as fibrils and phosphorylated α-Syn. Although we did not identify the specific enzymes or proteasomal components that mediate α-Syn degradation in pericytes, identifying the molecules involved in α-Syn degradation in pericytes is important for the identification of therapeutic targets and diagnostic markers, which enable early diagnosis and treatment. Furthermore, we did not investigate the receptors or binding sites for α-Syn internalization into pericytes and the subsequent activation of intracellular signaling pathways. In microglia, extracellular α-Syn interacted with Toll-like receptor (TLR) 2 or 4, triggered the activation of NF-kB, and then induced α-Syn degradation by autophagy [31]. Studies using microglia and astrocytes isolated from TLR4-knockout mice showed that TLR4 is involved in α-Syn-induced activation of microglia and astrocytes but not in α-Syn internalization [31,76]. Melatonin receptor 1 regulated LC3-associated phagocytosis, which is involved in α-Syn clearance by microglia [77]. Further research is required to identify the cell surface receptors or binding sites in pericytes that interact with α-Syn.

4. Materials and Methods

4.1. Cell Culture

All experiments were approved by the Laboratory Animal Care and Use Committee of Fukuoka University (Approval number: 2204002 and 2315117). All animal experiments complied with the ARRIVE guidelines and were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Primary cultures of pericytes and RBECs were prepared as previously described [41, 78]. Cells were isolated from 3–4-week-old Wistar rats. Astrocytes were prepared from 1–2 day old Wistar rats, as previously described [79]. Pericyte medium (DMEM (Wako, Osaka, Japan; 048-29763) supplemented with 20% FBS (Gibco/Thermo Fisher Scientific, Waltham, MA, USA; A3160602), penicillin (100 units/mL), streptomycin (100 μg/mL; penicillin–streptomycin mixed solution, Nacalai Tesque, Kyoto, Japan; 09367-34) and gentamicin (50 μg/mL, Biowest, Nuaillé, France; L0012)), RBEC medium (Dulbecco's Modified Eagle Medium/F12 (DMEM/F12; Wako, 042-30555) supplemented with 10% fetal bovine serum (FBS; Biosera, Kansas, MO, USA; FB-1365/500), basic fibroblast growth factor (1.5 ng/mL, R&D, Minneapolis, MN, USA; 2099-FB-025), heparin (100 μg/mL, Sigma, St. Louis, MO, USA; H3149), insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenite (5 ng/mL; insulin–transferrin–sodium selenite media supplement, Sigma; I1884), penicillin (100 units/mL), streptomycin (100 μg/mL; penicillin–streptomycin mixed solution, Nacalai Tesque), gentamicin (50 μg/mL, Biowest, L0012-100), hydrocortisone (500 nM, Sigma; H0135)), and astrocyte medium (DMEM (Wako) supplemented with 10% FBS (Biowest, S1820-500), penicillin (100 units/mL), streptomycin (100 μg/mL; penicillin–streptomycin mixed solution, Nacalai Tesque), and gentamicin (50 μg/mL, Biowest)) were used for maintaining pericytes, RBECs, and astrocytes, respectively.

4.2. α-Synuclein

Recombinant human α-Syn was prepared as previously described [80]. HiLyte488-conjugated human recombinant α-Syn (HL-α-Syn) was purchased from AnaSpec Inc. (Fremont, CA, USA; AS-55457).

4.3. α-Syn Uptake by Pericytes, RBECs, and Astrocytes

Primary cultures of pericytes, RBECs, and astrocytes were seeded at 10–20 × 10^4 cells per 35 mm dish. After the cells reached confluency, pericytes, RBECs, and astrocytes were treated with or without recombinant human α-Syn or HL-α-Syn (1 μg/mL each) for 1 or 24 h in serum-free medium or physiological buffer (141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$ anhydrous, 1 mM $MgSO_4$ $7H_2O$, 1 mM NaH_2PO_4 $2H_2O$, 10 mM D(+)-glucose, 10 mM HEPES, pH 7.4). To remove α-Syn that binds to the cell surface, cells were washed once with 1 mL of acid wash buffer (28 mM sodium acetate, 120 mM NaCl, 20 mM sodium barbital, pH 3.0) at 4 °C. Then, cells and the collected supernatants (1000 μL) were subjected to α-Syn measurement using Western blotting.

4.4. Drug Susceptibility Testing

Pericytes were treated with bafilomycin A1 (10–100 nM, Selleck, Houston, TX, USA; S1039), rapamycin (10–100 nM, Selleck; S1413), MG132 (10–100 nM, EMD Millipore Corporation, Billerica, MA, USA; 474790), LY294002 (10 μM, Tocris, Ellisville, MO, USA; 1130/5), U0126 (10 μM, Tocris; 1144/5), and SB203580 (10 μM, Tocris; 1202/1) in serum-free DMEM or physiological buffer for the indicated time at 37 °C. Drugs were dissolved in dimethyl sulfoxide (DMSO, Wako; 045-24511) to prepare stock solutions. Stock solutions were diluted with serum-free DMEM or physiological buffer to a final concentration of 0.1% DMSO. An equal volume of DMSO was diluted with appropriate media for the vehicle-treated control.

4.5. siRNA Transfection

siRNA for rat cathepsin D (50 nM, siCtsd, Silencer® Select Pre-designed siRNA, Ambion®/Invitrogen, Carlsbad, CA, USA; 4390771), rat DJ-1 (10 nM, siDJ-1, TriFECTa® DsiRNA Kit, Integrated DNA Technologies, Coralville, IA, USA), and rat Lrrk2 (25 nM, siLrrk2, Dharmacon™ siGENOME SMARTpool, Horizon Discovery Ltd., Cambridge, UK) was used. Silencer® Select Negative Control (siNC, 50 nM, Life Technologies/Thermo Fisher Scientific; 4390843), Negative Control DsiRNA (DsiNC, 10 nM, Integrated DNA Technologies; 51-01-14-03), or Dharmacon™ siGENOME Non-Targeting Pool #2 (siNT, 25 nM, Horizon Discovery Ltd.; D-001206-14-05) was used as an siRNA negative control. Pericytes were transfected with various siRNAs using Lipofectamine™ RNAiMAX Transfection Regent (Invitrogen by Thermo Fisher Scientific; 13778075), according to the manufacturer's protocol. siRNA–lipid complex containing siRNA and Lipofectamine™ RNAiMAX (4 μL) was prepared in Opti-MEM™ I Reduced Serum Medium (Gibco/Thermo Fisher Scientific; 31985062). Pericytes were incubated with siRNA–lipid complex in pericyte medium for 72 h. After transfection, pericytes were subjected to uptake experiments, Western blotting, or RT-qPCR.

4.6. Sample Preparation for Western Blot Analysis

Cells were lysed with 100 μL of lysis buffer (10 mM Tris-HCl, pH 6.8, 100 mM NaCl, 1 mM EDTA, pH 8.0, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM Na_3VO_4, 1 mM sodium fluoride, 20 mM sodium pyrophosphate decahydrate, and 50 μg/mL phenylmethanesulfonyl fluoride) containing 1% phosphatase inhibitor cocktail 2 (Sigma-Aldrich), 1% phosphatase inhibitor cocktail 3 (Sigma-Aldrich), and 1% protease inhibitor cocktail (Sigma-Aldrich)). Cell lysates were stored at −80 °C until

use. The total protein concentration in the cell lysates was determined using a BCA Protein assay kit (Thermo Fishier Scientific, Rockford, IL, USA), according to the manufacturer's protocol. Aliquots (350 µL) of the supernatants were concentrated to approximately 25 µL using Amicon® Ultra-0.5 Centrifugal Filter Devices (3 kDa cut-off, Millipore Corporation, Billerica, MA, USA; UFC500396). The filter units were pre-coated with 1% bovine serum albumin in PBS to minimize α-Syn adsorption.

4.7. Western Blot Analysis

Samples were mixed with 3× sample buffer (0.03% bromophenol blue, 6% sodium dodecyl sulfate (SDS), 30% glycerol, 187.5 mM Tris-HCl) or 6× sample buffer (Nacalai Tesque; 09500-64) containing 2-mercaptethanol and boiled at 95 °C for 5 min. Equal amounts of protein were electrophoretically separated on 13 or 15% SDS-polyacrylamide gels (0.03 A/gel, 50–60 min). After electrophoresis, the proteins were transferred to PVDF membranes (ClearTrans® SP; Wako; 033-22453) in transfer buffer (24.8 mM Tris HCl, 191.8 mM glycine, 20% methanol; 100 V, 180 min). Membranes for α-Syn detection were incubated with 4% paraformaldehyde (Wako) for 15 min at room temperature. Then, the membranes were blocked with Blocking One (Nacalai Tesque; 03953-95) or Blocking One-P (Nacalai Tesque; 05999-84) for phosphorylated proteins for 30 min at room temperature. The membranes were incubated with primary antibodies diluted in 5% Blocking One or Blocking One-P in Tris-buffered saline (10 mM Tris HCl, pH 8.0, 136 mM NaCl, 2 mM sodium orthovanadate, 50 mM sodium fluoride, pH 7.85) containing 0.1% Tween 20 (TBS-T) overnight at 4 °C. α-Syn, p62, LC3, cathepsin D, DJ-1, Lrrk2, and β-actin were detected using anti-α-synuclein antibody (MJFR1) (ab138501, 1:1000; Abcam plc, Cambridge, UK), anti-SQSTM1/p62 antibody (ab56416, 1:1000; Abcam plc), LC3B Rabbit antibody (2775S, 1:1000; Cell Signaling Technology, Danvers, MA, USA), cathepsin D (E179) Rabbit antibody (69854S, 1:1000; Cell Signaling Technology), DJ-1 (D29E5) XP® Rabbit mAb (5933S, 1:1000; Cell Signaling Technology), anti-LRRK2 antibody (MJFF2) (ab133474, 1:1000; Abcam plc), and anti-β-actin antibody (clone CA-15; A1978, 1:8000; Sigma-Aldrich, MO, USA). Phosphorylation of p42/p44 MAPK (Erk), p38 MAPK, and Akt was detected using primary antibodies against phospho-p44/42 MAPK (Erk1/2) (Tyr202/Tyr204) (D13. 14. 4E) XP® Rabbit mAb (4370S, 1:1000; Cell Signaling Technology), phospho-p38 MAPK (Tyr180/Tyr182) (D3F9) XP® Rabbit mAb (4511S, 1:1000; Cell Signaling Technology), and phospho-Akt (Tyr308) (C31E5E) Rabbit mAb (2965S, 1:1000; Cell Signaling Technology). The membrane was washed three times with TBS-T for 10 min and then incubated with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG (170-6516 or 170-6515, 1:5000 or 10,000; BIO-RAD, Hercules, CA, USA) diluted in 5% Blocking One in TBS-T for 1 h at room temperature. The membrane was washed five times with TBS-T for 5 min, and target protein bands were visualized using a Clarity Western ECL Substrate (BIO-RAD). To re-probe total p42/p44 MAPK, p38 MAPK, and Akt, membranes were incubated in stripping buffer (0.2 M glycine, 0.1% SDS and 1% Tween 20, pH 2.2) for 15 min, and this step was repeated. Total p42/p44 MAPK, p38 MAPK, and Akt were detected using primary antibodies against p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb (4695S, 1:1000; Cell Signaling Technology), p38 MAPK antibody (9212S, 1:1000; Cell Signaling Technology), and Akt (pan) (C67E7) Rabbit mAb (4691S, 1:1000; Cell Signaling Technology). The band images were digitally captured using a Multi Imager II ChemiBOX (BioTools, Gunma, Japan), and band intensities were quantified using the ImageJ 1.48 software (NIH, Bethesda, MD, USA).

4.8. Real-Time Quantitative PCR

Total RNA was extracted from cells using the FastGene RNA Basic Kit (FastGene Co., Ltd., Tokyo, Japan; FG-80250), according to the manufacturer's protocol. Equivalent

amounts of RNA from each sample were reverse-transcribed with FastGene cDNA Synthesis 5× ReadyMix OdT (FastGene Co., Ltd.; NE-LS65). Real-time PCR was conducted in a Light Cycler 96 System (F. Hoffmann-La Roche, Ltd., Basel, Switzerland) using a KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Inc., London, UK; KK4602), according to the manufacturer's protocol. After pre-incubation at 95 °C for 3 min, PCR was performed using the following conditions: 45 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 1 min, using specific primers (purchased from Takara Bio Inc. (Shiga, Japan), Sigma, and Integrated DNA Technologies, Inc.) to amplify the genes of interest. Primer sequences were as follows: Cathepsin D (Ctsd; NM_134334, Integrated DNA Technologies, Forward: 5′-TGACAAGTCCAGCACCTATG-3′, Reverse: 5′-TTCTCCACCTTGATACCTCCTA-3′), DJ-1 (Park7; NM_057143, Sigma, Forward: 5′-AAGGACAAAATGATGAACGG-3′, Reverse: 5′-CTCTCTAGTCTTTGAGAACAAG-3′), Lrrk2 (NM_001191789, Integrated DNA Technologies, Forward: 5′-GACCTTCATTCCCGACTCTTC-3′, Reverse: 5′-GTCCCTGTAAACTTCTCCTACAC-3′). Ywhaz (NM_013011, Takara, Forward: 5′-GAGTCGTACAAAGACAGCACGCTAA-3′, Reverse: 5′-GTGGGACAGCATGGATGACAA-3′). Ywhaz was used as the reference gene; therefore, the threshold cycle value (Cq) of the target genes was normalized to that of Ywhaz. Total RNA isolated from rat brain was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA; Premium Total RNA, Rat Brain, cerebral cortex; 636677) and used as a calibrator for relative quantification of gene expression.

4.9. Statistical Analysis

The results are expressed as the means ± standard error of the mean (SEM). All data were assessed and statistically analyzed using GraphPad Prism 10.1.2 (GraphPad Software, San Diego, CA, USA). Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test or the Tukey–Kramer test. The unpaired *t*-test was applied to compare two groups. $p < 0.05$ was considered statistically significant.

5. Conclusions

We demonstrated that pericytes possess the ability to degrade α-Syn via Akt, ERK, and p38 MAPK signaling pathway activation. In pericytes, α-Syn degradation is mediated by the ALP and UPS. Notably, this α-Syn clearance ability in pericytes was more efficient than that observed in other BBB-constituting cells, such as brain endothelial cells and astrocytes, suggesting a pericyte-specific role in α-Syn clearance at the BBB. Our findings highlight the importance of α-Syn clearance by pericytes in understanding the pathogenesis and progression of PD. This mechanism serves as a potential therapeutic target for disease-modifying therapies aimed at preventing the propagation of α-Syn pathology in the brain.

Author Contributions: Conceptualization, S.D.; methodology, M.Y. (Miki Yokoya) and S.D.; investigation, M.Y. (Miki Yokoya), F.T., T.I. and J.M. (Junichi Matsumoto); resources, T.I., J.M. (Junichi Matsumoto), Y.T., H.A., M.Y. (Miho Yasunaga), J.M. (Junko Mizoguchi) and K.S.; writing—original draft preparation, M.Y. (Miki Yokoya); writing—review and editing, F.T., T.I., J.M. (Junichi Matsumoto), Y.T., K.S. and S.D.; supervision, S.D.; project administration, M.Y. (Miki Yokoya) and S.D.; funding acquisition, F.T., T.I. and S.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partly supported by the Japan Society for the Promotion of Science (https://www.jsps.go.jp/ (accessed on 10 January 2025)) [KAKENHI; grant numbers JP21K06813 to F.T., JP23K14401 to T.I., JP21H04863 and JP23K18205 to S.D.] and the Central Research Institute of Fukuoka University (http://www.suisin.fukuoka-u.ac.jp/home1/ (accessed on 10 January 2025)) [grant numbers 221032 to S.D.].

Institutional Review Board Statement: The animal study protocol was approved by the Laboratory Animal Care and Use Committee of Fukuoka University [permit No: 2204002 (approved: 11 April 2022) and 2315117 (approved: 19 March 2024)].

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: We thank Yuka Tofuku, Nanami Tokunou, and Mana Nemoto for their technical assistance. M.Yokoya and M.Yasunaga were supported by Nagai Memorial Research Scholarship from the Pharmaceutical Society of Japan (N-226902 to M.Yokoya and N-246901 to M.Yasunaga). F.T. was supported through the Fukuoka University Program to support the research activities of female researchers (http://www.suisin.fukuoka-u.ac.jp/jyosei/ (accessed on 10 January 2025)).

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

ALP	Autophagy–Lysosome Pathway
α-Syn	α-Synuclein
BBB	Blood–Brain Barrier
Ctsd	Cathepsin D
ERK	Extracellular signal-regulated kinase
HL-α-Syn	HiLyte488-conjugated human recombinant α-Syn
MAPK	Mitogen-Activated Protein Kinase
Lrrk2	Leucine-Rich Repeat Kinase 2
MSA	Multiple System Atrophy
NC	Negative Control
PD	Parkinson's disease
PSP	Progressive Supranuclear Palsy
RBECs	Rat Brain Endothelial Cells
TLR	Toll-Like Receptor
UPS	Ubiquitin–Proteasome System

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International Journal of
Molecular Sciences

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Review

Decoding Neurodegeneration: A Review of Molecular Mechanisms and Therapeutic Advances in Alzheimer's, Parkinson's, and ALS

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Abstract: Neurodegenerative diseases, such as Alzheimer's, Parkinson's, ALS, and Huntington's, remain formidable challenges in medicine, with their relentless progression and limited therapeutic options. These diseases arise from a web of molecular disturbances—misfolded proteins, chronic neuroinflammation, mitochondrial dysfunction, and genetic mutations—that slowly dismantle neuronal integrity. Yet, recent scientific breakthroughs are opening new paths to intervene in these once-intractable conditions. This review synthesizes the latest insights into the underlying molecular dynamics of neurodegeneration, revealing how intertwined pathways drive the course of these diseases. With an eye on the most promising advances, we explore innovative therapies emerging from cutting-edge research: nanotechnology-based drug delivery systems capable of navigating the blood–brain barrier, gene-editing tools like CRISPR designed to correct harmful genetic variants, and stem cell strategies that not only replace lost neurons but foster neuroprotective environments. Pharmacogenomics is reshaping treatment personalization, enabling tailored therapies that align with individual genetic profiles, while molecular diagnostics and biomarkers are ushering in an era of early, precise disease detection. Furthermore, novel perspectives on the gut–brain axis are sparking interest as mounting evidence suggests that microbiome modulation may play a role in reducing neuroinflammatory responses linked to neurodegenerative progression. Taken together, these advances signal a shift toward a comprehensive, personalized approach that could transform neurodegenerative care. By integrating molecular insights and innovative therapeutic techniques, this review offers a forward-looking perspective on a future where treatments aim not just to manage symptoms but to fundamentally alter disease progression, presenting renewed hope for improved patient outcomes.

Keywords: Parkinson's disease (PD); Alzheimer's disease (AD); Huntington's disease (HD); protein misfolding; neuroinflammation; mitochondrial dysfunction; gene therapy; precision medicine; pharmacogenomics

1. Introduction

1.1. Overview of Neurodegenerative Diseases

Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD), present a unique scientific challenge due to their complexity and the devastating effects they have on cognitive and motor function [1]. Though each disease has its distinct characteristics, they share underlying molecular mechanisms such as oxidative stress and protein misfolding. For instance, Alzheimer's disease and Parkinson's disease are characterized by the accumulation of misfolded proteins like amyloid-beta and alpha-synuclein, respectively. These misfolded proteins propagate toxicity and disrupt cellular function, highlighting a shared pathological hallmark [2]. These diseases highlight an urgent need for deeper understanding as they continue to affect millions worldwide, straining healthcare systems and impacting families profoundly. For example, Parkinson's disease is increasingly prevalent, affecting over 10 million people worldwide, and Alzheimer's disease alone contributes to an economic burden exceeding $1 trillion annually due to healthcare costs and lost productivity [3].

In Alzheimer's disease, a major culprit is the amyloid-beta (Aβ) peptide, a fragment derived from the amyloid precursor protein (APP) [4]. When APP is cleaved by enzymes known as beta- and gamma-secretases, Aβ fragments are produced, which are prone to clumping together outside cells [5]. These clumps, or plaques, have long been considered a hallmark of AD, but recent research has taken a closer look at the soluble forms of Aβ, suggesting that these smaller aggregates might be the real neurotoxic agents [6]. Soluble Aβ oligomers can interact directly with cell membranes, disrupt ion channels, and trigger internal signaling pathways that interfere with synaptic function, eventually leading to neuronal death [7]. Adding to this, tau protein—a stabilizing component of the neuronal skeleton—undergoes hyperphosphorylation in AD, transforming into tangled filaments within neurons [8]. These tau tangles not only weaken neuronal structure but also appear to spread across brain regions in a manner that mirrors the disease's clinical progression [9].

In Parkinson's disease, the loss of dopamine-producing neurons in the substantia nigra region of the brain leads to the characteristic motor symptoms of tremors and rigidity [10]. At the heart of this loss lies alpha-synuclein, a protein that, under certain conditions, misfolds and accumulates inside neurons, forming structures called Lewy bodies. Studies have revealed that alpha-synuclein has an intrinsic ability to change shape, creating beta-sheet-rich structures that can seed aggregation in nearby cells, a behavior often described as "prion-like" [11]. This propagation potentially explains how Parkinson's pathology progresses through interconnected brain regions. Alongside protein misfolding, Parkinson's pathology includes significant mitochondrial dysfunction [12]. Key genes, such as PINK1 and Parkin, which are involved in mitochondrial quality control, become dysfunctional, impairing the cells' ability to clear damaged mitochondria [13]. This disruption leads to increased oxidative stress, further endangering the survival of dopamine-producing neurons [14].

In ALS, nearly all cases show an accumulation of a protein known as TDP-43 within motor neurons [15]. Normally, TDP-43 is found in the nucleus where it plays a vital role in RNA processing [16]. However, in ALS, it becomes abnormally modified and forms toxic aggregates in the cytoplasm [17]. This mislocalization disrupts essential cellular functions, creating stress that leads to motor neuron degeneration [18]. Genetic studies have also identified mutations in *SOD1*, a gene associated with familial ALS that contribute to mitochondrial dysfunction and oxidative damage, core features of ALS pathology [19]. Mitochondrial dysfunction in neurodegenerative diseases stems from disrupted oxidative phosphorylation, which reduces ATP production and generates excess reactive oxygen species (ROS). These ROS damage lipids, proteins, and mitochondrial DNA (mtDNA), perpetuating a cycle of oxidative stress. Impaired dynamics, characterized by disrupted fusion and fission processes, further fragment mitochondria, compromising their transport and energy distribution in axons and synaptic terminals [20].

Defective mitophagy exacerbates the problem, as seen in Parkinson's disease, where impaired PINK1-Parkin recruitment prevents the clearance of dysfunctional mitochondria. Similarly, in ALS, mutations in SOD1 amplify oxidative damage and disrupt calcium buffering. Suppressed mitochondrial biogenesis, regulated by the PGC-1α/NRF1/TFAM axis, limits the replacement of damaged organelles, deepening the energy crisis. Together, these processes create a critical vulnerability in neurons, driving excitotoxicity, synaptic failure, and cell death [21].

Advances in cell modeling, particularly using stem cell-derived neurons, have enabled scientists to observe how ALS-related proteins interfere with cellular transport, RNA processing, and stress responses, all of which contribute to the decline of motor neurons [22].

Huntington's disease has its own distinctive molecular feature: an expansion of CAG repeats in the *HTT* gene, leading to an extended polyglutamine tract in the huntingtin protein [23]. This mutant huntingtin (mHTT) misfolds and accumulates in neurons, primarily within the striatum, a region central to motor function and coordination. These aggregates disrupt normal cellular processes by binding to essential transcription factors, leading to widespread changes in gene expression [24]. Moreover, mHTT is known to interfere with mitochondrial function, impairing energy production and increasing oxidative stress, compounding the damage to vulnerable neurons [25].

Common Molecular Hallmarks

Although these diseases stem from different genetic and molecular origins, they converge on shared pathways that reflect common mechanisms of neurodegeneration. Protein misfolding and aggregation, for example, are central to each disease's progression [26]. In Parkinson's disease, α-synuclein exhibits prion-like behavior, propagating through interconnected brain regions by inducing misfolding in neighboring neurons. Similarly, in Alzheimer's disease, tau protein spreads via synaptic connections, highlighting a prion-like mechanism for disease progression [27].

Mitochondrial dysfunction and oxidative stress also play crucial roles as neurons rely heavily on energy and are particularly vulnerable to mitochondrial impairments [28]. When mitochondria malfunction, they produce ROS, leading to lipid peroxidation, protein oxidation, and DNA damage—each a destructive force that accelerates cellular aging and death. Neuroinflammation plays a dual role in neurodegeneration. While acute neuroinflammation serves protective functions by clearing debris and repairing tissue, chronic activation of microglia and astrocytes releases pro-inflammatory cytokines that exacerbate neuronal damage. This context-dependent role underscores the complexity of targeting neuroinflammation in therapeutic strategies [29].

This convergence of molecular pathways across neurodegenerative diseases emphasizes the need for a holistic, systems-based approach to understanding these interconnected mechanisms. Increasingly, researchers are looking for biomarkers and therapeutic targets that cut across these shared pathways, with the aim of developing treatments that could potentially benefit multiple neurodegenerative diseases [30].

1.2. The Importance of Molecular Understanding

The molecular exploration of neurodegenerative diseases has revolutionized the field, shifting our perspective from broad disease categories to precise, mechanistic understanding [31]. By dissecting the fundamental cellular and molecular pathways involved in diseases like AD, PD, ALS, and HD, scientists are uncovering how these intricate biological processes converge, sparking innovations in diagnostics and therapeutic strategies [32].

One of the pivotal advancements in recent years has been the identification and development of biomarkers. Biomarkers for AD, for instance, now include specific protein signatures detectable in cerebrospinal fluid (CSF) and blood, providing insight into disease progression long before symptoms arise [33]. Elevated levels of Aβ and phosphorylated tau—key pathological proteins in AD—are now measurable through advanced techniques such as ultrasensitive immunoassays and liquid chromatography–mass spectrometry [34]. These biomarkers provide a critical glimpse into the "silent" phases of neurodegeneration,

often detectable as early as 20 years before cognitive symptoms manifest and, thus, hold enormous potential for early intervention and tracking disease progression in clinical settings [35].

Moreover, molecular insights have clarified the interwoven pathways that drive neurodegeneration, revealing how initial cellular disturbances propagate across interconnected networks [36]. When proteins like tau in AD or alpha-synuclein in PD misfold and aggregate, they not only form toxic intracellular clumps but also disrupt surrounding cellular systems [37]. These aggregates place substantial stress on mitochondria, the cell's energy factories, impairing their function and leading to the generation of ROS. This oxidative stress damages essential cellular components like DNA, proteins, and lipids, creating a vicious cycle where protein misfolding and cellular damage fuel each other [38]. This cascade, involving both mitochondrial dysfunction and oxidative stress, is a theme across many neurodegenerative diseases and has underscored the importance of developing multi-target therapies that can simultaneously stabilize protein structures and protect mitochondrial health [39].

Targeted therapeutics represent another area transformed by molecular research. Traditional treatments often aimed to alleviate symptoms but did not address the disease's root molecular causes. Now, therapies are being designed with unprecedented precision [40]. For example, small molecule inhibitors have been developed to specifically prevent amyloid and tau aggregation in AD and alpha-synuclein aggregation in PD [41]. These inhibitors bind selectively to misfolded proteins, blocking the formation of toxic aggregates and, in preclinical studies, have shown potential to reduce plaque formation and improve neuronal health [42]. Another promising avenue involves molecular chaperones—proteins that assist in proper folding and prevent misfolding—showing efficacy in reducing neurotoxic aggregates in experimental models of diseases like HD and ALS [43]. Such approaches mark a significant departure from earlier symptomatic treatments as they focus on halting or even reversing molecular dysfunction at its source [44].

Gene-editing technologies, particularly CRISPR-Cas9, have also introduced transformative possibilities, especially for diseases with a clear genetic component. In HD, for instance, CRISPR has been experimentally used to silence the mutant *HTT* gene, which encodes the misfolded huntingtin protein that drives the disease [45]. By selectively editing the gene responsible for this toxic protein, CRISPR offers a path to not only slow disease progression but also potentially correct the underlying genetic defect [46]. In ALS, similar CRISPR applications target mutations in genes like *SOD1* and *C9orf72*, directly addressing the genetic basis of familial cases [47]. The precision of CRISPR technology allows for targeted interventions at the DNA level, effectively altering the trajectory of these diseases in preclinical models [48]. Despite its promise, CRISPR technology faces significant challenges, including off-target effects that may inadvertently alter non-disease-causing genes, and delivery issues that limit its effectiveness in targeting specific brain regions. Ethical considerations, such as the long-term consequences of gene editing and its potential misuse, must also be addressed before widespread clinical application [49].

Epigenetic mechanisms further enrich our understanding of neurodegeneration, revealing how gene expression can be influenced by factors beyond DNA sequence alone. DNA methylation, histone modification, and non-coding RNAs are dynamic regulatory elements that respond to cellular signals and environmental changes [50]. In neurodegenerative diseases, these epigenetic modifications can either exacerbate or protect against neuronal damage. For instance, histone deacetylase (HDAC) inhibitors have been shown to increase the expression of neuroprotective genes and reduce protein aggregation in animal models of HD and ALS. By altering histone acetylation levels, HDAC inhibitors loosen tightly packed chromatin, allowing protective genes to be more actively expressed, which enhances neuron survival [51]. Additionally, non-coding RNAs, particularly microRNAs (miRNAs), play a crucial role in gene regulation by influencing RNA stability and translation. Studies show that specific miRNAs are dysregulated in AD, PD, and ALS, impacting processes such as apoptosis, inflammation, and oxidative stress [52]. These miRNAs are

now being explored both as biomarkers for early and precise diagnosis and as therapeutic targets where adjusting miRNA levels could help re-establish cellular equilibrium [53].

Neuroinflammation has also been re-evaluated through a molecular lens, revealing its role as a driving factor rather than merely a byproduct of neurodegeneration [54]. In AD and PD, the brain's immune cells—microglia—are activated in response to the accumulation of misfolded proteins and damaged cellular components [55]. This activation triggers inflammatory signaling pathways, such as the NF-κB and MAPK pathways, which leads to the release of pro-inflammatory cytokines and chemokines [56]. These molecules, while initially intended to clear cellular debris, sustain a chronic inflammatory state that damages neurons and perpetuates cellular stress [57]. The prolonged activation of microglia thus transforms the brain environment into one that is neurotoxic, hastening neurodegeneration [58]. By targeting specific inflammatory pathways—such as through inhibitors that block cytokine production or through compounds that modulate microglial activation—researchers are exploring anti-inflammatory therapies that could disrupt this cycle and reduce the inflammatory burden in neurodegenerative diseases [59]. Experimental therapies are showing promise in preclinical studies and are moving into clinical trials, marking an exciting step forward in leveraging molecular insights for therapeutic development [60].

Altogether, the molecular understanding of neurodegenerative diseases has set the stage for precision medicine. By integrating data across proteomics, genomics, epigenetics, and immunology, researchers are constructing a detailed molecular map that guides diagnosis and treatment. The vision is not only to identify those at risk through biomarkers but to tailor therapies based on an individual's unique molecular profile, a personalized approach that holds promise for fundamentally altering the course of neurodegenerative diseases. As molecular techniques continue to evolve, this precision approach is likely to deepen, offering new hope in the fight against these challenging conditions.

1.3. Purpose and Scope of the Review

This paper delves into the intricate molecular landscape of neurodegenerative diseases, unraveling the profound connections that bind together conditions like AD, PD, ALS, and HD. Though each of these diseases displays its own distinct clinical features, recent molecular research reveals a fascinating convergence in their underlying mechanisms. Shared hallmarks—such as protein misfolding, mitochondrial dysfunction, oxidative stress, neuroinflammation, and epigenetic changes—suggest that these disorders are bound by common threads, offering a fresh perspective that unites our understanding of neurodegeneration.

At the heart of this exploration is the concept of interconnected cellular dysfunction where one molecular disturbance sets off a cascade of effects across different systems within the cell. For example, a single misfolded protein may trigger oxidative stress, impair mitochondria, and activate inflammatory pathways, creating a feedback loop that further drives protein misfolding. Through this lens, neurodegeneration emerges not as a sequence of isolated failures but as a deeply interconnected network of molecular disruptions, each amplifying the others in a downward spiral toward cellular collapse.

What sets this work apart is its emphasis on the extraordinary translational potential of these molecular insights, opening new doors for diagnostics and therapy. With recent breakthroughs, scientists can now go beyond merely understanding these pathways to actively intervening. Gene-editing techniques like CRISPR-Cas9, for instance, allow for the selective silencing of mutant genes in HD or ALS, while molecular chaperones are showing promise in reshaping misfolded proteins in AD and PD. Once seen as speculative, these approaches are advancing rapidly, with initial studies suggesting that they could redefine the trajectory of these diseases and bring a new level of hope to patients. This paper examines these emerging strategies, shedding light on their potential to reshape the landscape of neurodegenerative treatment.

In addition to therapeutic advances, this analysis highlights how molecular insights are fueling a revolution in personalized diagnostics. The discovery of biomarkers, once

a distant ambition, is now making it possible to detect neurodegeneration at its earliest stages. Ultra-sensitive tests now reveal changes in biomarkers like Aβ and tau in AD, or phosphorylated alpha-synuclein in PD, long before symptoms appear, providing a precious opportunity for early intervention. The capacity to diagnose these diseases years before they fully manifest opens the door to proactive, individualized care, marking a shift from late-stage management to pre-emptive action.

In essence, this work bridges the worlds of foundational molecular research and practical application, demonstrating how these insights could drive transformative change. For researchers, it offers a map of interconnected pathways and untapped cross-disease targets. For clinicians, it introduces powerful tools for early diagnosis and precision treatment. For patients and their families, it paints a picture of hope—a future where these insights may lead to early detection, effective intervention, and perhaps, one day, even cures for diseases once considered untreatable. Through this unified approach, the paper captures the extraordinary potential of molecular neurobiology, showing how it is reshaping our understanding and transforming the future of neurodegenerative disease treatment.

2. Protein Misfolding and Aggregation

At the core of many neurodegenerative diseases lies a fundamental breakdown in protein folding, a delicate cellular process that normally allows proteins to adopt precise shapes, enabling them to carry out their specific tasks. But in diseases like AD, PD, ALS, and HD, certain proteins misfold and clump together, creating aggregates that disrupt cellular balance and drive the progression of neurodegeneration [61]. These toxic accumulations of Aβ and tau in AD, alpha-synuclein in PD, and huntingtin in HD overwhelm the cell's natural defenses, activating pathways that, over time, destabilize neurons and lead to their eventual death [62].

Each of these proteins has a unique story—a pathway that explains how they go from functional molecules to toxic agents within the brain [63]. By examining these misfolding pathways in detail, we gain a deeper understanding of the biochemical transformations that lead to disease and, ultimately, how to intervene.

2.1. Molecular Pathways of Protein Misfolding

Each neurodegenerative disease is driven by a specific misfolding pathway that turns an otherwise normal protein into a damaging aggregate. These pathways are shaped by a combination of genetic predispositions, cellular stress responses, and environmental factors that, together, push these proteins over the threshold from order to chaos [64]. Below, we explore the molecular journey of Aβ and tau in AD, alpha-synuclein in PD, and huntingtin in HD, focusing on the unique ways they drive neuronal dysfunction.

2.1.1. Amyloid-Beta in Alzheimer's Disease

In Alzheimer's disease, the protein Aβ plays a key role in the buildup of toxic plaques. Under normal circumstances, APP, from which Aβ is derived, is cut by enzymes in a way that does not lead to harm [65]. However, in AD, an alternative pathway is activated, causing APP to be cut by beta- and gamma-secretases, which release Aβ fragments, particularly Aβ42. This specific form of Aβ is highly prone to aggregation due to its structure, allowing it to quickly form toxic clusters [66].

Aβ aggregation starts with the formation of small oligomers, or "seed" particles, which have the unique ability to insert themselves into cell membranes. Once embedded, these oligomers disrupt calcium regulation by creating pore-like structures, letting calcium ions flood into the cell [67]. This disruption destabilizes the neuron, affecting processes like synaptic plasticity, which is essential for memory and learning. Moreover, oligomers interact with cell-surface NMDA receptors, overactivating them and leading to excitotoxicity—an excessive calcium influx that damages or kills the cell [68]. Over time, these oligomers join together into larger fibrils and, eventually, amyloid plaques, which set

off an immune response that calls in microglia and astrocytes. These immune cells release inflammatory cytokines, creating a neurotoxic environment that further harms nearby neurons [69].

Interestingly, metal ions like zinc and copper bind to Aβ and stabilize its misfolded structure, accelerating the plaque-forming process. Current therapeutic strategies target early steps in this pathway, aiming to block beta- and gamma-secretase activity, chelate metals to slow Aβ aggregation, and use antibodies to clear Aβ oligomers before they accumulate into plaques [70].

2.1.2. Tau Protein Hyperphosphorylation

Tau, another protein central to Alzheimer's pathology, normally supports the cell's microtubules, which act like highways within the neuron to transport nutrients and signals. In AD, tau undergoes excessive phosphorylation—a process where phosphate groups attach to the protein [71]. When tau is hyperphosphorylated, it loses its affinity for microtubules, causing them to destabilize. Detached tau proteins then begin to self-associate, creating twisted structures known as paired helical filaments (PHFs) [72]. These filaments grow into neurofibrillary tangles (NFTs) within neurons.

The formation of NFTs disrupts the cell's internal architecture, leading to failures in nutrient transport and waste removal. Beyond this structural disruption, hyperphosphorylated tau exhibits a prion-like behavior, spreading from cell to cell and seeding misfolding in neighboring neurons. This spread is thought to drive the progression of AD across different brain regions, correlating with worsening cognitive symptoms [73]. Recent research shows that stress responses, like the unfolded protein response (UPR), play a role in tau aggregation. When misfolded proteins accumulate, cells activate the UPR, attempting to restore normal conditions, but this response becomes overactive in AD, perpetuating tau aggregation [74].

Therapeutic approaches for tau pathology include kinase inhibitors, which aim to prevent excessive phosphorylation, and immunotherapies that target tau as it moves between cells. The hope is that by halting tau's prion-like spread, these therapies can slow or stop the disease's progression [75].

2.1.3. Alpha-Synuclein in Parkinson's Disease

In PD, the protein alpha-synuclein misfolds and forms clumps that accumulate inside neurons as Lewy bodies. Alpha-synuclein normally helps regulate dopamine release, but mutations in the *SNCA* gene and environmental toxins can trigger it to misfold. Once misfolded, alpha-synuclein adopts a beta-sheet structure that is prone to aggregation, forming oligomers that are highly toxic [76].

These oligomers of alpha-synuclein integrate into cellular membranes, disrupting their integrity by creating pores. These pores allow unregulated calcium influx, which disrupts cellular homeostasis and strains mitochondria, leading to excessive production of ROS and activation of apoptosis, or programmed cell death [77]. Dopaminergic neurons are especially susceptible to this toxicity because they rely heavily on energy-demanding processes and have relatively low antioxidant defenses. As oligomers grow into fibrils and Lewy bodies, they interfere with cellular recycling processes like autophagy, causing damaged proteins and organelles to accumulate within the cell [78].

Furthermore, alpha-synuclein exhibits prion-like properties, allowing misfolded forms to travel between neurons. Extracellular alpha-synuclein is taken up by neighboring cells, inducing aggregation and spreading pathology across brain regions in a way that mirrors PD's clinical progression [79]. Therapies for PD focus on stabilizing alpha-synuclein in its native form, using antibodies to clear misfolded proteins, and enhancing autophagy to improve the cell's ability to degrade alpha-synuclein aggregates [42].

2.1.4. Mutant Huntingtin in Huntington's Disease

Huntington's disease arises from a mutation in the *HTT* gene, leading to an abnormal expansion of CAG repeats that encode a polyglutamine (polyQ) tract in the huntingtin protein. This extended polyQ region makes the mHTT sticky and prone to forming toxic aggregates [23]. The aggregation of mHTT is particularly harmful in the striatum, a brain region critical for motor function, where it interferes with essential cellular processes [80].

Mutant huntingtin disrupts transcription by binding to transcription factors and coactivators, interfering with the normal expression of genes essential for neuronal survival [81]. mHTT also impairs autophagy, the cell's main pathway for clearing damaged components. By disrupting autophagy, mHTT prevents the cell from effectively removing itself, allowing damaged proteins and aggregates to accumulate. Additionally, mHTT interacts with mitochondria, impairing their function and leading to energy deficits, which compounds cellular stress [82].

Therapeutic strategies for HD include approaches that silence mutant *HTT* expression through RNA interference and antisense oligonucleotides (ASOs), reducing the production of the toxic protein. Other therapies aim to enhance autophagy, helping cells clear mHTT aggregates or stabilize mitochondrial function, thereby alleviating some of the cellular damage caused by mHTT [83].

Each of these pathways reveals a unique aspect of protein misfolding, but together, they paint a picture of how small molecular changes can lead to devastating consequences. Misfolded proteins disrupt essential cellular processes, overwhelm protective pathways, and, as they accumulate, create an environment where neurons struggle to survive [84]. By targeting these early stages of misfolding and aggregation, researchers hope to develop therapies that can interrupt the cascade of cellular dysfunction, offering new avenues for treating neurodegenerative diseases at their source [85].

The following table (Table 1) provides an overview of the fundamental molecular mechanisms associated with neurodegenerative diseases, emphasizing high-impact studies that have shaped our understanding of each mechanism's role. The table categorizes key processes such as protein misfolding, neuroinflammation, and mitochondrial dysfunction, linking each mechanism to specific therapeutic targets. By synthesizing data from highly cited research, this table illustrates the multifaceted nature of neurodegenerative disease pathology and highlights current therapeutic approaches aimed at modulating these critical molecular pathways.

Table 1. Key molecular mechanisms and their therapeutic targets in neurodegenerative diseases.

Mechanism	Neurodegenerative Disease	Key Molecular Targets	Therapeutic Approaches	Notable Studies (High Citations)
Protein misfolding and aggregation	Alzheimer's, Parkinson's	Amyloid-beta, tau, alpha-synuclein	Anti-aggregation agents, tau inhibitors, immunotherapy	Selkoe (2002) [86], Spillantini et al. (1998) [87]
Neuroinflammation	All (AD, PD, ALS, HD)	Microglia, cytokines (TNF-α, IL-1β), NLRP3 inflammasome	Anti-inflammatory agents, cytokine inhibitors	Heneka et al. (2015) [88], Amor et al. (2010) [89]
Mitochondrial dysfunction	ALS, Huntington's	Electron transport chain complexes, mtDNA	Antioxidants, mitochondrial enhancers	Lin and Beal (2006) [90], Bhatt et al. (2021) [91]
Genetic and epigenetic regulation	Alzheimer's, ALS, Huntington's	APOE ε4, SOD1, HTT, DNA methylation, histone modifications	Gene silencing (RNAi, ASOs), CRISPR, epigenetic modulators	De Strooper and Karran (2016) [92], Finkbeiner et al. (2011) [93]
Synaptic dysfunction	Alzheimer's, Parkinson's	Cholinergic (ACh), dopaminergic systems, NMDA receptors	Synaptic modulators, neurotrophic factors	Stanciu et al. (2020) [94], Dauer and Przedborski (2003) [95]

2.2. Cellular Consequences of Protein Aggregation

In neurodegenerative diseases, the buildup of misfolded proteins within neurons does much more than just accumulate; it disrupts the cell's natural rhythm and resilience. These protein clumps overwhelm the cell's protective systems, overloading its capacity to handle protein folding, degradation, and calcium balance [96]. Neurons, with their intricate structure and high energy needs, are especially vulnerable to this kind of stress. Over time, the presence of these aggregates creates a toxic environment, setting off a cascade of dysfunction that leaves neurons struggling to survive. Understanding how protein aggregation disturbs cellular balance and proteostasis reveals the deep-seated challenges neurons face, highlighting how these disruptions ultimately push cells toward degeneration.

2.2.1. Disruption of Cellular Homeostasis

One of the first and most severe impacts of protein aggregation in neurodegenerative diseases is the stress it places on the endoplasmic reticulum (ER), the cell's central station for protein folding, lipid synthesis, and calcium storage. The ER is finely tuned to detect misfolded proteins, and when these start piling up, it activates the UPR, a defense mechanism designed to temporarily boost the cell's folding capacity and promote the clearance of misfolded proteins [97]. But when protein misfolding is constant, as it is in these diseases, the UPR remains switched on, eventually shifting from a protective to a harmful role as the cell's resources are stretched beyond capacity [98].

In AD, Aβ oligomers wreak havoc on calcium regulation within the ER. These toxic clumps latch onto calcium channels in the ER membrane, causing calcium to spill into the cytoplasm and disrupt essential signaling pathways that neurons rely on for communication [99]. As calcium floods into the cell, it also overloads mitochondria, which attempt to buffer the imbalance but become overwhelmed, swelling and releasing ROS. These excess ROS, in turn, damage cellular components and push the neuron closer to apoptosis, or programmed cell death.

PD presents a similar situation, with alpha-synuclein aggregates triggering stress responses within the ER. These misfolded proteins bind to BiP, an ER chaperone that plays a central role in managing protein folding [100]. With BiP compromised, the ER is left unable to handle the growing load of misfolded proteins, leading to prolonged UPR activation. This chronic stress response triggers inflammatory cytokines, which invite immune cells and create an environment of neuroinflammation [101]. Over time, ER stress induces CHOP, a transcription factor that activates pro-apoptotic genes, setting the neuron on a path toward programmed death [102].

These disruptions in calcium regulation and persistent ER stress create a cycle that is particularly harmful in neurons. As calcium leaks from the ER, it not only damages mitochondria but also erodes the neuron's ability to sustain essential functions. This constant strain weakens the cell's defenses, leaving neurons less able to handle additional stress and making degeneration almost inevitable [103].

2.2.2. Impairment of Proteostasis

Proteostasis, or the balance of protein maintenance, is a delicate process essential for cellular health, especially in neurons where proper protein turnover is crucial for survival [104]. This balance relies on two main pathways: the ubiquitin–proteasome system (UPS) and the autophagy–lysosomal pathway, both of which work together to identify, break down, and clear misfolded proteins and cellular waste [63]. In neurodegenerative diseases, however, these systems are overwhelmed, leaving the cell unable to handle the mounting accumulation of damaged proteins, which results in a toxic buildup that further stresses neurons [105].

Ubiquitin–Proteasome System Dysfunction

The UPS is responsible for identifying and degrading small, damaged, or short-lived proteins. Proteins marked for disposal are tagged with ubiquitin molecules, directing them

to the proteasome where they are broken down for recycling. But in neurodegenerative diseases, protein aggregates inhibit the UPS, leaving ubiquitinated proteins to accumulate within the cell [106].

In Alzheimer's disease, tau aggregates directly interfere with the proteasome's function. When hyperphosphorylated, tau binds to the proteasome's 20S core, blocking the entry of proteins marked for degradation. As these proteins back up, they form secondary aggregates, adding further strain to the cell's quality control systems. This UPS impairment disrupts normal cellular functions that rely on regulated protein turnover, leading to additional stress on the neuron [107].

In Parkinson's disease, alpha-synuclein clogs the proteasome by obstructing its entry points, preventing ubiquitinated proteins from being processed. Studies show that alpha-synuclein oligomers interact with the 19S regulatory particle, blocking substrate entry and leading to a buildup of damaged proteins within dopaminergic neurons [108]. This failure of the UPS not only accelerates the aggregation of alpha-synuclein but also adds to the cellular backlog, creating a cycle of rising toxicity and stress within the cell [109].

Autophagy–Lysosomal Pathway Defects

The autophagy–lysosomal pathway handles the degradation of larger protein aggregates and damaged organelles. Through autophagy, cells create vesicles known as autophagosomes that capture cellular debris, which are then fused with lysosomes to break down and recycle the contents [110]. In neurodegenerative diseases, however, this pathway becomes impaired, preventing the cell from removing toxic aggregates [111].

In HD, the mHTT protein directly impairs autophagy by sequestering key receptors like p62 and LC3, which are essential for identifying and capturing cargo for degradation. When these receptors are trapped within mHTT aggregates, autophagosomes cannot form properly, leaving toxic aggregates and damaged organelles to build up within the cell [112]. This accumulation strains the mitochondrial function, raises oxidative stress, and accelerates cellular decline, contributing to the characteristic cell death seen in HD [113,114].

In PD, alpha-synuclein disrupts lysosomal acidification, which is crucial for activating lysosomal enzymes [115]. Without the proper acidic environment, these enzymes cannot degrade alpha-synuclein fibrils or other cellular waste effectively. This impairment leads to a backlog of undegraded material in autophagosomes, which then leak into the cell, increasing oxidative stress and triggering inflammation [116]. Alpha-synuclein also affects chaperone-mediated autophagy (CMA), a pathway that degrades proteins through direct lysosomal delivery [117]. By blocking LAMP-2A, a critical lysosomal receptor, alpha-synuclein further reduces the cell's capacity to clear misfolded proteins, leading to a progressive toxic buildup that further destabilizes the neuron [118].

In ALS, TDP-43 aggregates block the fusion of autophagosomes with lysosomes, preventing the clearance of cellular waste [119]. TDP-43 also disrupts mitophagy, a form of autophagy specifically responsible for clearing damaged mitochondria [104]. The inability to clear dysfunctional mitochondria leads to a buildup of ROS and depletes ATP reserves, creating an energy crisis that strains motor neurons, particularly susceptible to high energy demands, and accelerates their degeneration [120].

Altogether, the failure of proteostasis—due to the impairment of both UPS and autophagy–lysosomal pathways—leaves neurons in a state of toxic overload, unable to clear out the cellular debris that continues to accumulate [121]. This chronic buildup of damaged proteins and organelles disrupts metabolic processes, heightens oxidative stress, and creates a toxic cellular environment that gradually drives neurons to the brink [122].

The breakdown in cellular homeostasis and proteostasis due to protein aggregation creates a cycle of stress that neurons cannot easily escape. As these cells struggle with constant misfolded proteins, oxidative damage, and metabolic instability, they become increasingly vulnerable to degeneration [123]. Understanding these complex interactions opens up new avenues for therapeutic research, as scientists work to reinforce these protective systems and develop treatments that might slow or even stop neurodegenerative progression at its roots.

2.3. Molecular Therapeutic Strategies

With the advances in understanding the molecular foundations of neurodegenerative diseases, there's now a push toward treatments that intervene directly at the roots of cellular dysfunction [124]. Traditional treatments often only address symptoms, but these novel approaches target the misfolded proteins and disrupted cellular pathways at the heart of diseases like Alzheimer's, Parkinson's, ALS, and Huntington's [125]. These therapies focus on stopping protein aggregation, enhancing cellular protein clearance, and supporting proper protein folding with molecular chaperones. Each strategy aims to restore cellular health, reduce toxic accumulations, and protect neurons from degeneration [126].

2.3.1. Inhibition of Protein Aggregation

A primary approach to neurodegenerative therapy involves small molecules designed to stop proteins from aggregating in the first place. These molecules work by binding to the misfolded proteins, either stabilizing them in a less toxic form or preventing them from clustering into larger, harmful assemblies.

Targeting Amyloid-Beta and Tau in Alzheimer's Disease

In AD, the aggregation of Aβ and tau is a well-known feature of disease pathology. Researchers have developed small molecules that bind to Aβ monomers, preventing them from joining into toxic oligomers that interfere with synaptic function [127,128]. One such compound, tramiprosate, binds to Aβ and stabilizes it in a way that blocks its self-assembly, while EPPS (4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid) has been shown to actually break down existing amyloid plaques in preclinical studies, suggesting potential to reduce Aβ burden in the brain [129].

Similarly, tau aggregation inhibitors focus on preventing tau from detaching from microtubules and forming neurofibrillary tangles. Compounds like LMTM (leuco-methylthioninium bis(hydromethanesulfonate)) stabilize tau's normal structure, thereby reducing its tendency to aggregate into the paired helical filaments that comprise neurofibrillary tangles [130]. LMTM works by inhibiting tau phosphorylation, which normally triggers its detachment from microtubules, and has shown promise in preclinical trials for preserving synaptic health and slowing cognitive decline [131,132].

The following figure (Figure 1) provides a visual overview of the steps involved in Aβ formation and plaque accumulation. It illustrates how APP is cleaved by β- and γ-secretases to release Aβ peptides, which, subsequently, aggregate in the extracellular space, forming the amyloid plaques that are characteristic of Alzheimer's disease pathology.

Preventing Alpha-Synuclein Aggregation in Parkinson's Disease

In PD, alpha-synuclein misfolding leads to the formation of Lewy bodies, which disrupt cellular communication and dopamine production. Small molecules like anle138b interact with alpha-synuclein early in the misfolding process, preventing it from forming toxic fibrils [133]. By stabilizing alpha-synuclein, these molecules reduce the buildup of Lewy bodies, preserving cellular function [134]. Additionally, NPT200-11 has shown the potential to reduce alpha-synuclein pathology by binding to alpha-synuclein oligomers, keeping them from forming the toxic aggregates that damage dopaminergic neurons [135].

Inhibiting Mutant Huntingtin Aggregation in Huntington's Disease

HD is marked by the aggregation of protein mHTT with extended polyQ sequences, leading to toxic inclusions in neurons [136]. Compounds such as EGCG (epigallocatechin gallate) and C2-8 target these polyQ regions, reducing mHTT's tendency to self-aggregate and potentially enhancing clearance of these protein clusters. Studies show that EGCG binds directly to huntingtin, stabilizing its structure and preventing aggregation [137]. This stabilization reduces cellular stress, making these inhibitors promising candidates for therapies targeting the root pathology of HD [138].

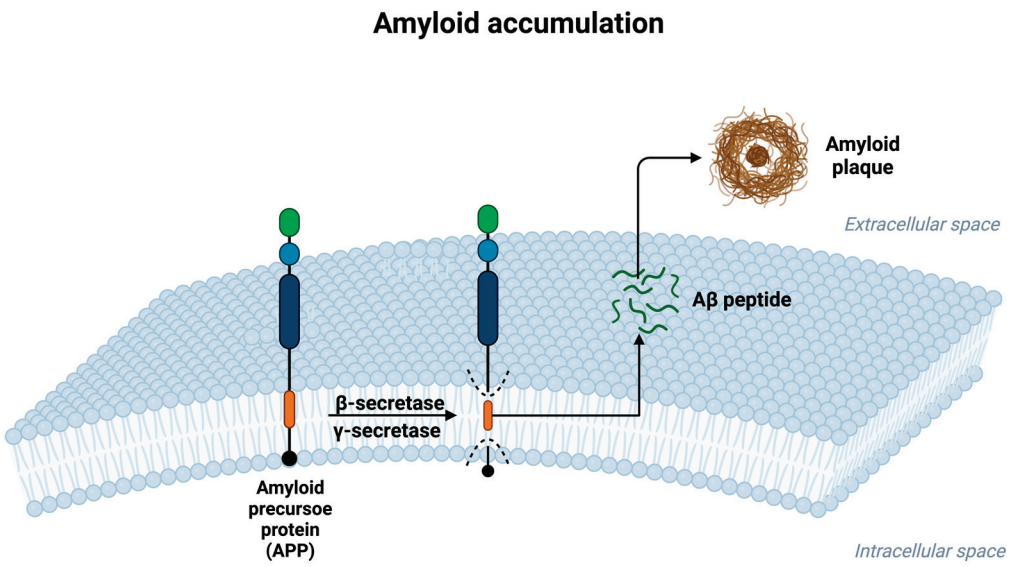


Figure 1. Amyloid accumulation in Alzheimer's disease. This figure illustrates the process of amyloid-beta (Aβ) peptide formation and accumulation, a hallmark of Alzheimer's disease pathology. Targeting Aβ oligomers is significant because these smaller aggregates are more neurotoxic than amyloid plaques. Aβ oligomers disrupt synaptic function, impair ion homeostasis, and activate detrimental signaling pathways, making them a critical focus for therapeutic interventions aimed at halting disease progression.

2.3.2. Enhancing Protein Clearance Pathways

The UPS and autophagy–lysosomal pathway are the cell's main methods for clearing out damaged and misfolded proteins. In neurodegenerative diseases, these systems become overwhelmed by toxic accumulations [139]. Therapies aimed at boosting these pathways work to enhance the cell's ability to clear misfolded proteins before they cause harm [140,141].

Enhancing the Ubiquitin–Proteasome System

The UPS handles the breakdown of short-lived or damaged proteins. In this system, proteins tagged with ubiquitin are directed to the proteasome for degradation. However, when misfolded proteins accumulate beyond the system's capacity, the UPS becomes overloaded [142]. Proteasome activators, like 3,4-dimethoxychalcone, show promise in restoring UPS activity in AD models where they promote the degradation of tau and prevent its buildup within neurons. By enhancing proteasome activity, these compounds can alleviate some of the toxic load within cells, reducing cellular stress and slowing disease progression.

Selective enhancement of E3 ligase activity is another approach to improving UPS efficiency. E3 ligases add ubiquitin tags to specific misfolded proteins, marking them for degradation. In ALS and AD, where proteins like TDP-43 and tau form damaging aggregates, activating specific E3 ligases helps the UPS selectively target these toxic proteins, enabling the cell to manage misfolded proteins more effectively and prevent them from accumulating in the first place [143].

Stimulating Autophagy and the Lysosomal Pathway

Autophagy is essential for clearing larger aggregates and damaged cellular components. By inducing autophagy, cells can encapsulate aggregates within autophagosomes, which then fuse with lysosomes to degrade their contents. Rapamycin, a well-known autophagy inducer, promotes the formation of autophagosomes by inhibiting mTOR, a regulator of cellular growth that normally suppresses autophagy [144]. In HD models, rapamycin has shown promise in clearing mutant huntingtin aggregates, thus improving cellular health and reducing motor symptoms [145].

In Parkinson's disease, lysosomal dysfunction plays a significant role in the buildup of alpha-synuclein. Ambroxol, a compound that promotes lysosomal acidification, restores

the activity of lysosomal enzymes like glucocerebrosidase, which is essential for breaking down alpha-synuclein fibrils [146]. By improving lysosomal function, ambroxol enhances the cell's ability to manage protein overload, which is essential for reducing cellular toxicity and preserving dopaminergic neuron function [147].

2.3.3. Molecular Chaperone Therapies

Molecular chaperones, particularly heat shock proteins (HSPs), are essential for helping proteins fold correctly and preventing misfolding [148]. In neurodegenerative diseases, chaperone support has become an area of interest as enhancing chaperone levels can stabilize misfolded proteins and prevent aggregation.

Supporting Chaperones in Alzheimer's and Parkinson's Disease

In Alzheimer's disease, the chaperones HSP70 and HSP90 are known to interact with tau, assisting in proper folding and preventing tangle formation. Arimoclomol, a drug that stimulates heat shock protein production, increases levels of HSP70, which stabilizes tau and reduces its aggregation potential. Studies suggest that arimoclomol's effects on HSP70 also help guide misfolded tau toward degradation pathways, reducing the buildup of neurofibrillary tangles [149].

In Parkinson's disease, small molecules like YM-1 help upregulate HSP70 and HSP27, which play key roles in stabilizing alpha-synuclein and preventing it from forming toxic oligomers. By promoting the expression of these chaperones, YM-1 reduces alpha-synuclein toxicity and helps maintain cellular integrity. This approach has shown promise in models of PD, suggesting that chaperone-based therapies could slow the spread of alpha-synuclein pathology within the brain [150].

Chaperone Enhancement in ALS and Huntington's Disease

In ALS, where the misfolding of TDP-43 and SOD1 is common, enhancing chaperone activity helps manage these proteins. Arimoclomol has demonstrated potential in increasing HSP levels, aiding the refolding or degradation of SOD1 aggregates and supporting neuronal health. The effect of arimoclomol in ALS models includes reduced protein aggregation, improved cell survival, and delayed disease progression [151].

In HD, chaperones like HSP40 and HSP70 have shown potential for reducing huntingtin aggregation. Geldanamycin, an HSP90 inhibitor, promotes HSP70-mediated pathways that guide mutant huntingtin toward degradation. This shift helps reduce the toxic burden of mHTT, with studies showing improved motor function and cellular resilience in HD models [152]. By supporting chaperones that keep proteins in proper shape or direct them toward clearance, these therapies offer a valuable strategy for countering the molecular disruptions of neurodegenerative diseases [153].

The therapeutic landscape for neurodegenerative diseases is rapidly evolving, with approaches that go beyond symptom relief to address the molecular drivers of disease progression. By preventing protein aggregation, enhancing cellular clearance systems, and leveraging chaperones for protein stability, these strategies aim to protect neurons from the cascading effects of misfolded proteins [154]. Though many of these treatments remain experimental, their potential to directly address the cellular imbalances that fuel neurodegeneration holds promise for interventions that could slow or halt the progression of diseases like Alzheimer's, Parkinson's, ALS, and Huntington's, offering a transformative impact on patient outcomes.

3. Neuroinflammation and Molecular Immune Responses

Neuroinflammation, while a natural defense mechanism, takes on a harmful role in neurodegenerative diseases. In these conditions, inflammation becomes a chronic and self-perpetuating process, driven largely by immune cells in the brain, such as microglia and astrocytes. Initially activated to clear damaged neurons and misfolded proteins, these cells eventually maintain a state of prolonged activation [155]. This chronic inflammation leads to a continuous release of pro-inflammatory molecules, cytokines, and reactive

oxygen species, creating a toxic environment that accelerates neuronal damage rather than alleviating it [156].

At the molecular level, neuroinflammation is fueled by specialized immune receptors, known as pattern recognition receptors (PRRs), which detect signs of cellular distress. These receptors respond to molecular signals called damage-associated molecular patterns (DAMPs), which are released by stressed or dying cells [157]. Together, PRRs and DAMPs drive the inflammatory response, transforming short-term immune activation into a long-term source of damage in neurodegenerative diseases. Understanding the molecular dynamics of these interactions offers a promising direction for therapeutic strategies that could disrupt the cycle of inflammation and protect neurons from further harm [158].

3.1. Molecular Triggers of Neuroinflammation

In neurodegenerative diseases, immune receptors on brain cells sense abnormalities, including protein aggregates and cellular debris. These abnormalities are detected by PRRs, which serve as cellular "sensors" that recognize DAMPs—molecules released by damaged or dying cells as distress signals. When PRRs are activated by DAMPs, they initiate inflammatory pathways that contribute to prolonged neuroinflammation [159]. The main PRRs implicated in this process include Toll-like receptors (TLRs), NOD-like receptors (NLRs), and the receptor for advanced glycation end-products (RAGE). These receptors recognize and respond to specific DAMPs, such as HMGB1, ATP, and mtDNA, amplifying immune responses that, over time, contribute to neuronal loss [160].

Pattern Recognition Receptors (PRRs)

PRRs are critical to the brain's immune response as they specialize in detecting signs of cell damage and stress. These receptors, including TLRs, NLRs, and RAGE, are integral to the inflammatory processes seen in neurodegenerative diseases. Each type of PRR detects unique molecular patterns and activates distinct inflammatory pathways [161].

TLRs are cell-surface receptors that recognize extracellular danger signals. For instance, TLR4, a key player in AD, binds to Aβ aggregates and activates the NF-κB signaling pathway, which leads to the production of pro-inflammatory cytokines like interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) [162]. In PD, TLR2 is activated by alpha-synuclein aggregates, setting off a cascade involving MAPK and NF-κB pathways, which increase the release of inflammatory mediators. Research suggests that blocking TLR2 and TLR4 activity could mitigate inflammatory damage, indicating their potential as therapeutic targets [163].

NLRs and the NLRP3 inflammasome respond to intracellular signals of cellular distress. The NLRP3 inflammasome, a multi-protein complex, is activated by factors like mitochondrial dysfunction, ROS, and protein aggregates such as Aβ and alpha-synuclein [164]. Upon activation, NLRP3 facilitates the activation of caspase-1, which processes cytokines like IL-1β and IL-18 into their active forms, intensifying the inflammatory response. In AD, NLRP3 activation near amyloid plaques contributes to neuron damage, while in PD, cytosolic alpha-synuclein activates NLRP3, promoting sustained inflammation [165]. Inhibiting NLRP3 has shown promising results in preclinical studies as it reduces neuroinflammation and slows disease progression, highlighting its potential as a therapeutic target [166].

RAGE recognizes advanced glycation end-products (AGEs) as well as other DAMPs, including Aβ and HMGB1. Once activated, RAGE promotes inflammation through NF-κB signaling, increasing cytokine production and ROS release. In AD, RAGE binding with Aβ enhances neurotoxic pathways and amyloid plaque formation, contributing to cognitive decline [167]. In ALS, RAGE activation by AGEs and oxidative stress compounds motor neuron degeneration. Experimental RAGE inhibitors are being studied for their potential to disrupt these harmful interactions and reduce inflammation, representing another promising direction for therapeutic intervention [168].

Damage-Associated Molecular Patterns (DAMPs)

DAMPs are endogenous molecules that act as distress signals, alerting the immune system to cellular damage. Released from injured cells, DAMPs activate PRRs on microglia

and astrocytes, maintaining a pro-inflammatory state that becomes chronic in neurodegenerative diseases. Key DAMPs in these diseases include HMGB1, extracellular ATP, and mtDNA, each contributing uniquely to the inflammation seen in neurodegeneration [169,170].

High mobility group box 1 (HMGB1) is a nuclear protein that helps regulate chromatin structure, but when released outside cells, it acts as a DAMP. In neurodegenerative diseases, HMGB1 is elevated in affected brain regions, such as the hippocampus in AD and motor neurons in ALS [171]. Once outside the cell, HMGB1 binds to receptors like TLR4 and RAGE, activating signaling pathways that lead to cytokine release and oxidative stress [172]. This sustained cytokine release disrupts synaptic function and accelerates neuronal death, particularly in areas surrounding amyloid plaques in AD. Blocking HMGB1 signaling has shown potential in reducing neuroinflammation and protecting against neurodegeneration [173].

Extracellular ATP and purinergic receptors serve as potent inflammatory signals when released from cells during injury. ATP binds to purinergic receptors like P2X7 on microglia, activating inflammasomes such as NLRP3, which further amplify inflammatory responses by releasing IL-1β and other cytokines [174]. In PD, elevated extracellular ATP levels enhance alpha-synuclein toxicity by activating P2X7. Experimental approaches that block P2X7 signaling show promise in reducing ATP-driven neuroinflammation and protecting dopaminergic neurons from sustained damage [175].

mtDNA is typically contained within mitochondria, but when mitochondria are damaged, mtDNA leaks into the extracellular space where it acts as a powerful DAMP [176]. Once outside the mitochondria, mtDNA binds to receptors like TLR9, which recognizes specific unmethylated DNA sequences. In ALS, mtDNA released from injured motor neurons triggers TLR9 activation in nearby glial cells, contributing to chronic inflammation and oxidative stress. Research into blocking TLR9 activation or limiting mtDNA release suggests potential therapeutic strategies to reduce inflammation in diseases driven by mitochondrial dysfunction [169].

Together, PRRs and DAMPs create a self-sustaining cycle of neuroinflammation in neurodegenerative diseases where prolonged immune activation results in progressive neuronal loss [177]. By targeting these molecular mechanisms, researchers are working to develop therapies that can break the cycle of chronic inflammation, protect neurons, and slow the progression of neurodegenerative diseases, offering hope for interventions that directly address the underlying drivers of neuroinflammatory damage [178].

3.2. Microglial Activation States

Microglia, the brain's resident immune cells, are essential for maintaining neural health and responding to injury. In neurodegenerative diseases, these cells often shift from protective roles to states that exacerbate inflammation and contribute to neuronal damage [179]. Microglial activation is dynamic, encompassing a spectrum of responses, but is often simplified into two main functional states: pro-inflammatory "M1" and anti-inflammatory "M2". This shift from protective to harmful roles is influenced by signals within the disease environment, making microglial activation a central focus for potential therapeutic intervention in neurodegenerative diseases [180].

The following figure (Figure 2) illustrates the differentiation of monocytes into M0 macrophages, which can then polarize into M1 or M2 phenotypes depending on environmental cues. By understanding the molecular pathways that drive macrophage polarization, researchers are exploring strategies to promote M2-like states in neurodegenerative diseases, aiming to harness their anti-inflammatory and protective functions to mitigate disease progression and support neuronal health.

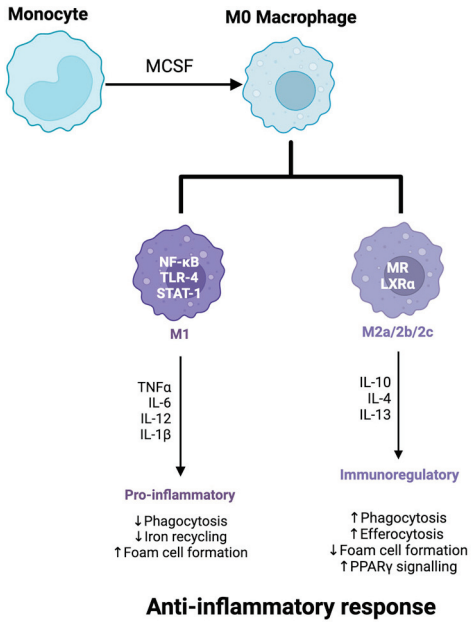


Figure 2. Macrophage polarization and anti-inflammatory response.

3.2.1. M1 vs. M2 Phenotypes

The M1 microglial phenotype is characterized by pro-inflammatory activity. When microglia adopt this state, they release high levels of cytokines such as IL-1β, TNF-α, and interleukin-6 (IL-6), as well as ROS and nitric oxide (NO) [181]. While these responses help protect the brain from pathogens and clear cellular debris, in neurodegenerative conditions, sustained M1 activation creates a neurotoxic environment [182]. Misfolded proteins, cellular stress, and inflammatory signals common in AD, PD, and ALS continuously fuel this state, overwhelming the brain's capacity to maintain homeostasis and contributing to ongoing neuronal loss.

Monocyte differentiation into macrophages and subsequent polarization to M1 or M2 phenotypes is driven by specific molecular signals. M1 polarization is induced by pro-inflammatory signals like interferon-gamma (IFN-γ) and lipopolysaccharides (LPS), leading to a pro-inflammatory state. Conversely, M2 polarization is promoted by anti-inflammatory cytokines such as IL-4 and IL-13, fostering tissue repair and resolution of inflammation [183].

On the other end of the spectrum, the M2 phenotype is associated with anti-inflammatory functions and tissue repair [184]. M2 microglia release cytokines such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β), which help resolve inflammation and support neuroprotection [185]. They also promote phagocytosis, aiding in the clearance of misfolded proteins and cellular debris. In neurodegenerative diseases, however, the shift toward an M2 phenotype is often suppressed, limiting the brain's ability to repair and recover [186]. Therapeutic strategies that promote M2 activation are being investigated as a way to reduce inflammation and protect against progressive neuronal damage [187]. PPAR-γ agonists and IL-10 enhancers are being explored to shift microglia toward an M2 phenotype, while agents targeting phagocytic pathways, such as TREM2 activators, aim to enhance the clearance of neurotoxic debris. By rebalancing microglial states, these approaches seek to alleviate chronic inflammation and create a supportive environment for neuronal repair [188].

3.2.2. Signaling Pathways in Activation

Microglial activation depends on intricate signaling networks that drive microglia toward either pro-inflammatory or protective states [189]. Among these, the NF-κB and MAPK pathways are key players, shaping microglial responses in the presence of pathological signals [190].

The NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathway is a primary regulator of inflammation in microglia. Activated by stimuli such as Aβ in AD and alpha-synuclein in PD, NF-κB translocates to the nucleus where it initiates the production of pro-inflammatory molecules like TNF-α, IL-1β, and IL-6 [191]. While NF-κB activity helps contain early threats, chronic activation in disease conditions drives a long-term inflammatory response. Modulating this pathway to reduce prolonged NF-κB signaling is being explored as a strategy to limit neuroinflammation [192].

The MAPK (mitogen-activated protein kinase) pathway is another critical pathway involved in microglial activation. Activated by external stress signals, the MAPK pathway includes kinases such as ERK, JNK, and p38, which regulate microglial responses to DAMPs [193]. For example, the activation of p38 MAPK has been linked to increased production of IL-1β and TNF-α, promoting inflammation in neurodegenerative disease contexts [56]. Targeting specific components of the MAPK pathway, especially p38, offers a potential therapeutic avenue to reduce pro-inflammatory microglial responses while preserving protective functions [194].

Other signaling molecules also influence the M1/M2 balance. Peroxisome proliferator-activated receptors (PPARs), particularly PPAR-gamma, are known to encourage M2 polarization by inhibiting NF-κB and suppressing the release of inflammatory cytokines [195]. Similarly, STAT6 (signal transducer and activator of transcription 6) activation supports M2 differentiation, enhancing the expression of anti-inflammatory molecules like IL-10 [196]. Therapies that target PPAR-gamma or STAT6 to promote M2 activation hold promise for shifting microglial responses away from sustained inflammation and toward protective roles in the brain [197].

By better understanding the molecular signals that drive microglial states, researchers aim to restore the balance between M1 and M2 functions, reducing the harmful inflammation that accelerates neurodegeneration. Encouraging a shift from the pro-inflammatory M1 state to the neuroprotective M2 state is a promising strategy, with the potential to alleviate chronic inflammation and enhance neural repair.

3.3. Cytokine Networks

Cytokines, small but powerful signaling proteins released by immune cells, orchestrate inflammation and communication within the brain's immune landscape. In healthy conditions, cytokine release is balanced, with pro-inflammatory signals initiating defense responses and anti-inflammatory signals resolving them [198]. However, in neurodegenerative diseases like AD, PD, ALS, and HD, this balance is disrupted. Chronic inflammation arises as pro-inflammatory cytokines dominate, fueling cellular stress and neuronal damage [199]. These intricate cytokine networks—encompassing both inflammatory and protective signals—reveal insights into the persistence of inflammation in these conditions and offer potential targets for modulating immune responses to protect neurons.

3.3.1. Pro-Inflammatory Cytokines

In neurodegenerative diseases, the persistent release of pro-inflammatory cytokines becomes a source of cellular distress. While intended to respond to injury or infection, these cytokines, when chronically elevated, create an environment that accelerates neurodegeneration. Among the most influential pro-inflammatory cytokines in these diseases are TNF-α, IL-1β, and IL-6 [200].

TNF-α is a central player in neuroinflammation, commonly elevated in affected brain regions. TNF-α binds to receptors on neurons and glial cells, activating signaling pathways like NF-κB, which further amplify inflammatory responses. In AD, TNF-α has been shown to impair synaptic function, while in PD, it contributes to the degeneration of dopaminergic neurons [55]. Animal studies suggest that blocking TNF-α signaling can alleviate inflammation and reduce neurotoxicity, and therapies targeting this cytokine are being developed as a means to mitigate its detrimental effects [201].

IL-1β is another potent pro-inflammatory mediator produced by activated microglia and astrocytes. It increases the permeability of the blood–brain barrier (BBB), allowing peripheral immune cells to enter the brain, thereby compounding inflammation [202]. In AD, IL-1β is released in response to Aβ plaques, intensifying neuronal damage around these aggregates. In ALS, IL-1β contributes to BBB breakdown and amplifies immune activity in ways that are particularly harmful to motor neurons. Targeting IL-1β or its pathways could offer a promising route to control neuroinflammation and protect the BBB in these diseases [203].

IL-6 has both pro-inflammatory and anti-inflammatory roles, but in neurodegenerative contexts, it typically promotes inflammation. Elevated IL-6 levels in cerebrospinal fluid and brain tissues of AD and PD patients correlate with increased glial activation and the release of other inflammatory signals [204]. By activating the JAK/STAT3 pathway, IL-6 sustains a feedback loop of inflammation, particularly detrimental in ALS where it activates astrocytes and microglia, accelerating motor neuron degeneration. Researchers are investigating therapies that inhibit IL-6 signaling as a potential means to reduce neuroinflammation and preserve neuronal health [205,206].

3.3.2. Anti-Inflammatory Mediators

In contrast to the destructive role of pro-inflammatory cytokines, anti-inflammatory mediators act to resolve inflammation and promote cellular repair. These cytokines work to counterbalance inflammation, yet in neurodegenerative diseases, their levels are often inadequate to mitigate chronic inflammatory responses. Two key anti-inflammatory cytokines with neuroprotective potential are IL-10 and TGF-β [207,208].

IL-10, produced by microglia, astrocytes, and infiltrating immune cells, inhibits the release of pro-inflammatory cytokines and reduces microglial activation via the STAT3 pathway, which represses inflammatory gene transcription [209]. IL-10's neuroprotective role is evident in its ability to limit excessive immune responses, yet its levels are often insufficient to counteract sustained inflammation in neurodegenerative diseases [210]. Strategies to enhance IL-10 activity are being explored as a way to promote an anti-inflammatory state in microglia, potentially slowing disease progression by creating a more protective environment for neurons [211].

TGF-β serves diverse functions within the CNS, modulating immune activity, promoting tissue repair, and helping maintain BBB integrity. By limiting pro-inflammatory cytokine production and supporting the M2 (anti-inflammatory) microglial phenotype, TGF-β plays a key role in controlling inflammation [212]. However, TGF-β signaling is often disrupted in conditions like AD and PD, diminishing its regulatory effects on immune activity. Efforts to restore or enhance TGF-β signaling are underway, with the aim of reinforcing the brain's natural anti-inflammatory mechanisms and providing support for neuronal survival [213].

The interplay between pro-inflammatory and anti-inflammatory cytokines shapes the immune environment within the neurodegenerating brain. When pro-inflammatory signals predominate, inflammation becomes a chronic, self-sustaining force, damaging neurons and accelerating disease progression [214]. Understanding these cytokine networks opens pathways for therapeutic interventions that rebalance inflammatory responses, reduce neurotoxicity, and promote protective immune activity. Targeting specific cytokines or modulating signaling pathways offers hope for treatments that could mitigate inflammation, preserve neuron function, and slow the advancement of neurodegenerative diseases.

3.4. Molecular Targets for Anti-Inflammatory Therapy

Chronic inflammation within the brain drives neurodegeneration in diseases. Anti-inflammatory therapies that target specific molecular pathways aim to restore immune balance, reduce neurotoxicity, and protect neurons [215]. By inhibiting harmful cytokine production, adjusting microglial activation, and enhancing protective signaling, these

therapies offer a focused approach to addressing inflammation as a root cause of neurodegeneration [216].

3.4.1. Inhibitors of Cytokine Production

Targeting the overproduction of pro-inflammatory cytokines is central to managing neuroinflammation. Inhibiting key pathways such as NF-κB and JAK/STAT offers a precise means to curtail cytokine signaling while preserving essential immune functions [217].

NF-κB Pathway Inhibitors: NF-κB is a key regulator of inflammation, often overactivated in neurodegenerative conditions. Small molecule inhibitors targeting IκB kinase (IKK), an activator of NF-κB, aim to reduce cytokine release (e.g., TNF-α, IL-1β) linked to neuronal damage in AD and PD [218]. These inhibitors selectively suppress NF-κB without broadly dampening immune response, showing potential in animal models for decreasing inflammation and preserving neuron function [219].

JAK/STAT Pathway Modulation: The JAK/STAT pathway, particularly STAT3 activation by IL-6, sustains inflammation in neurodegenerative disease. Janus kinase (JAK) inhibitors such as tofacitinib, which have been effective in autoimmune disorders, are being studied in neurodegenerative contexts [220]. These inhibitors target the JAK/STAT axis to selectively mitigate cytokine signaling and its downstream inflammatory effects, offering a way to protect neurons from chronic inflammation [221].

3.4.2. Modulation of Microglial Activation

Microglia, the brain's immune cells, play a central role in neuroinflammation. Therapies that shift microglia from a pro-inflammatory state to a neuroprotective profile hold promise for alleviating inflammation and supporting tissue repair [222].

PPAR-gamma Agonists: PPAR-γ agonists, such as pioglitazone, encourage microglia to adopt a protective role by activating PPAR-γ, a nuclear receptor regulating inflammation [223]. These agonists reduce the release of inflammatory cytokines and improve microglial clearance of toxic aggregates. In AD and PD models, PPAR-γ activation reduces oxidative stress and enhances microglial function, suggesting its potential for restoring immune balance in the brain [224].

Colony-Stimulating Factor 1 Receptor (CSF1R) Inhibitors: CSF1R is critical for microglial survival and activation [225]. CSF1R inhibitors, such as PLX3397, selectively modulate microglial activity, reducing their neurotoxic effects without eliminating them. These inhibitors limit excessive microglial activation, which can help control inflammation and prevent neurodegeneration [226].

3.4.3. Enhancing Protective and Anti-Inflammatory Pathways

Enhancing the brain's natural anti-inflammatory responses supports a more resilient neural environment, helping to counterbalance chronic inflammation.

IL-10 and TGF-β Enhancers: IL-10 and TGF-β have potent anti-inflammatory effects, limiting pro-inflammatory cytokine release and promoting microglial states that protect neurons [227]. IL-10, delivered through gene therapy in AD models, has shown promise in reducing inflammation near amyloid plaques. TGF-β helps maintain BBB integrity and promotes anti-inflammatory microglial phenotypes, making it a compelling target to reinforce immune regulation [185].

HDAC Inhibitors: HDAC inhibitors reduce inflammation by modulating gene expression. By altering chromatin structure, these inhibitors selectively suppress pro-inflammatory genes while promoting protective responses. In preclinical studies, HDAC inhibitors have improved mitochondrial function and enhanced neuronal resilience, positioning themselves as a promising approach to managing neuroinflammation in neurodegenerative diseases [228,229].

These strategies—precise inhibition of cytokine production, modulation of microglial activation, and enhancement of protective pathways—represent promising approaches to reducing inflammation while supporting neuron health. Anti-inflammatory therapies

focusing on specific molecular targets hold the potential to address the underlying drivers of neurodegeneration, offering a pathway toward preserving cognitive and motor function in neurodegenerative diseases [230].

4. Genetic and Epigenetic Mechanisms

Genetic and epigenetic factors shape the landscape of neurodegenerative diseases, intricately influencing how and why these conditions develop and progress. Mutations in specific genes disrupt essential cellular processes, often leading to the production of abnormal proteins or impairing cellular health [231]. Meanwhile, epigenetic modifications—heritable changes in gene expression that occur without altering the DNA sequence itself—further influence disease pathways by regulating genes tied to inflammation, cellular resilience, and synaptic function [232]. Together, genetic and epigenetic factors provide a framework for understanding disease mechanisms and uncovering potential therapeutic strategies aimed at modifying gene activity or correcting genetic errors at their source [233].

4.1. Genetic Mutations and Molecular Consequences

In neurodegenerative diseases, certain genetic mutations directly contribute to the pathological processes driving disease. These mutations can interfere with protein stability, disrupt cellular communication, and, ultimately, lead to the accumulation of toxic proteins or cellular dysfunction. Two well-studied examples of genetic drivers in neurodegeneration include familial AD and HD where mutations in key genes have been identified as central players in disease progression [234].

4.1.1. Familial Alzheimer's Disease Genes

Familial Alzheimer's disease (fAD) is a rare, inherited form of AD often linked to mutations in the APP and presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes [235]. These mutations disrupt the normal cleavage of amyloid precursor protein, increasing the production of the Aβ42 peptide—a particularly sticky form of amyloid-beta prone to aggregation. This tendency to clump together leads to the formation of amyloid plaques, a hallmark of AD pathology that disrupts neural communication and contributes to cell death [236].

Beyond increasing Aβ42 production, mutations in presenilin genes may also affect cellular calcium regulation and other vital signaling pathways, heightening cellular stress and promoting neurodegeneration [237]. This connection between genetic mutations and amyloid pathology has guided therapeutic approaches aiming to reduce amyloid buildup. For instance, gamma-secretase modulators and beta-secretase inhibitors target the enzymes involved in amyloid processing, with the goal of lowering Aβ42 levels and slowing plaque formation [238]. These interventions offer a targeted way to address the molecular effects of familial Alzheimer's mutations, providing insight into how altering specific pathways might impact disease progression [239].

4.1.2. Huntington's Disease and the HTT Gene

HD is directly caused by a mutation in the HTT gene, which encodes the huntingtin protein. This mutation involves an abnormal expansion of CAG trinucleotide repeats, leading to an extended polyQ sequence in the huntingtin protein. The result is a mutant form of huntingtin (mHTT) that misfolds and aggregates within neurons, disrupting essential cellular processes [240].

The presence of mHTT in neurons triggers a cascade of dysfunction. As mHTT accumulates, it interferes with mitochondria, disrupts intracellular transport, and hinders the cell's protein degradation systems, such as the ubiquitin–proteasome pathway and autophagy [241]. Beyond its structural impact, mHTT also influences gene expression, binding abnormally to transcription factors and disrupting the regulation of genes crucial for neuron survival. These disruptions ultimately lead to widespread neuronal damage, particularly within the striatum, which is prominently affected in HD [242].

To address the root cause of HD, gene-silencing therapies, including ASOs and RNA interference (RNAi), are being explored to reduce mHTT levels. These approaches aim to directly decrease the production of the mutant protein, targeting the disease at its genetic origin. Additionally, small molecules that enhance autophagy are under investigation to promote the clearance of mHTT aggregates, alleviating some of the cellular stress induced by protein accumulation [243]. Together, these approaches represent efforts to modify disease progression by addressing the molecular foundation of HD, opening possibilities for interventions that go beyond symptom management to potentially alter the course of the disease.

4.2. Gene Therapy at the Molecular Level

Gene therapy is revolutionizing neurodegenerative disease treatment, offering the capability to directly target genetic mutations that drive disease processes. Techniques such as CRISPR-Cas9, ASOs, and RNAi enable precise modulation of gene expression, while emerging methods like prime and base editing, CRISPRa/CRISPRi, and exosome-based delivery systems are enhancing specificity, reducing risk, and broadening therapeutic applications [244]. Each approach seeks to reduce toxic protein accumulation, protect neurons, and alter the course of neurodegenerative conditions such as HD, AD, PD, and ALS at their genetic core [245].

4.2.1. CRISPR-Cas9 and Advanced Precision Editing

CRISPR-Cas9, a gene-editing tool adapted from bacterial immune systems, enables precise targeting of specific DNA sequences for gene disruption or correction. By using a guide RNA to direct the Cas9 enzyme to designated sites within the genome, CRISPR can introduce double-strand breaks, allowing for the removal or repair of faulty genes [246]. For instance, in HD, CRISPR-Cas9 has been employed to excise expanded CAG repeats in the HTT gene, thereby reducing the production of mHTT protein [247].

Newer precision techniques like prime editing and base editing offer even greater accuracy without causing double-strand breaks, which can sometimes lead to unintended mutations. Prime editing, which allows for the direct insertion of new genetic sequences, has potential applications in correcting single-nucleotide mutations, such as those in the SOD1 gene implicated in ALS. Base editing, capable of changing a single nucleotide (e.g., converting A to G or C to T), could be used to directly repair mutation sites in neurodegenerative genes, providing a safer alternative with reduced off-target effects—particularly beneficial for the brain's delicate cellular environment [248].

4.2.2. Antisense Oligonucleotides (ASOs)

ASOs are synthetic nucleic acid strands that bind specifically to RNA, promoting its degradation or blocking translation to reduce protein production. This approach allows for the selective silencing of disease-associated genes by preventing the synthesis of harmful proteins [249]. ASOs are particularly promising in conditions like ALS and HD. For example, in ALS, ASOs targeting SOD1 mRNA reduce levels of the toxic SOD1 protein, alleviating oxidative stress and improving mitochondrial function in motor neurons. In HD, ASOs targeting HTT mRNA effectively reduce mutant huntingtin levels, easing cellular stress from protein aggregation. Delivered intrathecally, ASOs enable localized, sustained reduction of toxic protein production directly in the central nervous system, providing a potent and precisely targeted therapeutic option [250].

4.2.3. RNA Interference (RNAi)

RNAi leverages small interfering RNAs or short hairpin RNAs to target and degrade specific mRNAs, blocking translation and, thereby, reducing the production of disease-related proteins. RNAi has shown potential in multiple neurodegenerative diseases. In PD, for instance, siRNAs targeting SNCA mRNA reduce alpha-synuclein protein levels, alleviating the neurotoxic effects associated with Lewy body formation. In HD, allele-specific

RNAi approaches target the mutant HTT gene, selectively silencing the disease-causing allele while preserving the normal protein necessary for neuronal function. RNAi therapy, delivered via viral vectors, can provide sustained effects within targeted brain regions, offering a highly selective way to address neurodegeneration linked to protein toxicity.

4.2.4. CRISPRa and CRISPRi for Gene Activation and Suppression

CRISPRa (activation) and CRISPRi (interference) represent non-cutting forms of CRISPR that allow for the modulation of gene expression without altering the underlying DNA sequence. CRISPRa enhances the expression of neuroprotective genes, while CRISPRi selectively suppresses pathogenic genes [251]. In PD, CRISPRa could be used to upregulate genes that protect mitochondrial health or enhance dopamine synthesis, potentially offering a way to boost the brain's resilience against cellular stress. In HD, CRISPRi could selectively silence the mutant HTT allele, decreasing toxic protein production without affecting normal huntingtin function. These CRISPR variants offer a reversible, fine-tuned approach to modulating gene expression dynamically, providing an adaptable tool for targeting complex neurodegenerative pathways [252].

4.2.5. Exosome-Based Delivery Systems

Exosomes—small vesicles naturally secreted by cells—offer an innovative delivery system for gene therapies, capable of crossing the BBB and delivering therapeutic molecules to specific brain regions. Engineered exosomes can carry ASOs, siRNAs, or CRISPR molecules directly to affected neurons, enhancing the reach of gene therapies without invasive methods [253]. For example, exosomes loaded with siRNAs targeting alpha-synuclein could reduce neurotoxic protein accumulation in PD, while exosomes carrying ASOs against APP mRNA could decrease amyloid-beta production in AD. Using patient-derived exosomes could further improve compatibility, reducing immune responses and enabling personalized treatments [254].

4.2.6. Synthetic mRNA for Transient Therapeutic Expression

Synthetic mRNA therapy offers a non-integrative gene therapy approach, delivering transient doses of therapeutic proteins without altering DNA. In neurodegenerative diseases, synthetic mRNA can be used to produce neuroprotective factors like brain-derived neurotrophic factor (BDNF) or glial cell line-derived neurotrophic factor (GDNF), supporting neuronal health and survival. In HD, synthetic mRNA coding for proteins that promote autophagy could enhance the clearance of toxic huntingtin aggregates, offering a reversible, flexible method to address protein accumulation in the brain [255].

4.3. Epigenetic Regulation: Novel Pathways for Neurodegenerative Intervention

Epigenetic regulation offers a groundbreaking approach in neurodegenerative disease research, revealing ways to dynamically adjust gene expression without altering DNA itself [256]. By modulating gene accessibility, protein synthesis, and cellular stress responses, these mechanisms hold the promise of not only managing symptoms but also slowing disease progression. Emerging strategies in DNA methylation, histone modification, and non-coding RNA modulation are opening new, adaptive avenues for treatment of neurodegenerative diseases [257].

4.3.1. DNA Methylation Patterns: Restoring Protective Gene Expression

DNA methylation has emerged as a crucial regulator in silencing or activating genes, with far-reaching effects in neurodegenerative diseases. What makes this approach novel is the potential to selectively "unlock" silenced protective genes or reduce overactive inflammatory genes, offering a targeted path to resilience [258]. In AD, for example, DNA hypermethylation in neural repair genes restricts the brain's natural defense mechanisms, while hypomethylation in inflammatory genes perpetuates chronic immune responses. DNA methyltransferase inhibitors are being explored to reverse these harmful patterns,

dynamically restoring gene activity associated with neuron protection [259]. Additionally, the ability to map methylation profiles in affected brain regions creates biomarkers that can track disease progression and treatment responses, pushing beyond traditional imaging methods [260].

4.3.2. Histone Modifications: Fine-Tuning Gene Accessibility

Histone modifications control how tightly DNA is wound around histone proteins, impacting which genes are "on" or "off". This process provides an epigenetic dial to adjust gene expression in response to neurodegenerative stressors [261]. HDAC inhibitors, which increase histone acetylation, are being developed to enhance the expression of genes vital for neuronal defense and repair. In HD, HDAC inhibitors have shown potential in reactivating pathways for protein degradation, helping to clear toxic protein aggregates that are otherwise devastating to neurons [262]. This reversible approach allows for fine-tuning, supporting cellular health without the risk of permanent genetic alteration. As histone modifications can be adapted in real time to environmental changes, they present a novel, flexible solution for neurodegenerative management [263].

4.3.3. Non-Coding RNAs: Master Regulators of Disease Pathways

Non-coding RNAs (ncRNAs), particularly miRNAs, are fast gaining attention as powerful gene regulators that act at the RNA level, directly controlling protein synthesis. In neurodegenerative diseases, specific miRNAs are often dysregulated, which leads to the uncontrolled expression of genes related to inflammation, protein misfolding, and apoptosis [264]. For example, in AD, miRNAs associated with tau and amyloid-beta processing are disrupted, allowing these proteins to accumulate. Antagomirs, synthetic molecules designed to inhibit specific miRNAs, are a novel approach in therapy, providing precise, sequence-specific intervention to reset gene expression networks that go awry in disease [265]. This approach allows for highly targeted therapeutic intervention, tailoring treatment to the disease's molecular signature and offering a method to fine-tune cellular responses without altering the DNA structure itself [266].

4.4. Epigenetic Therapeutic Approaches: Precision in Gene Regulation

Epigenetic therapies open a fascinating new frontier in neurodegenerative treatment by allowing us to dynamically adjust gene expression in response to disease progression. Unlike traditional gene-editing techniques, these approaches do not alter the DNA sequence itself; instead, they modify how genes are read and expressed, offering reversible and adaptive control over cellular pathways implicated in neurodegenerative disease. By fine-tuning histone acetylation, DNA methylation, and non-coding RNA activity, these therapies provide a new way to enhance neuroprotection and resilience [267].

4.4.1. HDAC Inhibitors: Unlocking Protective Genes

One of the most compelling aspects of HDAC inhibitors is their ability to "unlock" protective genes by promoting histone acetylation, which makes DNA more accessible for gene expression. This enables the reactivation of pathways critical for cellular repair and protein degradation, essential for countering the toxic buildup seen in HD and AD. The flexibility of HDAC inhibition is intriguing: these changes are reversible, meaning treatment can be fine-tuned to the patient's needs and disease stage, allowing a truly adaptive approach to gene regulation in real time [268].

4.4.2. DNA Methyltransferase Inhibitors: Reactivating Silenced Pathways

DNA methylation often shuts down genes that would otherwise protect neurons from stress and inflammation. DNMT inhibitors offer a way to "reactivate" these genes by removing methyl groups that silence them [269]. This approach is especially interesting because it allows for selective demethylation, meaning only the most beneficial genes are reactivated while minimizing potential side effects. In diseases where aberrant methylation

restricts essential cell functions, DNMT inhibitors could offer a controlled reset of gene expression, restoring pathways critical for neuronal health and repair [270].

Epigenetic therapies represent a novel, adaptable approach to tackling neurodegenerative diseases by regulating gene expression in real time. They offer a unique combination of precision and flexibility—allowing us to target disease-specific pathways, boost protective gene activity, and track changes through biomarkers—all without permanently altering the genome [271]. These approaches pave the way for highly personalized, responsive treatments that could transform the management of neurodegeneration, bringing us closer to therapies that are as dynamic and adaptable as the diseases themselves [258].

5. Mitochondrial Dysfunction and Oxidative Stress

Mitochondrial dysfunction and oxidative stress are central drivers in neurodegenerative diseases. Mitochondria, essential for energy production and cellular health, become compromised in these conditions, leading to energy deficits and excess ROS. This imbalance damages cellular components, accelerating neuronal death. By understanding the specific mitochondrial breakdowns in these diseases, researchers aim to develop therapies that enhance mitochondrial stability and reduce oxidative stress, potentially slowing disease progression [272].

5.1. Molecular Basis of Mitochondrial Dysfunction

Mitochondrial dysfunction in neurodegeneration often stems from defects in the electron transport chain (ETC) and mutations in mtDNA, both of which disrupt ATP production and increase ROS, creating a toxic cycle within neurons [273].

5.1.1. Electron Transport Chain Defects

The ETC is a critical pathway for ATP synthesis, moving electrons across mitochondrial complexes to generate energy. In neurodegenerative diseases, defects in the ETC—particularly in complexes I, III, and IV—impede this energy flow, leading to ATP shortages and heightened ROS production [274]. For instance, PD is characterized by complex I impairment in dopaminergic neurons, leading to energy failure and oxidative stress. Similarly, ETC deficits in AD contribute to a crisis in cellular energy supply, impacting synaptic function and neuron viability. Treatments like coenzyme Q10, which supports ETC function and reduces ROS, are being investigated as ways to restore mitochondrial health and protect neurons [275].

5.1.2. Mitochondrial DNA Mutations

Mitochondrial DNA is highly vulnerable to mutations, partly due to its proximity to ROS production in the ETC and limited repair capacity. Over time, accumulated mtDNA damage leads to defective mitochondrial proteins that worsen mitochondrial function. In ALS, mtDNA mutations disrupt motor neuron energy production, while in HD, mutant huntingtin exacerbates mtDNA instability, triggering faulty mitochondrial dynamics like impaired fusion and fission [276]. Damaged mtDNA also increases neuronal susceptibility to calcium overload and apoptosis, making affected neurons more vulnerable to degeneration. Targeted therapies that promote mtDNA repair or clear damaged mitochondria (via mitophagy) offer novel avenues for mitigating mitochondrial decline in neurodegeneration [277].

Mitochondrial dysfunction represents a critical area in understanding and treating neurodegenerative diseases. By stabilizing ETC function and enhancing mtDNA resilience, researchers are exploring targeted strategies to reduce oxidative damage, support cellular energy demands, and protect neurons from progressive degeneration [278].

5.2. Generation of ROS: Novel Insights and Theories

In recent years, innovative theories have reshaped our understanding of ROS generation and oxidative damage in neurodegenerative diseases, revealing previously un-

recognized pathways and potential therapeutic targets [279]. These insights focus on the unique roles of ROS in disease-specific mechanisms and explore how manipulating ROS production or clearance could alter disease progression. Here are some emerging ideas and theories that highlight the novel aspects of ROS involvement in neurodegeneration [280].

5.2.1. Unique ROS Pathways in Mitochondria and Cellular Compartments

Localized ROS Signaling and Compartmentalization: Traditional views of ROS treat oxidative stress as a global cellular issue, but recent findings suggest that ROS function and damage may vary across cellular compartments. In neurons, for example, mitochondria produce ROS not only in the ETC but also through other internal stress-sensitive systems [281]. Specific regions within neurons, such as synaptic terminals, show distinct ROS dynamics, affecting neurotransmission and synaptic plasticity in a way that may selectively impact memory-related functions in AD. The theory of "compartmentalized ROS signaling" suggests that targeted antioxidant strategies could be developed for specific cellular locations, potentially allowing for finer control over ROS without impacting beneficial ROS-dependent processes elsewhere in the cell [282].

Mitochondria-Associated Membranes (MAMs): Specialized structures connecting mitochondria and the ER have emerged as key sites of ROS production and calcium signaling. In neurodegenerative diseases, disruptions in MAM function contribute to abnormal calcium exchange, increasing ROS levels and sensitizing cells to stress [283]. This interplay is particularly relevant in PD where MAM dysfunction exacerbates dopaminergic neuron vulnerability. Targeting MAMs could offer a novel strategy for modulating ROS production and calcium signaling in a way that restores cellular balance, providing a promising avenue for therapeutic intervention [284].

5.2.2. Dual Role of ROS in Neuroprotection and Damage

Adaptive ROS Response Theory: While excessive ROS are harmful, recent research suggests that low levels of ROS may trigger protective cellular responses—a phenomenon referred to as "mitohormesis". This adaptive ROS response activates cellular defense mechanisms such as antioxidant enzyme production and mitochondrial biogenesis that improve cell resilience [285]. In this context, ROS act as signaling molecules that stimulate cellular repair and protection pathways. For neurodegenerative diseases, harnessing this adaptive response could involve controlled ROS production to bolster cellular defenses against more severe oxidative damage [286]. For example, low-dose ROS-inducing agents or mild mitochondrial stressors could precondition cells, making neurons more resilient to the heightened oxidative stress seen in diseases like AD and HD [287].

ROS as Signaling Molecules in Immune Modulation: ROS are now recognized as important modulators of immune responses, particularly in the brain's innate immune system [288]. ROS produced by microglia and astrocytes in response to damage can signal neighboring cells to initiate repair processes or clear debris. In neurodegeneration, however, this signaling function can become dysregulated, with chronic ROS production fueling a harmful cycle of inflammation [289]. Novel therapies could aim to recalibrate ROS levels within the brain's immune cells, potentially dampening harmful inflammation while preserving the beneficial signaling functions of ROS. This approach could represent a breakthrough in managing neuroinflammatory aspects of diseases like ALS and PD [290].

5.2.3. Role of Redox-Sensitive Proteins and Molecular Sensors

Redox-Sensitive Protein Pathways: Advances in redox biology have highlighted specific proteins that act as molecular "sensors" for ROS, adjusting their function in response to changes in oxidative stress. Proteins like DJ-1 in PD and Nrf2 in various neurodegenerative diseases can detect shifts in ROS levels and activate antioxidant defenses [291]. Nrf2, for example, regulates the expression of genes that neutralize ROS, while DJ-1 helps protect cells from oxidative stress by stabilizing mitochondrial function. Novel therapies targeting these redox-sensitive proteins aim to enhance the cell's natural antioxidant

response without completely suppressing ROS, which are necessary for normal cell signaling [292]. By focusing on these sensors, researchers hope to activate protective pathways only when needed, offering a finely tuned approach to oxidative stress management in neurodegeneration [293].

Mitochondrial ROS Sensors and Feedback Loops: Emerging theories suggest that mitochondria contain feedback mechanisms that detect ROS levels and regulate energy production in response. For example, mitochondrial uncoupling proteins (UCPs) act as ROS regulators by dissipating the mitochondrial membrane potential, reducing electron flow through the ETC, and lowering ROS production [294]. Targeting UCPs in a controlled way could offer a method to manage ROS without shutting down energy production entirely. This strategy could be particularly useful in conditions like HD and ALS where mitochondrial function is progressively compromised, and managing ROS without energy disruption is critical [295].

5.2.4. Targeted Therapies Leveraging Novel Antioxidant Approaches

Selective Antioxidants for Compartmentalized ROS: Traditional antioxidants target ROS broadly across cells, but new approaches focus on compartment-specific antioxidants that accumulate in specific organelles, such as mitochondria. Compounds like MitoQ and SkQ1 are engineered to localize within mitochondria, directly neutralizing ROS at their main source [296]. The selective antioxidant approach minimizes interference with ROS signaling in other cellular regions, maintaining necessary ROS functions while protecting sensitive areas. This precision strategy represents a novel way to balance ROS levels, particularly in the energy-intensive environments of neurons affected by diseases like AD and PD [297].

Gene Therapy for Antioxidant Enzymes: Another emerging approach involves the use of gene therapy to enhance the production of endogenous antioxidants like superoxide dismutase (SOD) and glutathione peroxidase, specifically in neurons. By delivering genes that increase antioxidant enzyme expression directly in affected brain regions, this method provides a sustained, localized defense against ROS [298]. In ALS, for instance, gene therapy targeting SOD1 has shown promise in animal models, demonstrating reduced oxidative damage and extended motor neuron survival [299]. This targeted delivery of antioxidant defenses offers a cutting-edge approach for managing oxidative stress at the source, potentially extending neuron health and slowing disease progression [300,301].

These novel insights into ROS production and their role in neurodegeneration underscore the complexity and dual nature of ROS in the brain. Emerging theories highlight the importance of precision in managing ROS: by selectively targeting ROS pathways, modulating immune responses, and using compartment-specific antioxidants, researchers are moving closer to therapies that can harness the beneficial aspects of ROS while minimizing their destructive effects [302]. These advanced approaches offer hope for a new generation of treatments tailored to the intricate oxidative landscape of neurodegenerative diseases [303].

5.3. Antioxidant Defense Mechanisms

Cells rely on a natural defense system of antioxidants to neutralize ROS and prevent oxidative damage. In neurodegenerative diseases these defenses often fall short, leaving neurons vulnerable to the damaging effects of excess ROS. Current research is exploring ways to enhance these natural defenses and introduce new molecular agents, aiming to better manage oxidative stress and protect neuron integrity [300].

5.3.1. Endogenous Antioxidants

The body's natural antioxidants are crucial in the fight against ROS as they work to neutralize reactive molecules and repair oxidative damage. Recent findings underscore the importance of supporting these antioxidant systems to strengthen cellular resilience. SOD is one of the primary endogenous antioxidants, converting the highly reactive su-

peroxide molecule into hydrogen peroxide, which is then further broken down by other enzymes [304]. In ALS, mutations in the SOD1 gene disrupt this process, contributing to motor neuron damage. Researchers are now investigating gene therapy strategies to boost SOD1 activity, aiming to restore ROS balance and reduce oxidative stress in neurons [305].

Glutathione peroxidase (GPx) is another key antioxidant enzyme, converting hydrogen peroxide into water and preventing further ROS formation. Lower GPx activity has been associated with increased vulnerability to oxidative stress in both PD and AD. To counteract this, scientists are exploring ways to enhance GPx production through gene editing and by introducing GPx-mimetic compounds that perform similar functions within neurons [306]. Catalase, another essential antioxidant enzyme, also breaks down hydrogen peroxide within cells. Recently, targeted catalase delivery systems have been developed to transport this enzyme directly to mitochondria, the main sites of ROS production [307]. This mitochondria-targeted approach, especially relevant in HD, is designed to prevent oxidative damage specifically where it originates, helping protect neurons from stress at a foundational level [308].

5.3.2. Molecular Therapeutic Agents

Alongside natural antioxidants, novel compounds are being developed to amplify or mimic their effects, offering neurons added protection against oxidative stress. Mitochondria-targeted antioxidants like MitoQ, SkQ1, and TEMPOL are engineered to accumulate within mitochondria where ROS production is the highest [309]. These compounds neutralize ROS directly at its source. By integrating into the mitochondrial membrane, MitoQ and SkQ1 capture ROS before it can cause damage, providing localized and efficient protection that traditional antioxidants lack [310].

Another innovative approach focuses on activating Nrf2, a transcription factor that regulates the expression of antioxidant genes. When triggered, Nrf2 enhances cellular defenses against ROS, bolstering resilience to oxidative stress [311]. Compounds like sulforaphane and bardoxolone methyl are currently under study for their ability to stabilize and activate Nrf2, increasing the production of endogenous antioxidants. This approach holds promise for neurodegenerative diseases like AD and HD where sustained antioxidant activity could alleviate cellular stress and slow disease progression [312].

In addition to direct antioxidants, small molecules that mimic the action of key antioxidant enzymes, such as SOD, catalase, and GPx, offer an alternative therapeutic route. MnTBAP, a synthetic SOD mimetic, has shown potential in PD models by reducing oxidative stress and protecting neurons [313,314]. Similarly, Ebselen, which mimics GPx function, neutralizes hydrogen peroxide and has demonstrated neuroprotective effects in preclinical studies of AD and ALS. These enzyme mimetics bypass the need for genetic modification and provide a versatile means of enhancing cellular defenses directly [315].

These cutting-edge antioxidant strategies reflect a shift toward precision-targeted interventions designed to enhance neuronal resilience against ROS. By boosting natural antioxidants, activating key protective pathways, and employing targeted synthetic compounds, these approaches aim to address oxidative stress at its roots [316]. This novel direction in antioxidant therapy not only provides general protection but also tailors interventions to meet the specific needs of neurons in neurodegenerative diseases, offering hope for more effective and sustainable treatment strategies [317].

6. Synaptic Dysfunction at the Molecular Level

Synaptic dysfunction has emerged as a pivotal factor in neurodegenerative diseases where disruptions in communication between neurons contribute to progressive cognitive, motor, and behavioral impairments [318]. Rather than mere neuron loss, it is now understood that neurodegeneration is marked by a complex breakdown of synaptic structure, signaling, and energy metabolism. This deeper understanding reveals nuanced mechanisms, offering promising therapeutic targets aimed at restoring or preserving synaptic health [319].

6.1. Molecular Mechanisms of Synaptic Loss

The molecular processes driving synaptic loss are highly specific and involve changes at multiple levels, from synaptic protein alterations to energy deficiencies and receptor imbalances [231,320]. Each offers a unique entry point for potential intervention.

6.1.1. Altered Synaptic Protein Networks and Post-Translational Modifications

Synaptic proteins are not only structural components but also active players in maintaining synaptic function and plasticity. In AD, phosphorylation of tau protein accumulates specifically in dendritic spines where it disrupts the actin cytoskeleton, which is critical for spine stability and synaptic signaling. This localized tau phosphorylation appears to weaken synaptic contacts before more extensive tau pathology develops, pointing to early mechanisms of cognitive impairment [321]. Moreover, in HD, abnormal SUMOylation of synaptic proteins, including receptors and scaffolding proteins, disrupts protein–protein interactions necessary for synaptic stability and neurotransmitter receptor clustering. Targeting these early post-translational modifications, such as through kinase inhibitors that block tau phosphorylation or enzymes that modulate SUMOylation, could help maintain synaptic architecture and function [322].

6.1.2. Mitochondrial Dysfunction and Synaptic Energy Imbalance

Synapses are exceptionally energy-demanding, requiring continuous ATP production for vesicle recycling, ion gradient maintenance, and neurotransmitter reuptake. Mitochondrial dysfunction at the synapse disrupts this energy supply. For instance, in PD, complex I impairment in dopaminergic neurons at the synaptic level leads to insufficient ATP production, weakening vesicle loading and release [323]. In AD, mitochondria in glutamatergic synapses show reduced capacity for calcium buffering, which is essential for neurotransmitter release and receptor activation. This loss of calcium regulation interferes with synaptic plasticity processes critical for memory [324]. Current therapeutic developments are exploring small molecules like nicotinamide riboside and other mitochondrial enhancers that increase ATP production and improve calcium handling specifically within synapses, aiming to support the energy-intensive demands of neuronal communication [325].

6.1.3. Impaired Synaptic Vesicle Recycling and Endocytosis

Synaptic vesicle recycling is essential for sustaining neurotransmitter release. In HD, mutant huntingtin binds abnormally to endocytic proteins such as dynamin and clathrin, reducing the efficiency of vesicle recycling and decreasing the availability of synaptic vesicles [326]. This disruption causes delays in neurotransmitter release, dampening the flow of signals through neural circuits. In ALS, C9orf72 mutations affect endosomal pathways, impairing vesicle trafficking and exacerbating excitotoxicity due to excessive glutamate release [327]. Novel strategies are targeting the clathrin-mediated endocytosis pathway to enhance vesicle recycling, either by stabilizing dynamin interactions or by modulating clathrin coat assembly, aiming to restore synaptic vesicle availability and support sustained neurotransmission [328,329].

6.1.4. Neurotransmitter Receptor Modulation and Synaptic Plasticity

Neurotransmitter receptors such as AMPA and NMDA (in excitatory transmission) and dopamine receptors are finely tuned for synaptic plasticity and are especially vulnerable to neurodegenerative changes. In AD, beta-amyloid oligomers promote NMDA receptor endocytosis, leading to fewer receptors on the postsynaptic surface, which impairs synaptic transmission and is linked to memory deficits [330]. Studies indicate that beta-amyloid affects NMDA receptor-binding sites, altering receptor internalization pathways and destabilizing excitatory signaling. In PD, dopamine receptor phosphorylation patterns are altered, reducing the sensitivity of D1 and D2 receptors and diminishing dopaminergic signaling, which impairs motor control [331]. Novel therapeutics, including allosteric modulators, are being investigated to stabilize these receptors' functional states

without overstimulation, aiming to preserve normal neurotransmission while avoiding the excitotoxic effects that often accompany receptor overactivation [332].

These detailed insights into synaptic dysfunction underscore the precision needed to develop effective treatments for neurodegenerative diseases. By addressing the specific molecular disruptions—whether through stabilizing synaptic proteins, enhancing mitochondrial function at synapses, restoring vesicle recycling, or modulating neurotransmitter receptors—these approaches hold promise for preserving the intricate communication networks that underlie cognition and motor control [333]. Together, these advancements bring us closer to therapies that not only delay neuron loss but actively protect the connections that define brain health [334].

6.2. Neurotransmitter Systems

In neurodegenerative diseases, disturbances in neurotransmitter systems are not only symptoms but key drivers of the cognitive and motor issues that define these conditions [335]. Recent breakthroughs have revealed detailed disruptions within the cholinergic, dopaminergic, and glutamatergic systems, highlighting potential pathways for treatments that could address both the visible symptoms and underlying causes [336,337].

6.2.1. Cholinergic System in Alzheimer's Disease

In AD, it is well known that neurons responsible for acetylcholine (ACh) release are gradually lost, but new findings show that the story is more complex. Beta-amyloid, a hallmark of AD, appears to directly interfere with α7 nicotinic acetylcholine receptors (α7-nAChRs), causing these receptors to be withdrawn from the cell surface, reducing their ability to transmit signals [338]. At the same time, tau protein, when altered, disrupts the enzyme choline acetyltransferase (ChAT), limiting ACh production in areas where it is most needed [339].

This deeper understanding has led to focused treatments. For instance, α7-nAChR agonists are being developed to sidestep beta-amyloid interference, with the aim of restoring cognitive processes without triggering excess receptor activity elsewhere [340]. Gene therapies are also in the works to increase ChAT expression, potentially boosting ACh levels directly within synapses. Additionally, advanced acetylcholinesterase inhibitors are being designed to specifically enhance ACh signaling in the most affected brain areas, offering a more precise approach to preserving memory and cognitive function in AD [341].

6.2.2. Dopaminergic System in Parkinson's Disease

In PD, dopamine loss is central to symptoms, yet recent research has uncovered additional layers to this deficiency. Alpha-synuclein aggregates, which build up within dopaminergic neurons, not only interfere with dopamine synthesis by binding to tyrosine hydroxylase (TH)—the enzyme responsible for dopamine production—but also disrupt dopamine storage by affecting the vesicular monoamine transporter 2 (VMAT2) [342].

Therapies are now being developed with these discoveries in mind. For instance, small molecules that prevent alpha-synuclein from forming harmful aggregates could help protect dopamine production. VMAT2 activators are also being explored to improve dopamine packaging within neurons, ensuring more dopamine is available for release [343]. On the cutting edge, gene therapies targeting TH aim to increase dopamine synthesis, while optogenetic techniques that use light to stimulate dopamine-producing neurons offer a novel, noninvasive way to potentially restore dopamine levels and relieve motor symptoms [344].

6.2.3. Glutamatergic System and Excitotoxicity

In conditions like ALS and AD, the excitatory neurotransmitter glutamate can become toxic due to disruptions in how it is managed. For instance, in ALS, astrocytes lose some of their glutamate-clearing capacity due to a reduction in the EAAT2 transporter. This leaves

excess glutamate in the synapse, overstimulating NMDA and AMPA receptors and causing calcium overload, which leads to cellular stress and damage [345].

New therapies are zeroing in on ways to restore balance in the glutamatergic system. Gene therapies to boost EAAT2 expression in astrocytes are being explored, aiming to clear excess glutamate and reduce excitotoxic stress [346]. Meanwhile, new NMDA receptor modulators are being developed to reduce harmful signaling without affecting normal function. For AD, targeting metabotropic glutamate receptors (mGluRs) offers a more nuanced approach as these receptors help control glutamate release indirectly, allowing for a reduction in excitotoxicity while preserving synaptic communication [347]. Additionally, peptide-based therapies that act as glutamate "scavengers" have shown promise, selectively binding to excess glutamate to prevent overstimulation without interrupting healthy glutamate activity [348].

These insights into neurotransmitter system dysfunction provide a foundation for more refined, targeted treatments. By addressing the specific molecular disruptions in each system—whether by stabilizing receptors, enhancing transporter function, or modulating glutamate activity—these new therapies aim to protect cognitive and motor functions [349]. This shift toward precision medicine represents a hopeful frontier in treating neurodegenerative diseases, one that promises more effective, sustainable improvements for those affected [350].

6.3. Molecular Strategies to Restore Synaptic Function

Restoring synaptic function is a promising direction in neurodegenerative research, aiming not only to slow decline but to reinstate essential neural connections. Recent advancements in synaptic plasticity modulation, neurotrophic factor support, and direct synapse-targeted therapeutics are paving the way for innovative treatments [351].

6.3.1. Modulation of Synaptic Plasticity

Synaptic plasticity, the ability of synapses to adapt, is critical for memory and learning. Novel modulators of NMDA and AMPA receptors aim to enhance plasticity while avoiding excitotoxicity. Positive allosteric modulators for AMPA receptors subtly increase receptor activity, strengthening synaptic responses and supporting memory processes without overstimulation [352,353]. Similarly, partial NMDA receptor agonists are under development to reinforce pathways crucial for learning and memory, offering a balanced approach that preserves adaptability without harming neurons [354]. These receptor-specific treatments could provide a new layer of cognitive resilience in neurodegeneration.

6.3.2. Neurotrophic Factors

Advanced delivery methods for neurotrophic factors are improving synaptic resilience. Gene therapies for BDNF focus on precise delivery to affected brain regions, with engineered viral vectors targeting hippocampal areas associated with memory [355]. This approach aims to maintain synaptic plasticity and protect against further synaptic loss. In PD, encapsulated cell implants releasing GDNF provide sustained support for dopaminergic neurons in the substantia nigra [356]. These implants bypass the blood–brain barrier, ensuring targeted, continuous delivery to preserve neurons over time, offering a lasting neuroprotective effect [357].

6.3.3. Synapse-Targeted Therapeutics

Directly stabilizing synaptic structures represents a novel therapeutic approach. Research into scaffolding proteins like PSD-95 and SHANK3, which maintain receptor alignment, has led to peptide-based drugs that bind these proteins, enhancing synaptic stability. By reinforcing the structural matrix, these therapies aim to sustain communication between neurons in conditions where synaptic architecture is compromised [358].

Efforts to improve neurotransmitter availability by enhancing synaptic vesicle dynamics are also underway [359]. Targeting proteins like synapsin, crucial for vesicle mobi-

lization, could improve neurotransmitter release and recycling, addressing transmission deficits seen in HD [360]. These interventions aim to restore efficient signal transmission, preserving connectivity and overall neural network health.

These molecular strategies represent a shift toward therapies that rebuild and strengthen synaptic architecture. By targeting receptor function, sustaining neurotrophic support, and directly bolstering synaptic structure, these approaches offer the potential for meaningful restoration of cognitive and motor function [361,362]. This new generation of treatments brings hope for durable, structural improvements in the management of neurodegenerative diseases.

7. Molecular Biomarkers and Diagnostics

The discovery of molecular biomarkers is redefining how we approach neurodegenerative diseases, offering pathways to early diagnosis, precise monitoring, and personalized treatment [363]. Advances in proteomics and metabolomics allow researchers to detect unique biochemical changes in CSF and blood, providing insights into the underlying mechanisms of neurodegenerative diseases [364]. This molecular-level approach is moving diagnostics beyond symptom observation and toward a future of targeted interventions.

7.1. Molecular Biomarkers in Body Fluids

CSF and blood biomarkers are becoming indispensable for diagnosing neurodegenerative diseases, offering a minimally invasive way to assess disease-specific molecular changes. Proteomic and metabolomic profiling provides comprehensive insights, capturing shifts in protein and metabolite levels that are uniquely associated with each condition.

This table (Table 2) compiles high-citation studies on molecular biomarkers critical for the early detection and diagnosis of neurodegenerative diseases. Each biomarker type, from protein aggregates to inflammatory markers, is associated with particular neurodegenerative conditions and is linked to specific detection methods.

Table 2. High-citation studies on molecular biomarkers for early detection and diagnosis.

Biomarker Type	Primary Marker(s)	Associated Disease(s)	Detection Method	Diagnostic Value	High-Citation Studies
Protein aggregates	Amyloid-beta, tau	Alzheimer's	CSF analysis, PET imaging	High sensitivity and specificity for early AD diagnosis	Chapleau et al. (2022) [365], Greenberg et al. (2022) [366]
Inflammatory cytokines	TNF-α, IL-1β, IL-6	ALS, Alzheimer's, PD	Blood-based biomarkers, CSF sampling	Elevated levels correlating with neuroinflammatory activity	Heneka et al. (2015) [88], Hu et al. (2017) [367]
Genetic mutations	SOD1, HTT, APOE ε4	ALS, Huntington's, AD	Genotyping, next-gen sequencing	APOE ε4 for AD risk; SOD1 mutations in familial ALS	Serrano-Pozo et al. (2021) [368], Renton et al. (2014) [369]
Oxidative stress markers	8-OHdG, MDA, protein carbonyls	ALS, Huntington's	Urine, blood assays	Indicative of oxidative damage in early neurodegeneration	Beal et al. (2005) [370], Chen et al. (2004) [371]
Synaptic loss indicators	Synaptophysin, PSD-95	Alzheimer's, Parkinson's	Immunohistochemistry, PET tracers	Correlates with cognitive decline and synaptic dysfunction	Tzioras et al. (2023) [372], Dauer et al. (2003) [95]

7.1.1. Proteomic Approaches

Proteomics examines protein changes that are specific to neurodegenerative processes. In AD, reduced amyloid-beta 42 (Aβ42), increased phosphorylated tau, and elevated neurogranin in CSF collectively reflect the early presence of plaques, tau pathology, and synaptic loss, which are key indicators before cognitive symptoms fully develop [363]. This combination of markers enables a nuanced view of AD onset, offering a more accurate diagnosis than single biomarkers.

In PD, phosphorylated alpha-synuclein at serine-129 is emerging as a reliable marker in CSF and blood, distinguishing PD's pathology from other movement disorders [373]. This phosphorylation pattern, along with neurofilament light chain (NfL), allows clinicians to differentiate PD from similar conditions, providing a refined diagnostic approach based on early protein modifications [374].

7.1.2. Metabolomic Profiling

Metabolomics analyzes small molecules within body fluids, uncovering metabolic disruptions that are distinctive to neurodegenerative diseases. In AD, lipid peroxidation markers like malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) indicate oxidative stress in the brain, potentially allowing for early detection [375]. Changes in glucose metabolism also reflect AD's impact on brain energy usage, providing additional diagnostic insights.

In PD, lower levels of urate in blood have been associated with faster disease progression, sparking interest in urate as a biomarker. This discovery has led to research on urate-boosting therapies as a potential way to slow PD's course [376]. In ALS, elevated glutamate levels in CSF highlight excitotoxicity, offering a molecular target for therapies aimed at reducing glutamate-related damage. Alterations in amino acid levels also underscore the metabolic imbalance associated with ALS progression [345].

These biomarker advancements in CSF and blood present a transformative approach to neurodegenerative diagnostics. By identifying disease-specific protein and metabolite changes, researchers are creating precise diagnostic tools that support early intervention and personalized treatment [377].

7.2. Molecular Imaging Techniques

Molecular imaging is opening up remarkable possibilities in neurodegenerative disease diagnostics, allowing us to see inside the brain at the molecular level. Unlike traditional imaging, which captures structural damage only after symptoms appear, molecular techniques like PET and targeted MRI contrast agents are designed to reveal disease-related molecules in real time [378]. This shift means that for conditions like AD, PD, ALS, and HD, we can detect disease earlier, track its progression with greater accuracy, and evaluate therapies in a more targeted way [379].

7.2.1. PET Ligands for Protein Aggregates

Positron emission tomography (PET) imaging has transformed our approach to tracking disease-specific proteins in the brain. In AD, PET tracers such as 18F-florbetapir and 11C-PiB bind directly to amyloid-beta plaques, providing an early and clear view of amyloid buildup that correlates with disease progression [380]. Tau-specific PET tracers, like 18F-flortaucipir, add another layer, allowing us to see where tau tangles are concentrated. Studies show that tau load aligns more closely with cognitive decline than amyloid, offering crucial insights into the symptomatic side of AD and enabling a more nuanced approach to diagnosis and treatment [381].

In PD, dopamine-targeted PET tracers like 18F-FDOPA are instrumental in capturing the decline in dopamine levels within the substantia nigra, even in the earliest stages [382]. With the development of alpha-synuclein-specific tracers, PET imaging is on the brink of being able to visualize Lewy body pathology directly. This would allow us to distinguish PD from other movement disorders that do not involve alpha-synuclein buildup, poten-

tially transforming diagnostic accuracy and paving the way for alpha-synuclein-targeted therapies [383].

7.2.2. Molecular MRI Contrast Agents

Molecular MRI is expanding beyond structural imaging, using specialized contrast agents to highlight disease-specific changes invisible to conventional MRI. In AD, new gadolinium-based agents designed to bind amyloid-beta plaques are offering an innovative, nonradioactive approach to tracking amyloid burden over time. Iron-sensitive MRI techniques are also emerging as powerful tools for detecting iron accumulation in the hippocampus and other brain areas affected by AD [384]. This buildup of iron, associated with oxidative stress and inflammation, provides insights into the biochemical environment that accelerates disease progression [385].

For PD, ultra-small superparamagnetic iron oxide (USPIO) nanoparticles are being explored as contrast agents that enhance iron detection in the substantia nigra. Since iron accumulation is strongly linked with PD pathology, these agents offer a targeted way to visualize and monitor disease progression. This technique also aids in distinguishing PD from other disorders where iron buildup is absent, enhancing diagnostic specificity [386].

Nanoparticle-based MRI agents targeting neuroinflammation are making significant strides in conditions like ALS and HD. These nanoparticles can cross the BBB and attach to activated microglia, the immune cells involved in neuroinflammatory processes. In ALS, where inflammation closely correlates with motor neuron damage, tracking neuroinflammation with MRI offers a new layer of insight into how the disease progresses and how therapies impact inflammation over time [387].

The advances in molecular imaging through PET ligands and MRI contrast agents are reshaping our approach to neurodegenerative diseases. By focusing on proteins like amyloid, tau, dopamine, alpha-synuclein, iron buildup, and neuroinflammation, these imaging agents allow for a deeper, more precise understanding of disease mechanisms. Molecular imaging is not only enhancing diagnostic accuracy but also opening up pathways for targeted treatments, bringing a promising future of personalized neurodegenerative care within reach [388].

7.3. Molecular Diagnostics and Personalized Medicine

Molecular diagnostics is opening the door to personalized medicine in neurodegenerative diseases where treatments are tailored to each patient's unique genetic and molecular profile. This approach transforms how we manage neurodegenerative conditions by allowing for more precise and proactive care. Advances in genomics, risk modeling, and molecularly targeted therapies mean that clinicians can now detect disease early, anticipate its progression, and personalize treatment plans, moving beyond a one-size-fits-all approach [389].

7.3.1. Genomic Sequencing

Genomic sequencing has become essential for identifying specific genetic variations that influence the onset and progression of neurodegenerative diseases. In AD, the APOE ε4 allele remains a well-known risk factor, but polygenic risk scores (PRS), which analyze multiple genetic markers, now provide a richer understanding of each person's susceptibility [390]. These scores can reveal high-risk profiles long before symptoms appear, helping to guide early interventions aimed at slowing or preventing cognitive decline.

In PD, sequencing has uncovered mutations beyond LRRK2, such as GBA and SNCA, each linked to different molecular pathways involved in the disease. This knowledge is driving targeted therapies designed to address the distinct impacts of each mutation [391]. Innovative gene-editing technologies like CRISPR/Cas9 are also being explored to modify the expression of specific genes, with the potential to stop the disease before it progresses by targeting its genetic roots.

ALS and HD also see breakthroughs from genomic insights. For ALS, mutations like C9orf72 and SOD1 are directly associated with harmful proteins and RNA byproducts. Therapeutic approaches such as ASOs are being developed to counteract these specific toxic molecules at their source [392]. In HD, the precise measurement of CAG repeat length in the HTT gene enables not only an accurate diagnosis but also the potential for targeted gene therapies that aim to reduce the production of the mutant huntingtin protein, a primary cause of HD's progression [393].

7.3.2. Molecular Risk Models

Molecular risk models are now integrating genetic data with other factors—such as biomarker levels and environmental influences—to predict disease trajectory more accurately. In AD, models that combine APOE genotype with cerebrospinal fluid markers like amyloid and tau give clinicians the tools to forecast cognitive decline with greater precision. This means at-risk individuals can begin preventive or slowing interventions well before symptoms arise [394].

In PD, risk models that include early, non-motor symptoms such as REM sleep behavior disorder, along with genetic factors like LRRK2 and GBA mutations, allow for earlier intervention strategies. This approach helps identify high-risk individuals long before motor symptoms appear, supporting neuroprotective measures aimed at preserving brain health [395]. In ALS, combining biomarkers like NfL with specific genetic mutations helps clinicians identify faster-progressing cases, enabling more personalized and intensive treatment strategies where needed [377].

7.3.3. Personalized Treatment Strategies Guided by Molecular Profiling

Personalized medicine enables therapies that are aligned with each patient's unique molecular markers. In AD, for example, patients with significant amyloid buildup can be targeted with anti-amyloid therapies, while those showing tau pathology may be better suited to tau-specific treatments. This targeted approach avoids generalized treatment, reducing unnecessary exposure and enhancing the likelihood of a positive response [396].

In PD, mutation-specific treatments are emerging as well. For example, patients with LRRK2 mutations may respond to LRRK2 kinase inhibitors, which directly address the cellular effects of this mutation. In ALS, ASO therapies that target specific mutations such as SOD1 are being developed to prevent the production of toxic proteins [397]. HD, too, is benefiting from precision medicine with RNA interference therapies designed to selectively lower mutant huntingtin protein levels, aiming to slow the disease at its genetic origin [398].

The advancements in molecular diagnostics and personalized medicine are creating a future where neurodegenerative diseases are managed according to each patient's unique molecular signature.

8. Gut–Brain Axis: Molecular Interactions

The gut–brain axis reveals surprising insights into how the gut microbiome might influence brain health and the course of neurodegenerative diseases. Once viewed as largely separate, it is now clear that the gut and brain engage in continuous molecular conversations, with the gut microbiome playing an active role in shaping processes that can either support or challenge neural health [399].

8.1. Molecular Communication Pathways

The gut communicates with the brain through a web of molecular pathways, with metabolites, immune modulators, and microbial byproducts all playing roles. These signaling mechanisms illustrate how changes in microbial composition can ripple through the body and impact neurodegenerative processes at their core [400].

8.1.1. Metabolites as Signaling Molecules

One of the most fascinating discoveries is that metabolites produced by gut bacteria can directly affect brain function [401]. For example, certain gut bacteria synthesize gamma-aminobutyric acid (GABA), a neurotransmitter crucial for maintaining a balance between excitation and inhibition in the brain. Researchers are now exploring whether boosting levels of GABA-producing bacteria might offer support to neural circuits affected by neurodegenerative diseases. This approach presents the potential to address not just cognitive symptoms but also mood-related symptoms, with microbiome adjustments providing a new avenue for neuroprotection [402].

Polyamines, small organic molecules produced by certain gut microbes, have also gained attention for their role in cellular repair and growth. In the brain, balanced polyamine levels are crucial for maintaining cellular resilience, but when dysregulated, they can contribute to cellular stress [403]. Targeting polyamine synthesis in the gut as a means to fortify brain cells against the stresses seen in neurodegeneration is a promising approach. By carefully modulating polyamine levels through probiotics or dietary adjustments, scientists hope to create a more resilient neural environment, potentially slowing the progression of neurodegenerative conditions [404].

8.1.2. Bacterial Components Influencing Neuroinflammation

Bacterial components, such as LPS and peptidoglycans, are now recognized as influential players in neuroinflammation. These molecules can escape the gut and travel through the bloodstream, particularly when the gut barrier becomes compromised [400]. Once in circulation, they may cross into the brain and trigger inflammation by activating microglial cells, which can then initiate a cascade of immune responses that contribute to neuronal stress and damage [405]. Efforts to reinforce the gut barrier—through specific probiotics, prebiotics, or dietary interventions—are being explored as ways to prevent these inflammatory molecules from entering the bloodstream, potentially providing a novel strategy for controlling neuroinflammation in neurodegenerative diseases [406].

Microbial-derived amines like p-cresol are also of special interest. This byproduct, produced by certain bacteria during protein fermentation, can disrupt the BBB, making it more permeable and allowing neurotoxic molecules to reach the brain. This discovery has led to research focused on selectively reducing p-cresol-producing bacteria within the gut [407]. By protecting the BBB in this way, it may be possible to limit neurotoxic damage and slow down disease progression. The insights emerging from gut–brain research offer a fresh perspective on neurodegenerative diseases, highlighting the microbiome as a powerful modulator of brain health [408].

8.2. Molecular Impact of Microbiota on the CNS

Discoveries reveal the remarkable influence of the gut microbiota on CNS health. Far from being limited to digestion, the gut's microbial communities actively engage in a two-way conversation with the brain, affecting everything from barrier integrity to immune responses and neural communication [409]. By exploring the molecular pathways that connect gut microbes to brain processes, scientists are beginning to understand how microbiome-based therapies might support brain resilience and even slow disease progression [410].

8.2.1. Tight Junction Proteins and Blood–Brain Barrier Integrity

The BBB acts as a selective shield, keeping harmful substances out of the brain while allowing essential molecules to pass. Tight junction proteins are vital for this barrier's function, and recent studies show that certain gut bacteria can enhance the expression of these proteins, strengthening the BBB and making it more resistant to neurotoxic infiltration [411]. A key player here is butyrate, a short-chain fatty acid produced by beneficial gut microbes, which promotes the production of claudin and occludin—proteins essential to maintaining the BBB's integrity. In cases of gut dysbiosis where butyrate levels drop, BBB

function can weaken, potentially letting harmful compounds into the brain. Interventions that boost butyrate levels, either through diet or supplementation, are being explored to fortify the BBB and reduce neurodegenerative risk [412].

Zonulin, a protein regulated by certain gut bacteria, also influences BBB permeability by loosening tight junctions when its levels rise. High zonulin can allow inflammatory molecules into the brain, which may accelerate neuroinflammation. Therapeutic approaches aimed at controlling zonulin-regulating bacteria offer a promising route to stabilizing the BBB and mitigating inflammatory responses tied to disease progression [413].

8.2.2. Vagus Nerve Signaling and Gut–Brain Communication

The vagus nerve forms a direct communication link between the gut and the brain, transmitting signals that impact mood, cognition, and immune health. Certain gut bacteria produce neuroactive compounds, including serotonin precursors, which activate the vagus nerve and influence brain regions involved in emotional regulation and stress. In neurodegenerative conditions, promoting bacteria that produce serotonin and related compounds is being explored as a way to enhance mood and cognitive function through this gut–brain pathway [414].

In Parkinson's disease, research suggests that misfolded alpha-synuclein proteins may originate in the gut and travel to the brain via the vagus nerve. Some bacterial strains appear to encourage this initial misfolding, offering a new therapeutic target by addressing the gut's role in the early stages of PD progression [415].

Moreover, the vagus nerve plays a role in immune modulation, carrying signals from gut bacteria that produce anti-inflammatory compounds. In diseases like ALS, boosting these anti-inflammatory bacteria could help reduce brain inflammation, protect neurons, and potentially slow the course of neurodegeneration. The gut microbiota's impact on the BBB and vagal signaling underscores a powerful connection between gut health and brain health [416].

8.3. Molecular Therapeutics Targeting the Gut–Brain Axis

The gut–brain axis has emerged as a novel therapeutic target in neurodegenerative disease, with therapies designed to modulate the gut microbiome showing promise for supporting brain health. Prebiotics, probiotics, and targeted antibiotics are being explored as potential interventions to balance gut microbial populations, reduce neuroinflammation, and positively influence neurotransmitter production [417,418].

8.3.1. Prebiotics and Probiotics

Prebiotics and probiotics are under intense study for their potential to nurture a gut environment that actively supports CNS function. Prebiotics, which serve as food for beneficial bacteria, can enhance the production of SCFAs like butyrate. Elevated butyrate levels have been associated with improved BBB integrity, which helps restrict harmful molecules from reaching the brain and triggering inflammation. Clinical trials are currently evaluating whether prebiotic-rich diets can boost butyrate levels in the gut, potentially reinforcing BBB stability and providing neuroprotective benefits in conditions marked by neuroinflammation [419].

Probiotics, introducing live beneficial bacteria, are also being studied for their effects on neurotransmitter pathways and brain health. Certain strains produce metabolites that support mood and cognitive function by increasing levels of neurotransmitters such as GABA and serotonin. Enhancing populations of these neurotransmitter-producing bacteria offers an innovative approach to managing mood and cognitive symptoms often seen in neurodegenerative disorders [420]. Studies suggest that specific strains, like Bifidobacterium and Lactobacillus, may contribute to a more supportive gut–brain environment by balancing immune responses and promoting neural health [421].

In Parkinson's, where evidence suggests that alpha-synuclein misfolding may start in the gut, probiotics are being examined for their potential to alter microbial populations

that influence this process. Researchers are exploring whether specific strains could help stabilize microbial composition in ways that reduce alpha-synuclein misfolding and its propagation to the brain, thereby intercepting disease progression early [408].

8.3.2. Antibiotics and Microbiota Modulation

Selective use of antibiotics is also being studied as a way to adjust gut microbial composition in neurodegenerative diseases. While antibiotics are generally used to target pathogenic bacteria, certain regimens may help selectively reduce pro-inflammatory bacterial strains linked to neuroinflammatory pathways. For instance, in Parkinson's disease, specific bacteria appear to contribute to alpha-synuclein aggregation, which then migrates from the gut to the brain. Short-term antibiotic treatments are being investigated to manage these specific bacterial populations, potentially lowering neuroinflammatory triggers associated with the progression of Parkinson's [422].

Given the risks of broad-spectrum antibiotics disrupting beneficial bacteria, precision antibiotics designed to selectively target strains linked to neurodegenerative pathways are a promising focus. These precision agents aim to modulate the microbiome without disturbing the broader microbial ecosystem, thus supporting a balanced gut environment. Early studies in ALS models show that selective antibiotics can effectively reduce inflammation and support neuronal function, demonstrating the potential of microbiome-modulating antibiotics as a supportive treatment for neurodegeneration [400]. Therapies like prebiotics, probiotics, and precision antibiotics open new therapeutic pathways for neurodegenerative diseases. These treatments aim to reshape microbial populations in ways that bolster BBB function, reduce inflammation, and support neuroprotective signaling [423].

9. Emerging Molecular Therapeutic Approaches

In the world of neurodegenerative treatments, molecular therapies are opening doors that once seemed unreachable. With our expanding knowledge of cellular mechanisms, these approaches aim to target the precise molecular causes of neurodegenerative diseases [424]. Advances in nanotechnology, stem cell applications, and pharmacogenomics are transforming treatment possibilities, offering tailored solutions that reach deep into the brain's complex networks. By addressing the intricate needs of affected neurons and crossing physical barriers, these novel therapies signal a new era of precision medicine in neurodegeneration [425].

9.1. Nanotechnology in Drug Delivery

Nanotechnology is reshaping how we approach drug delivery in the brain. With its ability to construct incredibly tiny carriers, nanotechnology enables targeted, highly controlled therapeutic interventions that reach areas in the brain once inaccessible due to barriers like the BBB. Nanocarriers, meticulously engineered to transport therapeutic agents to specific regions within the brain, offer new hope for reaching disease hotspots with minimal side effects [426].

9.1.1. Nanocarriers for Enhanced Brain Penetration

The BBB has long posed a formidable challenge, blocking many potentially beneficial drugs from entering the brain. Nanocarriers—such as liposomes, polymeric nanoparticles, and dendrimers—are designed to bypass this barrier, acting as tiny vessels that can shield drugs as they make their way into the brain [427]. These carriers can be modified with specialized molecules on their surfaces, allowing them to interact with the BBB and enter precisely where they are needed, sparing other areas of the body from unnecessary exposure [428].

In Alzheimer's, researchers are exploring nanocarriers loaded with drugs aimed at breaking down amyloid plaques and preventing tau protein aggregation. These carriers bring the medication directly to the affected areas, enhancing its impact and reducing the systemic side effects often seen in traditional treatments [429]. In Parkinson's, dopamine-

loaded nanocarriers are being tested for their ability to reach specific brain regions, offering a more effective way to replace dopamine and address motor symptoms with precision and efficiency [430].

Newer biodegradable nanocarriers add another layer of innovation, breaking down in the body once their therapeutic payload has been delivered. This reduces any long-term accumulation of materials in the body, minimizing risks [431]. Moreover, researchers are creating nanocarriers that release drugs in response to specific signals, such as oxidative stress or pH changes, making treatment timing more precise and adaptable to the unique conditions within the diseased brain [432].

9.1.2. Targeted Delivery Systems for Precision Therapy

Building upon the BBB-crossing capabilities of nanocarriers, targeted delivery systems are taking precision to an even greater level by directing treatments specifically to diseased cells [433]. These systems use ligands—small molecules designed to bind selectively to receptors found only on certain brain cells—ensuring that treatments focus directly on affected areas, preserving healthy cells from exposure [434].

Nanoparticles traverse the BBB using specialized mechanisms tailored for efficient and precise delivery. Receptor-mediated transcytosis is a prominent strategy where ligands such as transferrin, lactoferrin, or apolipoprotein E bind to receptors like transferrin or LDL receptors on endothelial cells, facilitating transport across the barrier. Surface functionalization with PEG minimizes immune detection and prolongs circulation time, while active targeting strategies use antibodies or peptides to engage transporters such as LRP1 or GLUT1 for precise delivery to neural tissues [435]. Stimuli-responsive nanoparticles further enhance specificity, releasing their therapeutic payload in response to localized triggers like acidic pH, oxidative stress, or elevated enzymatic activity within the brain microenvironment. Additionally, lipid-based systems, such as liposomes and solid lipid nanoparticles, exploit endogenous carrier-mediated pathways to mimic natural substrates and improve permeability [435].

The long-term safety of nanoparticles depends on their composition, clearance mechanisms, and immune compatibility. Nonbiodegradable materials, such as metallic or carbon-based nanoparticles, risk accumulation in organs like the liver, spleen, and kidneys, potentially causing chronic toxicity. Immune responses, including complement activation-related pseudoallergy (CARPA), can be triggered by surface properties, leading to inflammatory or allergic reactions [436]. To address these concerns, biodegradable materials such as lipids or PLGA are preferred, and PEG coatings are used to reduce immune recognition and enhance clearance. Rigorous long-term in vivo studies are critical to evaluating biodistribution, toxicity, and the potential for bioaccumulation, ensuring the safe application of nanoparticles in clinical settings [437].

In ALS where motor neurons progressively deteriorate, researchers are developing targeted nanoparticles to deliver neuroprotective treatments right to these cells. This focused approach allows for the delivery of therapeutic agents directly to the cells that need them the most, potentially slowing degeneration and preserving motor function [438].

In HD, targeted nanoparticles are being designed to transport gene-editing tools to cells expressing the mutant huntingtin protein, selectively modifying only the damaged cells [439]. This could allow for a reduction in harmful protein levels without impacting other healthy cells, directly targeting the root of disease progression with minimal off-target effects [440].

Further innovations are pushing the boundaries of nanotechnology with multifunctional nanoparticles that address multiple aspects of neurodegeneration simultaneously. For instance, a single nanoparticle might be designed to carry both an anti-inflammatory agent and a neuroprotective compound, offering a dual approach for treating diseases like Alzheimer's and Parkinson's where inflammation and neurodegeneration occur hand in hand [441]. These versatile carriers provide a holistic treatment in one single package, addressing several facets of complex disease mechanisms [442].

Personalized medicine can tailor treatments by leveraging individual amyloid or tau profiles. For instance, patients with predominant amyloid pathology may benefit from anti-amyloid monoclonal antibodies, whereas those with tau-related dysfunction might respond better to tau aggregation inhibitors or kinase inhibitors targeting tau hyperphosphorylation [443].

Another promising direction is theranostics where nanoparticles integrate both therapeutic and diagnostic functions. These specialized nanocarriers can not only deliver treatments but also allow real-time tracking of drug distribution and therapeutic effects [444]. This capability offers physicians an unprecedented ability to monitor how well the treatment is working, adjusting doses and timing as needed, enabling a level of precision and adaptability in treatment that is especially valuable in progressive diseases [445]. The potential of nanotechnology to reshape neurodegenerative treatment lies in its unparalleled ability to deliver therapies with remarkable accuracy. By targeting specific cells, navigating biological barriers, and combining therapeutic actions in a single nanocarrier, these advances represent a transformative approach to tackling complex brain diseases. These precise, carefully designed interventions have the promise to significantly improve treatment efficacy and open new pathways to protect and sustain brain health [446].

9.2. Molecular Basis of Stem Cell Therapy

Stem cell therapy opens up exciting new possibilities in the fight against neurodegenerative diseases. With their unique ability to regenerate damaged neurons, release protective molecules, and modulate immune responses, stem cells offer a fresh approach. Advances in guiding stem cells to become specific types of neurons, delivering them directly to affected brain regions, and even enhancing them genetically bring hope for treatments that go beyond symptom relief to address the root causes of these diseases [447,448].

9.2.1. Differentiation into Neuronal Lineages

A key focus in stem cell research is guiding these versatile cells to become the specific types of neurons lost in neurodegenerative diseases. The latest breakthroughs in induced pluripotent stem cells (iPSCs) make it possible to take a patient's own cells, reprogram them, and turn them into exactly the kinds of neurons that are missing [449].

In Parkinson's disease, for instance, scientists are working to turn iPSCs into dopaminergic neurons—the cells that produce dopamine, which is essential for movement [450]. Early studies show that these lab-grown neurons can be transplanted into the brain where they can integrate with existing networks, produce dopamine, and, potentially, help restore motor control [451].

For ALS, researchers are refining ways to produce motor neurons from stem cells. These specially developed cells are designed to survive the inflammatory environment of ALS and re-establish lost connections with muscles [452]. By focusing on resilience and functionality, these motor neurons could, one day, help ALS patients retain motor function longer [453].

In HD, where a specific type of neuron called the medium spiny neuron is lost, scientists are developing ways to generate these cells in the lab. By tailoring differentiation protocols to produce neurons that function just like those affected in Huntington's, researchers hope to restore both cognitive and motor abilities [454].

9.2.2. Paracrine Effects of Stem Cells

Stem cells offer another valuable function beyond simply replacing lost cells—they can release molecules that support and protect surrounding neurons. This ability to influence the brain's environment is known as the paracrine effect. Mesenchymal stem cells (MSCs), for example, naturally secrete neurotrophic factors like BDNF and GDNF, which help neurons survive, form connections, and resist stress [455]. In Alzheimer's, where neuronal support is critical, these neurotrophic factors could help protect brain cells from the effects of the disease [456].

Exosomes are emerging as a clever way to deliver these protective molecules. These tiny, naturally occurring particles, which stem cells release, carry proteins, RNAs, and other therapeutic molecules [457]. Because exosomes can cross the blood–brain barrier, they can deliver neuroprotective factors directly to the brain. Studies have shown that exosomes might help reduce amyloid and tau deposits in Alzheimer's models, enhancing neuronal resilience without the need for full cell transplantation [458].

In ALS, stem cells are explored as a way to release anti-inflammatory molecules that calm overactive immune responses. By reducing inflammation, stem cell-derived factors can help create a healthier environment for motor neurons, potentially slowing the progression of the disease [459].

A further innovation involves genetically enhancing stem cells to make them even more effective. Using CRISPR gene editing, scientists are boosting stem cells' ability to release protective molecules and resist the conditions of the disease environment. This combined approach not only fortifies the transplanted cells but also allows them to support the brain more effectively, addressing both inflammation and neuronal damage at once [460]. Stem cell therapy holds transformative potential in neurodegenerative diseases by combining precise neuronal replacement, powerful paracrine support, and genetic enhancement [461].

9.3. Pharmacogenomics

Pharmacogenomics is redefining neurodegenerative disease treatment by leveraging genetic and molecular insights to tailor therapies to each patient's unique biology. Rather than relying on standard protocols, pharmacogenomics allows for a customized approach where treatments are selected based on how a patient's genetic makeup affects drug efficacy, safety, and disease progression [462]. This level of precision is especially valuable in complex diseases like Alzheimer's, Parkinson's, ALS, and Huntington's where individual responses to treatment can vary widely. By pinpointing the genetic and molecular factors that drive each person's disease, pharmacogenomics opens the door to more effective, patient-centered care [463].

9.3.1. Molecular Profiling for Drug Response

Pharmacogenomic profiling begins with identifying genetic variations that influence how patients process and respond to medications. Key genes involved in drug metabolism, such as those in the cytochrome P450 (CYP) family, play a central role. Variations in these genes can determine how quickly or slowly drugs are broken down in the body, which impacts both therapeutic outcomes and side effect profiles [464]. In Alzheimer's disease, for example, specific CYP2D6 variants affect how patients metabolize cholinesterase inhibitors, a commonly prescribed class of drugs [465]. Patients with certain genetic profiles may experience reduced drug efficacy or increased side effects, making it essential to adjust dosages based on genetic information for optimal results.

In Parkinson's, genetic variations in dopamine-processing genes like COMT and MAO-B affect how patients respond to dopamine replacement therapies. Patients with certain COMT polymorphisms may require customized levodopa dosing to achieve effective symptom control while minimizing motor complications [466]. Additionally, patients with mutations in genes like LRRK2 or GBA, which are associated with different PD subtypes, may respond better to specific treatments tailored to their genetic backgrounds [467,468].

In ALS, where genetic mutations play a major role in disease variability, pharmacogenomic insights are used to guide drug selection. Certain ALS patients carry mutations in the SOD1 gene, which are linked to faster disease progression [469]. For these patients, targeted therapies that specifically address the effects of SOD1 mutations, such as gene-silencing approaches, are under investigation [470]. By identifying patients with these and other relevant genetic variations, pharmacogenomics allows for more precise intervention strategies.

9.3.2. Tailoring Therapies Based on Molecular Signatures

Pharmacogenomics also involves analyzing molecular signatures—biomarkers, protein profiles, and gene expression patterns that reveal unique characteristics of a patient's disease. These signatures provide insight into the specific biological processes active in each case, allowing clinicians to select therapies that directly target the disease mechanisms most relevant to the individual [471].

In Alzheimer's, for instance, patients with particular biomarker profiles may benefit more from treatments targeting amyloid or tau pathology. Molecular profiling of biomarkers like APOE ε4 status, phosphorylated tau, and neurofilament light chain levels can guide clinicians in selecting amyloid-clearing drugs that patients are likely to respond to [472]. This approach enables the choice of targeted therapies that are better suited to each patient's distinct disease pathology.

The listed therapies (Table 3) span cutting-edge approaches such as nanotechnology-based drug delivery, stem cell therapy, and pharmacogenomics. Each therapeutic approach targets specific molecular processes within these diseases, with clinical outcomes demonstrating potential benefits and areas for further research.

Table 3. Therapeutic innovations and clinical trials in neurodegenerative disease treatments.

Therapeutic Approach	Molecular Target	Disease Focus	Therapeutic Agent(s)	Trial Phase	Key Outcomes	Cited Studies
Nanotechnology-based drug delivery	Blood–brain barrier penetration, tau/amyloid-beta targeting	Alzheimer's, Parkinson's	Liposomal encapsulation, PEG-modified nanoparticles	Phase II (various)	Improved brain bioavailability, reduced plaques/tangles in animal models	Hajjo et al. (2022) [473], Saraiva et al. (2016) [474]
Stem cell therapy	Dopaminergic neurons, motor neurons	Parkinson's, ALS	iPSCs, MSCs	Phase I/II	Motor symptom improvement, neuron survival post-transplant	Takahashi et al. (2007) [475], Glass et al. (2016) [476]
Gene therapy	SOD1, HTT, APOE ε4	ALS, Huntington's, AD	Antisense oligonucleotides, CRISPR	Phase I/II	Reduced toxic protein levels, slower disease progression	Kordasiewicz et al. (2012) [477], Bennett et al. (2019) [478]
Pharmacogenomics	CYP2D6, COMT, APOE ε4	Alzheimer's, Parkinson's	Genotype-guided dosing for existing drugs	Phase II/III	Reduced side effects, improved therapeutic outcomes	Singh et al. (2017) [479], Müller et al. (2015) [480]
Microbiome modulation	Gut–brain axis, inflammation	Parkinson's, Alzheimer's	Probiotics, prebiotics, antibiotics	Phase I	Reduced neuroinflammation markers, improved cognitive/motor symptoms	Sampson et al. (2016) [481], Cattaneo et al. (2017) [482]

In ALS, molecular signatures associated with inflammation or oxidative stress help doctors make more informed treatment decisions [483]. Patients with elevated inflammatory markers may benefit from anti-inflammatory therapies, while those showing signs of oxidative damage might respond better to antioxidant interventions [484]. Pharmacogenomics enables the alignment of treatment strategies with the underlying molecular drivers of each patient's ALS, optimizing treatment effectiveness and potentially slowing disease progression [485].

For HD, pharmacogenomics is particularly valuable in the development of gene-silencing therapies. By examining HTT gene variations, which affect the mutant huntingtin protein production, clinicians can identify patients likely to benefit from antisense oligonucleotides—therapies designed to reduce harmful protein levels [85]. This molecularly tailored approach holds promise for slowing HD progression, targeting the root cause, and offering the potential for long-term impact in genetically compatible patients. Pharmacogenomics is ushering in a new era of precision medicine for neurodegenerative diseases where treatments are chosen based on each patient's specific genetic and molecular landscape [486]. By aligning therapies with individual disease characteristics, pharmacogenomics enhances treatment effectiveness, minimizes adverse effects, and brings a new level of personalization to neurodegenerative care.

10. Conclusions

Molecular research is illuminating new pathways in the treatment of neurodegenerative diseases, leading to a deeper understanding that goes beyond managing symptoms to targeting the fundamental drivers of conditions like Alzheimer's, Parkinson's, ALS, and Huntington's. This review has delved into the intricacies of neurodegeneration, exploring mechanisms from protein misfolding and inflammation to genetic mutations and cellular energy deficits. These insights are not just theoretical; they guide cutting-edge therapeutic approaches aimed at intervening at critical junctures in these diseases.

10.1. Integration of Molecular Mechanisms

The complex interplay among the mechanisms driving neurodegeneration is becoming clearer, revealing potential for therapies that address these intertwined pathways simultaneously. Protein misfolding, for example, does more than disrupt cellular function; it also triggers chronic inflammatory responses that exacerbate neuronal damage. Similarly, mitochondrial dysfunction creates oxidative stress that accelerates protein aggregation and impacts energy-dependent cellular processes. Emerging theories suggest that by targeting these interconnected pathways, it may be possible to "reset" the cellular environment, reducing neurotoxicity while promoting resilience.

For instance, there is growing interest in therapies that simultaneously target both protein aggregation and inflammation. Studies on dual-inhibitor compounds—designed to disrupt tau or amyloid-beta accumulation while dampening neuroinflammation—demonstrate early promise in animal models. These approaches reflect a novel shift toward multi-target treatments that consider the cascading effects of protein pathology on cellular inflammation, energy dysregulation, and neuronal survival, aiming for a broader therapeutic impact.

10.2. Implications for Future Research

Despite substantial progress, significant questions remain about the exact molecular drivers that set neurodegeneration in motion. Future research is likely to focus on early cellular changes that precede visible disease symptoms, aiming to identify key biomarkers or "molecular signatures" that predict disease onset. Single-cell sequencing and CRISPR screens, now widely adopted, are shedding light on the earliest transcriptional and epigenetic changes in vulnerable neurons, identifying potential points of intervention long before clinical symptoms appear.

One of the most compelling areas of exploration is the influence of the gut–brain axis, which is increasingly linked to neurodegenerative processes. High-citation studies point to gut microbiota as a modulator of systemic inflammation and immune signaling, with microbial imbalance linked to heightened neuroinflammation and even direct impacts on protein misfolding. For example, recent findings suggest that certain bacterial metabolites may cross the blood–brain barrier where they interact with glial cells to influence neuroinflammatory states. Ongoing research examines whether balancing the gut microbiota through diet, probiotics, or prebiotics could serve as an adjunct therapy to reduce neuroinflammatory triggers and support brain health.

Another emerging area is the study of senescent cells in the brain, often referred to as "zombie cells", which persist in a dysfunctional state, secreting inflammatory signals that impact neighboring neurons. Senolytic therapies, which selectively target and clear these cells, are investigated as a way to reduce chronic inflammation and improve neural health. Early studies show that removing senescent glial cells can improve cognitive function in neurodegenerative animal models, offering a new approach to disease modification.

10.3. Translational Potential

The translational potential of these molecular discoveries is enormous, bridging laboratory findings with clinical applications that could fundamentally alter the approach to neurodegenerative diseases. Advanced drug delivery methods, particularly those involving nanotechnology, make it feasible to cross the BBB with greater precision, delivering therapies directly to affected brain regions. Nanoparticles and exosomes are engineered not only to carry drugs but to release them in response to specific cellular conditions, such as oxidative stress or inflammation, which are prevalent in neurodegenerative environments. This type of "smart" delivery could enhance the therapeutic effects of drugs by timing their release with the most active disease processes.

Stem cell and gene therapies are also moving forward with groundbreaking potential. Beyond replacing lost neurons, stem cells are now engineered to release neurotrophic factors or anti-inflammatory molecules, creating a more supportive environment for remaining brain cells. Gene-editing technologies, such as CRISPR-Cas9, are explored for their potential to silence or repair mutant genes directly in the CNS. High-profile trials examine gene therapies that target the root genetic causes in diseases like Huntington's and familial ALS, offering hope for treatments that go beyond managing symptoms to modifying the underlying disease.

Pharmacogenomics is accelerating this movement toward personalized neurodegenerative therapies, allowing for treatments to be tailored to each patient's unique genetic makeup. By predicting drug response through genetic testing, pharmacogenomics enables a more individualized approach, minimizing trial-and-error prescribing and reducing adverse effects. Advances in molecular risk modeling further enhance this field, integrating patient data across genomics, proteomics, and clinical history to refine personalized treatment recommendations. Such precision models are poised to revolutionize care by selecting the best treatment for each patient based on a comprehensive view of their molecular profile.

In sum, molecular research is driving a paradigm shift in neurodegenerative treatment, uncovering innovative therapeutic targets and enabling more precise, multi-targeted approaches. By addressing the complex network of interconnected mechanisms that drive these diseases, we are moving closer to developing treatments that not only alleviate symptoms but intervene at key points in the disease process. With continued advances in molecular understanding and targeted technology, there is a real promise that neurodegenerative diseases may become more manageable, and, one day, even preventable.

Author Contributions: Conceptualization, C.T., A.V.C. and C.P.T.; data curation, R.-A.C.-B., M.E. and C.P.T.; formal analysis, A.V.C. and M.S.; investigation, M.S. and R.-A.C.-B.; methodology, C.T. and M.E.; resources, C.T., M.S. and A.V.C.; supervision, C.T., M.S. and A.V.C.; validation, O.M., C.T. and A.V.C.; visualization, C.T., O.M. and R.-A.C.-B.; writing—original draft, R.-A.C.-B., M.S. and A.V.C.; writing—review and editing, M.S. and R.-A.C.-B. All authors have read and agreed to the published version of the manuscript.

Funding: Publication of this paper was supported by the University of Medicine and Pharmacy Carol Davila, through the institutional program Publish not Perish.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

4-HNE	4-hydroxynonenal
ACh	Acetylcholine
AGEs	Advanced glycation end-products
AD	Alzheimer's disease
Aβ	Amyloid-beta
Aβ42	Amyloid-beta 42
APP	Amyloid precursor protein
ALS	Amyotrophic lateral sclerosis
α7-nAChRs	α7 nicotinic acetylcholine receptors
ASOs	Antisense oligonucleotides
BBB	Blood–brain barrier
BDNF	Brain-derived neurotrophic factor
CARPA	Complement activation-related pseudoallergy
CSF	Cerebrospinal fluid
CMA	Chaperone-mediated autophagy
ChAT	Choline acetyltransferase
CSF1R	Colony-Stimulating Factor 1 Receptor
CARPA	Complement activation-related pseudoallergy
CYP	Cytochrome P450
DAMPs	Damage-associated molecular patterns
ETC	Electron transport chain
ER	Endoplasmic reticulum
EPPS	4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid
fAD	Familial Alzheimer's disease
GABA	Gamma-aminobutyric acid
GDNF	Glial cell line-derived neurotrophic factor
GPx	Glutathione peroxidase
IKK	IκB kinase
iPSCs	Induced pluripotent stem cells
IL-1β	Interleukin-1β
IL-10	Interleukin-10
IL-6	Interleukin-6
JAK	Janus kinase
LPS	Lipopolysaccharides
LMTM	Leuco-methylthioninium bis(hydromethanesulfonate)
MDA	Malondialdehyde
MAMs	Mitochondria-Associated Membranes
mtDNA	Mitochondrial DNA
MAPK	Mitogen-activated protein kinase
MSCs	Mesenchymal stem cells
mGluRs	Metabotropic glutamate receptors
miRNAs	MicroRNAs
mHTT	Mutant huntingtin
NfL	Neurofilament light chain
NFTs	Neurofibrillary tangles
NLRs	NOD-like receptors
ncRNAs	Non-coding RNAs
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PHFs	Paired helical filaments
PRRs	Pattern recognition receptors
PPARs	Peroxisome proliferator-activated receptors
PET	Positron emission tomography
PSEN1	Presenilin 1
PSEN2	Presenilin 2

polyQ	Polyglutamine
ROS	Reactive oxygen species
RAGE	Receptor for advanced glycation end-products
RNAi	RNA interference
STAT6	Signal transducer and activator of transcription 6
SOD	Superoxide dismutase
TLRs	Toll-like receptors
TGF-β	Transforming growth factor-beta
TNF-α	Tumor necrosis factor-alpha
TH	Tyrosine hydroxylase
USPIO	Ultra-small superparamagnetic iron oxide
UCPs	Uncoupling proteins
UPR	Unfolded protein response
UPS	Ubiquitin–proteasome system
VMAT2	Vesicular monoamine transporter 2

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International Journal of
Molecular Sciences

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Review

Regulation of *NR4A2* Gene Expression and Its Importance in Neurodegenerative and Psychiatric Diseases

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Abstract

Nuclear receptor subfamily 4 group A member 2 (NR4A2) is a transcription factor that regulates the expression of different genes involved in essential biological processes, including cell proliferation, neuronal development, immune response, cellular stress, apoptosis, DNA repair, and angiogenesis. The gene encoding this transcription factor is called NR4A2 and has been identified as an immediate early gene. Moreover, research in animal models and clinical trials has suggested an association between reduced *NR4A2* gene expression and some neurodegenerative diseases and psychiatric disorders. These include Parkinson's disease, Alzheimer's disease progression, schizophrenia, substance abuse (alcohol and amphetamines), neurodevelopmental disorders, and cognitive imairment. NR4A2 activity is controlled at multiple levels, including transcriptional and post-transcriptional regulation of its gene expression, such as translational and post-translational processes. This review summarizes the current knowledge of the *NR4A2* gene, encompassing its structure and the molecular mechanisms that regulate its expression. The key epigenetic mechanisms that regulate its gene expression are emphasized, including DNA methylation, histone deacetylation, and regulation by microRNAs. It also addresses its role in central nervous system pathologies associated with dysregulation of *NR4A2* gene expression. Finally, we discuss the potential of these regulatory mechanisms as biomarkers and therapeutic targets for neurodegenerative diseases and psychiatric disorders.

Keywords: *NR4A2* gene expression; *NR4A2* epigenetic mechanisms; neurodegenerative diseases; psychiatric disorders; gene expression regulation; transcription factors; epigenetic mechanisms; neurodegenerative diseases; psychiatric disorders; central nervous system

1. Introduction

NR4A2 (also known as NURR1) belongs to the NR4A subfamily of nuclear receptors, along with NR4A1 (NUR77) and NR4A3 (NOR-1). These transcription factors (TFs) are activated in response to a wide range of stimuli and stressors. They are expressed in a variety of tissues and play important roles in regulating numerous and diverse physiological and pathological processes, including metabolism, cell cycle, carcinogenesis, inflammation, vascular processes, immune response and neuronal function [1–4]. These nuclear receptors are regarded as "orphans" due to the occupation of the ligand-binding site (LBD) by bulky and compact hydrophobic residues, limiting the capacity of ligands to binding. Nevertheless, some endogenous and exogenous molecules have been identified that can modulate the activity of these TFs [5,6].

The mechanisms regulating the transcriptional activity of this family of nuclear receptors are not yet fully understood, but they have been shown to involve post-translational processes and regulation of their gene expression [1,7]. In the context of neurodegenerative diseases and psychiatric disorders, it is therefore important to highlight the role of the *NR4A2* gene and its gene regulation.

NR4A2 plays an important role in various biological processes, such as cell proliferation, neuronal development, immune response, cellular stress response, apoptosis, DNA repair, and angiogenesis [8–10]. In the nervous system, NR4A2 participates in neuroprotection and plays a critical role in the differentiation, maintenance and function of dopaminergic (DAergic) neurons [11]. Dopamine is an important neurotransmitter involved in the modulation of motor and cognitive functions, as well as motivation and reward [12]. NR4A2 also participates in hippocampal synaptic plasticity [13], which is crucial for diverse cognitive functions, particularly learning and memory. In particular, NR4A2 has been implicated in Alzheimer's disease (AD) progression, Parkinson disease, schizophrenia, substance abuse (alcohol and cocaine), neurodevelopmental disorders and cognitive impairment among others [7,13–18]. This TF is also involved in anti-inflammatory, and homeostatic processes in neurons and non-neuronal cell [19].

Given its importance, understanding the mechanisms that regulate *NR4A2* gene expression and identifying potential therapeutic targets for neurological diseases and psychiatric disorders is a priority.

This review is therefore organized as follows: The first section provides an overview of the NR4A2 TF characteristics. The second section analyzes *NR4A2* gene expression across different tissues to provide a comprehensive overview of its expression patterns. Subsequently, we analyze the regulation of *NR4A2* gene expression, including its gene structure, and the cis (promoter) and trans regulatory factors involved in its gene expression. This section also highlights the epigenetic and post-transcriptional mechanisms that regulate *NR4A2* gene expression. These mechanisms are key for understanding the complex process of gene expression. Finally, we describe the dysregulation of *NR4A2* gene expression in various neurodegenerative and psychiatric diseases. This allows us to discuss NR4A2 as a potential therapeutic target and the challenges of potential treatment involving NR4A2.

2. Methods

This review was conducted using electronic databases including PubMed, Scopus, and Google Scholar, focusing on peer-reviewed literature published in English between 2020 and 2025. Furthermore, relevant references were selected based on their contribution to the understanding of NR4A2-related mechanisms in neurobiology and disease. Although the initial search criteria were limited to recent publications, several of these sources cited foundational studies from earlier decades, which were subsequently incorporated for the importance of supporting mechanistic interpretations. The search strategy included the used of specific key terms such as NR4A2 gene expression; NR4A2 and expression regulation; NR4A2 and epigenetic mechanisms; *NR4A2* and DNA methylation; *NR4A2* and histone modification; *NR4A2* and genetic variants; NR4A2 and neurodegenerative diseases; NR4A2 and psychiatric disorders; NR4A2 and Parkinson's disease; NR4A2 and Alzheimer's disease; NR4A2 and neurodevelopmental disorders; NR4A2 and schizophrenia; NR4A2 and major depressive disorder; NR4A2 and substance use disorders.

3. NR4A2 Protein

TFs are proteins that regulate the process of transcription through the binding of specific DNA sequences, known as response elements, located throughout the genome. Once bound to DNA, they allow the direct recruitment of TFs to the pre-initiation complex or the binding of intermediate factors, also called co-regulator proteins which are positive modulators (co-activators) and negative modulators (co-repressors) in order to activate or repress the expression of target genes [20]. These co-regulators can form subunits of multiprotein complexes that act at different functional levels, such as chromatin remodeling, histone modification or preinitiation complex modulation. Consequently, co-regulators regulate the transcription of their target genes [21].

Nuclear receptors interact with co-regulatory proteins through activation domains. Many nuclear receptors have at least two activation domains: a ligand-independent activation domain (AF-1), located in the N-terminal region, and a ligand-dependent activation domain (AF-2), in the C-terminal region [22].

As a member of the NR4A family, NR4A2 shares structural homology with nuclear receptor domains (Figure 1A). These include: (a) a modulatory domain called the activation function AF-1 N-terminal domain; (b) a conserved double zinc finger DNA-binding domain (DBD); (c) a flexible hinge region; (d) a ligand-binding domain (LBD) composed of 12 helices; and (e) the AF-2 C-terminus, which interacts with a co-regulatory protein [23]. NR4A2 lacks a ligand binding pocket and a canonical nuclear receptor binding site for co-regulatory proteins, leading to changes in gene expression [24]. The available studies suggest that the LBD of NR4A2 may function in an atypical ligand-independent manner, behaving dynamically and likely capable of expanding to bind ligands. Furthermore, some NR4A2 ligands function independently of binding to its LBD, and it has been proposed that NR4A2 transcriptional activity is modulated by ligands that target its heterodimer binding partner, such as retinoid X receptor alpha [25].

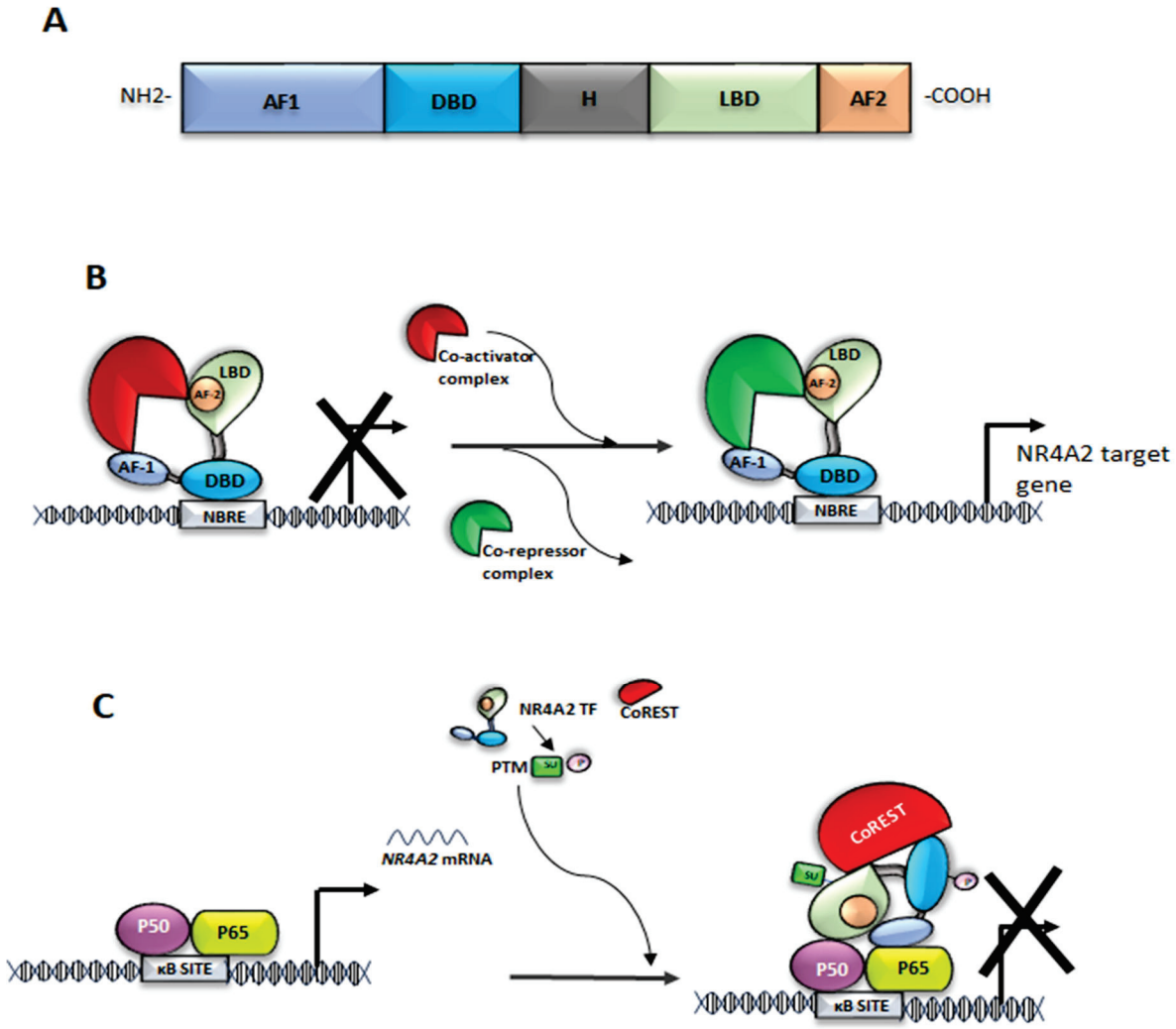


Figure 1. Structure and function of NR4A2 TF. (**A**) Schematic representation of the NR4A2 functional domains: N-terminal region AF-1, a conserved DBD, a H region and a C-terminal LBD-AF2 domain [23]. NR4A2 TF regulates gene expression through two main processes. (**B**) NR4A2 TF binds to specific DNA sequences through its DBD, thereby regulating transcription. Once bound to DNA, NR4A2 can bind to a co-regulator to regulate gene expression [24,26]. (**C**) NR4A2 binds to other TFs or co-regulators, without directly interacting with DNA. NR4A2 interacts with the p65 subunit of NF-κB in a manner determined by its post-translational modifications, such as sumoylation (mediated by STAT4) and phosphorylation (mediated by a Nemo-like kinase). NR4A2 then binds to the phosphorylated p65 subunit of NF-κB (mediated by GSK-3) in the promoter regions of target genes. This interaction leads to the recruitment of a repressor complex and affects the transcription of NF-κB target genes [27]. TF, transcriptor factor; DBD, DNA-binding domain; H, hinge region: LBD, ligand-binding domain; AF1, activation function 1; AF2: activation function 2; SU, sumoylation; P, phosphorylated; κB SITE, response element for NF-κB; (p50, p65), NF-κB, nuclear factor kappa B; NBRE, NGFI-B response element; CoREST repressor complex, co-repressor for element-1-silencing transcription factor.

The NR4A2 TF binds DNA sequences functioning as monomers (Figure 1B), homodimers or heterodimers, enabling regulation of multiple genes. The structure of the NR4A2 TF permits the regulation of gene expression of several target genes, which are essential for the development, maintenance, and survival of DAergic neurons [26]. Additionally, this nuclear receptor is also involved in maintaining cellular homeostasis, mitochondrial function and regulating the anti-inflammatory response in adult cells [2,28,29]. Therefore,

this important TF is essential for different functions, which underlines the need for effective regulation of its activity across diverse mechanisms in tissues and cells.

The mechanisms that regulate NR4A2 activity include its genetic expression, post-transcriptional, translational, and post-translational processes such as ubiquitination, phosphorylation, and SUMOylation. The last three mechanisms mentioned have been revised by other authors [30,31]. In addition, *NR4A2* expression and localization have been described in both the cytoplasm and nucleus, changes in their cellular localization or heterodimerization with specific proteins would also make it possible to control their stability and thus their activity [31]. In this review we describe the *NR4A2* gene and the molecular mechanisms that regulate the transcription and post-transcriptional processes of the gene encoding NR4A2 TF.

4. *NR4A2* Gene Expression

NR4A2 TF is encoded by an immediate early gene (IEG), called *NR4A2*. This gene is also known as *NURR1*, *NOT*, *HZF-3*, *TINUR*, and *RNR1*. It is important to note that the expression of IEGs occurs rapidly (in minutes) and is transient. Furthermore, IEGs respond to a variety of extrinsic and intrinsic stimuli in diverse cell types, suggesting a general response mechanism [32,33]. Many IEGs encode TFs and DNA-binding proteins that regulate the expression of target genes, which are referred to as late response genes [34]. In the central nervous system (CNS), IEG expression plays an important role in various processes, including brain development, learning, neuronal plasticity, memory formation, and response to drugs abuse [33,35]. The *NR4A2* IEG is particularly involved in all these processes.

Therefore, in response to cellular stimulus, the *NR4A2* gene is expressed ubiquitously with rapid transcription. However, dysregulated expression of this IEG has been associated with the understanding of molecular mechanisms and therapeutic targets in neurological, psychiatric, and metabolic disorders [36,37].

Notably, gene expression mapping in 40 different normal human tissues identified *NR4A2* messenger ribonucleic acid (mRNA) expression (GTEX project; HPA project) [38].

Accordingly, *NR4A2* mRNA expression has been documented in a range of tissues across diverse species. For example, the Human Protein Atlas version 23 [38] has reported *NR4A2* gene expression in mammals, including human, pig and mouse. In particular, *NR4A2* mRNA expression in human tissues, such as the adrenal gland, ovary and bone marrow, is considered enhanced by at least four-fold relative to the average expression levels observed in other tissues, regions, and cell types. Additionally, its expression has been observed in human tissues associated with metabolic functions, including the pancreas and liver, as well as in cardiac muscle tissue. However, mRNA expression of this gene in the human, pig, and mouse brain is considered to be of low regional specificity (normalized protein-transcripts per million = nTPM $\geq$ 1 in at least one tissue/region/cell type but not elevated in any tissue/region/cell type) [38–40].

Notably, the distribution of *NR4A2* mRNA expression in different brain regions of three mammalian species (human, pig, and mouse) differed when subregions were analyzed (Figure 2). For example, *NR4A2* mRNA expression in the cerebral cortex is the highest in the human brain and its protein expression is high in neuronal cells and glia, as well as medium in endothelial cells in that brain region. The second brain region with the highest expression of *NR4A2* mRNA is the cerebellum, which also exhibits a high expression of the protein, particularly in the cells of the molecular layer. Also, the protein is expressed at a medium level in Purkinje and granular layer cells. Thus, the mRNA expression of human CNS shows the following order: cerebral cortex > cerebellum > hippocampal

formation > midbrain > choroid plexus > white matter > basal ganglia > medulla oblongata > hypothalamus > pons > thalamus > amygdala > spinal cord [38,39].

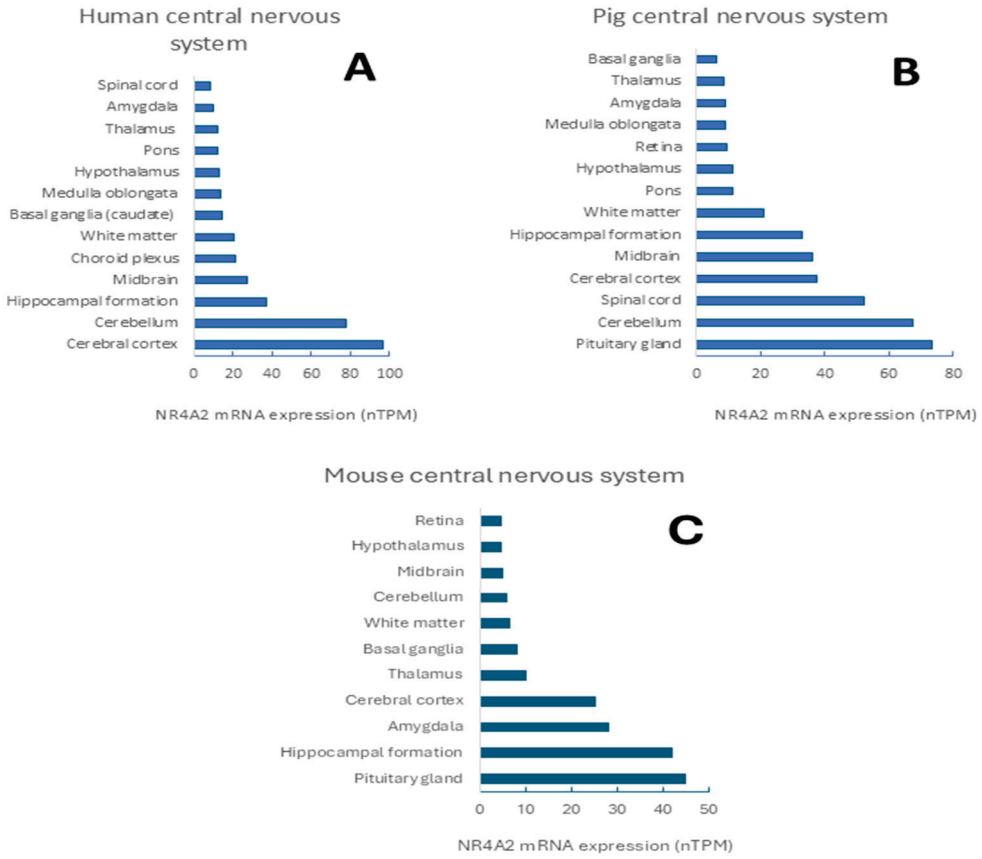


Figure 2. Regional variation in *NR4A2* mRNA expression in the central nervous system of human (**A**), pig (**B**) and mouse (**C**). The reported data were obtained from the Human Protein Atlas (HPA) based on an RNA-sequencing (RNA-seq) dataset for the brains of human, pig and mouse. Data are based on normalized expression (nTPM) values according to HPA RNA-seq brain gene data, which are available on the open-access resource http://v20.proteinatlas.org/humanproteome/brain (accessed on 7 August 2025) [38]. nTPM = normalized protein-coding transcripts per million.

The mRNA expression pattern of *NR4A2* mRNA for the pig CNS is as follows: pituitary gland > cerebellum > spinal cord > cerebral cortex > midbrain > hippocampal formation > white matter > pons > hypothalamus > retina > medulla oblongata > amygdala > thalamus > basal ganglia. In the case of the mouse CNS, the following pattern is observed: pituitary gland > hippocampal formation >amygdala > cerebral cortex > thalamus > basal ganglia > white matter > cerebellum > midbrain > hypothalamus > retina [38].

5. Expression Regulation *NR4A2*

Transcription is the process by which information from a specific DNA segment is copied into an RNA molecule. This represents the initial step of gene expression and plays a crucial role in determining both timing and the mechanism by which functional products, such as proteins and some RNAs, are produced [41].

Transcription involved a complex cascade of events, orchestrated by a multitude of molecular players that form interconnected regulatory networks. These events range from specific DNA-protein interactions to the recruitment and assembly of nucleoprotein complex. Since most signaling pathways converge on the transcriptional machinery, transcriptional dysregulation has been implicated in a variety of human diseases [42].

The regulation of transcription is multifactorial, involving cis-regulatory elements (e.g., promotor region) and trans-acting factors (e.g., transcription factors, co-regulators and chromatin-modifying enzymes). Additionally, epigenetics mechanisms -such as DNA methylation, histone modification, and non-coding RNA including MicroRNA (miRNAs)-, play essential role in modulating gene expression without altering the underlying DNA sequence [43].

5.1. NR4A2 Gene and Cis Regulatory Elements (Promoter)

5.1.1. *NR4A2* Gene

In humans, this TF is encoded by the *NR4A2* gene (also known as *NURR1*), located on chromosome 2q24.1 [44,45]. This gene exhibits high homologous with its murine and porcine orthologs [46,47]. Structurally, *NR4A2* consists of a promoter region, eight exons, and seven introns (Figure 3). The open reading frame spans from exon 3 to the 5′-end of exon 8, encompassing 1794 base pairs, and encodes a 598 amino acid with a molecular weight of approximately 66 kDa [45,48]. The translation initiation site is located at the start of exon 3, and the stop codon is situated at the 5′ end of exon 8. The 5′-untranslated region (UTR) encompasses the initial two exons. The 3′UTR at the 3′ end of exon 8 contains ATTA repeats [47]. These UTR sequences are essential for stabilizing mRNA and giving it a short half-life, a characteristic of IEG [49].

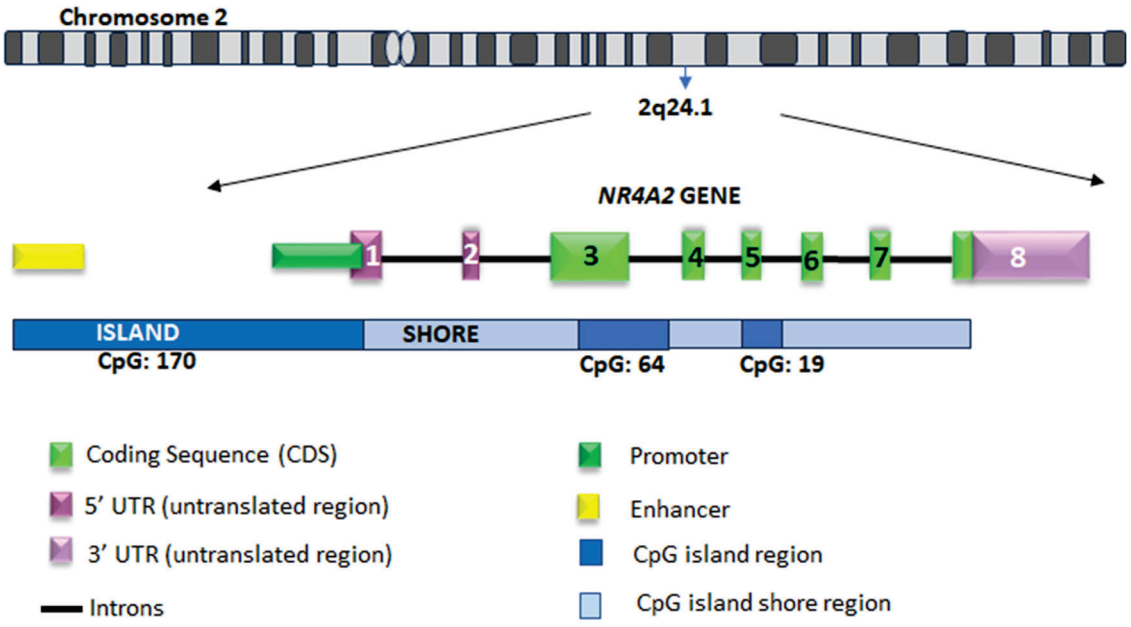


Figure 3. *NR4A2* gene representation. Gene is located on chromosome 2, band q24.1. It consists of 8 exons and 7 introns. The *NR4A2* gene contains different CpG islands that extend through its regulatory regions (promoter and enhancer), as well as in the body of the gene [50,51]. The numbers represent exons 1 to 8.

5.1.2. Promoter Region

The *NR4A2* promoter region exhibits a high degree of sequence conservation among humans, mice, and pigs [47]. It is located upstream of the transcription start site and characterized by a high concentration of CpG dinucleotides [46]. Specifically, the *NR4A2* promoter contains five CpG islands, while the first exon contains one additional island. Furthermore, the gene body harbors a differential methylation region (DMR) between exon 2 to 5, where two CpG islands overlap [47,52,53].

Among the transcriptional regulatory elements of the *NR4A2* promoter, the cAMP response elements (CREs) are particularly relevant, as they serve as binding sites for the

CRE binding protein (CREB). These sequence in the *NR4A2* promoter region are critical for memory and learning processes, particularly in hippocampus through the CREB signaling pathway [13]. In addition, these sequences have been associated with the expression of neuroprotective genes, such as *NR4A2* gene, which play key role in preserving neuronal integrity following stress or injury [54].

In the mouse, six CRE sites have been identified in the *NR4A2* promoter region. Three of these are distal CRE half sites (sequences located between −3 Kpb to −500 bp from the transcription start site, TGACG/CGTCA). In the proximal position (−500 bp to 300 bp), there is one full CRE site conserved between species (TGACGTCA/TGACGTCA) and two half CRE sites. These three proximal CRE sites have a TATA box within 300 bp and together are called the CRE/TATA promoter region. These regulatory sequences are important in activity-dependent gene regulation, particularly in response to neuronal activity and cAMP signaling. A notable example is the involvement of these sequences in *NR4A2* gene expression in neurons. The presence of these elements allows preferential transcription of the *NR4A2* gene when neurons are stimulated by depolarization and cAMP [55].

In the human *NR4A2* promoter region, consensus sequences for the binding of both Nuclear Factor Kappa B (NF-κB) (−585/−576) and CREB (−171/−163) have been identified. Furthermore, CREB and NF-κB consensus sites exhibit high conservation within the *NR4A2* promoter region of both human and mouse. This finding suggests that these binding sites play a critical functional role. This is supported by their very high evolutionary conservation [56].

Additionally, the promoter also contains response elements for transcription factors such as GATA1, SRY, MZFI, Sox5 and CArG-like [47].

5.1.3. *NR4A2* Genetic Variants

A genetic variant is a change in the DNA sequence. There are different types of genetics variants, such as single nucleotide variants (SNVs), and structural changes [57,58]. SNV are the most common variants and have been identified as risk factor for disease [58,59]. The biological effects of variants genetics depend on whether they are located in coding or non-coding regions. This may influence changes in the structure of the encoded proteins, post-transcriptional modifications, or changes in gene expression [59,60]. In addition, the context of these genetic variants is critical for identifying their effects or predicting their risk [61]. Therefore, the effect of a variant also depends on genetic interactions (epistasis), tissue and developmental stage-specific effects, as well as environmental influences [61,62]. There is increasing evidence that environmental components trigger responses that are modulated by genetic variants, especially during embryonic development [63].

In this respect, pathogenic variants in the *NR4A2* gene have been identified as a risk factor for alterations in the development of the nervous system [64]. These variants are associated with mechanisms of *NR4A2* haploinsufficiency, leading to a reduction in NR4A2 expression [65]. Similarly, genetic variants associated with familial Parkinson's disease have been identified to reduce *NR4A2* gene expression [66].

Furthermore, *NR4A2* genetic variants have been implicated as a risk factor for several neurological and psychiatric disorders, including Parkinson's disease, depression, schizophrenia and substance abuse [67–70].

5.2. Transcription Factors Regulating NR4A2 Gene Expression

CREB and NF-κB are among the most important TFs regulating *NR4A2* gene expression (Figure 4), highlighting the importance of these TFs in orchestrating cellular responses to different stimuli. The binding of CREB and NF-κB to the *NR4A2* promoter

region is not constant, but both TFs regulate *NR4A2* expression in a cell-, tissue- and time-specific manner [27,71].

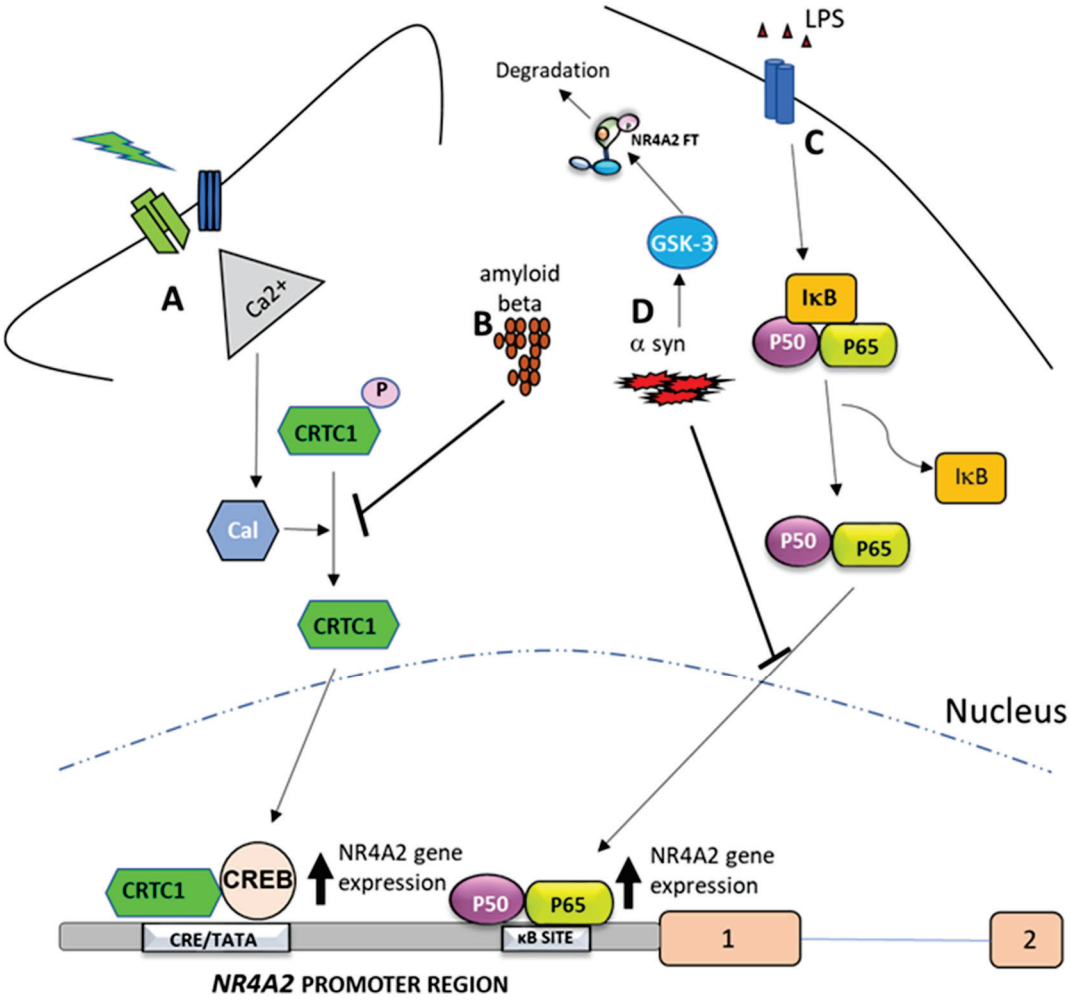


Figure 4. CREB and NFκB are transcription factors that regulate NR4A2 gene expression. (A) Activity-dependent induction of *NR4A2* gene expression in hippocampal neurons depends on Ca^{2+} influx through iGluRs and requires the CREB-CRTC1 signaling pathway. Neuronal activity induces CRTC1/CREB-dependent transcription through a mechanism involving the CRTC1 dephosphorylation, nuclear translocation, and CREB binding, preferentially into CRE/TATA-containing promoters [14,72]. (B) The CRTC1-dependent regulation of *NR4A2* gene expression is suppressed by the presence of amyloid beta. This mechanism is mediated by a reduction in calcium influx and disruption of CRTC1 dephosphorylation [72,73]. (C) TLR4 activation using LPS promotes NF-κB activation in nuclear localization, followed by the induction of *NR4A2* gene expression [56,74]. (D) α-synuclein inhibits NF-κB activity through the NF-κB related nuclear pathway. This reduces the binding of NF-κB to the *NR4A2* promoter, thereby affecting *NR4A2* gene expression. Moreover, GSK3 is promoted to degrade the NR4A2 protein by α-synuclein [75–77]. CREB, cyclic AMP response element-binding protein; (p50, p65), NF-κB, nuclear factor kappa B; α-syn, alpha synuclein; numbers 1 and 2, exons; Cal, calcineurin; LPS, lipopolysaccharides; IκB, inhibitory protein. CRTC1, CREB-regulated transcription co-activators 1; GSK3, Glycogen synthase kinase 3; CRE/TATA, sequences in the regulatory region that contain both CRE and TATA response elements.

CREB is a TF that is activated by various stimuli, including cAMP and other signaling pathways or stimuli such as neuronal activity. The binding of CREB to the *NR4A2* promoter region activates its expression, particularly in areas involved in synaptic plasticity and memory, such as the hippocampus. This process is crucial for memory and learning mechanisms (Figure 4A) [13,72]. Furthermore, in the context of *NR4A2*, CREB has been

identified as a regulatory factor for *NR4A2* gene expression in response to stimuli such as stress and hypoxia [54].

CREB regulated transcription co-activator 1 (CRTC1), is a transcriptional co-activator that enhances CREB-mediated transcription by interacting with the bZIP domain of CREB. It activates transcription through both consensus and variant CRE sites and increases CREB binding to RNA polymerase II [78]. Neuronal activity has been shown to induce CRTC1/CREB-dependent transcription through a mechanism involving the co-activator CRTC1, its dephosphorylation, nuclear translocation and binding to CREB preferentially into proximal CRE/TATA-containing promoters [55,72,73]. Thus, depolarization and cAMP signals induce the preferential transcription of activity-dependent genes containing promoters with proximal CRE/TATA sequences, such as *NR4A2* [55]. The binding of CRTC1 to CREB/CBP in the proximal CRE/TATA-containing *NR4A2* promoter region is an important step for robust activity-dependent *NR4A2* gene transcription in neurons. This mechanism has been involved in NR4A2-mediated glutamatergic synaptic plasticity in the hippocampus (Figure 4A) [14,72].

Notably, the CRTC1-dependent regulation of *NR4A2* gene expression is suppressed by the presence of amyloid beta in an animal model of Alzheimer's disease [73]. This mechanism is mediated by a reduction in calcium influx and disruption of CRTC1 dephosphorylation, a process that is required for the activation of *NR4A2* gene expression (Figure 4B) [72]. These findings suggest that CRTC1 plays a key role in coupling synaptic activity to gene transcription required for hippocampal-dependent memory, and that beta-amyloid may alter cognition by affecting CRTC1 function on *NR4A2* expression [73].

On the other hand, NF-κB and NR4A2 are involved in the regulation of inflammation and immune responses [2,79]. Several studies suggest that NR4A2 and NF-κB are involved in a feedback mechanism [27,56,74,80–86], in which NF-κB regulates *NR4A2* expression by binding to the *NR4A2* promoter (Figure 4C) [56,74]. Conversely, NR4A2 FT modulates NF-κB activity through direct binding to response elements at the promoters of NF-κB target genes or through co-regulators of NF-κB activity (Figure 1C) [82–85]. Depending on the specific context and the association of NR4A2 with co-repressors or co-activators, this can lead to either increased or decreased expression of NF-κB target genes such as *NR4A2* [86]. Another mechanism by which NR4A2 regulates NF-κB is through its interaction with NF-kB/p65 and inhibition of its nuclear translocation [27]. Consequently, NR4A2 plays a critical role in the TLR4-NF-κB signaling pathway, which is activated by α-synuclein in BV-2 cells, a type of glial cell [87].

On the other hand, α-synuclein is an abundant pre-synaptic protein that can misfold and polymerize to form toxic fibrils that coalesce to form pathological inclusions such as Lewy bodies and Lewy neurites [88]. These pathological inclusions are present in neurodegenerative diseases such as Parkinson's disease. In this context, the relationship between NR4A2 and α-synuclein has been reported [29,89]. During normal human aging, *NR4A2* and α-synuclein follow an inverse expression relationship in dopaminergic neurons. Thus, α-synuclein expression increases in the substantia nigra [90], while *NR4A2* expression levels progressively decrease with age [91]. Furthermore, it has been reported that α-synuclein inhibits *NR4A2* expression through mechanisms involving the NFκB TF (Figure 4D) [75]. Specifically, studies show that overexpression of α-synuclein can attenuate the activity or reduce the protein levels of NF-κB and reduce the binding of NF-κB to the *NR4A2* promoter [75,76].

In addition, glycogen synthase kinase 3 (GSK-3), which can phosphorylate NR4A2, is also activated by α-synuclein. This post-translational modification can lead to the degradation of FT NR4A2 via the ubiquitin-proteasome pathway [77]. Additionally, GSK3β regulates NF-κB signaling. Specifically, it modulates the activity of IKKγ/NEMO, a critical

component of the NF-κB activation complex. GSK3β phosphorylates NEMO, affecting its stability and interactions with other proteins involved in NF-κB activation, ultimately affecting the overall activity of the signaling pathway [92]. These data suggest that α-synuclein may disrupt the NF-κB pathway, leading to reduced *NR4A2* gene expression, such as degradation of FT NR4A2 by GSK-3 (Figure 4D).

A potential feedback loop has been identified between the NR4A2 and α-synuclein. This loop suggests a reciprocal relationship in which α-synuclein can affect *NR4A2* expression. Conversely, NR4A2 activity has been shown to affect α-synuclein levels [93]. Activation of the NR4A2 TF by an agonist has been shown to attenuate altered α-synuclein gene expression [94]. Taken together, these data show that NR4A2 has a dual role in regulating the inflammatory response and α-synuclein levels. This establishes NR4A2 as a critical neuroprotective factor whose dysfunction contributes to the progression of Parkinson's disease (PD) [95].

It is important to note that the initiation of *NR4A2* gene transcription occurs with TFs and co-regulators, described above, bind to specific sequences within the *NR4A2* gene regulatory regions such as promoters and enhancers to control NR4A2 gene expression (Figure 5A).

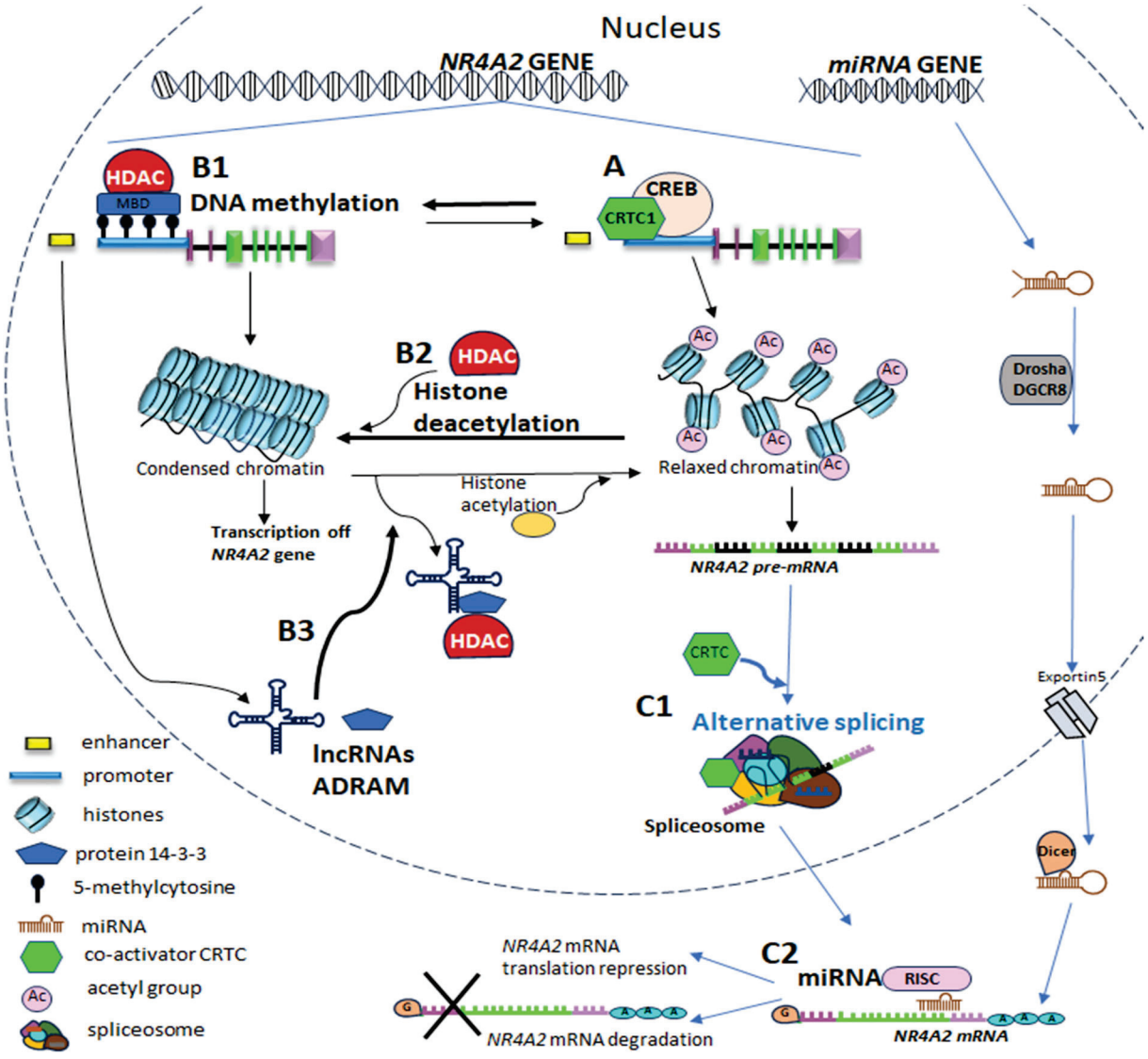


Figure 5. The *NR4A2* gene expression is regulated by transcriptional, epigenetic, and post-transcriptional mechanisms. (A) Transcriptional mechanisms. Regulatory sequences, such as promoters and enhancers, as well as transcription factors and co-regulators, control transcription of the *NR4A2*

gene. These bind to specific sequences within the regulatory regions of the *NR4A2* gene. (B) Epigenetic mechanisms are a form of gene expression regulation that occurs without any change to the DNA sequence. (B1) DNA methylation in specific regions, such as the promoter, facilitates the binding of DNA to proteins like MBD (methyl-CpG binding domain) and HDAC. This results in the condensation of chromosomes, leading to a reduction in gene expression. In contrast, DNA methylation in the gene body can increase expression or influence alternative splicing. (B2) HDAC3, a histone deacetylase, reduces *NR4A2* expression by deacetylating histones in the *NR4A2* promoter region [96–102]. (B3) Non-coding RNAs, such as *ADRAM* LncRNA, can regulate enzymes or proteins that modify chromatin condensation. *ADRAM* acts in conjunction with a chaperone to move HDAC3 from the promoter region of *NR4A2* [103]. (C) Post-transcriptional mechanisms are implicated at the level of the mRNA molecule. (C1) The co-activator CRTC1 is involved in the alternative splicing of *NR4A2* mRNA [104]. (C2) Several microRNAs (miRNA) have been identified that affect in *NR4A2* expression by either inhibiting translation or contributing to *NR4A2* mRNA degradation [105–109]. Various proteins and protein complexes are involved in microRNA regulation, including Drosha, DGCR8, Exportin5, Dicer, and RISC. MBD, methyl-CpG binding domain, HDCA 3, Histone deacetylase 3; CRTC, CREB-regulated transcription co-activators 1; LncRNA, long non-coding RNA; ADRAM, activity-dependent lncRNA associated with memory; CREB, cyclic AMP response element-binding protein.

5.3. Epigenetic Mechanisms in NRA42 Gene Expression Regulation

5.3.1. DNA Methylation

The process of DNA methylation consists of the covalent addition of a methyl group at the 5′ positions of DNA cytosine, resulting in the formation of 5-methylcytosine (5mC).

This mechanism occurs mainly in genomic regions rich in CpG dinucleotides, commonly referred to as CpG islands. These regions are found primarily within gene promoters in mammals, where methylation is associated with transcriptional inactivation (Figure 5(B1)) [110]. Nevertheless, DNA methylation in gene bodies is generally associated with gene expression and can influence transcription, splicing and other aspects of gene expression. The process of DNA methylation of exons plays a regulatory role in the alternative cleavage and polyadenylation of mRNAs, and this is facilitated by a protein known as CCCTC-binding factor (CTCF), which is a methylation-sensitive insulator protein. Of the various epigenetic modifications, 5mC has been the subject of the most extensive research.

In this particular context, the DMR of *NR4A2* gene has shown specific differences based on the brain region. DMR is more methylated in the hippocampus (71.6%) than in the hypothalamus (39.9%) [53]. The hippocampus is a brain region that requires constant modifications in gene expression and robust alternative splicing in order to satisfy its synaptic activity, especially in cognition [111]. These findings suggest that methylation plays a significant role in regulating *NR4A2* alternative splicing and that gene expression *NR4A2* may be influenced by region-specific epigenetic modifications. This regulation occurs through multiple mechanisms, including the direct influence of methylation on splicing factor binding and the indirect influence through altering chromatin structure, which in turn affects splicing [112].

Accordingly, it has been reported in animal models, that exposure to dieldrin, a pesticide, during development can result in differential sex-specific body methylation of the *NR4A2* gene. Specifically, dieldrin-induced *NR4A2* hypomethylation was observed exclusively in female mice and was accompanied by increased *NR4A2* expression [113].

In human studies, the methylation profiles in peripheral blood leukocytes were compared between experienced meditators with over 10 years of experience and individuals with no meditation experience [52]. The study showed a reduction in *NR4A2* gene methylation levels in experienced meditators compared to controls [52].

This evidence supports the hypothesis that methylation can influence the tissue- and time-dependent mechanisms of NR4A2 transcription and splicing.

5.3.2. *NR4A2* Genetic Expression Through Histone Modification

Post-translational modifications of histones affect gene transcription and cause changes in chromatin structure. One of the most important and widely studied mechanisms of histone modification is histone acetylation. This process involves the addition of an acetyl group to a lysine residue at the amino terminus of a histone. It primarily affects chromatin relaxation [114]. The addition of an acetyl group neutralizes the positive charge of lysine, reducing the binding between histones and DNA. This results in a more open and accessible structure for the transcriptional machinery, which in turns leads to transcriptional activation. In contrast, histone deacetylases (HDAC) are a class of enzymes that remove acetyl groups from lysine residues on histones, thereby reducing gene expression (Figure 5(B2)) [115].

The regulation of transcription is indeed crucial for long-term memory formation, but not for short-term memory. Short-term memory can be established by local synaptic changes, like phosphorylation or addition of receptors, while long-term memory requires transcription and translation of new proteins, which are needed to grow new synaptic connections [116,117]. Long-term memory formation requires at least two waves of transcription after learning. This suggests that NR4A2 FT may contribute to the second of these waves of gene expression [118]. Thus, the expression of the IEG and NR4A2 TF plays a role in synaptic plasticity and memory formation by influencing the expression or regulation of other genes or pathways, such as *BDNF* and NF-κB [72]. Therefore, it is important to note that NR4A2 is crucial for long-term memory consolidation. In contrast, the initial encoding and retention of short-term memories seem less affected by NR4A2 activity [96]. In this regard, the regulation of *NR4A2* gene expression in memory-related processes has been emphasized particularly during learning, formation and consolidation of memories in brain regions such as the hippocampus, amygdala, nucleus accumbens, and cortex (insular and perirhinal) [97–99]. Thus, the mechanisms that regulate *NR4A2* gene expression are crucial for memory processes, particularly in memory formation in the hippocampus [13].

NR4A2 expression is regulated by several mechanisms, including: (i) regulation by the CREB signaling pathway, a key pathway involved in memory formation [72]; (ii) the epigenetic mechanism of histone modification, such as acetylation. In this mechanism, histone deacetylases (HDACs), particularly HDAC3, remove histone acetyl groups from histones (deacetylation) in the *NR4A2* promoter region, thereby restricting *NR4A2* gene expression (Figure 5(B2)) [100].

This epigenetic mechanism of *NR4A2* gene expression regulation has been demonstrated in various studies, which have shown that: (i) *NR4A2* expression is promoted by memory-related processes accompanied by reduced HDAC3 occupancy at IEG promoters [98,101]; (ii) inhibition of HDAC3 activity (either pharmacologically or genetically) restores or continues cognitive functions related to memory processes and increases *NR4A2* expression [96,102,119]; (iii) decreased or altered expression (siRNA, shRNA knockdown) or activity (antagonist) of NR4A2 does not allow cognitive restoration associated with HDAC3 inhibition [102]. Taken together, these data show that HDCA3 decreases *NR4A2* expression, affecting long-term memory processes. Therefore, HDAC3 is a potent negative regulator of memory [120].

In particular, *NR4A2* regulation by the HDAC3 deacetylase domain has a selective role in specific brain regions that underlie long-term memory formation. This regulation influences different types of memory processes [98,121]. Thus, researchers have identified that HDCA3 negativity regulates *NR4A2* expression that impacts hippocampus-dependent long-term memories, including aversive [98], incidental [102] and reward [101,119] memories. These processes occur in brain regions such as the hippocampus, amygdala and nucleus accumbens, which are involved in learning, memory formation, consolidation and related behaviors.

Epigenetic regulation of *NR4A2* has been implicated in the mechanisms underlying age-related cognitive decline, and further research is needed to fully understand its role in this process. Kapris et al. (2019) [100] showed that aged rats exhibit greater variability in their cognitive abilities compared to young rats. Two groups were identified within the aged rats: those that were cognitively preserved (CP) and cognitive impaired (CI). CP rats performed similarly to young rats, while CI rats perform significantly worse. The *NR4A2* gene was induced during learning in young and CP rats, but not in CI rats. The study shows that HDAC3 limits *NR4A2* expression in the hippocampus of older rats, contributing to memory impairment. But removing HDAC3 restored *NR4A2* expression and improved memory in aged rodents. Additionally, overexpressing *NR4A2* in the hippocampus improves memory in hippocampal-dependent tasks, but not in hippocampal-independent ones. These findings suggest that memory deficits in cognitively impaired aged rats are associated with changes in genetic and epigenetic regulation of *NR4A2*. These results indicate that the epigenetic regulation of *NR4A2* by HDAC3 plays a significant role in age-related cognitive decline [100]. These findings suggest that increasing the expression or activity of the NR4A2 through therapeutic strategies may be effective in improving memory in the elderly [96].

5.3.3. Epigenetic Regulation of *NR4A2* Gene Expression by Non-Coding RNA

Non-coding RNAs (ncRNAs) are functional RNA molecules that do not code for proteins but play a crucial role in regulating gene expression. They can be divided into infrastructural ncRNAs and regulatory ncRNAs. Constitutively expressed infrastructural ncRNAs include ribosomal, transfer, and small nuclear RNAs. Regulatory ncRNAs influence gene expression through a variety of mechanisms, including transcriptional, epigenetic and post-transcriptional regulatory processes. Among the regulatory ncRNAs are microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs) and long non-coding RNAs (lncRNAs) [122].

The ncRNAs can mediate the interaction between the epigenetic machinery and DNA by sequestering chromatin modifiers, guiding them to specific genomic locations or serving as molecular scaffolds to coordinate the binding of epigenetic complexes [123].

In particular, lncRNA are non-coding RNA molecules that are more than 200 nucleotides long. They are characterized by unique and specific expression patterns among tissues, as well as by the fact that they do not conserve sequence between species. LncRNAs regulate gene expression depending on their subcellular location (nucleus or cytoplasm) and are categorized according to their genomic origin and orientation. Enhancer-associated lncRNAs (elncRNA) are transcribed from enhancer regions of DNA [124].

Researchers have discovered that lncRNAs play a significant role in regulating gene expression at the epigenetic level. LncRNAs can interact with HDACs directly or indirectly through other proteins, forming complexes that influence gene expression. LncRNAs can influence the acetylation state of histones by recruiting HATs or HDACs to specific locations in the genome, thereby modulating gene transcription [124].

In particular, the LncRNA called ADRAM (activity-dependent lncRNA associated with memory) regulates the expression of *NR4A2* IEG in the context of neuronal activity and fear extinction learning [103]. Wei et al. (2022) [103] identified experience-induced *ADRAM* lncRNA that acts as a scaffold and a combinatorial guide. This lncRNA recruits the brain-enriched chaperone protein 14-3-3 to the promoter of the memory-associated *NR4A2* IEG, being a required process for fear extinction memory. *ADRAM* has been identified as an enhancer-derived RNA (eRNA). This lncRNA is upregulated in response to fear extinction learning and regulates *NR4A2* expression by directly interacting with its promoter. *ADRAM* guides 14-3-3 to the *NR4A2* promoter, which results in the time-dependent removal of HDAC3 and HDAC4. This is followed by the coordinated deposition of CBP

(Figure 5(B3)) [103]. This increases *NR4A2* gene expression. The researchers emphasize that ADRAM is also expressed in other brain regions, including the hippocampus, cerebellum, and somatosensory cortex. This suggests that the regulation of *NR4A2* expression in other memory processes may be influenced by this eRNA [103].

5.4. Post-Transcriptional Mechanisms in NRA42 Gene Expression Regulation

Post-transcriptional mechanisms are crucial for regulating gene expression after the pre-RNA transcription but before mRNA translation into protein. These mechanisms fine-tune gene expression by affecting various aspects of mRNA processing, stability, localization, and translation efficiency.

5.4.1. Alternative Splicing of *NR4A2* Pre-RNA and Its Regulation by CRTC

Alternative splicing (AS) is a process in which a single gene can produce multiple mRNA transcripts, leading to the creation of different protein isoforms. In this process, a pre-mRNA is processed in more than one way This results in the removal of different introns or the inclusion of different combinations of exons, and the consequent production of mature mRNAs with different sequences [125]. This mechanism allows for a single gene to encode for a variety of proteins, each of which may have a different function. AS can also affect the stability and other features of the mRNA. The majority of human genes undergo AS, and defects in AS are associated with disease [125,126].

On the other hand, different alternative splicing variants of the *NR4A2* mRNA have been identified in different species, such as humans, rodents and pigs [46–48,105,127]. Furthermore, at least 15 transcript variants of the *NR4A2* gene have been identified. Of these, 11 encode a protein and 4 encode nonsense mediated decay; however, their biological actions remain unclear [50]. These isoforms are generated by alternative splicing of exons 3, 5 and 7 [47,105,127]. In neurons, these isoforms have been observed to account for 20–35% of *NR4A2* transcripts. The resultant proteins show reduced transcriptional activity of the NR4A2 TF [127]. Most of the isoforms produced by the alternative splicing of *NR4A2* RNA can lead to the production of mRNA with different 3′UTRs lengths.

Alternative splicing is an important post-transcriptional mechanism that regulates *NR4A2* gene expression. This mechanism is influenced by CREB-regulated co-activators (CRTCs) [104].

In the brain, CRTCs act as sensors and integrators of hormonal, metabolic and neural signals, contributing to brain plasticity and communication between brain–body. This co-activator is both a potential genetic factor and a therapeutic target for psychiatric and neurodegenerative diseases [126,128]. CRTCs act as signal-dependent co-activators that regulate gene expression in response to cAMP. Under basal conditions, CRTCs are phosphorylated and are retained in the cytoplasm by 14-3-3 proteins. Increases in cAMP lead to the dephosphorylation of CRTCs, allowing them to translocate to the nucleus and bind to CREB. This enables the transcriptional regulation of CRE-containing genes [21].

In addition to acting as co-activators in transcriptional activation, Amelio et al. (2009) [104] showed that CRTCs selectively control pre-mRNA splicing alternative. This was identified by: (1) cAMP and calcium stimulation increased the process of alternative splicing of *NR4A2* in differentiated neuronal cells (ND-PC12); (2) the use of shRNA directed against CRTC reduced the selection of excision sites in these cells; (3) in hepatocytes, activation of *NR4A2* transcription does not result in significant changes to alternative splicing. Thus, researchers demonstrated that CRTC co-activators mediate alternative splicing of the *NR4A2* gene in a specific cellular context (Figure 5(C1)).

Furthermore, mutations in the functional domains of CRTCs demonstrate that they have distinct domains for transcriptional activation and AS [104]. These co-activators can

induce AS without increasing transcriptional activation and vice versa. The alternative splicing of certain CREB target genes appears to be modulated by CRTCs via a conserved proline-rich domain. Despite lacking conserved RNA-binding domains, which are found in splicing factors, the CRTC splicing domain can act as a scaffold for assembling the protein complex involved in alternative splicing.

CRTCs are essential for regulating gene expression in response to environmental signals, demonstrating a dual regulatory function by acting in both transcriptional activation and pre-mRNA splicing site selection. However, these functions depend on the cell type or context of regulatory regions.

5.4.2. *NR4A2* Gene Expression Through MicroRNAs

MicroRNAs (miRNAs) are non-coding RNA molecules comprising 18 to 25 nucleotides that play an important role in post-transcriptional gene expression [129]. In most cases, miRNAs interact with the 3′UTR of target mRNAs in order to induce mRNA degradation and translational repression (Figure 5(C2)) [130]. In this regard, several reports have described the transcriptional regulation of *NR4A2* by miRNA in DAergic neurons, cell death and cancer [105–109].

In embryonic pluripotent cells, miR-132 miRNA has been demonstrated to negatively regulate the differentiation of DAergic neurons by directly suppressing *NR4A2* expression. In this regard, the overexpression of miR-132 in a neurodegeneration model has been observed to result in a decrease in the expression levels of both *NR4A2* and brain-derived neurotrophic factor called BDNF in the midbrain [106,131]. Furthermore, increased miR-132 expression and diminished *NR4A2* mRNA levels were identified in peripheral blood cells of PD patients as compared to control group, as well as a negative correlation between these two RNAs [132].

On the other hand, studies have identified miR-145-5p miRNA as a regulator of *NR4A2* in microglia in an animal model of hypoxia and ischemia. In this animal model, a reduction in *NR4A2* expression resulted in neuronal death via TNF-α [19,133].

Moreover, different miRNAs have been identified that directly and negatively regulate *NR4A2* expression in cancer [108,109]. In particular, it has been demonstrated that miRNA-34c-5p directly and negatively regulates *NR4A2*. It is part of a possible feedback mechanism involving p53, miRNA-34 and *NR4A2* [108].

6. Dysregulation of the Expression of the *NR4A2* Gene in Animal Models and in Clinical Studies of Neurological and Psychiatric Disorders

The level of *NR4A2* gene expression is significantly associated with age-dependent neurodegeneration and cognitive function in animal models of PD, schizophrenia (SZ), AD, and attention deficit disorder [134–137]. Furthermore, clinical studies have identified *NR4A2* as a potential contributor to a range of dopamine neuron-related pathologies, including PD, cognitive deficits, developmental disorders, SZ, and AD [13,14,29,64,138]. In this manner, alterations in the expression of the *NR4A2* gene have been associated with various neurological and psychiatric diseases. However, altered *NR4A2* mRNA expression seems to occur in a disease-specific manner, suggesting that specific mechanisms are involved in regulating *NR4A2* expression and activity in each disease.

6.1. Parkinson's Disease

Parkinson's disease (PD) represents the second most prevalent neurodegenerative disease, following AD, and typically affects people over the age of 60. It is characterized by progressive loss of DAergic neurons in the substantia nigra, an important brain region of the nigrostriatal DAergic pathway [139]. The disease is characterized by both motor

and non-motor symptoms [140]. A number of preclinical and clinical studies suggest that the *NR4A2* gene plays a significant role contributing to the pathogenesis of PD. In this context, several authors have suggested *NR4A2* regulation as a biomarker and potential PD therapeutic target.

In animal models, heterozygous *NR4A2* (*NURR1* (+/−)) mice showed a range of reductions in *NR4A2* gene and protein expression across different brain regions. In particular, a 39% decrease was observed in the substantia nigra pars compacta and the ventral tegmental area [141]. As a result, *NR4A2* (*NURR1* (+/−)) mice showed an increase in susceptibility to age-related dysfunction [142] and using MPTP neurotoxin [143], which is used as an animal model of PD.

A reduction in NR4A2 expression was observed in post-mortem samples of patients with PD, specifically in the substantia nigra pars compacta [144], in individuals with α-synuclein aggregates [145]. In this regard, a reduction in the level of expression of the *NR4A2* gene has also been observed in peripheral blood samples from patients with PD, which has been reported in several populations [132,146–150]. Furthermore, this decreased expression was accompanied by elevated levels of inflammatory cytokines and miRNA that regulate the *NR4A2* gene in PD patients [132,149].

The mechanisms that have been identified for the deregulation of *NR4A2* gene expression include α-synuclein, which negatively regulates *NR4A2* expression via NF-κB [75], as well as DNA methylation [113], and miRNAs [132].

With regard to DNA methylation, Kochmanski et al. (2022) [151] performed a genome-wide analysis of DNA methylation in enriched neurons obtained from the post-mortem parietal cortex of patients with PD [151]. The parietal cortex was used because this brain region shows pathological alterations in the late stages of PD. This study identified hypermethylation in an exon just downstream of an exon-intron boundary within the body of the *NR4A2* gene in male PD patients, but not in female PD patients. This hypermethylation of *NR4A2* gene in men with PD suggests that *NR4A2* gene dysregulation plays a role in the pathogenesis of PD and suggests the possibility for sex-specific differences in the development of this disease. Gender-related differences in *NR4A2* gene DNA methylation have also been observed in laboratory animals administered with insecticide dieldrin, a substance associated with an increased risk of PD. The study also identified specific differential methylation of the *NR4A2* gene in females [113] after exposure to the environmental toxin dieldrin, suggesting a mechanism that links environmental factors to altered *NR4A2* gene expression. Taken together, these studies suggest that sex-specific epigenetic regulation of the *NR4A2* gene may play a role in the risk of idiopathic PD.

On the other hand, altered *NR4A2* gene expression has been proposed as a potential therapeutic target for PD. In this context, it has been shown that NR4A2 agonist can restore the transcriptional activity of NR4A2 as a transcription factor, as well as the expression levels of this gene in an animal model of PD [152]. In this sense, neuroprotective treatments for DAergic neurons, such as EGb761 (a standardized extract of *Ginkgo biloba* leaves), have demonstrated that restoring *NR4A2* expression levels could be part of a neuroprotective mechanism [153].

Although the functional role of *NR4A2* is well known, it is not surprising that it is also involved in functions outside those of dopamine. Indeed, recent findings indicate that *NR4A2* exerts significant functions in numerous brain regions, encompassing neuroprotection and cognition [54,97,134].

6.2. Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder that is the primary cause of dementia, which most individuals develop after the age of 65. It is characterized by a significant decline in cognitive abilities that results in an increased need for assistance with

daily activities [154]. Glutamatergic neuronal activity is compromised in AD due to synapse destruction and neuronal death, and this deficit can affect memory, cognition and behavior, including cortical and hippocampal processing. Glutamatergic neurons, which use glutamate as their primary neurotransmitter, are particularly vulnerable in the hippocampus due to their critical role in excitatory neurotransmission and synaptic plasticity [155]. On the other hand, the pathological accumulation of glutamate can induce neurotoxicity.

The relationship between NR4A2 and AD has been investigated in animal models of this disease. For example, in a mouse model of AD known as 5X-FAD, *NR4A2* is highly co-expressed with amyloid β (Aβ) in the early stages, particularly in the subiculum and the frontal cortex [156]. In control and 5X-FAD mice, *NR4A2* expression is high in neurons but not in glia. Specifically, *NR4A2* expression in glutamatergic Aβ-positive hippocampal neurons degenerates in an age-dependent manner in 5X-FAD mice [134]. It has also been shown that *NR4A2* expression is significantly reduced when Aβ is administered to neuronal cells [157]. In addition, pharmacological activation of NR4A2 reduced Aβ plaque deposition in the subiculum and significantly ameliorated AD-like pathology in 5X-FAD mice, including neuronal loss, microglial activation and impaired neurogenesis in the adult hippocampus [134]. Taken together, these data suggest that a reduction in *NR4A2*-expressing glutamatergic neurons in the hippocampal formation may be associated with AD and that NR4A2 activation may be a promising therapeutic strategy for the treatment of AD [134].

In post-mortem samples from patients diagnosed with AD, a reduction in *NR4A2* expression was observed in the hippocampus and in individuals with Aβ deposition [158]. In comparison to control individuals without AD, patients with AD also exhibited a downregulation of the *NR4A2* gene in blood samples [146].

The precise mechanisms underlying altered *NR4A2* expression in AD remain unclear. However, in animal models of cognition, particularly memory and learning, histone deacetylases have been shown to be part of the mechanism involved in changes in the expression levels of this gene [100]. Similarly, the administration of NR4A2 agonists has been shown to improve memory and learning in animal models [100,159].

6.3. Neurodevelopmental Disorders

Neurodevelopmental disorders (NDDs) are a group of health conditions characterized by abnormal development of CNS, leading to brain malfunction later in life. Such health conditions may also present neuropsychiatric problems or impairments in motor skills, learning, language or nonverbal communication [160]. NDDs impose a significant burden on individuals, their families, and society in general due to their chronic and severe nature [161]. NDDs comprise intellectual disability (ID), communication disorders, autism spectrum disorder (ASD), attention-deficit hyperactivity disorder (ADHD), neurodevelopmental motor disorders (including tic disorders), and specific learning disorders.

A meta-analysis by Du et al. (2023) [162] identified significant alterations in neurometabolites such as N-acetyl-aspartate (NAA), gamma-aminobutyric acid (GABA), and glutamate + glutamine (Glx), in individuals with autism spectrum disorder. These alterations suggest dysfunctions in key processes such as neurotransmission, synaptic plasticity, and neuronal metabolism [162]. While the study itself does not address NR4A2 directly, previous research has implicated it with the regulation of genes involved in synaptic plasticity and the expression of glutamatergic receptors. In particular, NR4A2 has been proposed to play a role in glutamatergic synaptic plasticity [72], which may influence synaptic mechanisms altered in the autism spectrum disorder. In this context, several studies conducted in animal models and in patients have identified the *NR4A2* gene as a factor associated with NDD. For example, the *NR4A2*-deficient male mouse was associated with ADHD-like phenotype which may serve as a reliable tool for studying the behav-

ioral phenotypes associated with ADHD and evaluating the clinical efficacy of potential therapeutic agents [135]. Furthermore, a prenatal stress model has implicated *NR4A2* expression as a mediator of the long-term effects of early life events. These changes, such as the dysregulation of *NR4A2* expression or the maintenance of *NR4A2* expression, may be associated with disease phenotypes [163] or resilience [164], respectively.

It should be noted that a number of genetic variants of NR4A2 have been linked to NDDs. Gabaldon-Albero et al. (2024) [64] conducted a recent systematic review of the literature [64] describing 26 patients with such variants who exhibit a heterogeneous neurodevelopmental phenotype. The phenotypic spectrum includes intellectual disability and/or developmental delay as the main features, as well as particular language impairment, dystonia or other movement disorders, and a high predisposition to neuropsychiatric disorders and/or epilepsy. These patients were also found to exhibit genetic heterogeneity in *NR4A2* variants ranging from single nucleotide variants (SNVs) to structural variants, such as copy number variants (CNVs) [65]. Among the SNVs, missense, frameshift, nonsense, and splicing variants have been identified. There do not appear to be any differences in the clinical presentation between structural variants CNVs and SNVs.

Studies using whole exome sequencing and functional validation have allowed us to understand the effects of *NR4A2* genetic variants on NDDs. Research has revealed that certain variants can lead to reduced gene expression or the production of a truncated protein. Basically, all of these variants lead to something called *NR4A2* haploinsufficiency [165], which is basically an alteration in its gene expression. Therefore, these variants are called pathogenic or likely pathogenic *NR4A2* variants. According to these data, the *NR4A2* gene could be considered a candidate gene for patients with suspected NDDs [165].

6.4. Schizophrenia

Schizophrenia (SZ) is a severe and chronic mental disorder, typically diagnosed in late adolescence. It led to significant disability and distress. According to the World Health Organization (WHO), it has a lifetime prevalence of ∼1%, and is considered one of the top ten causes of disability in developed countries. SZ is characterized by disturbances in thought, perception, and behavior that include hallucinations, delusions, paranoia, depression and cognitive impairments, among others [166]. Cognitive deficits are a core feature of schizophrenia, significantly contributing to the functional impairment associated with the disorder and remaining largely resistant to current treatments [167]. The development of these impairments, much like schizophrenia itself, arises from a complex interplay between genetic and environmental factors. It has been proposed that the expression of key genes must be linked to cognitive function in individuals with schizophrenia [168]. Alterations in gene expression can disrupt normal brain development and function, leading to dysregulation of neuronal signaling and synaptic dysfunction. These molecular changes may be associated with different neurotransmitters and neural circuits that are linked to cognitive deficits observed in schizophrenia [167,169], many of which are related to *NR4A2* gene expression.

In particular, *NR4A2* heterozygous (+/−), also known as *Nurr1* (+/−), mice have been proposed as an animal model to study some of the behavioral and molecular mechanisms underlying SZ [136,137]. These mice exhibit abnormal dopamine neurotransmission in the striatum and prefrontal cortex [137]. This DAergic imbalance-associated behavior has been linked to impaired emotional memory, increased spontaneous locomotor activity, heightened sensitivity to stress, and depression-like behavior [136,137]. Furthermore, male *NR4A2* (+/−) mice have been reported to have deficits in sensory gating using prepulse inhibition of the acoustic startle reflex [136], which is a common feature found in SZ patients [170].

In schizophrenia (SZ) patients, *NR4A2* expression in the dorsolateral prefrontal cortex (DLPFC) was found to be reduced in postmortem samples [171]. In addition, RNA-seq

data from postmortem DLPFC tissue of SZ patients and healthy controls found that *NR4A2* expression levels were significantly reduced in patients [138]. This reduction may contribute to understanding the mechanisms involved in SZ pathophysiology. It is important to emphasize that DLPFC is a key part of the brain that controls executive functions, such as attention, behavior, and impulse inhibition. In SZ patients, the DLPFC is less activated during tasks that assess working memory and cognitive control.

Furthermore, genetic variants in the *NR4A2* gene have been found to be associated with the cognitive alterations present in the SZ [69,172]. Thus, genetic risk variants in the promoter, enhancer and intronic regions may be among the mechanisms involved in the dysregulation of *NR4A2* gene expression observed in SZ. Genetic variants found in a linkage disequilibrium (LD) block, together with variants in the promoter, enhancer and intronic regions within the *NR4A2* gene, have been associated with sustained attention in patients with SZ [69]. In addition, reduced *NR4A2* gene expression was correlated with impaired working memory in patients homozygous for a genetic variant located in the promoter region [172]. Based on these findings, the *NR4A2* gene has been proposed as a potential biomarker for cognitive endophenotypes in SZ.

6.5. Major Depressive Disorder

Major depressive disorder (MDD) is a chronic illness that affects an individual's emotional, physical, and cognitive health [173]. MDD was the third leading cause of disease burden worldwide in 2018, and the WHO projects that it will become the leading cause by 2030 [174]. It is characterized by persistent depressed mood, anhedonia or loss of interest or pleasure in previously enjoyable activities, recurrent thoughts of death, feelings of guilt or worthlessness, lack of energy, poor concentration, changes in appetite, psychomotor slowing or restlessness, sleep problems, and other physical and cognitive symptoms [175]. Dysfunction of serotonin (5-HT) and dopamine signaling has been implicated in the etiology of MDD [176]. The dorsal raphe nucleus (DR) is a major source of 5-HT in the brain, and its dysfunction in 5-HT-related gene expression plays a significant role in the development of MDD [177]. It is a multifactorial and probably polygenic disorder. In this context, a significant decrease in *NR4A2* mRNA in DR has been reported in MDD patients [178]. Furthermore, genetic variants of *NR4A2* have been identified with susceptibility to MDD in the Caucasian population [68].

In particular, there are several preclinical models for studying depression in which the role of *NR4A2*, also known as *Nurr1*, has been described. One of the most widely used models is the forced swim test, a well-known and established preclinical animal model of depression-like behavior induced by an acute stressful event, since stress is an important factor in inducing depression. In this regard, it has been reported that *NR4A2* (*Nurr1*) heterozygous (+/−) mice exhibited a depression-like behavior, indicating difficulties in coping with stress [137]. Furthermore, it has been reported that using this particular model of depression-like behavior in adult mice led to a rapid increase in *NR4A2* (*Nurr1*) mRNA expression in specific brain regions following a second exposure to the forced swim test [179]. This response could be indicative of a compensatory mechanism that is developed to cope with depression-like behavior.

The findings of research studies have shown that animals responded differently to the forced swim test, which were classified as either vulnerable or resilient. After stress exposure, there was a significant increase in NR4A2 (Nurr1) protein levels in the hippocampus of vulnerable animals compared to the resilient group [180]. This differential response, which is associated with the behavioral phenotypes, may be attributed to the molecular response to stress, with NR4A2 (Nurr1) acting as a mediator of stress coping mechanisms. The molecular mechanisms underlying these two behavioral phenotypes are currently under study.

6.6. Substance Use Disorders

Substance use disorders (SUDs) are chronic, relapsing conditions in which substance use causes potential harm to physical or mental health, disability, and functional impairment for those affected and around them [181]. These conditions represent an important health, economic and social burden, being among the most significant causes of premature morbidity and mortality [182]. According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5-TR), SUDs are diagnosed using 11 criteria that fall into four basic categories: impaired control, physical dependence, social problems and high-risk use [183]. The causes, course, comorbidities, treatment use, and retention of SUDs differ between women and men, for a variety of reasons. These include biological, genetic, environmental and behavioral factors [184]. Worldwide, alcohol use disorders are the most common of all substance use disorders, followed by cannabis, opioid, and cocaine dependence [185]. A better understanding of the genetic mechanisms involved in the neurobiology of SUDs provides opportunities for the development of effective treatments and prevention strategies.

Cocaine addiction induces long-term neuroplastic changes [186] that, in turn, contribute to maladaptive behaviors, which are associated with transcriptional reprogramming of brain reward circuits [187].

In this regard, chronic cocaine administration has been shown to down-regulate the *NR4A2* gene in the rat ventral midbrain [188]. These results are consistent with the findings in postmortem human cocaine abusers, where the *NR4A2* gene is also reduced in the ventral tegmental area [189,190]. This decrease in *NR4A2* expression can be considered a regulatory response to the excess of dopamine neurotransmission caused by repeated cocaine exposure, which persists in the midbrain for at least two weeks after cocaine withdrawal [188]. This finding contributes to our understanding of the long-lasting brain changes associated with chronic drug use increased neural plasticity associated with prolonged exposure to drugs of abuse [191].

Prefrontal cortex (PFC)-dependent functions, such as executive function, explicit learning, and memory, are negatively affected in individuals with cocaine abuse as well as in experimental animal models of cocaine treatment [192,193]. Thus, *NR4A2* has been shown to exhibit dynamic expression changes in the PFC after repeated cocaine exposure in mice. An initial increase in *NR4A2* expression was observed, followed by a subsequent decrease after the last cocaine exposure. These transient changes after cocaine withdrawal may have played a role in the different stages of cocaine addiction [194]. The medial prefrontal cortex (mPFC) has been identified as a key region involved in the phenomenon of relapse after cocaine withdrawal. However, the underlying cellular mechanisms involved in these processes remain to be fully elucidated [195].

An important region of integration of the reward system, the nucleus accumbens (NAc), is the major component of the ventral striatum. Repeated cocaine exposure has been shown to induce coordinated changes in gene expression, which in turn drive plasticity in the NAc and promoting the development of addiction-like maladaptive behavior. Several studies have identified the role of transcription factors as critical mediators of cocaine-induced plasticity [196]. Most studies of epigenetic mechanisms involved in cocaine drug-taking and drug-seeking behaviors have focused on histone post-translational modifications in the NAc [197]. In this regard, it has been reported that memory formation is highly dependent on the regulation of the *NR4A2* gene by HDAC3 [100]. HDCA3 is one of four members of the human class I HDCAs. It regulates gene expression by deacetylating of histones and non-histone proteins [198]. Normally, HDAC3 binds to the promoters of *NR4A2*, repressing its expression. This regulatory function of HDAC3 may classify *NR4A2* as an important epigenetic effector gene [100]. In the NAc, *NR4A2* expression is increased

in HDAC3 flox/flox (HDAC3 selectively depleted in the NAc) mice that are exposed to cocaine [101]. In wild-type mice, acquisition of a cocaine-induced place preference leads to a reduction in HDAC3 occupancy at the *NR4A2* promoter [101]. This phenomenon is associated with the increase in *NRA42* expression, thereby a link between *NR4A2* and cocaine-related behaviors is suggested. This heightened expression of *NR4A2* is involved in HDAC3-mediated cocaine context memory formation in mice [101].

The medial habenula (MHb), is one of several regions associated with depression, addiction, and nicotine withdrawal. This brain region may provide further insight into the mechanisms underlying relapse to cocaine use [199]. Studies have demonstrated that the expression levels of HDAC3 and *NR4A2* were highly expressed in this brain region [200, 201]. Moreover, cocaine exposure in mice has been shown to elicit epigenetic regulation of the *NR4A2* gene during the reinstatement of cocaine-associated behaviors [201]. This suggests that HDAC3 is uncoupled from the *NR4A2* promoter in MHb as response to cocaine-induced reinstatement, which would imply an increase in *NR4A2* expression levels. These results suggest that HDAC3 acts as a molecular brake on memory by negatively regulating *NR4A2* [201]. It is also noteworthy that the expression of the dominant negative splice variant of *NR4A2*, termed *NURR2C*, in MHb cholinergic neurons provides a model to assess the effects of reduced *NR4A2* function in animal models [200,201]. Accordingly, Nurr2c expression in MHb has been shown to block cocaine-seeking reinstatement in mice. This finding facilitated the identification of the role of *NR4A2* in MHb in cocaine-induced associative memory processes. Furthermore, potential NR4A2 target genes associated with alterations in transcriptional networks related to addiction, neuroplasticity, GABAergic, and glutamatergic signaling have been identified by characterizing the molecular cascade resulting from reduced NR4A2 function [200]. These results further support the role of epigenetic regulation of *NR4A2* in MHb and its involvement in cocaine relapse.

7. Therapeutic Potential of Modulating *NR4A2* Gene Expression in Neurodegenerative Diseases and Psychiatric Disorders

NR4A2 is a promising neuroprotective and anti-inflammatory target in neurodegenerative and psychiatric diseases. However, because the effects of *NR4A2* dysregulation vary by brain region and disease, therapeutic strategies must be specific to each disease.

Among the most extensively studied approaches in preclinical research is the use of NR4A2 activators, which act at the NR4A2 TF or protein level. Examples of activators include prostaglandins (endogenous ligands), antimalarial drugs such as chloroquine and amodiaquine (synthetic ligands), and bicyclic compounds such as SA0025. Most studies on NR4A2 activators in neurodegenerative and psychiatric diseases have focused on PD, AD and cognitive impairments [134,152,159,202]. Comprehensive reviews of these NR4A2 activators have been carried out by other authors [13,203]. Nevertheless, these activators do not specifically target the brain regions involved in each pathology, and potential adverse effects cannot be ruled out due to the pleiotropic effect of NR4A2.

The mechanisms that regulate *NR4A2* gene expression have significant therapeutic potential for the treatment of neurodegenerative and psychiatric diseases. As shown in Table 1, various preclinical and clinical studies of *NR4A2* expression provide evidence for supporting the identification of its regulatory mechanisms in these diseases. Although some mechanisms of *NR4A2* genetic dysregulation may be shared across diseases, the affected brain regions appear to be specific to each disease. Therefore, understanding the timing and tissue-specific patterns of *NR4A2* dysregulation is a key factor for elucidating the pathophysiology of each disease. It is also critical to consider the influence of other genetic and environmental factors contributing to these pathologies.

Table 1. Comparison of NR4A2 gene expression dysregulation in neurodegenerative and psychiatric disorders.

Neurodegenerative Disease/ Psychiatric Disorder	*NR4A2* Expression in Patients	Relevant Studies in Animal Models	Potential Mechanisms Implicated in Gene Regulation
Parkinson's disease (PD)	In patients with PD: • Postmortem brain samples showed decreased in *NR4A2* mRNA expression in regions such as the substantia nigra, which plays a key role in movement, and in individuals with α-synuclein aggregates [144,145]. • Reduced *NR4A2* mRNA levels in peripheral blood were accompanied with elevated levels of inflammatory cytokines and miR-132 [132,149]. • Neurons from the postmortem parietal cortex exhibited sex-specific DNA methylation changes in the *NR4A2* gene [151].	• *NR4A2* heterozygous mice are more susceptible to the neurotoxin MPTP, which is a model of PD [143]. • An NR4A2 agonist restores both the transcriptional activity of NR4A2 as a transcription factor and its gene expression levels in a PD animal model [152]. • In mice administered insecticide dieldrin, a compound associated with an increased risk of PD, sex-specific DNA differential methylation of the *NR4A2* gene was observed [113].	• The presence of synuclein, which negatively regulates *NR4A2* expression via NF-κB, therefore decreases *NR4A2* mRNA expression [29,89]. • The microRNA miR-132 directly targets and represses *NR4A2* gene expression [106,131]. • The impact of alterations on DNA methylation is not yet fully understood. It is unclear how these alterations impact *NR4A2* expression or if they play a part in disease progression. No research has been conducted on this subject.
Alzheimer's disease (AD)	In patients with AD: • Postmortem samples revealed reduced *NR4A2* expression in the hippocampus and in individuals with Aβ deposition [158]. • Peripheral blood samples from AD patients also showed downregulation of the *NR4A2* gene [146].	• *NR4A2* expression is significantly reduced when Aβ is administered to neuronal cells [157]. • In 5X-FAD mouse model of AD, *NR4A2* is highly co-expressed with amyloid β (Aβ), particularly in the subiculum and the frontal cortex [134]. • Pharmacological activation of NR4A2 reduced Aβ plaque deposition in the subiculum and significantly ameliorated AD-like pathology in 5X-FAD mice [134].	The precise mechanisms underlying altered *NR4A2* expression in AD remain unclear. However, in animal models of cognition, particularly memory and learning, histone deacetylases have been shown to be part of the mechanism involved in changes in the *NR4A2* gene expression levels [100].

Table 1. *Cont.*

Neurodegenerative Disease/ Psychiatric Disorder	*NR4A2* Expression in Patients	Relevant Studies in Animal Models	Potential Mechanisms Implicated in Gene Regulation
Neurodevelopmental Disorders (NDD)	• In patients with NDD, evidence suggests the presence of pathogenic or likely pathogenic *NR4A2* variants. These variants lead to reduced gene expression or the production of a truncated NR4A2 protein [64].	• *NR4A2*-deficient male mice were associated with ADHD-like [135].	• *NR4A2* haploinsufficiency [64].
Schizophrenia (SZ)	In SZ patients: • Reduced *NR4A2* expression was observed in the dorsolateral prefrontal cortex (DLPFC) of postmortem simples [138,171]. • Variants in *NR4A2* gene have been associated with the cognitive alterations observed in the SZ [69,172]. • Hypermethylation of the *NR4A2* gene was found in females with major psychosis, compared to female control subjects [204].	• *NR4A2* heterozygous (+/−) mice, also known as Nurr1(+/−) have been proposed as an animal model for studying some of the behavioral and molecular mechanisms underlying SZ [136,137].	It is unclear how DNA methylation impacts *NR4A2* expression or if they play a part in disease progression. No research has been conducted on this subject. However, in animal models of cognition, particularly memory and learning, have shown that histone deacetylases are part of the mechanism involved in changes in the *NR4A2* gene expression levels [100].
Major depressive disorder (MDD)	In MDD patients: • A significant reduction in *NR4A2* mRNA expression has been reported in the dorsal raphe nucleus [178]. • Genetic variants of the *NR4A2* gene have been identified as risk factors for MDD in Caucasian populations [68].	• *NR4A2* (NURR1) heterozygous (+/−) mice exhibited a depression-like behavior, indicating difficulties in coping with stress [137].	• The impact of genetic variants on *NR4A2* expression is not fully understood. No research has been conducted on this subject.
Substance use disorders	Postmortem human individuals with history of cocaine and heroin abuse: • The ventral tegmental area showed reduced *NR4A2* gene expression [189,190]. This decrease may be considered a regulatory response to excessive dopaminergic neurotransmission induced by repeated cocaine exposure.	• In mice, heightened *NR4A2* expression is involved in the formation of cocaine context memory in the nucleus accumbens via HDAC3 [101]. • Cocaine exposure in rats has been shown to elicit epigenetic regulation of the *NR4A2* gene by HDAC3 during the reinstatement of cocaine-associated behaviors [200,201].	• The role of HDAC3 in the epigenetic regulation of *NR4A2* in medial habenula and nucleus accumbens [101,200,201].

With the advent of new genome editing and genetic engineering technologies such as CRISPR/Cas, CRISPR/dCas, and gene therapy, a new window of opportunity has opened not only for investigating gene expression mechanisms but also for developing tools that enable specific treatments for neurodegenerative and psychiatric diseases [205–207]. These technologies offer the potential to act on specific tissues or even a specific region of the brain [208–210]. This represents a major advantage over conventional systemic pharmacological approaches.

In general, gene therapy, in its most elementary form involves introducing genetic material into target cells using either non-viral or viral vectors, with the aim of treating or preventing diseases by correcting or supplementing defective genes [207]. Adeno-associated viruses (AAV) are among the most widely used viral vectors. This is because they are safe, naturally efficient at delivering genes to cells, and can produce sustained transgene expression in multiple tissues [208]. On the other hand, the CRISPR-Cas system consists of clustered regularly interspersed short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas), was discovered to be a prokaryotic defense mechanism that leads to an adaptive immune response [205]. CRISPR/Cas is a gene editing system that enables precise DNA modification at a specific site within a living organism (deleting, inserting or correcting sequences). It uses a Cas protein, such as Cas9, as molecular scissors and a customizable guide RNA (gRNA) to locate and cleave the target DNA in a sequence-specific manner. This DNA can then be replaced or repaired [211].

In contrast, CRISPR/dCas-based systems provide a powerful approach to modulate gene expression and genome function without altering the DNA sequence. This technology uses a genetically modified Cas enzyme that has lost its cutting activity (dCas) but retains its ability to recognize specific DNA via RNA guidance (gRNA) [206]. The dCas can bind to functional domains of other proteins important in epigenetic mechanisms, including those involved in the acetylation, deacetylation or methylation of histones, as well as DNA methylation. Thus, this system can perform epigenetic regulation of a specific gene [207].

In particular, the study of gene expression mechanisms and like potential therapeutic of *NR4A2* has been carried out using CRISPR/Cas technology and VAA vectors, especially in the field of oncology, but also in PD, AD and substance use disorders [199,201,212–214]. However, this field offers endless possibilities for future research into the mechanisms that regulate *NR4A2* expression [201], especially epigenetic mechanisms involved in neurodegenerative and psychiatric diseases, along with potential therapeutic interventions targeting the regulatory pathways of the *NR4A2* gene.

8. Discussion

The NR4A2 TF plays an important role in regulating the expression of several genes involved in mechanisms related to proper brain function, including neurodevelopment, protection and homeostasis of different neuronal cell populations, with the highest levels reported in dopaminergic neurons [8,9,19]. In particular, the regulation of gene expression by NR4A2 TF occurs through genomic and non-genomic pathways. However, the mechanisms that regulate its transcriptional activity are diverse, with the regulation of *NR4A2* gene expression being one of the most important.

The gene that encodes this TF, *NR4A2*, also known as *NURR1*, exhibits homology across different species [46,47]. This gene consists of a promoter region, eight exons and seven introns [48]. Depending on the cellular context, this gene can be stably regulated or induced as an IEG, leading to pleiotropic physiological effects. Furthermore, its expression is tightly regulated by extracellular signals. Consequently, the mechanisms that regulate *NR4A2* gene expression occur at multiple levels, through transcriptional, epigenetic and post-transcriptional mechanisms. Among the epigenetic mechanisms that regulate *NR4A2*

expression are DNA methylation, histone deacetylases (HDACs) and non-coding RNA (ncRNA) (Figure 5) [100,105,113].

Several animal models and clinical studies have linked *NR4A2* gene dysregulation to neurodegenerative diseases and psychiatric disorders. Thus, *NR4A2* gene dysregulation has been implicated in PD, AD progression, SZ, substance abuse (alcohol and cocaine), NDDs and cognitive impairment [7,13–18]. However, these changes in *NR4A2* expression occur in a disease-specific, cell type-associated manner and can be associated with the disease stage, suggesting the existence of disease-specific mechanisms underlying *NR4A2* dysregulation.

As discussed in this review, it is important to note that the mechanisms associated with *NR4A2* regulation are still being explored. In particular, some neurodegenerative diseases and psychiatric disorders have been studied more than others. Thus, we can highlight mechanisms regulating *NR4A2* expression in some CNS diseases.

This review has the following limitations: first, because of the limited number of studies conducted in humans, the review predominantly relies on studies conducted in animal models, which could restrict the generalization of the findings to human biological systems. It is also important to remember that the studies performed in animal models of the different diseases are the basis for the hypothesis that *NR4A2* is involved in different human diseases, but as we know, this does not mean that animal models reflect the reality of human diseases. Likewise, excluding literature not written in Spanish or English may have limited the representativeness of the available evidence. The narrative approach introduces a risk of bias in the selection and synthesis of studies. Finally, the scarcity of recent data from single-cell RNA sequencing techniques related to NR4A2 could limit our understanding of its functional specificity according to cell type. These limitations underscore the need for future research with broader coverage to strengthen the validity and applicability of the findings.

9. Conclusions

This revision encourages us to continue to further explore the diverse mechanisms that regulate *NR4A2* expression in different diseases and disorders, including those associated with neurodevelopmental, neurodegenerative, addiction, and mental disorders. Understanding these mechanisms will allow us to identify therapeutic targets for neurological diseases and psychiatric disorders.

The accumulated evidence supports the translational relevance of *NR4A2* as a potential biomarker and a therapeutic target. However, significant gaps remain in our understanding of its specific mechanisms of action, epigenetic regulation, and function in different cell types.

In this context, the combination of genetic and epigenetic large-scale data with bioinformatic tools represents a significant breakthrough in identifying genetic regulatory mechanisms. One example is TRAPT (Transcription Regulator Activity Prediction Tool) tool, which uses epigenomic data to predict transcriptional regulators (TRs) in specific contexts, such as neurodegenerative diseases [215]. This tool could contribute not only to characterizing the regulatory activity of NR4A2 TF, but also to inferring the TRs involved in *NR4A2* gene expression. These TRs, such as transcription cofactors and chromatin regulators, may mediate regulatory signals between the promoters and distal enhancers of the *NR4A2* gene, thereby regulating its expression, particularly in the context of neurodegenerative and psychiatric diseases.

On the other hand, it is a priority to advance toward specific clinical applications. Thus, it is necessary to conduct preclinical studies with NR4A2 agonists, evaluating their efficacy and safety in animal models of the aforementioned diseases and disorders. Likewise, longitudinal studies in humans, especially in relevant clinical cohorts, are required to validate the potential of *NR4A2* as a diagnostic and or prognostic biomarker.

At the same time, the impact of epigenetic modifications on the expression and function of NR4A2 should be investigated, considering its possible reversibility as a therapeutic strategy. It is also key to explore cell-type-specific delivery technologies that allow NR4A2-based therapies to be directed toward the involved neuronal or glial populations, reducing off-target effects in uninvolved tissues.

These lines of research could open the way for personalized treatment of neurodegenerative diseases and psychiatric disorders, reinforcing the role of NR4A2 in the field of translational medicine.

Therefore, we can conclude that *NR4A2* is a potential therapeutic target for neurological and/or psychiatric diseases, due to its key role in the survival of dopaminergic neurons, as well as in the regulation of neuroinflammation and neuronal plasticity, among other functions. In this context, it is important to highlight that recent advances in network pharmacology are providing a deeper understanding of drug mechanisms of action, especially in complex diseases such as neurological and psychiatric diseases.

Currently, the development of new drugs requires an interdisciplinary approach that integrates biology, pharmacology and bioinformatics, as proposed by network pharmacology. This discipline allows us to explore how drug affect multiple genes, proteins, and signaling pathways simultaneously in complex diseases. This contrasts with traditional pharmacology, which focuses on a single molecular target. This type of study has been performed, for example, for the treatment of intracerebral hemorrhage [216]. This represents a valuable contribution to the search for drugs in complex neurological and psychiatric diseases associated with NR4A2 dysfunction.

Author Contributions: Conceptualization, E.R.-S. and P.R.; investigation, E.R.-S., P.R., N.M.-R., C.R., Á.A.R.-C., G.I.-C., P.Y.G., A.A.R.-A., I.M.A.-M. and C.N.-R.; writing—original draft preparation, E.R.-S. and P.R.; writing—review and editing, E.R.-S., P.R., N.M.-R., C.R., Á.A.R.-C., G.I.-C., P.Y.G., A.A.R.-A., I.M.A.-M. and C.N.-R.; visualization, E.R.-S.; supervision, P.R. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this article.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Aβ	amyloid β
AD	Alzheimer's disease
ADHD	Attention-deficit hyperactivity disorder
AF1	Ligand-independent activation function domain 1
AF2	Ligand-dependent activation function domain 2
ASD	Autism spectrum disorder
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CP	Cognitively preserved
CI	Cognitive impaired
CNVs	Copy number variants
CREs	Cis-regulatory elements

CREB	cAMP response element-binding protein
CRTC	CREB-regulated transcription co-activators
CRTC1	CREB regulated transcription co-activator 1
CTCF	CCCTC-binding factor
DAergic	Dopaminergic neurons
DBD	DNA-binding domain
DMR	Differential methylation region
DR	Dorsal raphe nucleus
DSM-5-TR	Diagnostic and Statistical Manual of Mental Disorders
eRNA	Enhancer-derived RNA
GSK-3	Glycogen synthase kinase 3
HDAC	Histone deacetylases
HDAC 3	Histone deacetylases 3
IEG	Immediate early gene
ID	Intellectual disability
LBD	Ligand-binding site
LBD	Ligand-binding domain
LD	Linkage disequilibrium
lncRNAs	long non-coding RNAs
MDD	Major depressive disorder
mPFC	Medial prefrontal cortex
5mC	5-methylcytosine
miRNAs	microRNAs
miRNA-132	microRNA 132
miRNA-145-5p	microRNA-145-5p
miRNA-34	microRNA-34
mRNA	Messenger ribonucleic acid
MHb	Medial habenula
NAc	Nucleus accumbens
ncRNAs	Non-coding RNAs
NDDs	Neurodevelopmental disorders
NF-κB	Nuclear Factor Kappa B
NR4A2	Nuclear receptor subfamily 4 group A member 2
NR4A2 mRNA	messenger RNA transcript of NR4A2
PD	Parkinson's disease
PFC	Prefrontal cortex
piRNAs	Piwi-interacting RNAs
5-HT	Serotonin
SZ	Schizophrenia
SNVs	Single nucleotide variants
SUDs	Substance use disorders
TF	Transcription factor
UTR	5′-untranslated region
WHO	World Health Organization

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International Journal of
Molecular Sciences

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Review

Role of Histone Lactylation in Neurological Disorders

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Abstract

Lactate is not only the end product of glycolysis but also plays a key role in epigenetic regulation. Recently, lactate-derived histone lactylation has been identified as a novel epigenetic modification that can directly influence gene transcription. Histone lactylation has been associated with various pathological conditions and shows significant therapeutic potential. However, studies on histone lactylation in central nervous system diseases are still quite limited. In this review, we summarize the latest research progress on histone lactylation, detailing the specific mechanisms and sites of histone lactylation, including lactylation and delactylation. We also discuss the role of histone lactylation in Alzheimer's disease (glycolysis/H4K12la/PKM2 feedback loop), depression (neuronal excitation), neuroinflammation (anti-inflammatory/pro-inflammatory balance of microglia), aging, stroke (infarct volume), and glioblastoma (activation of oncogenes), pointing out the research directions for the future. This may provide new ideas for the diagnosis and treatment of neurological diseases.

Keywords: histone lactylation; Alzheimer's disease; depression; neuroinflammation; aging

1. Lactate and Lactylation

Lactic acid is the end product of cells undergoing anaerobic glycolysis, and its production regenerates the NAD^+ consumed during the process of glycolysis [1]. L-lactic acid, a metabolic intermediate that can be converted through gluconeogenesis, is transported between different cells and organs in the body. It plays an important role in many physiological processes. Under pathological conditions such as hypoxia or certain cancers, cells may produce L-lactic acid at higher rates. In hypoxic conditions, cells switch to anaerobic metabolism, which leads to increased L-lactic acid production [2]. Similarly, cancer cells often exhibit heightened glycolytic activity even in the presence of adequate oxygen (the Warburg effect), resulting in increased L-lactic acid production as a metabolic adaptation [3]. Moreover, L-lactic acid is also a vital signaling molecule in the brain, and elevated levels of lactate have been observed in many neurological disorders [4].

Recent research has found that lactic acid has a new role in the field of epigenetic research. Epigenetics refers to changes in gene function that can be inherited without changing the DNA sequence, ultimately leading to changes in phenotype. Post-translational histone modifications comprise part of the hierarchy of epigenetic regulatory mechanisms [5]. Various histone acylation marks derived from cellular metabolites have been discovered in

recent years, such as propionylation, butyrylation, 2-hydroxyisobutyrylation, succinylation, malonylation [6], and so on. These histone modifications alter the spatial structure of proteins, regulating many physiological and biochemical processes in cells [7]. Histone lactylation, derived from lactate, was recently identified as a novel histone modification [8].

Lysine lactylation (Kla) is a post-translational modification that is stimulated and regulated by lactic acid [8]. There are three isomers of Kla: L-lactyl-lysine ($K_{L\text{-}la}$), N-ε-(carboxyethyl)-lysine (K_{ce}), and D-lactyl-lysine ($K_{D\text{-}la}$). It has been proven that neither K_{ce} nor $K_{D\text{-}la}$ are present on histones in wild-type cells [9]. Meanwhile, $K_{L\text{-}la}$ is susceptible to glycolysis. The initial evidence of histone Kla comes from the discovery of three protein hydrolysis peptides with a mass shift of 72.021 daltons on the lysine residue when analyzing core histones digested with trypsin in MCF-7 cells using high-performance liquid chromatography (HPLC)–tandem mass spectrometry (MS/MS) [8]. This mass shift is identical to that caused by the addition of a lactate group to the ε-amino group of the lysine residue [8]. Under physiological conditions, lactylation fine-tunes neuroplasticity genes (e.g., Egr1, c-Fos) in response to synaptic activity. Baseline H3K18la levels in hippocampal neurons modulate spatial memory consolidation, while exercise-induced lactylation promotes neurogenesis [10].

Lactic acid, as a precursor, is converted to lactic acid coenzyme A and transferred to histone under the action of acetyltransferase [11]. In M1 macrophages, lactate is a signaling molecule that stimulates gene transcription through histone Kla [8]. Zhang and others have shown that the regulation of intracellular lactate production leads to histone Kla levels changing in a dose-dependent manner. In addition, intracellular metabolic disorders are associated with the functionality of epigenetic writers and erasers, while epigenetic writers and erasers can also regulate histone acetylation and lactylation [12]. Critically, lactate shuttling between astrocytes and neurons regulates synaptic plasticity via lactylation-dependent gene expression such as BDNF [13]. Dysregulated lactylation in neurological contexts may thus disrupt neurometabolic coupling, exacerbating disease progression.

While prior reviews have summarized histone lactylation in cancer and immunology, this is the first comprehensive synthesis focusing on its mechanistic roles in neurological disorders (AD, depression, neuroinflammation, aging), highlighting novel feedback loops (e.g., PKM2/H4K12la in AD) and therapeutic implications specific to the central nervous system (CNS).

2. Addition and Removal of Lactyl Groups

2.1. Lactylation: Enzymatic and Non-Enzymatic Reactions

Lysine acetyltransferase (KAT) enzymatic, P300, and lactyl-CoA jointly result in enzyme-catalyzed Kla [8]. It has also been found that the nuclear receptor Nur77 recruits P300, reduces HDAC1 (histone deacetylase 1) expression, enhances the transcriptional activity of signal transducer and activator of transcription 3 (STAT3) through Nε-acetylation, and regulates the expression of the downstream gene *Pomc* in the hypothalamus, thereby creating a positive feedback loop that promotes the expression of STAT3. Both pathways mentioned above jointly regulate histone lactylation and form the enzymatic parts [14]. Although Nur77-P300-STAT3 primarily regulates acetylation, this pathway exemplifies how acetyltransferases like P300 can be recruited for lactylation, given P300's dual role in both modifications (Figure 1) [11]. Zhu et al. recently reported on lactyl-CoA synthetase and lactyltransferase as well as their role in histone lactylation. They found acetyl-CoA synthetase 2 (ACSS2) functions as a lactyl-CoA synthetase and converts lactate to lactyl-CoA [15]. Lactyl-CoA is subsequently transferred to histones by KAT, primarily P300 and KAT2A [8,15]. Specifically, KAT2A catalyzes site-specific lactylation at H3K14 and H3K18, while P300 may function as a complementary lactyltransferase recruited by nuclear

receptors (e.g., Nur77) [15]. In addition to the enzyme-dependent transfer of lactyl-CoA to lysine residues, lysine acylation has been shown to originate from a non-enzyme reaction with lactic glutathione (LGSH) [11,16]. Methylglyoxal is a byproduct of sugar metabolism that reacts enzymatically with glutathione to form LGSH. LGSH is then hydrolyzed by GLO2 to produce glutathione and D-lactate. Gaffney and others recently discovered that non-enzymatic lactate transfer from LGSH to protein lysine residues can lead to "LactylLys" modification in the protein, which can be inhibited by GLO2, thereby regulating glycolysis [16]. In addition, in mitochondrial proteins, the non-enzyme-promoted lysine acetylation of acetyl-CoA and acetylglutathione is promoted by proximal S-acetylation thiol intermediates, which can be restrained by GLO2 [17].

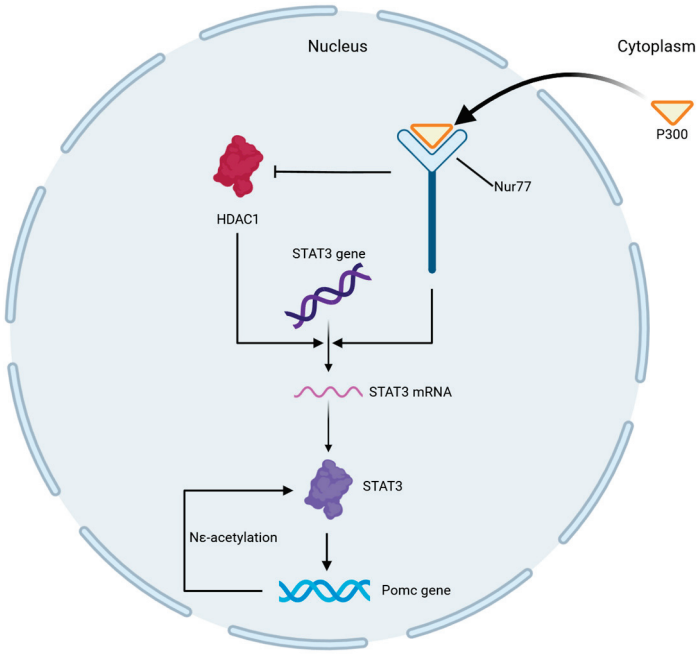


Figure 1. Regulation of histone lactylation enzymatic reactions through nuclear receptor pathways. The nuclear receptor Nur77 recruits P300, reduces HDAC1 expression, and enhances the transcriptional activity of STAT3 through N-ε acetylation. P300, a key lactyltransferase, is recruited here for acetylation; its role in lactylation may operate via analogous mechanisms. Nur77: nuclear receptor 77; P300: lysine acetyltransferase enzymatic protein 300; STAT3: signal transducer and activator of transcription 3.

2.2. Delactylation

Mammals express two families of lysine deacetylases, comprising a total of 18 enzymes. The histone deacetylases (HDAC1-11) rely on Zn^{2+} [18], while sirtuins (SIRT1-7) depend on NAD+ as a cosubstrate [19]. Histone delactylation is dynamically regulated by class I histone deacetylases (HDAC1-3), which exhibit specific lysine delactylase activity [20,21]. Among these, HDAC1 and HDAC3 are the primary delactylases that target lactylation sites such as H4K5la and H3K18la [20]. This functional specificity is attributed to their integration into multi-subunit complexes that confer substrate selectivity [22]. These complexes contain scaffold proteins that interact with other epigenetic enzymes, such as demethylases, as well as with transcription factors [22]. When HDACs bind to their respective complexes, their activity is enhanced [23]. HDAC1-3 show different substrate selectivity in cells, possibly due to interactions with different complex partners [24]. It has also been found that HDAC1 and HDAC3 can significantly change the lactylation level of the H4K5 site, while HDAC2 cannot. The study suggests that the impact of each HDAC isoform on overall histone lactylation levels is limited. HDAC1/2 are often present in the

same multi-protein complex in the cell nucleus and are believed to compensate for each other in the regulation of histone Kla [22]. HDAC1/3-mediated delactylation demonstrates that histone Kla is dynamically regulated, akin to acetylation, offering new targets for epigenetic therapeutics. These enzymes in the cell nucleus delactylate histones, thereby achieving reversible and dynamic regulation of histone lactylation [25]. This result, along with the chromatin immunoprecipitation sequencing observation of histone lactylation, strongly demonstrates the potential genome-wide localization specificity of histone lactylation [8], providing powerful evidence that histone l-lactylation may be a major pathway for nuclear lactylation [20]. In summary, these findings not only provide new insights into the novel activity of Class I HDACs, but also validate the pharmacologically targetable enzyme regulation of histone K, laying the foundation for future functional studies of this modification and its related regulatory pathways [20].

3. Pathology Manifestations

Histone lactylation plays an important regulatory role under normal physiological conditions as well as in pathological conditions (such as Alzheimer's disease (AD) and depression [26]).

3.1. AD

AD is a debilitating neurodegenerative condition characterized by a progressive decline in cognitive function [27]. It manifests through impairments in memory, cognition, and behavior. The etiology of AD is mainly attributed to the accumulation of Amyloid-β(Aβ)plaques and tau neurofibrils within the brain parenchyma. These pathological aggregates disrupt neuronal integrity, leading to the characteristic symptoms associated with the disease [28,29]. AD is also a chronic neuroinflammatory disease, accompanied by the abnormal activation of microglial cells, which progressively leads to the dysfunction of microglial cells [30]. Microglia are the resident immune cells of the central nervous system, providing immune protection under pathological conditions [13].

In AD, several histone Kla sites, including H4K8, H4K12, H3K14, and H3K18 sites, have been identified in both microglia and neurons in the hippocampus [31]. It has been found that histone lactylation increases in the amyloid β-plaque-associated microglia of AD patients, particularly H4K12la and H3K18la [31,32]. In AD, histone lactylation activates the transcription of glycolysis genes, forming a positive feedback loop that further exacerbates microglial cell activation and functional impairment (Figure 2). This loop involves active glycolysis, H4K12la, and pyruvate kinase M2 (PKM2). Further study has also found that inhibition of PKM2 disrupts the glycolysis/H4K12la/PKM2 positive feedback loop in microglia, which can alleviate microglial activation and neuroinflammatory responses. 5XFAD mice represent a commonly utilized genetic model for AD. They are generated through transgenic technology, which involves the introduction of human amyloid precursor protein (APP) and presenilin 1 (PSEN1) mutant genes into the mouse genome [33]. These mutant genes cause the mice to produce large amounts of Aβ plaques, which are one of the typical pathological features of AD. In 5XFAD mice, specific deletion of the Pkm2 gene in microglial cells can reduce β-amyloid deposition and improve spatial learning and memory [32]. Thus, inhibiting the glycolysis/H4K12la/PKM2 feedback loop may be considered a potential strategy for the treatment of AD. Similarly, research also suggests that a positive feedback loop exists for H3K18la. H3K18la enhances NFκB signaling, and facilitates the senescence-associated secretory phenotype (SASP) components IL-6 and IL-8, thereby promoting AD progression through inflammatory signaling pathways [31].

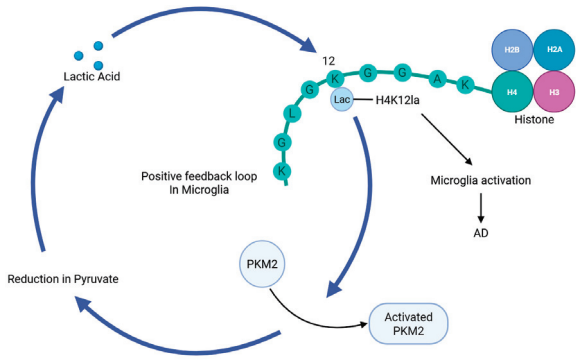


Figure 2. The positive feedback loop in microglia leads to AD by promoting histone H4K12la. Upon activation of PKM2, a key enzyme in glycolysis, it promotes anaerobic respiration to produce pyruvate, which is subsequently reduced to lactate. Once lactate enters the nervous system, it induces histone H4K12 lactylation in microglia, leading to the activation of microglia. Lactylation activates PKM2, creating a positive feedback loop that amplifies lactylation, ultimately leading to AD. PKM2: Pyruvate kinase isozyme type M2; AD: Alzheimer's disease.

3.2. Depression

Depression can lead to a decrease in the number and density of astrocytes in the prefrontal cortex (PFC) of patients, suggesting that astrocyte dysfunction plays a role in the pathogenesis of depression [34]. There is evidence that suggests that fluoxetine and paroxetine promote lactate release from cortical astrocytes [35] and that peripheral administration of lactate can produce antidepressant-like effects, such as reduction in behavioral despair, anhedonia-like behaviors, and social avoidance [36,37]. Chronic corticosterone administration can induce a depressive-like state in mice [38], and peripheral lactate treatment can reverse this effect of corticosterone [36]. In a corticosterone model of depression, antidepressant effects of lactate require adult hippocampal neurogenesis, using the anti-mitotic drug Temozolomide, which impairs neurogenesis in the hippocampus [39]. Corticosterone exerts an augmentative effect on neuronal oxidative stress [40–42]. Hippocampal stem cells that have been treated with corticosterone exhibit diminished cell proliferation and an elevated production of reactive oxygen species (ROS). However, co-administration of corticosterone and lactate mitigates these detrimental effects. The above results suggest that the conversion of lactate to pyruvate, accompanied by the generation of NADH, is essential for the neuroprotective properties of lactate in depression. These effects are partially mediated by corticosterone-induced inhibition of ROS [39]. Both lactate and pyruvate enter the central nervous system via monocarboxylate transporter 1 (MCT1) across the blood–brain barrier, but chronic administration of pyruvate failed to produce antidepressant-like effects, in sharp contrast to lactate [43]. This suggests that NADH generated by lactate dehydrogenase during lactate is oxidized to pyruvate may be involved in the neurogenic and antidepressant effects of lactate [39].

Stress, especially chronic stress, has been shown to alter neural activity in the brain [44,45]. To explore the mechanism of brain lactate regulation in depression, the researchers applied an in vitro neuronal excitation model with high-K. They found that the levels of lactate have a time-dependent increase after high-K treatment, and a high-K-induced increase in Kla levels was inhibited by sodium oxamate (OX), which inhibits lactate dehydrogenase (LDH) activity. LDH can inhibit the production of lactate in the central nervous system [46,47]. Thus, neuronal excitation may induce Kla via intracellular metabolism and the glycolytic pathway. The inhibitor of monocarboxylate transporter 2 (MCT2), α-cyano-4-hydroxycinnamate (4-CIN), dose-dependently attenuated the lactate-induced increase in Kla immunoreactivity, blocking the transport of lactate into neurons, indicating that MCT2 may also be involved in the effect of lactate on Kla [48]. Further

study in mice with electroconvulsive stimulation (ECS) in vivo found that both intracellular lactate production and extracellular lactate uptake are involved in acute neuronal activation in vivo [48]. C-Fos is an immediate early gene product used as a marker for neuronal activation [49]. Double-immunostaining analysis of c-Fos showed a significant positive correlation between Kla and c-Fos immunoreactive intensities, suggesting that more Kla is induced in the cell when more neurons are activated [48]. Finally, they examined whether Kla was affected by behaviorally induced neuronal excitation associated with SDS. The results showed that Kla levels in the PFC were associated with anxiety-like behaviors, suggesting a potential behavioral significance of Kla in the brain. In addition, they highlighted that histone H1 lactylation, out of 63 candidate lactylated proteins, increases in the mouse PFC in response to chronic stress. Overall, this study provides evidence for the role of neuronal activity-induced lactate mediated by protein lactylation (Figure 3) [48].

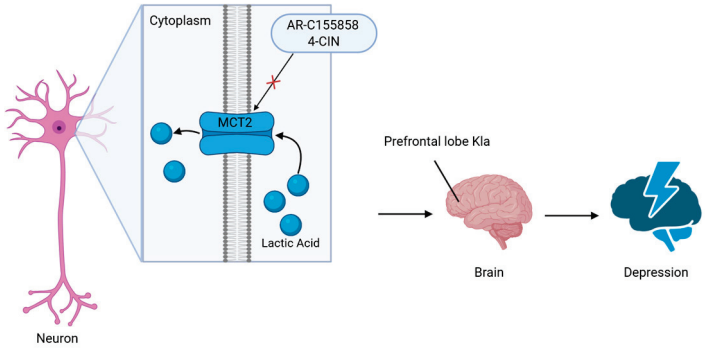


Figure 3. Lactate transport into prefrontal cortex neurons promotes depression-associated protein lactylation. Lactic acid molecules produced in the body are transported into prefrontal cortex neurons by MCT2, contributing to Kla, which is associated with depressive symptoms. The inhibitor of MCT2, 4-CIN, dose-dependently attenuated the lactate-induced increase in Kla immunoreactivity, blocking the transport of lactate into neurons, indicating that MCT2 may be involved in the effect of lactate on Kla. The selective MCT1/2 inhibitor AR-C155858 also inhibited the lactate-induced increase in Kla, further supporting the role of MCT1/2 in lactate regulation of Kla. MCT2: monocarboxylate transporter 2; 4-CIN: α-cyano-4-hydroxycinnamate; AR-C155858: $C_{21}H_{27}N_5O_5S$, the inhibitor of MCT1/2.

3.3. Neuroinflammation and Aging

Neuroinflammation is a common pathological feature of many acute and chronic neurological diseases. It exerts a devastating impact on cells and brain function and accelerates the progression of long-term neurodegenerative diseases [50]. Neuroinflammation may be a cause or a consequence of disease progression at different stages of the disease. Inflammation can cause damage to cells and brain function by suppressing neurogenesis and accelerating neurodegeneration [51].

To investigate the role of lactate-induced Kla in promoting a reparative microglial phenotype in neuroinflammation, researchers incubated the BV2 microglial cell line with Aβ1-42 to induce activation and examined the mRNA expression of pro-inflammatory gene IL-1β and reparative genes Arg 1 and VEGF [10]. The results revealed that the IL-1β gene was sharply upregulated at the initial stage of Aβ 1-42 inflammatory stimulation, and then gradually declined over time, indicating that exogenous lactate treatment promotes the transformation of pro-inflammatory microglia to anti-inflammatory/reparative phenotype by lactylation [10].

In addition to in vitro experiments, lactate molecules mediated by anaerobic respiration during exercise also play a crucial role in neuroinflammation in vivo. Lactic acid is a major product of exercise and is transported by the blood to various tissues throughout the body [52]. Also, lactate levels rise in muscle cells after running exercise [53]. Lactic acid

produced by exercise amplifies microglial transition from a detrimental to a reparative phenotype via H3K18 lactylation of microglial histone, which improves neural and cognitive function in a mouse model. Lactylation enrichment and increased arginase 1 expression in macrophages was observed when these cells were treated with exogenous lactate [8,53]. In this process, lactate plays the role of a "timer" in the transition of macrophages from a pro-inflammatory phenotype to an anti-inflammatory/reparative phenotype [10]. Finally, the researchers demonstrated that the addition of lactate accelerated the same transition of the detrimental to the reparative phenotype as cultured BV2 microglia. Thus, exercise training or lactate administration may act as an "accelerator" for the microglial intrinsic "lactate timer", with histone lactylation improving cognitive function via modulating the anti-inflammatory/pro-inflammatory balance of microglia in conditions such as neuroinflammation and aging (Figure 4) [10,54]. Researchers demonstrated that exercise-induced lactate elevation in the mouse brain also acts as an endogenous accelerator of the "lactate timer" in microglia in various aged mouse models induced by D-gal and $AlCl_3$ [10].

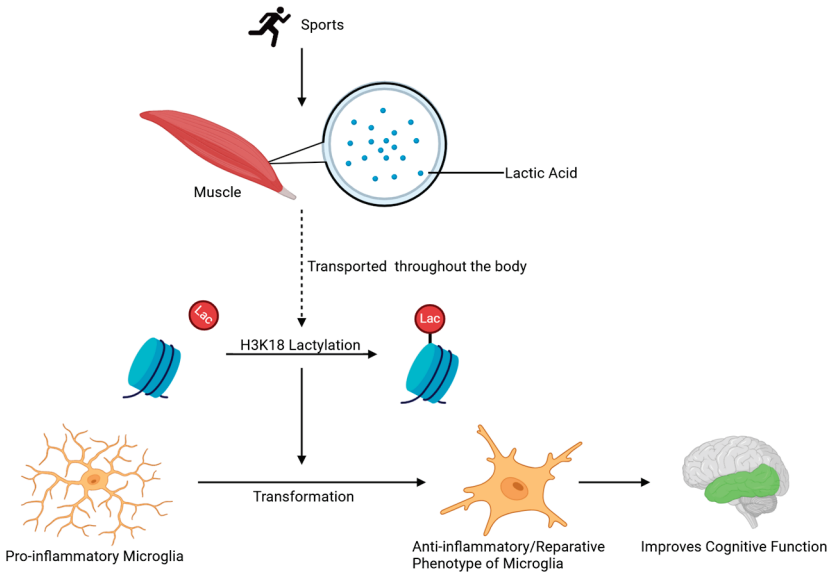


Figure 4. Lactic acid produced during exercise is transported from muscle cells into microglia, promoting their phenotype transformation through histone lactylation. During exercise, lactic acid is produced in muscle cell cytoplasm through anaerobic respiration and amplifies microglial transition from a detrimental to a reparative phenotype via H3K18la lactylation of microglial histone H3.

Aging is a risk factor for neurodegenerative diseases [55]. D-galactose (D-gal) is an aging agent, while aluminum is a known neurotoxin associated with the onset of AD. There is abundant evidence that D-gal/aluminum chloride ($AlCl_3$) combined treatment can induce AD-like symptoms, including cognitive and memory impairment, amyloid-β overexpression, oxidative damage, microglia activation, and neuroinflammation [56,57].

3.4. Histone Lactylation in Other Neurological Disorders

Stroke is one of the leading causes of mortality and disability globally, presenting substantial burdens to society and healthcare systems [58]. Among stroke subtypes, ischemic stroke constitutes the most prevalent form [59]. Evidence indicates that lactate production is closely linked to the progression of ischemic stroke [60]. In ischemic stroke, lactate accumulation during reperfusion injury exacerbates blood–brain barrier disruption and neuronal death. A recent study revealed that the knockdown of the lactate dehydrogenase A (LDHA) protein could reduce histone lactylation levels at the HMGB1 promoter, as well as decrease the expression levels of IL-18, IL-1β, cleaved caspase-1, and GSDMD-N proteins in both in vivo and in vitro models of cerebral ischemia/reperfusion. This intervention

was associated with a reduction in infarct volume and an improvement in neurological function in MCAO rats [61]. In addition, lactate-driven histone lactylation (particularly H3K9la and H3K18la) dysregulates gene expression in the neurovascular unit, amplifying pro-inflammatory pathways and impairing tissue repair [62].

Glioblastoma is the most common and aggressive primary brain tumor in adults [63]. For glioblastoma, the Warburg effect creates a hyperlactate microenvironment that epigenetically fuels tumor progression [64]. H3K9la directly activates oncogenes by recruiting bromodomain-containing protein 4 (BRD4) to enhancer regions, driving glioblastoma cell proliferation and therapy resistance [26,65,66]. As a lactylation "reader", BRD4 recognizes H3K9la/H4K8la marks and recruits transcriptional machinery to activate oncogenes [26,66]. In subarachnoid hemorrhage (SAH) models, BRD4-mediated binding to H4K8la drives astrocyte pro-inflammatory polarization [66]. The study confirmed that BRD4 mediates A1 polarization of astrocytes after SAH by regulating histone lactylation, particularly H4K8la. Knockdown of BRD4 significantly reduced histone lactylation levels but did not affect glycolysis or lactate production. Experiments confirmed that BRD4 directly binds to H4K8la. Under OxyHb stimulation, the absence of BRD4 inhibited the upregulation of H4K8la and the expression of the pro-inflammatory marker C3, leading astrocytes to secrete more IL-1β, TNF-α, and IL-6. These inflammatory factors further reduced neuronal viability and exacerbated neuronal death [66]. These findings collectively highlight histone lactylation as a critical metabolic–epigenetic nexus across diverse neurological disorders, offering novel targets for therapeutic intervention.

3.5. Cell-Type-Specific Regulation of Histone Lactylation in the CNS

Emerging evidence reveals that histone lactylation exerts cell-type-dependent functions across neural populations, with distinct pathological and therapeutic implications (Table 1).

Table 1. Comparative analysis of histone lactylation mechanisms across major neurological disorders.

Disorder	Key Lactylation Sites	Core Pathological Mechanism	Disease Phenotypes
Alzheimer's disease	H4K12la, H3K18la	PKM2/glycolysis positive feedback loop → microglial hyperactivation	Aβ deposition, cognitive decline, neuroinflammation
Glioblastoma	H3K9la	BRD4 recruitment to enhancers → oncogene activation	Tumor proliferation, therapy resistance, Warburg effect amplification
Ischemic stroke	H3K9la, H3K18la	LDHA-mediated HMGB1 promoter lactylation → NLRP3 inflammasome activation	Infarct volume expansion, blood–brain barrier disruption, neuronal death
Depression	H1Kla	MCT2-mediated neuronal lactate uptake → prefrontal cortex protein lactylation	Neuronal hyperexcitability, behavioral despair, social avoidance
Aging/Neuroinflammation	H3K18la	Exercise-induced lactate → microglial phenotype switching (pro- to anti-inflammatory)	Cognitive dysfunction, accelerated neurodegeneration, synaptic impairment

In Aβ-activated microglia, exogenous lactate promotes H3K18la-mediated upregulation of reparative genes (Arg1, VEGF), facilitating phenotype switching via a "lactate timer"

mechanism [10]. AD-associated microglia exhibit elevated H4K12la/H3K18la, driving *PKM2*-dependent glycolytic feedback loops that exacerbate neuroinflammation [31,32]. Exercise-induced lactate further amplifies H3K18la-enriched anti-inflammatory microglial states, improving cognition in aging models [10,54]. Astrocyte–neuron lactate transport regulates synaptic plasticity through BDNF lactylation, essential for long-term memory formation [13]. Cortical astrocytes release lactate under fluoxetine/paroxetine treatment, while chronic stress reduces astrocyte density in the PFC—suggesting that astrocytic lactate dysregulation is a core component of depression etiology [34,35]. Neuronal excitation (high-K^+ stimulation or electroconvulsive shock) induces time-dependent Kla, particularly on histone H1, correlating with c-Fos activation markers [48]. MCT2-mediated lactate uptake in prefrontal neurons promotes Kla modifications linked to anxiety-like behaviors, whereas corticosterone-induced oxidative stress is mitigated by lactate-derived NADH generation [39,48]. Cell-specific lactylation signatures may enable precision targeting of neuropsychiatric disorders. Future studies should leverage single-cell epigenomics to resolve spatial heterogeneity in lactylation landscapes [67].

4. Drug Development

Based on the core regulatory mechanisms of histone lactylation in neurological disorders, future drug development should focus on synergistic interventions targeting pathological feedback loops, lactylation enzyme systems, and lactate transport pathways [8]. For the critical link of aberrant microglial activation in AD, highly selective small-molecule PKM2 inhibitors can be developed to disrupt the "glycolysis/H4K12la/PKM2" positive feedback loop, while designing HDAC1/3-specific cyclic peptide inhibitors to block the neuroinflammatory cascade mediated by the H3K18la/NFκB pathway [31,68,69]. At the enzymatic regulation level, allosteric modulators of ACSS2 (lactyl-CoA synthetase) should be constructed to precisely control lactyl-CoA biosynthesis, and competitive KAT2A inhibitors should be developed to block pathological lactylation modifications at H3K14/H3K18 [20,70]. For lactate metabolism and transport, optimization of blood–brain barrier-penetrant MCT1/2 dual-function modulators is required to achieve brain region-specific lactate flux regulation, alongside designing "exercise-mimetic compounds" to directly elevate brain lactate concentration and promote H3K18la-dependent reparative microglial phenotype transformation [71]. Additionally, integrated diagnostic-therapeutic strategies should be implemented, developing novel PET tracers based on H4K12la/H3K18la epigenetic maps to provide dynamic monitoring for disease stratification and efficacy evaluation [67]. This R&D framework necessitates combining cryo-electron microscopy to resolve lactyl–CoA–histone complex structures for rational drug design, utilizing single-cell epigenomics to screen cell-type-specific targets, and employing nanocarrier delivery systems to overcome blood–brain barrier limitations. Ultimately, validation in models will demonstrate translational value, driving breakthroughs in histone lactylation regulation, from mechanistic research to clinical therapeutics.

5. Limitations and Future Perspectives

Histone lactylation represents a druggable node in neurological diseases, such as suppressing H4K12la via PKM2 inhibitors ameliorates AD pathology, while exercise mimetics that boost H3K18la may combat depression. Precision targeting of lactylation dynamics could yield disease-modifying therapies. The burgeoning field of histone lactylation has unveiled its critical roles in neurological disorders, yet numerous questions remain unanswered. Future research should prioritize the following directions to bridge existing gaps and translate findings into clinical applications.

Firstly, there is a critical scarcity of cell-type-specific lactylation data within the human brain. This gap impedes the precise delineation of lactylation's physiological roles and pathological contributions across distinct neuronal and glial populations. Secondly, the crosstalk and potential regulatory interplay between lactylation and other major post-translational modifications (PTMs), such as acetylation, remains poorly defined. This lack of clarity regarding competitive, cooperative, or hierarchical relationships complicates the interpretation of lactylation's functional consequences within broader signaling networks. Furthermore, a major translational barrier exists due to the absence of lactylation-specific positron emission tomography (PET) tracers. The lack of such non-invasive imaging tools severely restricts the ability to monitor lactylation dynamics in vivo, assess their relevance regarding disease progression, or evaluate the efficacy of lactylation-targeting therapies in clinical settings. Addressing these limitations is essential for advancing the field.

Secondly, while enzymatic and non-enzymatic pathways for lactylation have been identified, their spatiotemporal regulation across diverse cell types and disease states remains poorly understood. Advanced techniques, such as single-cell multi-omics and cryo-electron microscopy, could resolve the structural basis of lactyl-CoA binding to histones and delineate isoform-specific functions of lactylation in neurological diseases.

Thirdly, the role of histone lactylation in Parkinson's disease, stroke, and glioblastoma warrants exploration. Furthermore, the glycolysis/H4K12la/PKM2 and H3K18la/NFκB feedback loops in microglia represent promising therapeutic nodes. Developing highly specific HDAC1/3 inhibitors or lactate-CoA synthase modulators can effectively intervene in the pathological lactylation modification cycle. Concurrently, exercise-mimetic compounds that enhance lactate flux without physical exertion might offer novel strategies to amplify reparative microglial phenotypes via H3K18 lactylation. Notably, the dual role of MCT1/2 in lactate shuttling and lactylation underscores their therapeutic potential. Engineering blood–brain barrier-penetrant MCT modulators or lactate prodrugs could spatially control lactylation in specific brain regions, mitigating off-target effects.

Beyond neurological pathologies, emerging evidence suggests potential roles of histone lactylation in developmental biology. In embryogenesis, dynamic metabolic shifts—such as the Warburg-like glycolysis prevalent in stem cells—may generate localized lactate pools that could epigenetically regulate gene expression via lactylation. Preliminary studies indicate that lactylation marks (e.g., H3K18la) are enriched in early embryonic tissues and pluripotent stem cells, where they may influence lineage specification by modulating chromatin accessibility at key developmental genes [72]. Further investigation into lactylation's impact on stem cell self-renewal, differentiation, and tissue morphogenesis could unveil novel mechanisms linking cellular metabolism to developmental programming, with implications for regenerative medicine and organoid models of disease.

By addressing these challenges, future research will refine the mechanistic landscape of histone lactylation and catalyze the development of lactate-centric diagnostics and therapies for neurological disorders.

Author Contributions: Y.-M.Z.: writing—original draft. J.-N.Z.: writing—original draft, writing—review and editing. Q.L.: writing—review and editing. F.Y.: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Natural Science Foundation of China (82371321 to Q. Li, 82271240 to F. Yang, 82301604 to J. Zhang) and the Beijing Natural Science Foundation Program and Scientific Research Key Program of Beijing Municipal Commission of Education (KZ202010025033 to Q. Li).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: No data were analyzed during the current study.

Acknowledgments: The figures were created with BioRender.

Conflicts of Interest: The authors have declared that no conflicts of interest exist.

Abbreviations

AD	Alzheimer's disease
$AlCl_3$	aluminum chloride
APP	amyloid precursor protein
Arg 1	arginase 1
Aβ	amyloid-β
4-CIN	α-cyano-4-hydroxycinnamate
CNS	central nervous system
D-gal	d-galactose
ECS	electroconvulsive stimulation
ERK	extracellular regulated protein kinases
GLO2	glyoxalase ii
HPLC	high-performance liquid chromatography
BNDF	brain-derived neurotrophic factor
HDAC	histone deacetylase
KAT	lysine acetyltransferase
Kla	lysine lactylation
$K_{L\text{-}la}$	l-lactyl-lysine
K_{ce}	n-ε-(carboxyethyl)-lysine
$K_{D\text{-}la}$	d-lactyl-lysine
LDH	lactate dehydrogenase
LGSH	lactic glutathione
MCT1	monocarboxylate transporter 1
MCT2	monocarboxylate transporter 2
ROS	reactive oxygen species
SIRT	sirtuins
STAT3	signal transducer and activator of transcription 3
OX	oxymate
PKM2	pyruvate kinase m2
PSEN1	presenilin
SASP	senescence-associated secretory phenotype
SAH	subarachnoid hemorrhage
PFC	prefrontal cortex
VEGF	vascular endothelial growth factor
LDHA	lactate dehydrogenase A
BRD4	bromodomain-containing protein 4

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International Journal of
Molecular Sciences

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Review

SLC1A4 and Serine Homeostasis: Implications for Neurodevelopmental and Neurodegenerative Disorders

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Abstract: The *solute carrier family 1 member 4* (*SLC1A4*) gene encodes a neutral amino acid transporter, also referred to as alanine-serine-cysteine transporter 1, ASCT1, that helps maintain amino acid balance in the brain and periphery. In the brain, SLC1A4 plays an important role in transporting levo (L) and dopa (D) isomers of serine. L-serine is required for many cellular processes, including protein and sphingolipid synthesis, while D-serine is a co-agonist required for normal neurotransmission through *N*-methyl-D-aspartate receptors. Through its roles transporting L-serine across the blood–brain barrier and regulating synaptic D-serine levels, SLC1A4 helps establish and maintain brain health across the lifespan. This review examines the role of SLC1A4 in neurodevelopment and neurodegeneration and assesses the therapeutic potential of serine supplementation to treat neurodevelopmental symptoms associated with mutations in *SLC1A4*, as well as schizophrenia, depression, traumatic brain injury, and Alzheimer's and Parkinson's diseases.

Keywords: *solute carrier family 1 member 4* (*SLC1A4*) gene; SLC1A4; serine homeostasis; SPATCCM; neurodevelopment; neurodegeneration; Alzheimer's disease

1. Introduction

The *solute carrier family 1 member 4* gene (*SLC1A4*) encodes the neutral amino acid transporter, SLC1A4 (also called alanine-serine-cysteine transporter 1, ASCT1), which is involved in amino acid homeostasis [1,2]. It directs neutral amino acids, including serine, alanine, and cysteine, across cell membranes, which is necessary for maintaining neuronal function, synaptic transmission, and osmotic balance. In the brain, *SLC1A4* is expressed in astrocytes, with the highest levels found in the cerebral cortex, hippocampus, and cerebellum [3,4]. These brain regions are critical for cognition, memory formation, and motor control, making them fundamental to normal neurodevelopment. SLC1A4 activity ensures availability of L-serine, which is required for the production of the *N*-methyl-D-aspartate (NMDA) glutamate receptor co-agonists, glycine and D-serine, and it further modulates NMDA receptor activity by regulating synaptic levels of D-serine [3,5,6]. SLC1A4 is also expressed in peripheral tissues, such as the liver, kidney, skeletal muscle, and placenta, and has recently been identified in brain endothelial cells and shown to play a critical role in transport of L-serine across the blood–brain barrier (BBB) in mice [7,8]. Mutations in *SLC1A4* can disrupt transporter activity, resulting in altered amino acid transport for serine, alanine, cysteine, and threonine, deficiency of BBB-mediated L-serine transport and brain L-serine levels, and modified neurotransmitter precursor availability and neural signaling [3,9,10]. Reduced SLC1A4 levels or activity is associated with neurodegenerative

disorders and conditions characterized by metabolic stress, suggesting a possible role in the pathogenesis of several neurologic and neurodegenerative disorders [3]. This review focuses on the importance of serine metabolism in the brain and the emerging insights into the role of SLC1A4 in maintaining serine homeostasis in neurodevelopment and brain aging.

2. SLC1A4 and Serine Metabolism

Serine exists in two enantiomers: L-serine and D-serine. Both play important roles in a variety of metabolic processes. L-serine can be synthesized de novo, but most is derived from diet. It is found in a number of natural compounds, such as dairy products, legumes, fish, meat, and eggs (Figure 1). L-serine plays key roles in purine and pyrimidine synthesis and sphingolipid formation, and serves as a precursor to dopamine, glycine, and tryptophan in addition to D-serine (Figure 1a) [11]. L-serine has demonstrated neuroprotective properties by reducing reactive oxygen species, suppressing microglial activation, and decreasing the production of inflammatory mediators such as TNF-α, IL-6, and IL-1 in the brain [12–15]. Conversely, D-serine must be synthesized in the body. Accumulating evidence suggests that in the brain, D-serine is predominantly synthesized in neurons, supported by the observation that mice homozygous for neuron-specific knock-out of serine racemase (the enzyme that isomerizes L-serine into D-serine) have endogenous D-serine levels < 15% of that of controls [16–18]. Neuronal synthesis of D-serine utilizes L-serine produced in astrocytes by the enzyme 3-phosphoglycerate dehydrogenase (PHGDH), suggesting a serine shuttle mechanism between astrocytes and neurons [19–25]. Recent work also supports a role for SLC1A4 in the transport of L-serine across the BBB (Figure 1) [8].

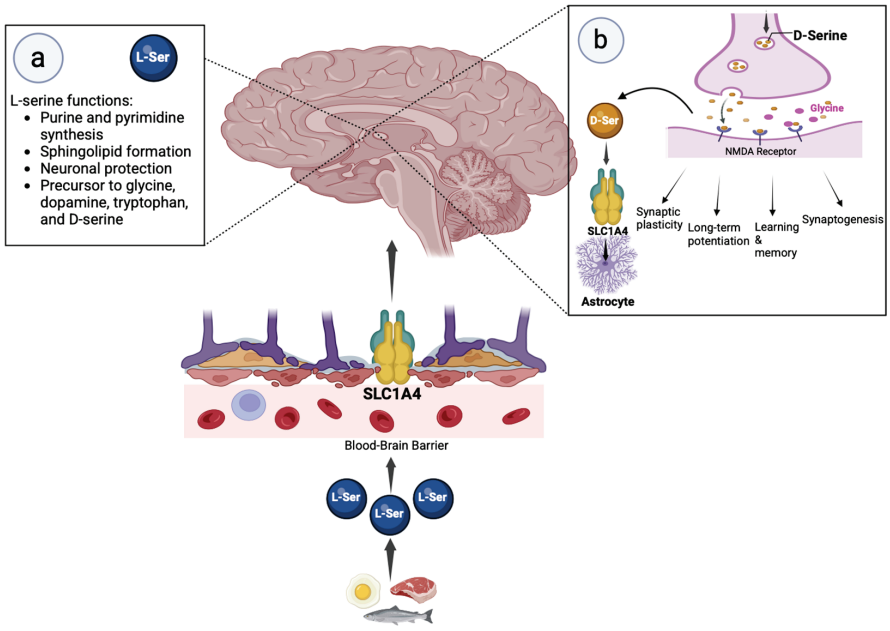


Figure 1. Serine transport into and function in the brain. L-serine is ingested via dietary sources such as meat, eggs, fish, and legumes and is transported into the brain across the BBB via SLC1A4 and other transporters. Panel (**a**) illustrates the key functions of L-serine (L-ser) in the brain and underscore its importance in both cellular metabolism and neurological health. L-serine is converted to D-serine (D-ser) by the enzyme serine racemase in neurons. Panel (**b**) illustrates D-serine secretion into the synaptic cleft where it functions as a co-agonist for NMDA receptors (right), serving as a critical component in synaptic plasticity, long-term potentiation, learning and memory, and synaptogenesis. SLC1A4, expressed on astrocytes (left), is the major uptake system to remove excess D-ser from the synaptic cleft. Not shown: astrocytic SLC1A4 can also heteroexchange L-ser for D-ser, providing neurons with the L-ser needed for D-ser synthesis.

D-serine functions as a neuromodulator in the central nervous system through its role as a co-agonist of NMDA receptors (Figure 1b) [26,27]. The activation of tetrameric NMDA receptors is triggered by the binding of synaptically released L-glutamate to GluN2 (or GluN3) subunits. Receptor activation also requires the binding of glycine (extrasynaptically) or D-serine (at synaptic NMDA receptors) to co-agonist sites on GluN1 subunits [27–29]. While the ambient extracellular concentration and relative contribution of each co-agonist appears to depend on age and brain region, evidence suggests that D-serine plays an equal if not greater role in the control of synaptic NMDA receptor signaling in adult cortices [26,27,30–32]. Membrane transport is likely to be critical in determining the ambient concentrations of NMDA receptor co-agonists. SLC1A4 is expressed by astrocytes and plays a major role in D-serine reuptake in the brain (Figure 1b) [5].

3. SLC1A4 in Neurodevelopment

SPATCCM

An important role for SLC1A4 in human brain development was revealed when recessive mutations in *SLC1A4* were identified in children with spastic tetraplegia, thin corpus callosum, and progressive microcephaly (SPATCCM) [9,33–40]. More recently, a dominant mutation was described that duplicates three amino acids (L86_M88dup), resulting in a dominant-negative N-glycosylation defect that reduces SLC1A4 plasma membrane localization and L-serine transport [41]. Primary clinical manifestations in these patients include intellectual disability, microcephaly, speech delay, hypotonia (which can range from mildly clumsy to wheelchair-bound), and movement disorders (Table 1). Patients may also show growth retardation and failure to thrive, behavioral abnormalities, ataxia, metabolic disturbances, and seizures. The variable severity of clinical phenotypes may reflect genetic modifiers, environmental factors, and/or epigenetic effects. The majority of SPATCCM patients have heterozygous consanguineous parents and most common mutations are point mutations that alter single amino acids critical for SLC1A4 function, including p.E256K and p.R457W, but loss-of-function mutations have also been identified, including a nonsense mutation (c.573T > G, p.Y191*) that most likely results in nonsense mediated decay and no production of SLC1A4 protein. Interestingly, p.E256K mutant SLC1A4 shows increased affinity for D-serine but a lower maximum velocity, consistent with saturation at a lower concentration [10]. To date, no mutations have been described that result in upregulation of *SLC1A4*.

Mouse models provide an opportunity to investigate the effects of specific genetic changes associated with human diseases and provide a better understanding of gene function. Two *Slc1a4* mouse models have been developed that, interestingly, show divergent phenotypes from each other and from human patients with SPATCCM (Table 1). *Slc1a4* knock-out mice were generated by the Knockout Mouse Project (KOMP) on a C57BL/6N background [42], while two knock-in models have been described that carry the p.E256K mutation described in several human SPATCCM patients: one generated using traditional gene-targeting approaches in 129B6 hybrid ES cells and backcrossed onto the C57BL/6J background for five generations [8] and another generated using CRISPR-Cas9 gene editing on a C57BL/6J background [10]. The knock-out model had lower levels of brain D-serine but higher levels of L-alanine, L-threonine, and glycine [3]. They also showed significantly decreased D-serine and L-serine transport across astrocytes, while the knock-in models demonstrated higher uptake of D-serine in homozygotes [8,10]. *Slc1a4* knock-out and p.E256K knock-in mice showed reduced striatal and hippocampal volumes, enlarged ventricles, and had reduced brain weight, but only p.E256K homozygotes displayed a thin corpus callosum (Table 1). *Slc1a4* knock-out mice displayed mild sensorimotor, behavioral, and learning deficits, showing poorer initial performance on the rotarod and in the Mor-

ris water maze but similar performance to controls at later timepoints, suggesting they may learn more slowly but retained information once it was learned. The two p.E256K models showed different behavioral phenotypes, perhaps reflecting differences in genetic background: at 5 months-of-age, the CRISPR-Cas9 generated mice showed no differences relative to control mice on the Barnes maze or rotarod but had reduced exploratory behavior in the novel object test, while 90 day-old homozygotes for the allele generated using a traditional gene targeting approach did display impaired performance on rotarod. Although patients with SPATCCM present with reduced comprehension and verbal skills, which may be related to impaired NMDAR signaling due to altered D-serine homeostasis, none of the *Slc1a4* mouse models reported to date show defects in long-term potentiation (LTP) in the hippocampus. Another difference in the clinical presentation of SPAATCC patients and the mouse models is seizure susceptibility (Table 1), although the fact that not all human patients have seizures suggest there may be modifier genes and/or environmental factors that influence the penetrance of this phenotype.

Table 1. Comparison of clinical phenotypes of SPATCC patients and *Slc1a4* mouse models.

Symptom	SPATCCM (Various *SLC1A4* Mutations)	*Slc1a4*$^{tm1e(KOMP)Wtsi}$ (Knock-out, C57BL/6N)	*Slc1a4*^{em2Tmg} (p.E256K, C57BL/6J)	*Slc1a4*^{tmColumbia} (p.E256K, B6;129B6)	*Slc1a4*$^{fl/fl}$; *Tg.Tie2-cre* (Endothelial Knock-out)	*Slc1a4*$^{p.E256K}$ + Maternal Serine Supplementation
Intellectual Disability	+	+/−	−	+	+	Improved
Microcephaly	+	+	+	+	+	Improved
Thin Corpus Callosum	+	N/A	+	+	N/A	N/A
Seizures	+/−	N/A	−	N/A	N/A	N/A
Impaired Motor Coordination	+	+	−	+	+	Improved
Growth Retardation	+	−	−	−	−	−
Ataxia	+	−	−	N/A	N/A	N/A
Metabolic Disturbances	+	+	N/A	+	+	+/−
Altered Serine Uptake	+	+	+	+	+	Normalized

Legend: present (+), absent (−), variable (+/−), not explicitly reported (N/A).

A key finding from mouse model studies is that SLC1A4 is expressed in brain endothelial cells and plays a significant role in transporting L-serine across the BBB, especially developmentally [8]. Mice homozygous for the *Slc1a4* knock-out or the p.E256K mutation demonstrated a reduced influx of serine through the BBB, and wildtype mice treated with a SLC1A4-specific inhibitor that can act on vascular cells but does not cross the BBB displayed a significant decrease in brain L-serine, whereas knock-out mice treated with the drug showed no change. Furthermore, conditional deletion of *Slc1a4* in *Tie2*-expressing endothelial cells recapitulated many of the *Slc1a4* mutant phenotypes. Consistent with a deficiency of BBB-mediated L-serine transport in the mutant mice, doubling oral intake of L-serine of mothers, starting two days before pups were born through to postnatal day 11 resulted in improved brain weight and motor performance in p.E256K homozygous mutant offspring. Brain L-serine levels were increased but, somewhat surprisingly, there was no effect on brain D-serine levels. Taken together, these data suggest that the clinical phenotypes of SPATCCM patients may also be predominantly due to defective transport of L-serine across the BBB and that serine supplementation may be beneficial, although it is unclear at what developmental stage supplementation would need to be initiated to have a significant impact.

The differences in the clinical presentation of human SPATCCM patients and mice homozygous for knock-out or knock-in mutations in *Slc1a4*, summarized in Table 1, suggest there may be species-specific compensatory mechanisms, perhaps reflecting differences in serine transport and metabolism. In mice, alternative pathways or regulatory networks might buffer the effects of *Slc1a4* mutations, maintaining partial D-serine regulation and mitigating more severe phenotypes observed in human SPATCCM patients. These mechanisms could include differences in the expression patterns of other amino acid transporters, enzyme activity, or alternative metabolic pathways that compensate for disrupted SLC1A4 function. Genetic or environmental factors may also modify the penetrance of SPATCCM phenotypes. Another consideration is species-specific differences in NMDA receptor subunit composition and D-serine regulation, particularly between rodents and humans, which influence behaviors, synaptic function, and responses to neurological disorders [43]. Further investigation into the cause of the species differences in phenotype associated with impaired SLC1A4 function could reveal novel therapeutic approaches. The limitations these differences impose must also be taken into consideration when using the mouse models to test treatment strategies for SPATCCM.

4. Neuropsychiatric Disorders

4.1. Schizophrenia

Schizophrenia is a mental disorder distinguished by symptoms of disordered thoughts, delusions, and hallucinations. It has been proposed that glutamatergic dysfunction underlies its pathogenesis [44–46]. This hypothesis is supported by the significant association of schizophrenia with glutamate receptor genes and genes related to glutamatergic neurotransmission [47,48], as well as the observation that exposing healthy individuals to drugs like phencyclidine (PCP) or ketamine, which block NMDA receptors, produces behaviors resembling the symptoms and cognitive deficits of schizophrenia, while treating individuals with schizophrenia with these drugs exacerbates their symptoms [49,50]. Deng and colleagues [44] assessed whether genes involved in glutamatergic transmission, including *SLC1A4,* were associated with schizophrenia using 100 Japanese case–control pairs. Although they found nominally significant associations between two single nucleotide polymorphisms (SNPs) in *SLC1A4,* the association did not survive controlling for the false discovery rate at level 0.05 and they concluded that this gene is not a major susceptibility locus in the Japanese population. Another study assessing a German population also failed to detect an association between *SLC1A4* and schizophrenia [51], while earlier studies that mapped schizophrenia susceptibility to the same chromosomal region as *SLC1A4* had failed to identify any variants within SLC1A4 that segregated with schizophrenia [47,48]. Studies have reported low serum D-serum levels in people with schizophrenia, however (see Cho et al., 2016 for a meta-analysis of 20 studies [52]), and although studies have failed to identify a genetic association between SLC1A4 and schizophrenia, *SLC1A4* was identified as a gene with dysregulated expression in the cortical brains of individuals affected with schizophrenia [53], suggesting that it may be involved in disease pathogenesis but acts downstream of causative genes.

4.2. Depression

Major depressive disorder (MDD; depression) is a common chronic disease with mental, emotional, and physical health effects [54]. Symptoms include sadness, low self-esteem, sleep disturbance, tiredness, attention deficit, and reduced motivation or ability to experience pleasure, and patients frequently show intention to self-harm or commit suicide. Altered serine metabolism has been implicated in the pathophysiology of MDD through its role in modulating NMDA receptor-mediated neurotransmission, and D-serine

and the NMDA antagonist ketamine have been shown to have anti-depressive effects in rodent models of depression [55–57] and clinical trials with human patients [58–65]. Furthermore, an integrative gene set-based GWAS meta-analysis of case–control studies of MDD revealed statistical enrichment for variants in genes involved in glutamatergic synaptic neurotransmission, including *SLC1A4* [66], and reduced SLC1A4 expression has been reported in the anterior cingulate cortex and hippocampus of patients with MDD [67]. Altered SLC1A4 levels or activity would lead to impaired inhibitory control of glutamatergic neurotransmission and result in hyperexcitability of cortical neurons, a phenomenon linked to disrupted affective processing and observed in MDD (reviewed in [68]). While studies report conflicting data on whether L- and D-serine levels are elevated in the serum or CSF of MDD patients compared to healthy controls, CSF D-serine concentration correlated with MDD severity (based on the Hamilton Depression Rating Scale) and baseline D-serine plasma concentration may predict ketamine responsiveness [69–71].

5. SLC1A4 and Serine Metabolism in Aging and Neurodegeneration

5.1. Normal Aging

Aging is characterized by a decline in various brain functions, including cognitive performance and neuronal plasticity [72,73]. Proper amino acid transport is essential for maintaining neuronal health and supporting neurotransmitter systems, including NMDA receptor signaling. During normal aging, there is reduced expression of serine racemase, leading to lower levels of D-serine and decreased NMDA receptor activity, and this down-regulation is believed to impair synaptic plasticity, resulting in deficits in learning and memory [43,73]. As discussed earlier in this review, SLC1A4 plays a crucial role in transporting neutral amino acids, which are critical for maintaining neurotransmitter balance and neuronal health. D-serine is a co-agonist of NMDA receptors, which are essential for synaptic plasticity and memory formation. Dysregulation of NMDA receptor activity has been implicated in age-related cognitive decline as well as AD [74]. Activation of NMDA receptors depends on the co-agonist site being occupied, consistent with the idea that endogenous D-serine levels significantly influence neuronal plasticity, both during development and in the mature brain [5]. Since SLC1A4-mediated reuptake of D-serine into astrocytes plays an important role in regulating synaptic D-serine levels, SLC1A4 levels and activity affect age-related cognitive decline and AD. The observations that plasma D-serine levels decrease with age and D-serine supplementation can reverse age-related cognitive decline in rats has led to the hypothesis that cognitive decline in older individuals may be associated with a deficiency of this critical co-agonist [75]. As discussed in the next sections, several studies have explored the relationship between serine levels and neurodegenerative diseases as well as the possible use of serine supplementation in disease management.

5.2. Alzheimer's Disease

Alzheimer's disease (AD) is the most common cause of late-onset dementia, affecting nearly 7 million Americans aged 65 and older [76]. While reduced D-serine levels have been linked to cognitive decline associated with normal aging, studies report conflicting results on D-serine levels in the brains of individuals with AD and AD mouse models. One study reported reduced levels of PHGDH in hippocampal astrocytes in AD brains as well as in a 3xTg transgenic mouse model that expresses three mutations associated with familial AD [77]. Consistent with PHGDH's role in catalyzing the first rate-limiting step in converting glucose to L-serine, both L- and D-serine levels were reduced in the brains of 3xTg mice; however, levels were not assessed in AD brain tissue. In the mice,

recordings of the NMDA receptor component of field excitatory post-synaptic potentials in acute hippocampal slices indicated a lower level of co-agonist binding site occupancy in 3xTg mice relative to controls. Conversely, elevated levels of D-serine have been detected in serum and CSF from AD patients compared to healthy controls [78–80]. Furthermore, SLC1A4 and D-serine levels were increased in the brains of two other mouse AD models. In 5xFAD transgenic mice, which overexpress mutant human amyloid precursor protein harboring five familial AD (FAD) mutations as well as human presenilin-1 harboring two FAD mutations, SLC1A4 levels were twice as high as in wildtype controls, as were cortical levels of serine [81]. Similarly, the brains of 12-month-old knock-in mice expressing human amyloid precursor protein harboring four FAD mutations also showed elevated levels of SLC1A4 and D-serine [82]. Taken together, these observations underscore the importance of astrocytes in serine metabolism and suggest that altered SLC1A4 activity could contribute to AD by impacting the availability of D-serine on NMDA receptors. It is unclear what triggers the increase in SLC1A4 observed in the brains of mouse AD models, or whether a similar increase occurs in human AD brains. The conflicting observations on D-serine levels in biofluids and PHGDH levels in AD brains, and in the brains of different mouse AD models, indicate that further studies of human AD brain tissue are needed. The dichotomy if D-serine levels decrease in the brain with normal aging but increase in AD brains could be explained by the fact that NMDA receptor hypo- and hyperactivity can both lead to synaptic dysfunction, neurotoxicity, and cognitive dysfunction [72,83].

5.3. Parkinson's Disease

Parkinson's disease (PD) is a progressive neurological condition characterized by motor symptoms, constipation, depression, memory loss, and difficulty swallowing and speaking [84]. It is associated with the degeneration of neurons in the brain that produce dopamine and norepinephrine. Altered NMDA receptor neurotransmission has been observed in the striatum of PD patients and is thought to contribute to the onset and progression of PD symptoms [85–87]. Disrupted serine homeostasis is further implicated in PD pathogenesis by several studies. One reported that, although there were no differences in total serum levels of D- or L-serine between PD patients and healthy controls, changes in serum D-serine levels correlated with age and age-of-onset in PD patients [14]. Another study demonstrated a significant reduction in SLC1A4 and a significant increase in D-serine levels in post-mortem caudate putamen samples from PD patients compared to healthy controls [88] and that D-serine was significantly increased in CSF of living PD patients. However, similar to the contradictory results reported for D-serine levels in AD, other studies have reported lower D-serine in the substantia nigra and cerebrospinal fluid of untreated PD patients [89]. These studies suggest a link between SLC1A4, serine homeostasis, and PD, but the underlying mechanisms remain unclear. Further evidence that serine levels are important in PD pathogenesis comes from studies examining serine supplementation as a treatment for PD, which will be discussed below.

5.4. Traumatic Brain Injury

Traumatic brain injury (TBI) is a form of acquired brain injury resulting from sudden trauma to the brain that disrupts homeostasis, with severity ranging from mild concussion to severe trauma with acute or chronic cognitive dysfunction [90]. Brain trauma is associated with neuroinflammation and a widespread gliotic response, involving alterations in astrocyte and microglial morphology and the proteins they express [91,92]. One of these changes includes an increase in D-serine production, associated with a switch in its production from neurons to astrocytes, which is thought to contribute to synaptic damage and dysfunction [93]. One study demonstrated that SLC1A4 was up-regulated in the

cortex of mice following TBI induced by controlled cortical impact, and treatment with a noncompetitive substrate inhibitor with high selectivity and potency for SLC1A4 or with genetic ablation of *Slc1a4* in astrocytes or microglia was protective [94]. That study also demonstrated up-regulation of SLC1A4 mRNA and protein in peri-lesional tissues resected from human TBI patients relative to cortical control specimens, further linking SLC1A4 and D-serine to TBI pathology.

6. Serine Supplementation

Given the connections between brain serine levels and cognition, as well as with multiple neurodevelopmental and neurodegenerative diseases, there is increasing interest in the therapeutic potential of serine supplementation. There are competing trains of thought regarding the safety and benefits of L-serine or D-serine supplements. L-serine is currently FDA approved, while D-serine is not. Moreover, L-serine is a neurotrophic factor, and its neuroprotective properties are thought to be superior to those of D-serine, the latter having more potential for possible side effects related to NMDA receptor-mediated excitotoxicity. The likelihood of encountering this undesirable side effect increases in patients with mutations that affect D-serine transport, whereas excess L-serine does not appear to be associated with adverse effects [15]. L-serine also plays important roles in the proliferation and survival of neural stem cells as well as microglial activation and cytokine production, processes central to protection against and recovery from nervous system injury and disease (reviewed in [12]). On the other hand, L-serine must be converted into D-serine to achieve the desired therapeutic outcome, and the pathway responsible for this process may be altered in some patients, which could weaken the efficacy of L-serine supplementation [15]. Below, we summarize the therapeutic potential of serine supplementation for the treatment or management of neurodevelopmental and neurodegenerative disorders associated with perturbed SLC1A4 function and/or serine homeostasis.

Serine supplementation has been suggested as a treatment for SPATCCM patients, with the caveat that it is unclear whether treatment started after diagnosis would be too late to have a beneficial effect [33,35,37,40]. Mouse models have provided some insight into this issue. Pregnant dams carrying *Slc1a4*-p.E256K homozygous pups were supplemented with L-serine in their drinking water starting 2–3 days before pups were born, and brain weight, ultrasonic vocalizations, and motor performance of the offspring were assessed on postnatal day 11 [8]. All three parameters were normalized and associated with increased levels of brain L-serine, but not D-serine. The latter observation is surprising, given that D-serine is synthesized from L-serine, but suggests that the phenotypes assessed specifically reflect a deficiency in brain L-serine. Further studies are needed to determine whether there are any beneficial effects of treatment at later stages, corresponding to the mouse equivalent of the age at which human SPATCCM patients are typically diagnosed, and whether individuals with null mutations (i.e., that lack SLC1A4 expression altogether) benefit from dietary serine supplementation.

Augmentation of antipsychotic treatments with NMDA receptor modulators, including D-serine, improved multiple schizophrenia symptoms, particularly negative ones, with satisfactory side effects and safety profile (see Goh et al., 2021 for meta-analysis of 40 trials involving 4937 patients with schizophrenia [95]). More recently, a double-blind, placebo-controlled study demonstrated a beneficial effect of D-serine supplementation in combination with auditory cognitive remediation treatment, with a dose-dependent improvement in plasticity [96]. These findings underscore the critical role of NMDA receptor function in schizophrenia and highlight D-serine supplementation as a promising therapeutic avenue to enhance neural plasticity and potentially mitigate disease symptoms. In fact, D-serine supplementation may be beneficial for a broad range of psychiatric disor-

ders as many are associated with altered signaling through NMDA receptors, including MDD [97,98]. The role of D-serine in depression is supported by studies in mice. Serine racemase knock-out mice have dramatically reduced D-serine levels and exhibited elevated levels of anxiety and reduced cognitive function [16]. Transgenic over-expression of serine racemase in astrocytes and acute or long-term administration of D-serine resulted in elevated brain D-serine levels and was associated with behavioral phenotypes similar to those observed in mice treated with anti-depressant drugs [55,56].

Oral serine supplementation has also been suggested as a "ready to use therapy for AD" [77]. In 3xTg AD mice, reduced levels of astrocytic PHGDH and hippocampal L-serine and D-serine correlated with impaired synaptic plasticity and deficits in learning and memory. Oral supplementation with D-serine in drinking water at levels known to increase D-serine in the brain rescued the cognitive deficits of 6- to 7-month-old female 3xTg-AD mice [77]. This study also showed that dietary L-serine supplementation restored D-serine levels, improved NMDA receptor activity, and protected against synaptic and cognitive deficits. These observations suggest that L-serine supplementation is as effective as D-serine in counteracting the metabolic deficiencies caused by AD and in supporting essential neuronal functions, an important finding since L-serine supplements are FDA approved and safe. However, further clinical research is needed to confirm these results in human patients and evaluate the long-term safety and efficacy of L-serine supplementation in treating AD. Furthermore, as described earlier in this review, there is conflicting data on D-serine and PDGDH levels in AD patients. Given the discrepancies that have been reported, it is not surprising that other studies have cautioned against the use of serine as a therapy for AD [99]. Moving forward, further research is needed to better understand the role of PHGDH and serine metabolism in AD progression and to inform therapeutic strategies.

PD is currently treated with levodopa (L-DOPA) supplementation, but chronic use is associated with a multitude of side effects, including deficits in motor function as well as L-DOPA-induced dyskinesia [84], and its effectiveness wanes over time. Thus, new or combinatorial therapeutic approaches are still much needed. A double-blind, placebo-controlled study conducted over a 6-week period suggested a positive correlation between a 30 mg/kg per day dose of D-serine and the relief of motor and extrapyramidal symptoms in thirteen PD patients [100]. Further studies are needed to replicate the results in a larger cohort, determine optimal dose, and test whether L-serine replicates the effects, but these preliminary findings hint at potential benefits of serine supplementation to PD patients.

7. Conclusions

While the importance of D-serine in neurotransmission and synaptic plasticity is well established, the neuroprotective and neurorestorative effects of D-serine supplementation to treat neurodevelopmental and neurodegenerative disorders remain somewhat controversial, perhaps reflecting the fact that too much or too little NMDA receptor signaling can be detrimental to brain health. Given the role of astrocytic SLC1A4 in mediating the exchange of intracellular L-serine for extracellular D-serine, this transporter may represent a key therapeutic target for maintaining serine homeostasis in the brain. While L- or D-serine supplementation may be beneficial in cases where D-serine levels are reduced, it may also be possible to reduce SLC1A4 activity using selective, high-affinity inhibitors such as the alkoxy hydroxy-pyrrolidine carboxylic acid compounds described by Lyda et al. [101]. These inhibitors pharmacologically modulate transporter function and, if they cross the BBB, could be used to treat AD, TBI, and other brain disorders associated with NMDA-mediated excitotoxicity due to increased SLC1A4-mediated D-serine efflux from inflammatory astrocytes. Furthermore, discovering pathways that regulate SLC1A4

levels and activity may reveal ways to up-regulate it to treat diseases associated with elevated extra-synaptic D-serine, although further studies are needed to assess potential side-effects.

Author Contributions: All authors contributed to writing the original draft, review, and editing; visualization, D.E. and T.M.G. All authors have read and agreed to the published version of the manuscript.

Funding: The APC was funded by Touro University College of Osteopathic Medicine—Montana and the McLaughlin Research Institute.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Figure 1 was created with BioRender.com version 2025.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

AD	Alzheimer's disease
ASCT1	Alanine-serine-cysteine transporter 1
BBB	Blood–Brain Barrier
CSF	Cerebrospinal fluid
D-ser	Dextro (D)-serine
FAD	Familial Alzheimer's Disease
GluN2	Glutamate ionotrophic receptor NMDA type subunit 2
GluN3	Glutamate ionotrophic receptor NMDA type subunit 3
KOMP	Knockout Mouse Project
L-DOPA	Levodopa
L-ser	Levo (L)-serine
LTP	Long-term potentiation
MDD	Major Depressive Disorder
NMDA	*N*-methyl-D-aspartate
PD	Parkinson's disease
PCP	Phencyclidine
PHGDH	Phosphoglycerate dehydrogenase
SLC1A4	*Solute carrier family 1 member 4 gene*
SLC1A4	Solute carrier family 1 member 4 protein
SNP	Single nucleotide polymorphism
SPATCCM	Spastic tetraplegia, thin corpus callosum and progressive microcephaly
TBI	Traumatic Brain Injury

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International Journal of
Molecular Sciences

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Review

The Biomarker Profile of Alzheimer's Disease for Disease-Modifying Treatment Eligibility: Questions and Debates

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Abstract

Alzheimer's disease (AD) is the most common cause of cognitive decline; currently, anti-amyloid monoclonal antibodies are available for clinical use as disease-modifying treatments, while many other substances are being tested in clinical trials. Molecular biomarkers for AD have been studied for more than two decades, and various guidelines and diagnostic recommendations have been published. However, there are still questions and controversies about the biomarker profile needed to confirm AD and the eligibility for such established treatments and clinical trials. Is amyloid positivity sufficient for eligibility, or is a biomarker for tau biochemistry/pathology also needed? What is the role of hybrid ratios combining amyloid and tau? Should we rely on plasma biomarkers alone? This review aimed to describe and discuss such questions and controversies.

Keywords: Alzheimer's disease; biomarkers; amyloid beta; tau protein; phospho-tau protein; lecanemab; donanemab; disease modifying treatments

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and cause of cognitive decline [1]. It accounts for ~60–70% of cases; by 2030, patients with AD are expected to reach 75 million worldwide, posing a major health and socioeconomic problem [2]. The main biochemical and pathological characteristics of AD include the extracellular polymerization of amyloid beta peptide with 42 amino acids ($A\beta_{42}$) forming amyloid plaques [3] and the intracellular polymerization of hyperphosphorylated tau protein in the form of paired helical filaments evolving to neurofibrillary tangles [4]. The interplay between the two converging mechanisms [5] leads to AD [6].

Until a few years ago, AD therapeutics were based on symptomatic treatments introduced ~25 years ago [7]. Since the amyloid pathway is an early and key pathogenic

mechanism, as well as a potential therapeutic target [8], anti-amyloid monoclonal antibodies have been recently introduced in AD therapeutics as the first disease-modifying treatments [7–10]. Currently, Lecanemab [11] and Donanemab [12] are the two anti-amyloid antibodies fully approved in the USA by the Food and Drug Administration and in Europe by the European Medicines Agency. They are approved for use in early AD, i.e., mild cognitive impairment (MCI) or mild dementia due to AD; appropriate use recommendations thought to be useful in clinical practice [13] have been published from authorities in various states, including the U.S.A. [14,15], France [16], Canada [17], and South Korea [18].

But how is AD diagnosed during life? It has been observed that clinical diagnosis may be accompanied by a high clinicopathological concordance (>90%) when typical cases without comorbidities, fulfilling even the older probable criteria of AD [19], are examined by specialists in tertiary/academic centers [20]. However, this is not always true; in early or atypical cases, in the presence of comorbidities, and in the community (approach by non-experts), diagnostic accuracy may decrease to <75% [21]. In general, it has been estimated that ~25–30% of patients clinically diagnosed with AD will suffer from a non-AD disorder at post-mortem pathological examination [22]. On the other hand, post-mortem examination will reveal the presence of AD (co)pathology in ~39% of cases of patients with a clinical diagnosis of non-AD disorder [23]. Thus, it is not surprising that the chance of in vivo misdiagnosis was ~1/3 in a more recent pathologically confirmed study from an academic center [24]. The newer disease-modifying anti-amyloid antibodies are (a) effective in cases with early AD, i.e., exactly in the population where clinical diagnostic accuracy may be relatively lower, and (b) not free of safety issues, including amyloid-related imaging abnormality (ARIA) [11,12,25]. Thus, the maximal possible diagnostic accuracy should be ensured before considering their administration. Over the last 25 years, classical cerebrospinal fluid (CSF) [26,27] and molecular biomarkers visualized via positron emission tomography (PET) [28] have become the gold standard in vivo diagnosis of AD. They have been incorporated into diagnostic criteria and guidelines [29–34], resulting in pathologically verified improvement of in vivo diagnostic accuracy [24]. Research on biomarker-based diagnosis has intensified in the context of anti-amyloid treatment approval [35–37].

There are many extensive, comprehensive, and very informative reviews and recommendations on the use of various biomarkers for AD, many of which are cited in this study. A search gave a total of 886, 626, and 1602 results for CSF [38], PET [39], and blood-based biomarkers [40], respectively. However, despite improved diagnostic abilities [37], there are still controversial issues and debates among various authorities that publish diagnostic and classification criteria for AD [41–45]. The aim of this work was not to present just another review but to present some critical points and questions related to the molecular biomarker-based approach in diagnosing AD and identifying patients eligible for approved or investigational disease-modifying treatments.

2. Classical Biomarkers with Molecular Specificity for AD

Classical biomarkers with molecular specificity for AD are related to the two fundamental biochemical and pathological hallmarks of AD: Aβ formation and deposition in amyloid plaques and tau hyperphosphorylation and the formation of paired helical filaments and neurofibrillary tangles.

2.1. CSF Markers

$A\beta_{42}$ levels are decreased in the CSF of patients with AD, inversely related to amyloid plaque burden [46]. Amyloid beta peptide with 40 amino acids ($A\beta_{40}$) can also be determined, and the $A\beta_{42}/A\beta_{40}$ ratio may be a better indicator of amyloid plaque formation [47],

being preferable to $A\beta_{42}$ alone [48]. Thus, $A\beta_{42}/A\beta_{40}$ shows molecular specificity for the amyloid component of AD.

Increased levels of hyperphosphorylated tau protein are considered a marker of the tauopathic component of AD [49]. Tau hyperphosphorylated at a threonine residue at position 181 ($\tau_{P\text{-}181}$) is the most widely used form; however, other hyperphosphorylated tau forms can be quantified in the CSF, including $\tau_{P\text{-}217}$ and $\tau_{P\text{-}231}$ [50], with $\tau_{P\text{-}217}$ having some possible advantages over $\tau_{P\text{-}181}$ [51]. All of the above hyperphosphorylated tau forms become abnormal early in the disease process, when tau molecules and small aggregates are still soluble (before neurofibrillary tangle formation), as a result of the amyloid cascade, due to amyloid-triggered activation of kinases such as cyclin-dependent kinase-5 (CDK-5), glycogen synthase kinase-3β (GSK-3β), and mitogen-activated protein kinases (MAPKs) [52,53]. Thus, these forms may be viewed as markers of the amyloid-triggered initiation of the tauopathic process and as "indirect markers" of amyloidogenesis. In this context, CSF $\tau_{P\text{-}217}$ (especially the ratio of the phosphorylated to total form) may be a slightly better indicator of amylogenesis than the $A\beta_{42}/A\beta_{40}$ ratio [54]. Recently, CSF $\tau_{P\text{-}212}$, which may be related to the action of dual-specificity tyrosine phosphorylation-regulated kinase 1A, was found to be increased early in the course of AD, either sporadic or due to Down syndrome [55].

As tauopathy progresses, tau aggregates become insoluble, and tangle pathology emerges. At this time, other tau forms increase in the CSF, including the tau microtubule-binding region containing residue 243 (MDBR243) [56] and $\tau_{P\text{-}205}$ [57], which enables AD staging [58].

2.2. PET Markers

PET for Aβ with various tracers, such as florbetaben, florbetapir, and flutemetamol, provides imaging evidence of amyloid deposition [59]. It can be interpreted visually or by Standardized Uptake Value Ratios (SUVRs) or centiloids for detecting neuritic plaque load corresponding to Thal phases 3–5 [60]. Thus, PET for Aβ generally correlates with the Thal phases; however, it cannot detect the very early phases of amyloidogenesis and early Thal phases, and a centiloid level of at least 28–30 should be reached for diagnostic purposes [61].

On the other hand, PET for tau deposition (neurofibrillary tangle formation) generally follows Braak stages, usually requiring a Braak stage of at least III [62], whilst for diagnostic purposes, ^{18}F-flortaucipir PET detects stages $\geq$ IV [63]. The severity of tau PET abnormality correlates positively with the AD progression rate [64]. Furthermore, while in PET for Aβ, the amyloid distribution pattern is usually similar in all AD patients, the tau load distribution in tau PET is not the same and may be either typical (as expected by Braak staging) or atypical, showing a posterior, left predominant, or hippocampal sparing pattern [65]. Thus, PET for tau deposition may correlate with both the clinical picture (typical amnestic or atypical presentations) and AD severity.

3. Other Biomarkers Typically Used

3.1. Markers of Neurodegeneration

Cerebrospinal fluid τ_T [66,67] and NfL [68] are long-known markers of neuronal/axonal degeneration, and their levels are usually increased in AD [4]. However, they are not specific for AD and may be increased in other neurodegenerative disorders [69].

Positron emission tomography (PET) with 18F-fluoro-deoxy-glucose (FDG) can reveal hypometabolic brain areas. In AD, hypometabolism occurs typically in the parietotemporal association cortices, posterior cingulate cortex, and precuneus [70], and this helps in the differential diagnosis from other neurodegenerative disorders [33]. However, deviations

from the typical topography occur in atypical clinical presentations of AD, posing difficulties in the diagnostic process, for example, between frontotemporal dementias and the frontal variant of AD [70,71]. Single-Photon Emission Computed Tomography (SPECT) with ^{99m}Tc-Hexamethylpropylen-amine Oxime (HMPAO) for assessing hypoperfusion may be a useful alternative, depending on availability issues [72]. Recently, 3-Tesla Magnetic Resonance Imaging (3T MRI) with arterial spin labeling (ASL) quantifying cerebral blood flow has emerged as another alternative [73], more affordable, and radiation-free imaging method, suitable for longitudinal follow-up and with a diagnostic accuracy that may be comparable to that of PET [74].

Brain atrophy observed in MRI or even CT is another useful marker of neurodegeneration, and the topography may be helpful in the (differential) diagnosis of AD. Volumetry may have some advantages [75], but visual scales assessing the atrophy of hippocampal formation, such as the long-known Medial Temporal Atrophy (MTA) scale [76] or, more recently, the Entorhinal Cortex Atrophy (ERICA) scale [77,78], are easy-to-use tools for everyday practice.

It is worth noting that the term neurodegeneration encompasses many components, including neuronal and/or axonal and/or neuropil dysfunction/loss, hypometabolism and hypoperfusion, and central and cortical atrophy [41]. Therefore, the above markers are not equivalent and may show different patterns of evolution during the disease process, with atrophy usually being the last to emerge [79].

3.2. Markers of Other Primary Co-Pathologies

For vascular co-pathology, MRI or CT are the usual imaging methods. In case of large vessels and/or lacunar infarcts, the vascular nature of the lesions is easily recognized. However, white matter hyperintensities should not always be attributed to cerebral small vessel disease in AD. It has been observed that anterior (frontal) white matter lesions may be associated with both vascular pathology and AD neurodegenerative changes (tau-related axonal loss and Aβ burden) [80,81]; thus, they may be related to mixed cognitive impairment (AD + vascular). On the contrary, posterior (parietal) periventricular lesions are related to cortical AD pathology [82] and, if associated with hippocampal and temporal atrophy, may even serve as early biomarkers of AD [81]. A juxtacortical multisport pattern of white matter MRI hyperintensities and lobar microbleeds points to cerebral amyloid angiopathy [81,83].

Various methods of assessing CSF α-synuclein (α-syn) have been tested [84–86], but the seeding amplification assay is the most promising [87–89]. PET for α-syn has also been studied [28]. Similarly, CSF and PET assessments of transactive-response DNA-binding protein-43 (TDP-43) have been studied [90,91], but it is unknown whether they can be used effectively to identify TDP-43 co-pathology in patients with AD [92]. TDP-43 accumulation is the hallmark of limbic predominant age-related TDP-43 encephalopathy (LATE), an entity characterized by AD-like symptoms mainly in patients over 80 years old, with distinct neuropathological stages initiated by the amygdala [91]. Co-pathology identification is significant since TDP-43 inclusions can occur up to 50% in AD and may be closely related to the disease's pathogenesis and prognosis [91,92].

4. Plasma Biomarkers

The above-described biomarkers are either CSF-based (requiring lumbar puncture, a generally safe but minimally to moderately invasive procedure) or imaging-based (mainly PET). Blood-based (plasma) biomarkers have received significant attention recently due to their noninvasiveness and reduced costs compared to CSF and PET biomarkers [93,94].

Plasma $A\beta_{42}/A\beta_{40}$ ratio [95,96], plasma $\tau_{P\text{-}181}$ [97,98], plasma $\tau_{P\text{-}231}$ [99], plasma $\tau_{P\text{-}212}$ [55], and especially plasma $\tau_{P\text{-}217}$ [95,96,99–102] are early markers of AD; as in CSF, the above phosphorylated tau forms are good indicators of not only amyloid-triggered tau hyperphosphorylation but also amyloidogenesis [95,96,99], with a sensitivity comparable to that of CSF Aβ markers and better than CSF $\tau_{P\text{-}181}$ [103]. It has been suggested that plasma levels of the endogenous cleaved tau microtubule-binding region containing residue 243 (eMBDR-tau243) increase when tangle pathology appears [104].

Plasma levels of NfL serve as a nonspecific marker of neurodegeneration in AD [105]. Therefore, NfL assessment could serve as a valuable prognostic marker in AD, considering its association with brain atrophy, especially if it were feasible to acquire repetitive values throughout the course of the disease [105].

Neuroinflammation markers, such as Glial Fibrillary Acidic Protein (GFAP), may be assessed in plasma [106], and it has been observed that plasma GFAP levels may perform diagnostically better than CSF levels [107]. Hence, the response of plasma GFAP levels to monoclonal antibodies has been studied in crucial randomized trials [11,12], and there is evidence that their increase, as a result of reactive astrocytosis, could accompany much earlier stages of AD pathology. More specifically, findings that contain uncertainty suggest a relation to the amyloid cascade, even in the preclinical phase of the disease [106,107].

5. Alternative Biomarkers

Some biomarkers have been introduced into research relatively recently or have been long known to have special advantages in studying various aspects of AD pathology [26]. They could have the potential to be helpful either clinically or to study (in clinical trials) the possible benefits of new treatments in aspects other than Aβ or tau proteinopathy.

5.1. Biomarkers of Amyloidopathy or Tauopathy

Soluble Aβ oligomers, formed very early in the amyloid cascade process, are thought to be neurotoxic through their ligand-like action [108]. They can be quantified in CSF [109] and plasma [110] and have the potential to offer very early diagnosis [111]. Recently, a portable apparatus detecting Aβ oligomers has been tested, promising easy diagnosis with low costs, even at the site of primary care [112]. In a similar context, CSF quantification of tau and α-syn oligomers is also being tested as a diagnostic tool, possibly useful in the differential diagnosis of dementia-related and Parkinsonian neurodegenerative disorders [113].

In addition to the only approved tracer for PET, ^{18}F-flortaucipir, other tracers are being tested [114], and there is evidence that some may outperform flortaucipir, showing increased selectivity for affected brain areas [115].

5.2. Biomarkers of Microglia Activation and Other Parameters of Neuroinflammation

Microglia are activated early in Alzheimer's disease in an attempt to remove aggregating Aβ but with ongoing increases in Aβ burden, impairment in microglial phagocytic activity, and chronic dysregulated neuroinflammation occur, leading to neurodegeneration [116]. However, this phenomenon is nonspecific and may occur in many other neurodegenerative conditions. Triggering Receptor Expressed on Myeloid cells 2 (TREM2) is a marker of microglia activation, which has received much attention recently due to its involvement in AD mechanisms, its potential as a diagnostic marker, and its role as a possible therapeutic target [106]. The CSF levels of soluble TREM2 (sTREM2) are increased in early AD [117,118] and could be related to discrepancies between $A\beta_{42}$ levels and the $\tau_{P\text{-}217}/A\beta_{42}$ ratio [119]. sTREM2 can also be measured in plasma [120] and may be associated with concomitant

cerebrovascular disease [121]. However, abnormal levels of sTREM2 may be observed in other neurodegenerative disorders [122].

CSF or plasma levels of chitinase 3-like protein 1 (YKL-40) [123–125] and numerous other markers, including Interleukin-1β (IL-1β), Tumor Necrosis Factor-alpha (TNF-α), and S100, involved in the interplay between microglia, astrocytes, and inflammation, are being extensively studied both as diagnostic biomarkers and possible targets of individualized therapies [126–128].

Recently, CSF levels of soluble α-Klotho (sαKl), a protein involved in antioxidant and neuroprotective mechanisms, have been reported to be lower in dementia due to AD compared to controls [129].

5.3. Biomarkers of Synaptic Loss

Synaptic dysfunction and loss are integral parts of Alzheimer's neuropathology [130]. Among other molecules, Synaptotagmin (SYT-1) [131], Synaptosomal-Associated Protein 25 (SNAP-25) [132,133], Postsynaptic Density Protein 95 (PSD-95) [133], Neurogranin [133,134], and Vesicle-Associated Membrane Protein 2 (VAMP-2) [135] have been tested as possible markers of synaptic loss in AD.

Brain-Derived Neurotrophic Factor (BDNF) is a neurotrophin supporting neuronal and synaptic integrity and plasticity. Low levels have been reported in Alzheimer's disease, associated with Aβ- and tau-related mechanisms, neuroinflammation, and apoptosis, but BDNF may be involved in many other neurological and psychiatric disorders [136].

It has been suggested that combining various classical biomarkers with markers of neuroinflammation and synaptic loss may prove diagnostically helpful and even outperform classical biomarkers alone [128]. However, in an umbrella review, only blood-based YKL-40 yielded convincing results, while many others were suggestive than established proof [137].

6. Genetic Biomarkers

Apolipoprotein E (*APOE*) genotyping is not uncommonly performed in cognitively affected patients. It is a susceptibility gene for AD, present in three allelic forms: ε2, ε3, and ε4 [138]. The presence of at least one ε4 allele is a major risk factor for AD [138], while the ε2 allele may be protective [139]. Recently, it has been suggested that although ε4 heterozygosity is a risk factor, ε4 homozygosity may represent a distinct genetic type of AD [140]. This notion has been challenged [44] since it has been observed that the concomitant presence of a loss-of-function mutation in the Sortilin-Related Receptor 1 gene (*SORL1*) is necessary for this to occur [141]. Thus, in addition to evaluating the risk of AD, *APOE* and/or *SORL1* testing could be diagnostically helpful.

Nevertheless, *APOE* genotyping before initiating anti-amyloid treatment is considered significant for AD patients since the presence of ε4 increases the risk of side effects (amyloid-related imaging abnormalities), and in the case of ε4 homozygosity, these drugs may be contraindicated [14–16]. It is also significant in clinical trials for newer drugs targeting ε4 [142].

Mutations in the gene encoding TREM2 may be related to neurodegeneration and increase the risk of AD [143].

Down syndrome is a specific genetic type of AD [144] for which anti-Aβ antibody treatment is contraindicated due to the increased presence of cerebral amyloid angiopathy [14]. These treatments are also contraindicated in Amyloid Precursor Protein gene (*APP*) mutations associated with cerebral amyloid angiopathy [14].

7. Profiles and Ratios

The National Institute of Aging—Alzheimer's Association (NIA-AA) research framework (2018) introduced the so-called AT(N) system for the diagnosis and classification of AD [41]. A stands for amyloid biomarkers, and A^+ and A^- indicate positive (abnormal) and negative (normal) results of the biomarker used for amyloids, respectively. T stands for tauopathy biomarkers (tau phosphorylation, tangle formation), with T^+ and T^- indicating abnormal and normal results of the biomarker tested, respectively. Finally, (N) stands for neurodegeneration (neuronal or axonal dysfunction or loss), with (N) and (N) indicating abnormal and normal results of the corresponding tests, respectively.

Biomarkers widely used (or suggested for use) currently in AD diagnosis and staging are summarized in Table 1, according to the 2018 NIA-AA research framework and the 2024 Alzheimer's Association (AA) revised classification system [41,43]. Since biomarkers for neurodegeneration or neuroinflammation are nonspecific, and vascular biomarkers or α-syn indicate possible co-pathologies, only those indicating amyloid- and tau-related biochemistry/pathology are currently considered suitable for AD diagnosis and staging [27].

Table 1. Biomarkers of various types of abnormal biochemistry/pathology, useful in the (differential) diagnosis of Alzheimer's disease and other dementia-related disorders, based on the classifications presented in Refs. [41,43] [1].

A (Amyloid)	**T (Tau)**		**N (Neurodegeneration)**	**V (Vascular)**	**S (α-syn)**	**I (Inflammation)**
Core 1	**T_1**	**T_2 Core 2**				
CSF $A\beta_{42}$	CSF $\tau_{P\text{-}181}$	CSF $\tau_{P\text{-}205}$	CSF τ_T		CSF α-syn	CSF GFAP
CSF $A\beta_{42}/A\beta_{40}$	CSF $\tau_{P\text{-}217}$	CSF MDBR-τ_{243}	CSF NfL			
	CSF $\tau_{P\text{-}231}$	Other tau forms				
PET Aβ		PET tau	PET FDG SPECT HMPAO MRI ASL Atrophy (MRI, CT)	MRI, CT		
Plasma $A\beta_{42}$	Plasma $\tau_{P\text{-}217}$	Plasma eMDBR-τ_{243}	Plasma NfL			Plasma GFAP
Plasma $A\beta_{42}/A\beta_{40}$	Plasma $\tau_{P\text{-}181}$ Plasma $\tau_{P\text{-}212}$		Plasma τ_T			

Hybrid ratios or combinations of different markers, including CSF $\tau_{P\text{-}181}/A\beta_{42}$, CSF $\tau_T/A\beta_{42}$, and plasma $\tau_{P\text{-}217}/A\beta_{42}$, are also in use.

CSF: cerebrospinal fluid; PET: positron emission tomography; Aβ: amyloid beta peptide with 42 ($A\beta_{42}$) or 40 ($A\beta_{40}$) amino acids; $\tau_{P\text{-}x}$: phospho-tau phosphorylated at residue x; eMDBR-τ_{243}: (endogenous cleaved) tau microtubule-binding region containing residue 243; τ_T: total tau protein; NfL: neurofilament light protein; FDG: fluoro-deoxy-glucose; SPECT HMPAO: Single-Photon Emission Computed Tomography with ^{99m}Tc-Hexamethylpropylen-amine Oxime; MRI: Magnetic Resonance Imaging; MRI ASL: MRI with arterial spin labeling; CT: Computerized Tomography; α-syn: alpha synuclein; GFAP: Glial Fibrillary Acidic Protein. [1] Other biomarkers may include CSF TDP-43 for transactive-response DNA-binding protein-43 (co)pathology and PET for α-syn or TDP-43.

Cumulative data from many studies [103,144–148] lead to the general notion that CSF or plasma $A\beta_{42}/A\beta_{40}$ are the first to become abnormal very early in the disease process and earlier than PET for Aβ up to ~19 years before symptom onset (Figure 1). The levels of CSF $\tau_{P\text{-}181}$, $\tau_{P\text{-}217}$, $\tau_{P\text{-}231}$, and $\tau_{P\text{-}212}$ may start to increase around the first indication of abnormality in Aβ PET, and they are usually already abnormal when Aβ PET reaches the diagnostic threshold (28–30 centiloids). Thus, the above phospho-tau ("T") forms [41] are now termed "T_1", and, together with Aβ (A) markers, they are classified as "core 1" biomarkers [43]. With disease progression, insoluble forms of tau accumulate in the form of neurofibrillary tangles, at which point CSF or plasma $\tau_{P\text{-}205}$ and MBDR-τ_{243} become

abnormal, and tangle pathology becomes evident in PET for tau. These "T" biomarkers are now classified as "T_2" and "core 2" biomarkers [43].

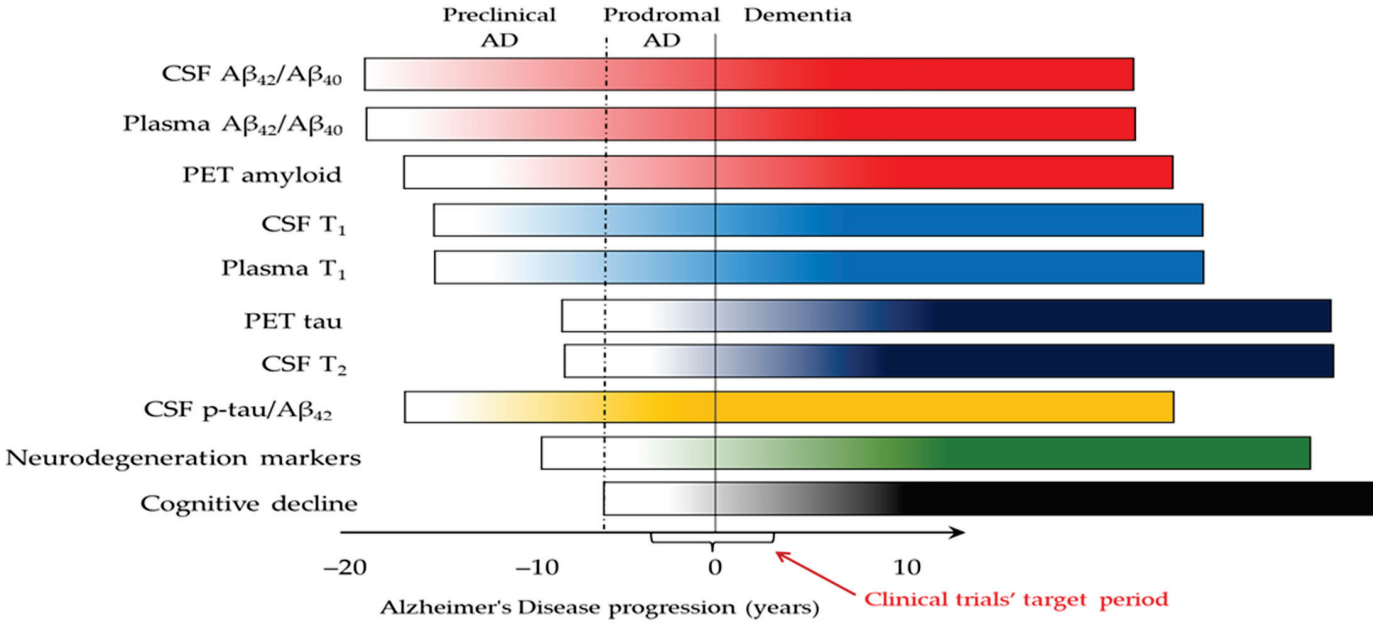


Figure 1. Theoretical diagram of biomarker changes during Alzheimer's disease evolution and progression [41,43,103,144–148]. The changes in biomarkers follow the smooth increase in color intensity throughout the course of AD. The red arrow shows the period that is the target of monoclonal antibodies' clinical trials. Deviations may occur in some patients based on genetic or other factors, as well as the biomarker tests used. Cerebrospinal fluid (CSF) T_1 refers to $\tau_{P\text{-}181}$, $\tau_{P\text{-}217}$, and $\tau_{P\text{-}231}$. Plasma T_1 refers to $\tau_{P\text{-}217}$, $\tau_{P\text{-}181}$, and $\tau_{P\text{-}212}$. CSF T_2 mainly refers to $\tau_{P\text{-}205}$ and MDBR-τ_{243}. For simplicity reasons, neurodegeneration markers are shown together in one bar, although they are not equivalent and may become abnormal at relatively different time points.

According to the 2018 NIA-AA criteria, AD can be diagnosed when the biomarker profile is A^+T^+, while the A^+T^- profile is classified as Alzheimer's continuum since only amyloid biomarkers may be positive very early in the disease process, with T biomarkers remaining negative [41]. According to the 2024 AA revised criteria, an abnormal core 1 biomarker either A (PET for Aβ, or CSF $A\beta_{42}/A\beta_{40}$) or plasma T_1 ($\tau_{P\text{-}217}$, good indicator of Aβ pathology) is sufficient for AD diagnosis since the vast majority of A^+ patients (~90% with positive PET for Aβ) will prove to have AD at autopsy [43]. Indeed, this is true, but it is not always the case.

Firstly, the A^+ profile is heterogeneous, comprising different subgroups of patients with typical amnestic AD, AD mixed with other pathologies, or vascular cognitive decline [149]. The A^+T^- profile is also heterogeneous. Many such patients may suffer from early presymptomatic or even mildly symptomatic AD in which the T biomarkers have not become abnormal yet [145]. Additionally, AD with atypical clinical presentations [150–152] or mixed AD [153] may present with marginal or normal CSF $\tau_{P\text{-}181}$ levels. The A^+T^- profile may also be observed in other non-AD disorders such as dementia with Lewy bodies (DLB) [154], subcortical small vessel disease, various tauopathies [155,156], Creutzfeldt–Jakob disease [157], limbic-predominant age-related TDP-43 encephalopathy [158], subcortical small vessel disease [159], and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [160]. In such clinical settings, with equivocal CSF profiles, the $A\beta_{42}/A\beta_{40}$ ratio, instead of $A\beta_{42}$ alone, led to a reliable diagnosis in ~50% of cases [161], but not in all, and diagnostic uncertainty may remain. A proportion of A^+T^- patients may remain A^+T^- for at least 5 years, showing some clinical

and genetic differences compared to those with A^+T^+ [162]. Recently, an amyloid predominant subtype of Alzheimer's neuropathological change (~10%) has been described, with patients progressing to amyloid Thal phase 5 while remaining in tau Braak stages I–II and not progressing up to stage VI, as would be expected for AD; these patients may present with clinical and genetic differences compared to those with typical AD pathology [163]. Furthermore, some patients with amyloid pathology may never progress clinically [164]. Taken together, the above findings indicate that a percentage of A^+ or A^+T^- patients, despite showing amyloid pathology, may deviate from what is expected in AD.

To increase the diagnostic performance of biomarkers, hybrid ratios of different biomarkers have been used, such as CSF $\tau_{P\text{-}181}/A\beta_{42}$, CSF $\tau_T/A\beta_{42}$, and plasma $\tau_{P\text{-}217}/A\beta_{42}$ [165–170]. Since the nominator increases and the denominator decreases, the ratio change is augmented, resulting in good discrimination between AD and non-AD disorders. The CSF $\tau_{P\text{-}181}/A\beta_{42}$ and $\tau_T/A\beta_{42}$ ratios may be helpful in cases with marginal or conflicting results from individual biomarkers [166] and in discriminating between likely AD and likely non-AD patients within the A^+T^- profile [171]. However, these ratios could sometimes become abnormal due to a significant reduction in only $A\beta_{42}$, i.e., in the absence of any evidence of tau-related biochemistry. These CSF hybrid ratios and the CSF $A\beta_{42}/A\beta_{40}$ ratio, also included in Table 1, are classified independently as "core 1" biomarkers according to the 2024 AA revised criteria, reflecting a well-established AD neuropathological change, even in asymptomatic individuals [43]. The importance of the CSF $A\beta_{42}/A\beta_{40}$ ratio, which identifies amyloid pathology with adequate accuracy, has already been mentioned as a key initial change over the course of AD in relation to classic fluid and imaging biomarkers [147,148]. Ultimately, plasma $\tau_{P\text{-}217}/A\beta_{42}$, which has recently received FDA approval, established encouraging data in the cognitive impaired population using the two-cut-off approach, but it needs further investigation to strengthen its clinical utility [170].

In the approval study of Lecanemab (Clarity AD), evidence of amyloid positivity was required for recruitment (PET or CSF $A\beta_{42}$) [11], while for Donanemab (TRAILBLAZER-ALZ 2), PET scans for both Aβ and tau were required [12]. According to appropriate use recommendations for Lecanemab, an abnormal PET for Aβ or CSF findings of increased p-tau and reduced $A\beta_{42}$ (increased p-tau/$A\beta_{42}$) is required [14], while for Donanemab, amyloid positivity by an abnormal PET for Aβ or by reduced CSF $A\beta_{42}/A\beta_{40}$ or, alternatively, by abnormal CSF $\tau_{P\text{-}181}/A\beta_{42}$ and $\tau_T/A\beta_{42}$ ratios are required [15]. Canadian [17] and Korean [18] guidelines are similar to the appropriate use recommendations for Lecanemab and Donanemab. However, the French appropriate use recommendations for Lecanemab show some deviations since they recommend the CSF A^+T^+ profile, and in cases with inconclusive CSF results or, alternatively, an abnormal PET for Aβ [16]. In fact, similar recommendations are advocated by the International Working Group (IWG) criteria for the diagnosis of AD [42,44].

Thus, there are still debates over the definition and biomarker profile of AD, i.e., amongst the 2018 NIA-AA criteria [41], 2024 AA revised criteria [43], and IWG criteria [42,44]. However, this may be very important since it has been observed that different criteria may result in discordant diagnoses in ~42% of patients, especially when only one biomarker is abnormal [172].

8. Discussion

Currently, the most widely used biomarkers in everyday practice for AD diagnosis are the classical or core biomarkers (CSF or PET). Depending on availability, familiarity, acceptance, contraindications, cost, and reimbursement issues, either CSF or PET may be preferred in various countries for confirming AD and eligibility for anti-amyloid treatments

or enrolment in experimental trials. However, after roughly 25 years of research on classical biomarkers, there are still controversies.

- Should we use the AT(N) classification system according to the 2018 NIA-AA guidelines [41] or the 2024 AA revised criteria [43]?
- Should we use the AA revised guidelines [43] or the IWG recommendations [44]?
- Should the profile be both A^+ and T^+ (either $T_1{}^+$ or $T_2{}^+$), or is A^+ sufficient?
- Should we rely on the $\tau_{P\text{-}181}/A\beta_{42}$ and $\tau_T/A\beta_{42}$ ratios and when?

In some countries, CSF studies are the first to be performed. An example of how we work with our patients (not a recommendation) is shown in Figure 2, based on different scenarios according to CSF findings. To maximize confidence, we prefer using the A^+T^+ profile for confirming AD presence [153]. In the case of inconclusive results, we use the $\tau_{P\text{-}181}/A\beta_{42}$ [171] or (if affordable due to high cost) a PET scan for $A\beta_{42}$. By doing so, we try to combine the various criteria and guidelines.

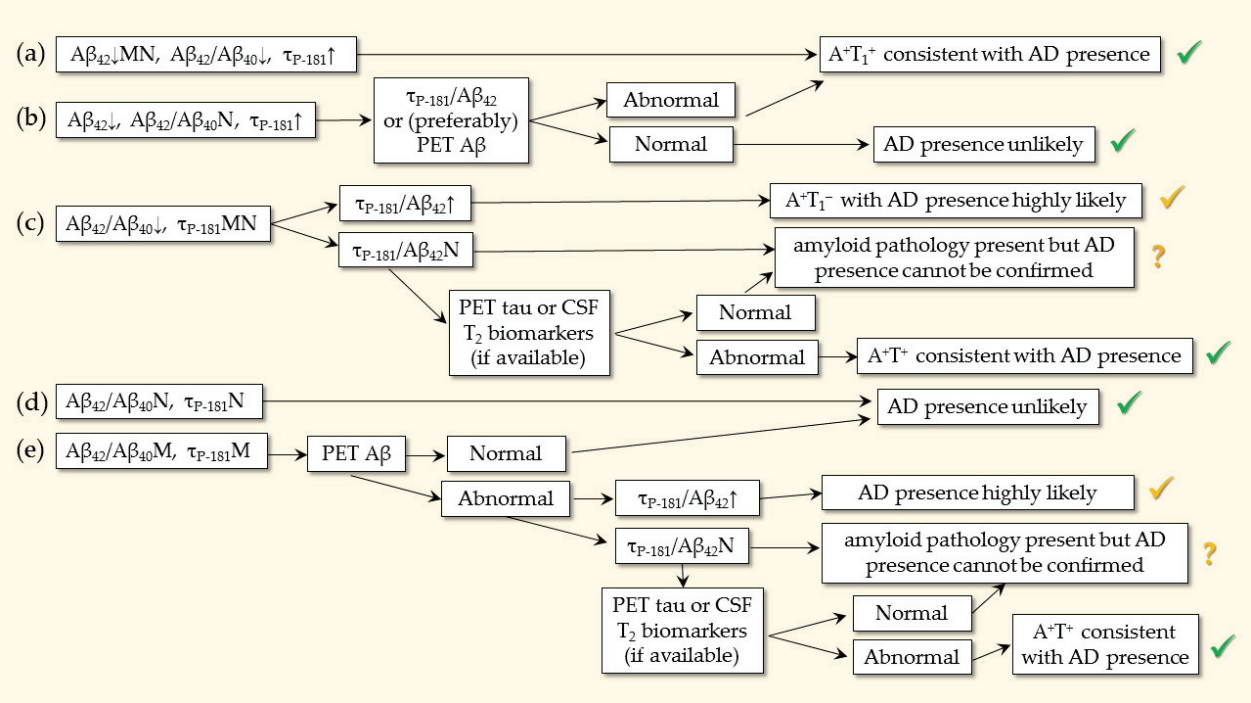


Figure 2. Use of biomarkers for the confirmation of AD presence in 5 scenarios, starting from CSF analysis. N: normal; M: marginal. Green "O.K. symbols" indicate confirmation. Yellow "O.K. symbols" indicate the likely presence of AD. Question marks indicate the presence of amyloid pathology, but AD (additional occurrence of tau pathology) cannot be verified. In scenario (**a**), the typical CSF profile of AD is present (A^+T^+ or $A^+T_1{}^+$). In scenario (**b**), $A\beta_{42}/A\beta_{40}$ is normal, raising questions about the presence of amyloid pathology, despite the abnormality of both $A\beta_{42}$ and $\tau_{P\text{-}181}$. In this case, a PET scan for Aβ or (if not available) the $\tau_{P\text{-}181}/A\beta_{42}$ ratio is required to reach a conclusion. In scenario (**c**), the A^+T^- profile is present with abnormality only in the $A\beta_{42}/A\beta_{40}$ ratio (or alternatively the amyloid PET). In this case, the $\tau_{P\text{-}181}/A\beta_{42}$ ratio can be used, but if normal, a T_2 biomarker (if available) may be required. In scenario (**d**), the A^-T^- ($A^-T_1{}^-$) profile is inconsistent with AD. In scenario (**e**), both molecular biomarkers are marginal. In such patients, the $\tau_{P\text{-}181}/A\beta_{42}$ ratio and/or PET scans for Aβ and/or tau may be needed according to availability and cost.

It is noteworthy to highlight that, accordingly to the AA revised guidelines, hybrid ratios encompass CSF $\tau_{P\text{-}181}/A\beta_{42}$, CSF $\tau_T/A\beta_{42}$, CSF $A\beta_{42}/A\beta_{40}$, and plasma $\%\tau_{P\text{-}217}$, without discrimination, even though the last two ratios represent distinct protein deposits,

tau, and amyloid beta protein, respectively. Conversely, the first ones demonstrate the change in tau protein in relation to amyloid beta, assuming that $\tau_{P\text{-}181}/A\beta_{42}$ is more specific for AD, instead of total tau, which is a general indicator of neuronal damage. The nomenclature is of great importance, considering it may help both the comprehension of ratio implications and their accurate utilization.

An AD diagnosis based only on biomarkers may not suffice for treatment eligibility. Some patients may have mixed pathologies, and AD may not be the main pathology responsible for the clinical picture. This may be true in cases with DLB [154] or significant cerebrovascular disease [159], and evidence or suspicion of such co-pathologies is considered a contraindication to anti-amyloid treatment [14].

The clinical phenotype of AD may also be important. For the common AD phenotypes (typical amnestic, logopenic-type primary progressive aphasia, and posterior cortical atrophy) [42], there are no concerns [16,173].

However, for the uncommon AD phenotypes (frontal variant, non-logopenic primary progressive aphasia, and corticobasal syndrome), AD may not be the only pathology, and, since the presence of other co-pathologies cannot be excluded, anti-amyloid treatments may not be indicated [16].

From this perspective, it is questionable whether blood-based biomarkers could be a standalone tool for AD diagnosis and evaluation of anti-amyloid treatment eligibility, despite the adequate diagnostic accuracy of some of them. We need further investigation into the plasma biomarker changes in the aforementioned mixed and atypical pathologies, as well as in other pathologies that can affect the central nervous system, including secondary causes of dementia. Moreover, a recent approach to their assessment, also classified according to the performed immunoassays [174], enlightens a new era with realistic expectations and skepticism.

There are some limitations in this review. We did not perform a systematic or umbrella review with or without meta-analyses. But this was not our aim. We preferred a simple narrative review to show the current uncertainties in biomarker-based confirmation of AD, not for theoretical reasons but for implementing anti-amyloid treatments or for recruiting patients for clinical trials of new drugs. Furthermore, the data enclosed and analyzed primarily come from White populations, limiting the potential of generalizability in different ancestries.

9. Future Directions

Currently, there are many criteria and guidelines for AD diagnosis [33,34,41–45] and for eligibility for anti-amyloid treatments [13–18]. Among them, there are many common features, but also some differences. Every effort should be made to reach a consensus and achieve homogeneous criteria.

To increase diagnostic accuracy, alternative biomarker testing for synaptic loss, microglia activation, and neuroinflammation should become more widely available since, in the appropriate clinical setting, they have the potential to offer additional information and explain some discrepancies between core biomarkers [119,128].

To simplify the diagnostic procedure and potentially decrease the cost, plasma biomarkers, especially $\tau_{P\text{-}217}$ and the $\tau_{P\text{-}217}/A\beta_{42}$, are promising and may enter everyday diagnostic algorithms in the near future [16]. However, the recently published AA Clinical Practice Guideline suggested caution since there may be significant variability in their diagnostic accuracy and moderate confidence [174]. The role of salivary, urinary, or ocular biomarkers in offering simple and accurate diagnosis remains to be established [137].

Author Contributions: Conceptualization, I.T., A.A., F.B., C.Z., V.C.C., S.G., J.S.T., E.K., G.T. and G.P.P.; data curation, I.T., A.A., F.B., A.T., A.M., P.-E.T., A.B., J.S.T., C.Z., S.G.P., S.G. and G.P.P.; formal analysis, I.T., V.C.C., A.M., F.B., A.T., A.A., C.Z., J.S.T., S.G.P., A.B., E.K., G.T. and G.P.P.; investigation, I.T., F.B., A.A., A.M., A.T., J.S.T., P.-E.T., C.Z., S.G.P., A.B., E.K., G.T. and G.P.P.; methodology, I.T., A.A., V.C.C., F.B., J.S.T., A.T., P.-E.T., S.G.P., S.G., E.K., G.T. and G.P.P. project administration, E.K., G.T. and G.P.P.; supervision, S.G., E.K., G.T. and G.P.P.; visualization, S.G., E.K., G.T. and G.P.P.; writing—original draft, I.T., V.C.C., P.-E.T., S.G.P., J.S.T., A.M., A.T. and G.P.P.; writing—review and editing, I.T., A.A., F.B., C.Z., A.B., S.G., J.S.T., E.K., G.T. and G.P.P. All authors have read and agreed to the published version of the manuscript.

Funding: The authors declare that this study received no funding. G.T., G.P.P., I.T., A.A., C.Z. and A.B. are clinical investigators in the "EVOKE" and "EVOKE plus" trials of semaglutide for early Alzheimer's disease (NovoNordisk, NCT04777396 and NCT04777409, respectively). G.P.P. received fees from Biogen International and from ITF Hellas as a consultant for advisory boards. These activities are not related to the present work and the funders were not involved in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to submit it for publication.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: No new data were created or analyzed in this study. Data sharing is not applicable to this review article.

Conflicts of Interest: None.

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ISBN 978-3-7258-8966-2