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Special Issue Reprint

# Brain Injury and Neurodegeneration

## Molecular, Functional, and Translational Approach 2.0

Edited by
Kumar Vaibhav, Pankaj Ahluwalia, Pankaj Gaur and Meenakshi Ahluwalia

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Guest Editors

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# About the Editors

**Kumar Vaibhav**

Kumar Vaibhav (MS, PhD) is an Assistant Professor in the Department of Neurosurgery, Medical College of Georgia at Augusta University, Augusta, GA, USA. Dr. Vaibhav is also affiliated with the Department of Oral Biology and Diagnostic Sciences, Center for Excellence in Research, Scholarship and Innovation, Dental College of Georgia, Augusta University, Augusta, GA and Transdisciplinary Research Initiative in Inflammaging and Brain Aging (TRIBA), Augusta University, Augusta, GA. Dr. Vaibhav is a Neuroscientist, Educator, Mentor, and Director of the Brain Injury, Senescence, and Translational Neuroscience laboratory. He has more than 15 years of research experience in brain pathologies. His current research focus is on the outcomes of traumatic brain injury and intracerebral hemorrhages, with emphasis on immune interaction, the systemic influence of diseases, inflammaging, neurodegeneration, and alteration in endocannabinoid system in acute and chronic pathology. Dr. Vaibhav is a seasoned researcher who has more than 4700 research citations with an h-index of 35.

**Pankaj Ahluwalia**

Pankaj Kumar Ahluwalia (MS, PhD) is a postdoctoral fellow in the Department of Pathology at Augusta University. His research focuses on applying genomics and cutting-edge technologies, such as spatial transcriptomics, to advance the understanding of various human disorders, ranging from neurological diseases to cancer. Additionally, he investigates immune responses in healthcare workers following SARS-CoV-2 vaccination as part of the larger SPARTA study. His contributions have been cited more than 890 times with an h-index of 16. Beyond his research, Dr. Ahluwalia is passionate about mentoring medical students and inspiring them to engage in research.

**Pankaj Gaur**

Pankaj Gaur (MS, PhD—Neuroscience) is a highly accomplished researcher, instructor, and scientist who has made significant contributions to the fields of neuroscience, immunology, and cancer research. Dr. Gaur is affiliated as a Research Instructor (Faculty) at Lombardy Cancer Center, Georgetown Medical College, Georgetown University, Washington DC. Dr. Gaur's primary research interest revolves around identifying innovative strategies to enhance specific immune responses against cancer, autoimmune diseases (such as multiple sclerosis and systemic lupus erythematosus), as well as various brain disorders including Parkinson's disease, epilepsy, traumatic brain injury, and neurodegeneration. His aim is to unravel the intricate mechanisms underlying immune responses, ultimately leading to the development of novel therapeutic approaches. He has 855 citations with an h-index of 11.

**Meenakshi Ahluwalia**

Meenakshi Ahluwalia (MS, PhD) is a researcher with expertise in human genetics. In her PhD, she studied the effect of pesticides in farm workers by analyzing chromosomal aberrations. She extended her molecular and genetic expertise in traumatic brain injury, hemorrhagic stroke, and glioma, and has completed her post-doctoral training in understanding mechanism behind neuropathology and their intervention at Medical College of Georgia, Augusta University, Augusta, GA. She has more than 720 overall citations with an h-index of 13. Her research interest is to develop non-invasive therapy for brain insult and oncolytic treatment for repression of glioma.

# Preface

We are extremely glad to introduce the Reprint of the Special Issue "Brain Injury and Neurodegeneration: Molecular, Functional, and Translational Approach 2.0" for the readers. This Special Issue includes 16 articles, including 1 editorial, 9 research articles and 6 reviews on the diverse topics of brain injury, pathology, cellular functionality, neurodegeneration and therapeutic interventions. The brain encompasses a variety of cells with diverse but integrated roles in brain health and physiology. However, these integrated cellular functions get disturbed by many environmental, occupational, pathological, and lifestyle factors. Even alterations in the health of distant organs or an altered level of hormones can affect brain homeostasis to a great extent. Authors from different parts of the world have contributed to this Special Issue and have added to our knowledge about CNS pathologies and health. Thus, this issue will be a good read for scientists, medical professionals, students, and for the public.

We would like to thank the authors who chose to publish their fascinating works in our Issue, and the reviewers for doing a wonderful job in timely evaluating the articles. We are also thankful to our Editor in Chief, Prof. Dr. Shaker A. Mousa, our Assistant Editor, Ms. Dora Xie, and the entire *Biomedicines* editorial staff for the support we received throughout this journey. Finally, I would like to praise our team of Guest Editors—Drs. Pankaj Ahluwalia, Pankaj Gaur and Meenakshi Ahluwalia—who helped and supported the cause throughout this journey.

**Kumar Vaibhav, Pankaj Ahluwalia, Pankaj Gaur, and Meenakshi Ahluwalia**
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*Editorial*

# Brain Injury and Neurodegeneration: Molecular, Functional, and Translational Approach 2.0

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The brain is composed of different cells, such as neurons, glia, endothelial cells, etc., that perform specialized functions. Glial cells are key cells in the brain and are dominated by two major populations—astrocytes and microglia. These cells communicate with each other and other cells in the CNS, support the function of neurons, secrete cytokines as per immune and environmental clues, and maintain CNS homeostasis [1]. Astrocytes perform a wide range of functions in CNS homeostasis, and its reactivity to insult or pathological conditions is a heterogenous, dynamic, and context-dependent response [2]. The transforming growth factor-β (TGF-β) is a pleiotropic cytokine with a wide range of essential cellular functions, such as tissue homeostasis, and immune response and remodeling [3–5]. TGF-β has been identified as a key regulator of astrocyte reactivity, which can be manipulated to alter pathological and functional outcomes in brain diseases [6]. The CNS not only regulates the function of different organs of the body but is also affected by different organs and their functional components. The oral microbiota is the second most diverse microbiome population in the body. Through their systematic review, Giordano-Kelhoffer et al. highlight the importance of oral microbiomes in healthy and diseased conditions. Dysbiosis of the oral microbiota is clearly related with cardiovascular and neurodegenerative diseases [7,8]. The authors emphasized that lifestyle, diet, stress, alcohol, smoking, and health conditions actively modify the oral microbiome and may increase health complexities. Thus, maintaining a healthy lifestyle, physical activity, and a healthy diet aids in maintaining a balanced microbiome and thus a healthy body [9].

With the advancement of neuroscience research, we have come across various brain pathologies such as traumatic brain injury (TBI), hypoxic/hypobaric insults, hemorrhages, strokes, and neurological disorders such as Parkinson's and Alzheimer's diseases. Any insult to the brain (mild or severe) is multifactorial and initiates a cascade of inflammation, necrotic, and apoptotic pathways. It has long been known and established that brain insult or injury to the brain may lead to neurological disorders such as Alzheimer's and Parkinson's disease as time elapses, and genetic or environmental factors play significant roles in disease progression [10–12]. A substantial body of evidence has shown that oxidative stress, mitochondrial dysfunctions, protein aggregation and phosphorylation, excessive iron accumulation in the brain, and neuro-inflammation play a pivotal role in neurodegeneration and brain injuries [13–15]. The absence of a specific cure to restrain injury progression after an insult has prompted the scientific community to study the process behind the degenerative cascade and explore various therapeutic strategies.

Brain damage with clinical implications can be caused in two instances: either at birth or later in life. Any brain damage that transpires after birth, except for those caused by congenital disorders or degenerative diseases, is termed as acquired brain injury (ABI).

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Damage incurred from an ABI can last from days to a lifetime, depending on the severity of the injury, and has psychological, neurological, and physiological consequences [13]. Among all ABIs, TBI is a common cause of accidental long-term disability and brain pathologies in the adult population [16–18]. Adults surviving moderate and severe TBI often experience long-lasting neuropsychological, cognitive, and neuropsychiatric disorders with a prevalence rate ranging from 25 to 88% [19–21]. Chronic traumatic encephalopathy (CTE) is a chronic disease that develops after TBI and leads to long-term memory and cognitive impairment [22,23]. TBI-induced chronic pathologies and CTE show similarities with Alzheimer's disease (AD) and other neurodegenerative diseases; therefore, a battery of diverse and innovative diagnosis tools is needed [23]. Advanced diagnostic tools such as diffusor tensor imaging, functional magnetic resonance imaging, positron emission tomography, and the fluid biomarkers t-tau, sTREM2, CCL11, NFL, and GFAP can be included to assess chronic effects post TBI [23]. Clinically, general cognitive tools such as the Mini Mental State Examination or more specific cognitive tests such as the Wisconsin Card Sorting Test and Trail Making Test could be implemented during the acute or chronic phase of TBI to assess the patient's neuropsychological and cognitive function level [24]. Since the antemortem diagnosis of CTE is poor, future efforts toward clinical, radiological, metabolic, and molecular biomarker development are keys to improved diagnosis and prognosis [22].

Human activities and occupations in diverse environments have varying effects on the brain, particularly for individuals working in extreme conditions. It is critical to elucidate the genetic and environmental factors that influence its function, including a wide range of pathological conditions. Military personnel are exposed to different intensities of blasts and environmental conditions in war zones. Repetitive low-level blast exposure is one of the major occupational health concerns among US military service members and law enforcement and is associated with whole-blood dysregulated gene signatures mostly related to chronic inflammation and immune response, suggesting that these pathways may relate to the risk of lasting neurological symptoms following extended exposure to blast over a career [25]. In addition, environmental factors may also influence neuropathology and inflammation in military personnel. During the Gulf War, military personnel were exposed to paraoxon, an organophosphate. In a study by Freidin et al., the authors found that cognitive and behavioral deficits with neuroinflammation in the dentate gyrus were prominent in mice subjected to both mild TBI and paraoxon exposure [26]. In addition to external factors, endogenous factors such as hormones or previous stress episodes may affect TBI outcomes. The hypothalamic–pituitary–adrenal (HPA) axis is the main endocrine system that plays a critical role in late post-traumatic pathology, such as synaptic activity and neuroinflammation in the hippocampus. TBI leads to an alteration of cortisol levels due to altered HPA axis function. This glucocorticoid (cortisol in humans or corticosterone in animals) regulates two basic systems—glucose metabolism and immune response. The effect of glucocorticoid on the hippocampal neuronal population depends on its concentration, the duration of exposure, and the local cell population. Elevated levels of glucocorticoid may increase neuroinflammation and may be involved in chronic traumatic pathologies, such as epilepsy, depression, and cognitive impairment [27]. Thus, the excessive GCs/dysfunctional HPA axis may be responsible for neuroinflammation and distant hippocampal damage in many brain diseases. Besides direct brain injuries, substances of abuse can also affect brain health. Alcohol is one of the most common substances of abuse and can contribute to chronic brain pathologies. Skuja et al. investigated the substantia nigra section of alcoholic postmortem human brains and observed increased expression levels of collagen-IV, laminin-111, and fibronectin but decreased $CD31^+$ vessels in the basement membrane of the blood–brain barrier (BBB) [28]. Waterpipe tobacco smoking (WPS) is prevalent in Asia and the Middle East but rapidly gaining popularity in other countries, especially among youths. A 6-month exposure study in mice revealed that WPS inhalation elevated the levels of DNA damage, lipid peroxidation, cytochrome C, IL-6, IL-1β, cleaved caspase-3, and nuclear factor-κB in the cerebellum. Thus, WPS may be

associated with cerebellar inflammation, gliosis, oxidative stress, and apoptosis via NF-κB activation [29].

The brain consumes more oxygen and energy than any other organ in the human body, and its disruption can lead to widespread damage. Brain hypoxia because of stroke or TBI or any means is detrimental to motor function. The authors of a study examining primary motor cortex slices from 16–18-day-old infant rats demonstrated a high incidence of hypoxia-induced seizures associated with epileptiform motor behavior when exposed to post-natal hypoxia. This was linked to reversible depression in glutamatergic synaptic transmission and neuronal excitability mediated by adenosine acting on pre-synaptic A1 receptors to decrease glutamate release and the nitric oxide (NO)/cGMP postsynaptic pathway [30]. A better understanding of these molecular pathways would improve therapeutic efforts to treat this condition in humans.

Ischemic disease is the second most prevalent health problem globally with high mortality and morbidity. Temporary occlusion of the common carotid artery causes 25% of ischemic stroke cases [31]. The results of an experimental study examining male Wistar rats with temporary and permanent occlusion of either the common carotid artery or both showed an interesting effect on sciatic nerve motor-evoked potential (MEP) as a measure of the efferent transmission of the corticospinal tract. MEP amplitude decreased by 23.2% to 41.6% in 5 min and 10 min occlusion, respectively, while 5 min of arterial blood flow recovery stabilized MEP. While temporary occlusion did not evoke total and permanent inhibition in the activity of corticospinal tract neurons, bilateral occlusion was histologically more prominent and caused alteration in the sensory and motor areas controlling the forelimb [31]. Many factors can influence ischemic stroke outcomes and associated events. Arterial stiffness and vascular calcification increase with age and with the occurrence of ischemic events. The European Society of Cardiology recommends the use of polyunsaturated fatty acids (PUFAs) to reduce blood pressure, LDL, and inflammation and to increase NO in the vascular wall [32,33]. Levels of serum fatty acids are good indicators of the risk of ischemic stroke [34,35]. However, the effects of saturated and unsaturated fatty acids on stroke pathology remain inconsistent. Drozd et al. have assessed the impact of free fatty acids and their metabolites on non-dipping blood pressure and sleep apnea in 64 ischemic stroke patients [36]. Patients with preserved physiological dipping (DIP) showed higher scores on the Epworth sleepiness scale (ESS) with high levels of anti-inflammatory mediators from EPA and DHA acids; in comparison, 31 patients with the non-dipping phenomenon (NDIP) showed high levels of C18:3n6 gamma linoleic acid, indicating advanced inflammation events [36].

Over the past decade, several therapeutic approaches have emerged as promising strategies for addressing brain pathologies. The recombinant tissue plasminogen activator (rt-PA) is still a cornerstone of acute ischemic stroke treatment but is associated with bleeding complications [37,38]. In a study involving immortalized brain-derived endothelial cells (bEnd5) as a model of the BBB, rt-PA was found to cause cytotoxic and BBB damage, while the NLRP3-specific inhibitor MCC950 minimized this effect of rt-PA under ischemic conditions [39]. However, it is worth mentioning that ischemia and hypoxia are significant contributors to chronic neurodegeneration. Sharifulina et al. observed increased cytoplasmic levels of N- and C-terminal fragments of APP at 24 h post photothrombotic stroke, with an increase in co-immunoprecipitation with caveolin-1. The authors further reported that a caveolin inhibitor (Diadzein) enhanced Aβ synthesis from APP, while the γ-secretase inhibitor (DAPT) inhibited astrogliosis and reduced infarct volume after photothrombotic stroke [40].

The brain is the central organ of the nervous system, vital for daily functioning [41], and is among the organs most affected by toxicants and trauma, during aging, and its associated disorders. This Special Issue has provided a multidisciplinary platform for discussing the cellular functionality, brain pathology, and intervention of brain disorders. This issue emphasizes the pathological findings, the effect of environmental factors on the CNS and disease, and the mechanisms in the development of preventive and therapeutic

strategies to limit brain injury and neurodegenerative disorders. In total, 15 articles were published as a part of this Special Issue, including 9 research articles, 5 reviews, and 1 systematic review, which complete the different aspects of this Special Issue and will provide a compelling read for the audience.

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*Review*

# TGF-β as a Key Modulator of Astrocyte Reactivity: Disease Relevance and Therapeutic Implications

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**Abstract:** Astrocytes are essential for normal brain development and functioning. They respond to brain injury and disease through a process referred to as reactive astrogliosis, where the reactivity is highly heterogenous and context-dependent. Reactive astrocytes are active contributors to brain pathology and can exert beneficial, detrimental, or mixed effects following brain insults. Transforming growth factor-β (TGF-β) has been identified as one of the key factors regulating astrocyte reactivity. The genetic and pharmacological manipulation of the TGF-β signaling pathway in animal models of central nervous system (CNS) injury and disease alters pathological and functional outcomes. This review aims to provide recent understanding regarding astrocyte reactivity and TGF-β signaling in brain injury, aging, and neurodegeneration. Further, it explores how TGF-β signaling modulates astrocyte reactivity and function in the context of CNS disease and injury.

**Keywords:** astrocytes; reactive astrogliosis; TGF-β; traumatic brain injury; stroke; aging; Alzheimer's disease; Parkinson's disease; amyotrophic lateral sclerosis; multiple sclerosis; epilepsy

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## 1. Introduction

Astrocytes perform a wide variety of complex and essential functions in maintaining central nervous system (CNS) homeostasis and are pivotal responders to a wide spectrum of pathological insults [1]. Astrocytes respond to insults to the brain by a process referred to as "reactive astrogliosis" and undergo a spectrum of changes in gene expression, as well as morphological, biochemical, metabolic, and physiological remodeling, which ultimately result in the gain of new function(s) or loss or upregulation of homeostatic ones (definition by a recently published consensus statement [1]). Astrocyte reactivity is highly heterogeneous and dynamic, and comprises a graded continuum of context-dependent responses that may result in adaptive or maladaptive effects [2]. Maladaptive astrocytes lose homeostatic functions and may also gain detrimental ones, thus exacerbating ongoing pathology and promoting disease progression [1–8], but the cellular and molecular mechanisms of maladaptive reactivity are not well understood. Dissecting underlying mechanisms and learning how to beneficially modulate astrocyte reactivity are therefore of great importance for astrocyte research.

The transforming growth factor-β (TGF-β) superfamily consists of a large group of pleiotropic cytokines with a wide range of essential functions, including cell development, differentiation, proliferation, and survival; tissue homeostasis, remodeling, and repair; morphogenesis and angiogenesis; and inflammation and immune responses [9–11]. Members of the TGF-β superfamily regulate multiple aspects of brain function during development and in the adult brain, and aberrant TGF-β signaling contributes to the pathogenesis of neurological disorders [11–14]. TGF-β has been identified as a key regulator of astrocyte reactivity and glial scar formation [5,15–17]. The manipulation of the TGF-β signaling pathway in astrocytes alters pathological and functional outcomes in models of neurological diseases. Recent advances in genomics and multi-omics have yielded novel insights into astrocyte reactivity and diversity, and TGF-β signaling in health and disease. This review will discuss recent insights into how astrocytes respond and function in response to

brain injury, aging, and neurodegeneration, focusing on how TGF-β signaling regulates astrogliosis and affects disease outcome.

## 2. TGF-β Signaling in the Brain

The TGF-β superfamily includes TGF-βs, bone morphogenetic proteins (BMP), growth differentiation factors (GDFs), activins, nodal, inhibins, and anti-Mullerian hormone proteins (Müllerian inhibiting substance) [9–11]. TGF-β isoforms (TGF-β1, 2, and 3) are synthesized as protein precursors with a 70–80% homology. They are secreted in a biologically inactive (latent) form as part of a latent complex stored in the extracellular matrix. Latent TGF-β must be activated before binding to the receptors [9,10]. The activation is a critical step in the regulation of TGF-β's activity. Activators of latent TGF-β include cell surface integrins ($\alpha_v\beta_6$ and $\alpha_v\beta_8$), proteases (calpain, cathepsin D, and matrix metalloproteinase), thrombospondin-1 (TSP-1), and reactive oxygen species (ROS) [18] (Figure 1). The TGF-β complex serves as an extracellular sensor and control switch responding to specific signals or perturbations by releasing active TGF-β [19].

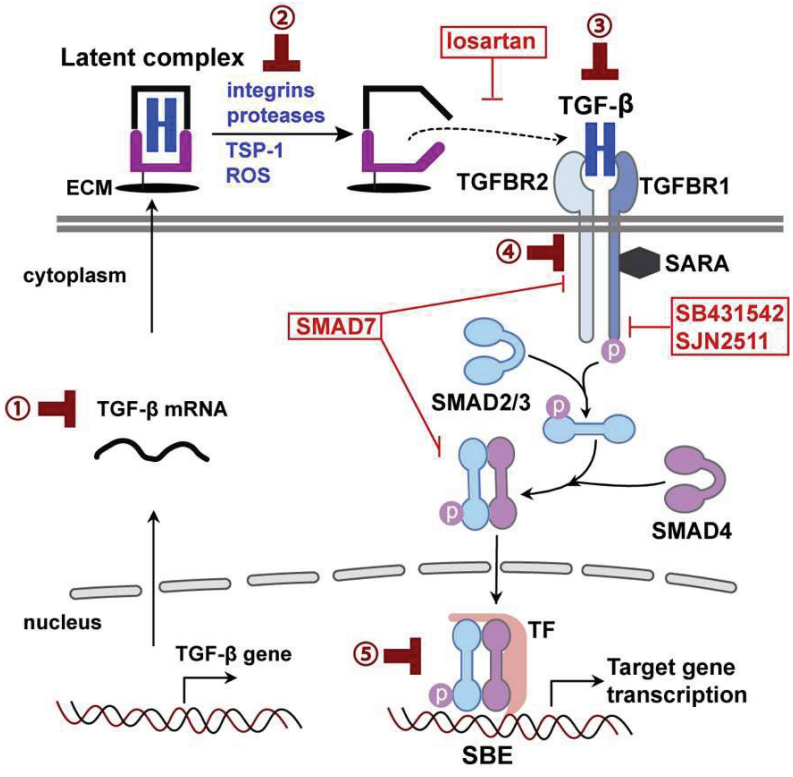


**Figure 1.** TGF-β activation and the canonical, SMAD-dependent signaling pathway. TGF-β is produced in a latent form as part of a latent complex tethered to ECM. The latent complex consists of mature dimeric TGF-β, associated with latency-associated peptide (black color) and a latent TGF-β-binding protein (magenta color). Latent TGF-β can be activated and released from the complex by integrins, proteases, TSP-1, and ROS. Active TGF-β binds to its receptors and initiates Smad signaling to exert its biological effects. The activities of TGF-β signaling can be modulated through (1) TGF-β translation and production, (2) TGF-β activation, (3) TGF-β neutralization using recombinant antibodies, (4) synthetic molecules that inhibit the phosphorylation of the TGFBR and SMAD, and (5) targeting downstream effectors. The red boxes show inhibitors and their action sites mentioned in this review.

Canonical TGF-β signaling is mediated by the binding of TGF-β1-3 to TGF-β receptor types 1 and 2 (TGFBR1/2), which are transmembrane serine/threonine kinase receptors (Figure 1). Upon TGF-β binding, TGFBR1 (also called activin receptor-like kinase 5, ALK5) and TGFBR2 form a heteromeric complex [9,10]. In the receptor complex, TGFBR2 subunits phosphorylate and activate TGFBR1, which phosphorylates SMAD2 and SMAD3. Phosphorylated SMAD2/3 then form a complex with SMAD4 and translocate into the nucleus, where they bind to the Smad binding element (SBE) to regulate the expression of TGF-β/Smad-responsive genes [9,10] (Figure 1). The complex signaling pathway with numerous signaling components presents a great deal of flexibility, as well as complexity, in manipulating TGF-β signaling in a disease context. As such, TGF-β signaling can be manipulated through different strategies, from production, activation, and receptor binding to downstream Smads and target genes (Figure 1).

The SBE has been demonstrated to confer TGF-β/Smad-responsive transcription in numerous in vivo and in vitro assays and has thus been used as a promoter for reporter systems specific to the TGF-β/Smad signaling pathway. We generated SBE-luc mice that express luciferase under the control of an SBE promoter [20–23]. These mice allow us to monitor the temporal, tissue-specific activation of Smad2/3-dependent signaling in living mice [20–23]. By analyzing the SBE-luc mice, we discovered that the brain has the highest basal TGF-β signaling among all organs, and that, within the brain, the hippocampus has the highest basal TGF-β signaling among all regions [21,23]. Basal TGF-β signaling is localized primarily to pyramidal neurons [21]. The hippocampus, which plays a critical role in learning and memory, is particularly vulnerable to a variety of insults [24]. These results indicate the importance of TGF-β signaling in maintaining the normal function of the hippocampus. The SBE-luc mice have also helped us to identify endogenous and synthetic modulators of the TGF-β/Smad pathway [25,26].

In the CNS, TGF-βs and their receptors are widely expressed among many cell types and they play an essential role in the differentiation, development, and function of glial, neuronal, and endothelial cells [12]. In astrocytes, TGF-β regulates radical glia (neuroglial progenitor) differentiation and astrocyte formation during development [14]. The expression of glial fibrillary acidic protein (GFAP), an intermediate filament protein commonly used as a classical marker for mature astrocytes, is tightly regulated during development and under pathological conditions [27,28]. TGF-β signaling is one of the main pathways that regulate GFAP promoter activity and expression [14]. Neurons, especially during the embryonic period, secrete TGF-β1 that induces GFAP expression in astrocytes [27,28]. Interestingly, by regulating TGF-β1 synthesis and secretion, neurons at different developmental stages and from different regions exert different effects on GFAP expression [27,28]. On the other hand, astrocyte-derived TGF-β mediates crosstalk between astrocytes and other cells in the brain and modulates their functions. The overexpression of TGF-β1 selectively in astrocytes reduces neurogenesis and astrogenesis in the GFAP-TGF-β1 mice [29]. During inflammation, astrocytes alone [30] or cooperating neurons [31] release TGF-β to promote microglial homeostasis and repress inflammatory responses. Therefore, TGF-β1 is not only a key regulator of astrocyte formation and maturation, but also an effector of astrocyte function, mediating crosstalk between astrocytes and other cells in the CNS.

## 3. Astrocytes Reactivity

Astrocytes are essential for brain function and homeostasis during development and in the adult brain [1,2]. During CNS development, astrocytes regulate synapse formation, function, plasticity, and elimination, a function that continues in the adult and pathological brain. In the adult brain, astrocytes play multiple roles in the maintenance of brain structure and function. They provide metabolic and structural support to neurons and dynamically regulate brain homeostasis, signal transmission, and synaptic plasticity, as well as blood–brain barrier (BBB) integrity [1,2,6].

In addition to their physiological roles, astrocytes play important roles in response to disease and injury [1,2,5,6,32]. Astrocytes react to pathological insults by becoming

reactive, and undergo a broad spectrum of morphological, molecular, and functional changes in a graded manner [1,2]. The changes that reactive astrocytes undergo include the downregulation of inwardly rectifying K+ (Kir) channels, the glutamate transporters GLT-1 and GLAST, the purinergic receptor P2Y1, adenosine kinase, and water transporter aquaporin 4 (AQP4); and the upregulation of metabotropic glutamate receptors [33]. These changes may cause glutamate and potassium imbalances, leading to neurotoxicity or epileptiform activity, but they do not always occur concurrently [33]. In the literature, many names have been used to describe this phenomenon. A recent recommendation suggests using "reactive astrogliosis," "reactive astrocytes," or "astrocyte reactivity" [1], all of which will be used interchangeably in this review.

Reactive astrogliosis is considered a defense response that aims to limit damage, control inflammation, and restore homeostasis [2,34]. However, like peripheral inflammatory responses, astrogliosis may become maladaptive and cause tissue damage under certain circumstances [33]. A recent transcriptomic analysis has revealed a high heterogeneity of reactive astrocytes and indicated that there may be spatial and temporal diversity in their response relative to the degree and site of injury [33]. The heterogeneity is thought to have functional implications, but, currently, how the heterogeneity is regulated and contributes to the adaptive and maladaptive effects of reactive astrocytes remains elusive. Efforts on identifying and categorizing beneficial and detrimental aspects of astrocyte reactivity led to the discovery of "A1" (neurotoxic and proinflammatory) and "A2" (neuroprotective) astrocytes [35,36], analogous to proinflammatory M1 and anti-inflammatory M2 macrophages. However, recent single-cell RNA sequencing (scRNA-seq) studies have demonstrated that the heterogeneity of reactive astrocytes extends beyond these two distinct states [37] and that the binary categorization of reactive astrocytes bears significant shortcomings [1]. Therefore, in the recent consensus statement, it is recommended to abandon the A1/A2 labels and the misuse of their marker genes [1]. Nevertheless, it is evident that, under certain pathological conditions, astrocytes can adopt an inflammatory phenotype and exert harmful and maladaptive effects, and markers for inflammatory reactivity are becoming established [37]. Accordingly, "inflammatory astrocytes" [37,38] or "neuroinflammatory astrocytes" [39,40] has been used to describe this type of astrocyte reactivity. "Neurotoxic reactive astrocytes" has also been used [35,41], but it is recommended to use it only when the observed neuronal death is due to identified toxic factors released by reactive astrocytes [1].

The complement system is a powerful modulator and effector of astrocyte function [42]. The activation of the complement pathway is a well-established feature of inflammatory astrocytes and can result in detrimental neuroinflammation [37]. Astrocytes are a major source of the complement system proteins [42], and the third complement component (C3) is particularly enriched in inflammatory astrocytes [37,42]. C3 mediates astrocyte toxicity to neurons, microglia, and endothelial cells [43–46]. A more recent study shows that $C3^+$ astrocytes release long-chain saturated lipids that mediate astrocyte toxicity and induce cell death via apoptosis-related pathways [41]. Therefore, C3 exerts toxicity in many cell types via a variety of mechanisms and thus is now frequently used to identify inflammatory astrocytes [37]. Of note, currently, there is no specific marker for inflammatory reactivity and current markers for reactive astrocytes can be expressed by other cell types [37,42]. It is therefore recommended to include multiple molecular markers, together with functional assessments, when characterizing reactive astrocytes [1,2].

Inflammatory (reactive) astrocytes can be induced by microglia [35,41,47] and endothelial cells [48]. The transformation of normal astrocytes to reactive phenotypes involves a variety of intrinsic and extrinsic molecular regulators and signaling pathways, including cytokines (IL-6, LIF, CNTF, IL-1, IL-10, TGF-β, TNF-α, and INF-γ) and transcription factors (NF-κB, Stat3, Olig2, mTOR, and AP-1) [6,15,49] (Figure 2). Among them, TGF-β signaling has been identified as a key regulator of astrocyte reactivity and glial scar formation [6,15,49].

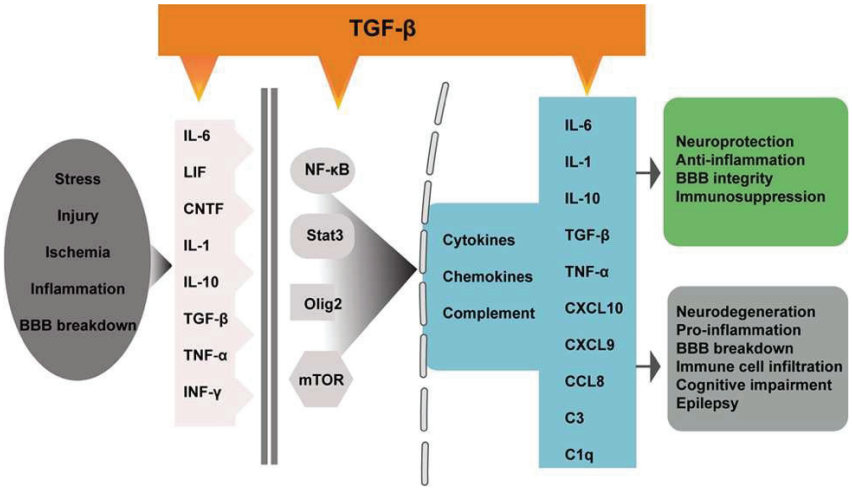


**Figure 2.** Regulators and signaling pathways of astrocyte reactivity. In response to insults such as stress, injury, ischemia, inflammation, and BBB breakdown, brain cells produce inflammatory factors that trigger astrocyte reactivity through transcriptional pathways involving NF-κB, Stat3, Olig2, and mTOR. These pathways regulate the production of cytokines, chemokines, and complements, which mediate the neuroprotective or neurodegenerative effects of reactive astrocytes, and are also involved in triggering and maintaining astrocyte reactivity. TGF-β regulates astrocyte reactivity through multiple mechanism: inflammatory factors, transcriptional pathways, and downstream target genes.

## 4. TGF-β Regulation of Astrocyte Reactivity

Astrocyte reactivity is strongly regulated by TGF-β signaling in both autocrine and paracrine fashion. Astrocytes have the ability to both synthesize and respond to TGF-β, enabling TGF-β to act as an autocrine or paracrine factor for astrocytes [14]. Astrocytes are a main contributor of endogenous TGF-β1 production in the CNS [50]. During CNS inflammation or injury, the expression of TGF-β ligands and receptors is rapidly upregulated in microglia and astrocytes, a typical hallmark of gliosis [11,51,52]. The action of TGF-β on astrocytes is highly context-dependent and can promote or inhibit astrogliosis. In primary astrocyte culture, treatment with TGF-β1 results in reactive astrogliosis characterized by the upregulation of GFAP, chondroitin sulfate proteoglycans (CSPGs), and other molecules that inhibit axon growth [53,54]. The TGF-β1-induced astrogliosis recaptures many aspects of astrogliosis and the inflammatory response in disease in vivo [55], and has thus been developed as an in vitro model of reactive astrogliosis [53]. In the presence of inflammatory stimuli, however, TGF-β1 inhibits reactive astrogliosis. Primary astrocytes cultured with IL-1α, TNF-α, and C1q show an inflammatory phenotype, which is reversed by TGF-β1 treatment [35]. The GFAP-TGF-β1 mice, which overexpress TGF-β1 specifically in astrocytes [56,57], provide an excellent tool for studying the function of astrocytic TGF-β1 in vivo. It has been shown that, depending on the disease context, increasing TGF-β1 in astrocytes in these mice can promote or inhibit astrogliosis [22,58,59].

There are several possible mechanisms by which latent TGF-β is activated in astrocytes. Many of the known activators of latent TGF-β (Figure 1) [18] have been demonstrated to function in astrocytes and activate latent TGF-β in a context-dependent manner. For example, $\alpha_v\beta_8$ is enriched in astrocytes. $\alpha_v\beta_8$-activated TGF-β mediates cell–cell contact and communication during development and disease [60]. The $\alpha_v\beta_8$-mediated activation of TGF-β is the major mechanism of activating TGF-β in primary astrocytes and is sufficient for inhibiting endothelial migration [61]. The selective ablation of $\alpha_v$ or $\beta_8$ in astrocytes leads to the diminished activation of latent TGF-β and defective TGF-β signaling in vascular endothelial cells, accompanied by impaired blood vessel sprouting and hemorrhage [62].

We have shown that TSP-1 is upregulated in astrocytes during neuroinflammation, leading to increased TGF-β signaling during the early stages of disease [63]. Under conditions where BBB integrity is impaired, blood constituents, such as albumin, fibrinogen, and immunoglobulins, can enter the parenchyma, accumulate particularly in astrocytes, and activate astrocytic TGF-β signaling [64–66].

Similarly, TGF-β may regulate astrocyte reactivity via different mechanisms. TGF-β can directly activate the GFAP promoter [14], which increases GFAP expression in astrocytes [67]. In addition, TGF-β activates many of the known molecular triggers and modulators (Figure 2) of reactive astrogliosis, including IL-6 [68], NF-κB, Jak-Stat, MAP-KKK, and the complement signaling pathways [69]. TGF-β induces the rapid activation of mTOR signaling through noncanonical (SMAD-independent) pathways [70]. Astrocytes protect neurons from serum deprivation-induced cell death by the release of TGF-β and activation of the c-Jun/AP-1 signaling pathway [71]. Finally, TGF-β may modulate reactive astrogliosis through its downstream effectors. KCa3.1 (a potassium channel protein) [72], repulsive guidance molecule a (RGMa) [54], response gene to complement 32 (RGC-32) [73], and connective tissue growth factors (CTGF) [55] are all known downstream targets and have been shown to mediate TGF-β-induced reactive astrogliosis and glial scar formation. Interestingly, the effects of CTGF and TGF-β are mediated by NF-κB and AP-1 through ASK1-p38/JNK pathways [55], implicating the extensive crosstalk among astrogliosis regulators. ASK1 (apoptosis signal-regulating kinase 1) is an important mediator of astrocyte reactivity and scar formation [74]. Other well-known downstream targets of TGF-β signaling, such as Cdkn1a (p21) and plasminogen activator inhibitor-1 (PAI-1), have not been shown to be involved in TGF-β-induced astrocyte reactivity. p21 can be induced by TGF-β independent of p53 [75]. p21 plays a critical role in the mediation of TGF-β downstream effects and in the control of the specificity of TGF-β responses [76]. p21 mediates the effects of TGF-β1 on cell proliferation and can exert neuroprotective effects via cell-cycle-independent pathways through noncanonical action [77]. An absence of p21 reduces lipopolysaccharide (LPS)-induced astrogliosis [78], but whether p21 mediates TGF-β-induced astrocyte reactivity remains to be investigated. Similarly, the Smad3-dependent induction of PAI-1 in astrocytes mediates the neuroprotective activity of TGF-β1 against NMDA-induced toxicity [79], but its role in TGF-β-induced astrocyte reactivity is not yet known. In summary, the precise signaling mechanisms by which TGF-β regulates astrocyte reactivity are not fully clear, but it is likely that multiple mechanisms are involved, in a disease- and context-dependent manner.

Whether TGF-β plays a role in the regulation of inflammatory marker C3 in astrocytes is not known; however, the crosstalk between the TGF-β signaling and complement system has been shown in many other cell types, including immune cells. In human whole blood cells, Toll-like receptor 9 (TLR9) activation stimulates C3 expression by inducing TGF-β1 production [80]. Alk5 inhibitor SB431542 abolishes TLR9 stimulation on C3 gene expression [80]. TGF-β signaling is responsible for iron-induced C3 expression in the retinal pigment epithelium [81]. The pharmacologic inhibition of SMAD3 phosphorylation or knockdown of SMAD3 decreases iron-induced C3 expression [81]. In pulmonary epithelium, the treatment of TGF-β1 causes a loss of complement inhibitory proteins, leading to complement activation, whereas treatment with C3 upregulates TGF-β1 transcripts and down-regulates SMAD7 (negative regulator of TGF-β signaling) [82]. C3 inducing factors IL-1α and TNF-α upregulate TGF-β1 in astrocytes [83,84], and pretreatment with morphine suppresses the TNF-α induction of C3 and TGF-β1 [83]. Importantly, scRNA-seq analysis identified one inflammatory astrocyte subpopulation (characterized by the upregulation of C3) enriched in TGF-β signaling in Alzheimer's disease brains [85]. These studies indicate a link between TGF-β1 and C3 induction, but whether TGF-β1 directly upregulates C3 has not been demonstrated in astrocytes.

Recent studies have suggested that TGF-β signaling may regulate astrocyte heterogeneity. During brain development, TGF-β signaling is a hallmark of astrocyte lineage diversity, and the response to TGF-β signaling drives astrocyte progenitors to generate

different astrocyte lineages [86]. In the adult brain, astrocytes from different brain regions display different synaptogenic properties [87], which is, in part, due to their differential expression of TGF-β1 [88]. An in vitro study using human-induced pluripotent stem cell (iPSC)-derived astrocytes further demonstrated that the regional heterogeneity of astrocyte function is due to differences in TGF-β2 secretion between astrocytes from different regions [89]. A scRNA-seq analysis of diencephalic astrocytes revealed that astrocyte heterogeneity and low-level astrogenesis are regulated by Smad4 [90]. Likewise, the identification of an inflammatory astrocyte subpopulation enriched in TGF-β signaling in Alzheimer's disease brains [85] suggests that the response to TGF-β signaling may drive astrocytes towards the inflammatory phenotype. As discussed above, TGF-β1 can either promote or inhibit inflammatory reactivity in vitro [35,53], depending on experimental conditions. Together, these findings point towards important roles for TGF-β signaling in astrocyte heterogeneity in the normal and diseased brain.

## 5. Astrocytic TGF-β Signaling in CNS Diseases

Reactive astrogliosis is observed in virtually all neurological conditions, including traumatic injury, stroke, aging, neurodegeneration, and epilepsy [91], as discussed in the following sections. TGF-β signaling is a powerful regulator and effector of astrocyte reactivity. The manipulation of this pathway in models of CNS injury and disease alters pathological and functional outcomes, highlighting the importance of TGF-β signaling and reactive astrogliosis in CNS diseases.

### *5.1. Traumatic Brain Injury*

Traumatic brain injury (TBI) is a leading cause of death and disability and represents a significant socioeconomic and public health burden worldwide [92]. Clinically, TBI can be categorized as mild (mTBI, also referred to as concussions), moderate, or severe based on the extent of damage to the brain. The damage can be focal (confined to one area of the brain) or diffuse (widespread). Moderate and severe TBI can lead to long-term physical, cognitive, emotional, and behavioral deficits. mTBI accounts for approximately 80% of all clinically diagnosed cases and is predominantly diffuse in nature. Most patients sustaining a single mTBI recover to full function without long-term neurological impairments [93,94]. However, patients with repetitive mTBI may experience long-lasting neurological symptoms because even mild recurrent brain injuries may induce cumulative effects and interfere with neuropsychological recovery [95]. TBI has been identified as a risk factor for neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD) [96]. Animal models have been essential for our understanding of the pathophysiology and cellular and molecular mechanisms of TBI. Commonly used animal models of TBI include fluid percussion injury, cortical impact injury, weight drop-impact acceleration injury, and blast injury for non-penetrating injury [97,98]; and stab lesion and penetrating ballistic-like brain injury [99] for penetrating head injury.

The underlying pathophysiology of TBI is complex and, in general, is divided roughly into primary and secondary injury [100,101]. The primary injury is severity-dependent and results from mechanical forces applied to the skull and brain at the time of impact [100,101]. Studies utilizing various experimental models of TBI indicate that the primary brain injury triggers a cascade of molecular and biochemical events leading to long-lasting secondary neuronal and glial damage, including excitotoxicity, oxidative stress, neuroinflammation, brain edema, and delayed neuronal death [100,101].

TBI can quickly trigger astrocyte reactivity through the activation of mechanosensitive ion channels and receptors [102]. Astrocyte reactivity is an early secondary response to TBI [103,104]. Reactive astrogliosis is highly heterogeneous and can range from reversible alterations in gene expression and cell hypertrophy to scar formation with substantial cell proliferation and permanent structure reorganization [103]. Therefore, reactive astrocytes play multiple roles in TBI pathogenesis and are a major determinant of TBI outcomes [104]. Astrocyte reactivity contributes to initial synaptic loss and BBB breakdown, later synaptic

remodeling [103,104], and aberrant neurogenesis in the hippocampus after TBI [105]. On the other hand, reactive astrocytes are the main cellular component of the glial scar, which is considered a protective mechanism to prevent the spreading of secondary damage [17,103]. The astroglial scar also releases inflammatory mediators to remove damaged tissue and to promote regeneration [17,103]. Thus, reactive astrocytes can have beneficial or detrimental effects following TBI.

Using the GFAP-luc mice, which express firefly luciferase reporter under the control of the GFAP promoter [106], we showed that astrocyte reactivity and GFAP expression (measured by the bioluminescence signal) correlate with injury intensity (controlled by the speed of the injury impactor) and can be used as a reliable surrogate biomarker for TBI [107,108]. The usefulness of GFAP and significance of astrocyte reactivity in TBI are highlighted by the fact that, in 2018, the FDA authorized a blood test of GFAP for the clinical evaluation of mTBI patients, because blood GFAP levels correlate with the clinical severity and extent of intracranial lesions following head trauma [109].

TGF-β is a major regulator of the injury response and has complex roles in TBI. Controversial results have been reported on the expression of TGF-β and the components of the pathway, as well as the effects of the modulation of TGF-β signaling in TBI. It is generally reported that, after traumatic injury, TGF-β signaling is activated and the components of TGF-β pathway are upregulated. For instance, increased levels of TGF-β are found in the brain following traumatic injuries in patients and in animal models [110]. TGF-β1, -β2, and -β3 [111], Tgfbr1 and Tgfbr2 [112], and Smads and p-Smads [113,114] are upregulated after injury and usually in astrocytes, oligodendrocytes, microglia, and neurons in a variety of TBI models. We observed that stab lesions resulted in the consistent induction of TGF-β1 and PAI-1 mRNA, as well as rapid activation of TGF-β signaling in SBE-luc mice [23]. On the contrary, multiple studies in preclinical models reported a reduced expression of TGF-β pathway components. The expression of Tgfb1 and Tgfbr1 is reduced in the rat brain after cortical contusion [114]. Similar results were obtained by employing NanoString gene expression analysis, where genes in the TGF-β signaling pathway (Tgfb1, Tgfbr1, Tgfbr2, Smad3, and Ski) were shown to be significantly downregulated in mouse microglia after cortical impact injury [115].

The modulation of TGF-β signaling in TBI has also shown both beneficial and detrimental effects. For example, the activation of the TGF-β signaling pathway restrains pro-inflammatory responses and boosts tissue reparatory responses of reactive astrocytes and microglia after stab wounds [113]. The intracerebroventricular (ICV) injection of TGF-β1 promotes functional recovery and alleviates axonal injury and neuroinflammation after weight drop-induced TBI [114]. Consistent with a protective role in TGF-β signaling, Tgfb1 knockdown worsens the neurological outcome in rats with weight drop TBI [116]. In contrast to these findings, a detrimental role in TGF-β signaling has also been shown by inhibiting TGF-β signaling. The inhibition of TGF-β signaling with SB431542 or transfection of Tgfb1 siRNA and inhibitory Smad7 has shown protective effects, diminishing neuroinflammation and apoptosis in a rat TBI model of fluid percussion injury [117]. Similarly, blocking TGF-β signaling with angiotensin receptor 2 antagonist losartan ameliorates secondary brain injury in a cortical impact injury model, decreases the brain lesion volume and neuronal apoptosis, and improves the neurological and motor function [118]. The reason for these contradictory results is not clear, but it is most likely due to the complex pathophysiology of TBI and/or differences in animal models employed, and the stages of injury and cell type affected.

The role of TGF-β signaling in astrocytes in TBI seems to be more defined. The TBI-induced astrocyte reactivity and activation of TGF-β signaling in astrocytes may be triggered by the exposure of astrocytes to blood-borne factors, such as albumin, fibrinogen, and thrombin. Albumin stimulates the secretion of TGF-β and IL-1β from astrocytes [64]. Fibrinogen is a carrier of latent TGF-β and induces p-Smad2 in astrocytes, which leads to astrocyte reactivity and the inhibition of neurite outgrowth [65]. The genetic or pharmacologic depletion of fibrinogen in mice reduces active TGF-β, p-Smad2, gliosis, and neurocan

deposition after TBI [65]. The stereotactic injection of fibrinogen into the mouse cortex induces astrogliosis, and the inhibition of TGF-β signaling abolishes fibrinogen-induced glial scar formation [65]. Similarly, the ICV infusion of thrombin activates TGF-β signaling [119] and drives astrogliosis and memory impairment [120]. Thrombin-induced astrocyte dysfunction contributes to depression [121] and seizure [122] following TBI. These results identify blood-borne factors as a primary activation signal for astrocyte reactivity and TGF-β signaling, and support a detrimental role of astrocytic TGF-β signaling in TBI. They point to TGF-β signaling as a molecular link between vascular damage and astrogliosis.

### *5.2. Stroke*

Stroke is the second leading cause of death and third leading cause of disability in adults worldwide [123]. Stroke can be classified into two major types: ischemic and hemorrhagic. Hemorrhagic stroke results from a ruptured blood vessel and the extravasation of blood into the brain parenchyma. Ischemic stroke accounts for approximately 80% of all strokes and results from the thromboembolic occlusion of a cerebral artery. Cerebral artery occlusion results in a decreased blood flow, which leads to neuronal dysfunction/death and clinical deficits [123]. The ischemic penumbra, the area of hypo-perfused brain tissue surrounding the ischemic core, could potentially be salvaged and is therefore the focus of stroke research and clinical practice [124]. Following ischemic stroke, the most pronounced pathological and cellular change is the glial response: microglia within and around the infarct engulf cellular debris, astrocytes around the infarct proliferate and form glial scars, and astrocytes in the ischemic penumbra become reactive in a spatial gradient [33].

Reactive astrogliosis is a prominent pathological feature after stroke [33]. The transcriptomic profile of astrocytes after ischemic injury shows a beneficial anti-inflammatory phenotype [36]. In the acute phase, astrocytes limit the tissue damage by promoting brain homeostasis. In the post-acute, recovery phase, reactive astrocytes modulate axonal sprouting and synaptic plasticity, and participate in CNS regeneration [125,126]. Accordingly, the ablation of a subset of reactive astrocytes disrupts vascular repair and remodeling, exacerbates vascular permeability, and worsens motor recovery [127]. However, the swelling endfeet of reactive astrocytes can compress brain microvessels and thereby decrease microvascular perfusion [128]. Therefore, astrogliosis and astrocyte dysfunction have been linked to post-stroke cerebral blood flow (CBF) reduction and BBB impairment [33]. Consequently, the inhibition of astrogliosis improves CBF and reduces cerebral microvessel damage and BBB injury in ischemic mouse brains [129], suggesting a detrimental role of astrogliosis after stroke. A number of key factors and intracellular signaling pathways have been discovered to govern astrocyte behavior. Among them, TGF-β is identified to support the protective phenotype of reactive astrocytes after stroke [15].

The neuroprotective function of TGF-β is most established in brain ischemia [130]. The mechanisms by which TGF-β mediates neuroprotection include the suppression of inflammation, apoptosis, and excitotoxicity, as well as the promotion of scar formation, angiogenesis, and regeneration [11,130]. For example, the ICV delivery of TGF-β1 reduces infarction size and suppresses neuronal apoptosis in rats after ischemic stroke [131]. TGF-β1 produced in the ischemic core is shown to diffuse toward the ischemic penumbra and drive microgliosis to eliminate degenerating neurons [132]. Accordingly, the overexpression of Smad3 in the rat brain reduces infarct volume through anti-inflammatory and anti-apoptotic pathways [133]. In summary, TGF-β exerts neuroprotective effects through multiple mechanisms, but it is not clear whether these effects are mediated through neurons or glial cells.

In the SBE-luc mice, the ischemic-stroke-induced activation of TGF-β signaling begins on day 1 and peaks on day 7 [134]. Microglia [134] and astrocytes [135] are the predominant source of TGF-β production after stroke. TGF-β signaling is activated in astrocytes and microglia in the stroke penumbra [134]. Like in TBI, TGF-β signaling in astrocytes after stroke can be activated by albumin through BBB breakdown [64]. The role of TGF-β signaling in astrocytes has been studied in the "Ast-Tbr2DN" mice, which express a

dominant negative mutant form of Tgfbr2 specifically in astrocytes. These mice display exacerbated neuroinflammation and worse motor outcomes after stroke [136]. These findings are in line with recent work reporting that the blockade of Tgfbr2 in astrocytes abolishes zinc finger E-box binding homeobox 1 (ZEB1)'s protective effects against acute ischemic brain injury [137]. Together, these experiments demonstrate that TGF-β signaling in astrocytes plays a protective role in stroke. Of note, astrocytes isolated from stroke mice show neuroprotective properties by transcriptomic analysis, which has led to the identification of neuroprotective A2 astrocytes [35,36]. TGF-β1 could revert A1 astrocytes to a non-reactive phenotype [35], but whether the activation of TGF-β signaling skews astrocytes toward a protective A2 phenotype remains to be determined.

### *5.3. Aging*

Astrocytes are vulnerable to age-associated dysfunction and stress [138] and undergo complex and region-specific morphological, molecular, and functional changes upon aging [138,139]. Astrocytes display a reactive phenotype with compromised homeostatic functions as the brain ages [138,140]. These include a decreased support of neurons and synapses, impaired synapse formation and synaptic transmission, and a decreased support of BBB integrity [138,140]. Transcriptomic studies have revealed that aged astrocytes display signatures indicative of inflammatory astrocyte reactivity, with highly up-regulated genes involved in the complement (C3 and *C4b*), peptidase inhibitor (*Serpina3n*), and cytokine (*Cxcl10*) pathways [141–143]. Interestingly, C3 and other members of the complement cascade are strongly upregulated in aging astrocytes in all brain regions [141]. A meta-analysis study of 591 gene expression datasets from human prefrontal cortices of distinct ages revealed that the most outstanding results were the age-related decline of synaptic transmission and activated expression of GFAP in the aging brains [144]. Interestingly, the regional expression patterns of astrocyte-specific genes also change upon aging, particularly in the hippocampus and substantia nigra [145], two key areas involved in Alzheimer's and Parkinson's disease. In summary, these results support that, with aging, astrocytes develop a region-dependent reactive and inflammatory phenotype [138,139]. The region specificity might contribute to the differential regional vulnerability to aging and age-related neurodegenerative disorders [138]. Besides reactive astrogliosis, it has been recently proposed that asthenia with a loss of function is a main feature of astrocyte dysfunction in the aging brain [146].

Age-related alterations of the TGF-β pathway in the brain include TGF-β and Smad expression and the TGF-β-induced glial response. Whereas TGF-β levels are increased with aging in the brain of humans [147] and mice [148], Smad expression is reduced with aging. Smad2(Δexon3), a splice form of Smad2 lacking exon3, directly binds to the DNA, resulting in a functional hybrid of Smad2 and Smad3. Smad2(Δexon3) is the most abundant Smad2 isoform in the brain, and is strongly increased prenatally and in early postnatal life, but it continuously diminishes as the brain matures and ages [149]. Such an age-related reduction in Smad expression could lead to impaired TGF-β signaling. Indeed, through longitudinal in vivo bioluminescence monitoring, we recently observed that brain TGF-β signaling decreases with age in the SBE-luc mice [26]. The aged brain not only has reduced TGF-β signaling, but it also fails to upregulate TGF-β1, Smad3, and p-Smad3 upon LPS stimulation [148], suggesting an impaired response to inflammatory stimuli. The impairment of TGF-β signaling could contribute to the persistent mild neuroinflammation [150] and reduced adult neurogenesis [151] during aging. In agreement with this view, the photoactivation of the TGF-β signaling pathway promotes adult hippocampal neurogenesis [152].

The age-related changes in TGF-β signaling are cell-type-dependent. TGF-β1 is reduced in aged neurons [28]. The expression of TGF-β1 is also reduced in oligodendrocytes with aging, which is thought to impair the differentiation of oligodendrocyte progenitor cells into myelinating oligodendrocytes [153]. In contrast, TGF-β1 is increased with aging in astrocytes [154]. In vitro, astrocytes from old animals consistently secrete higher

amounts of TGF-β in vitro compared with the cells from postnatal or young animals [155]. Collectively, these studies suggest that the increased TGF-β observed in the aged brain may be predominantly from astrocytes. The activation of TGF-β signaling in astrocytes has been linked to BBB breakdown in aged human and rodent brains. BBB breakdown is an early biomarker of human cognitive dysfunction [156]. When BBB integrity is compromised, blood-borne factors such as albumin, fibrinogen, and immunoglobulins accumulate in aging human and rodent brains, particularly in astrocytes [157]. These blood-borne factors trigger the activation of TGF-β signaling in astrocytes, which is necessary and sufficient to cause neuronal dysfunction and age-related pathology in rodents [66]. The toxic effects of albumin extravasation have been demonstrated by showing that a direct infusion of albumin into the young mouse brain induces astrocytic TGF-β signaling and an aged brain phenotype [66]. In addition, the conditional genetic knockdown of Tgfbr2 in astrocytes or pharmacological inhibition of Tgfbr1 attenuates age-related cognitive decline and vulnerability to seizures in mice [66]. These observations establish TGF-β as a novel link between reactive astrocytes and cognitive decline. In summary, TGF-β signaling is activated in astrocytes during aging and promotes age-related cognitive deficits. The inhibition of TGF-β signaling may offer therapeutic benefits against cognitive impairments during aging.

### *5.4. Alzheimer's Disease*

Alzheimer's disease (AD) is the most common form of dementia and one of the top global health concerns [158]. Clinically, AD is characterized by progressive cognitive impairments, mainly learning deficits and memory loss [158]. Pathologically, AD is characterized by extracellular amyloid plaques of amyloid β (Aβ) and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau (p-tau), accompanied by reactive gliosis [158]. AD risk genes are mainly expressed by glial cells [3]. For example, apolipoprotein E (APOE), clusterin (CLU), and fermitin family member 2 (FERMT2) are predominantly expressed by astrocytes, supporting a critical role of astrocytes in AD pathophysiology [159].

Reactive astrogliosis is a common and widespread pathological feature of AD brains [8,49,160]. Reactive astrocytes are detected during the early phases of AD, before the presence of characteristic AD pathology, and their reactivity increases with disease progression [3,8]. Recent studies demonstrate that there may be a causal relationship between reactive astrocytes and neurodegeneration. In an animal model where astrocyte reactivity is finely controlled, mild reactivity naturally reverses its reactivity, whereas severe reactivity causes irreversible neurodegeneration and cognitive deficits [161]. The severe reactivity also induces neurodegeneration in APP/PS1 mice, an AD model known for a lack of neurodegeneration [161]. In another study, the overexpression of 3R tau, specifically in hilar astrocytes, leads to reactive astrogliosis and impaired adult neurogenesis and spatial memory performances [162]. Accordingly, the inhibition of astrocyte reactivity through the STAT3 pathway attenuates amyloid deposition and synaptic and spatial learning deficits in AD model mice [163]. These studies demonstrate that reactive astrocytes are sufficient to cause neurodegeneration and may be key players in the etiology of AD [3,5,15,164].

One potential mechanism in which reactive/inflammatory astrocytes contribute to neurodegeneration is through C3 [35]. The upregulation of C3 is observed in AD astrocytes from bulk RNA-seq [165] and snRNA-seq [85] analyses. In the cortex of AD patients, around 60% of the astrocytes are $C3^+$ [35]. Exposure to Aβ activates NF-κB and C3 release in astrocytes [45]. C3 mediates communication/neurotoxicity from astrocytes to other brain cells, contributing to AD pathogenesis. C3 binding to the receptor C3aR in microglia attenuates microglial phagocytosis [44], binding to C3aR in neurons disrupts dendritic morphology and network function [45], and binding to C3aR in endothelial cells promotes vascular inflammation and BBB dysfunction [46]. Accordingly, the genetic deletion of C3 mitigates the Aβ and tau pathology, neurodegeneration, and functional deficits in AD models [44,45,166–168]. Together, reactive astrocytes contribute to maladaptive effects in part through proinflammatory reactions, where C3 acts as a perpetrator of neuroinflammation and neurotoxicity.

An early microarray and RNA-seq analysis of acutely isolated astrocytes from APP mouse models [169] and astrocytes microdissected from AD patients [165] revealed that astrocytes acquired an inflammatory phenotype, with less supportive capacity to neurons. Recent scRNA-seq/snRNA-seq studies of human AD brains have revealed a high heterogeneity and identified many astrocyte subpopulations/clusters. These AD pathology-associated astrocyte subpopulations display different gene expression signatures characterized by, for example, (1) the upregulation of GLUL and CLU, and downregulation of APOE [170]; (2) enrichment for TGFβ signaling and immune responses, with upregulation of C3 [85]; (3) downregulation of genes implicated in metabolic coordination [171]; (4) expression of Aβ plaque-induced genes [172]; (5) enriched expression of stress response-associated genes [173]; (6) GFAP-high subpopulation with upregulation of genes involved in the extracellular matrix and proteostasis [174]. In the 5xFAD transgenic mouse model of AD, astrocytes undergo dynamic responses as the disease progresses, from a GFAP-low to a GFAP-high state, and an AD-specific population (termed "disease-associated astrocytes") [175]. These "disease-associated astrocytes" are enriched in Gfap, Serpina3n, Ctsb, ApoE, and Clu. Overall, these studies provide complementary snapshots of astrocytic responses to AD pathology [159]. They suggest that reactive astrocytes in AD are highly heterogenous and may contribute to different aspects of AD pathology. A recent systemic review of 306 publications further supports this notion, showing that AD reactive astrocytes undergo a wide range of functional changes [176]. Interestingly, 3 of the 196 AD astrocyte proteins, TGFB2, TGFB3, and TGFBR2 [176], are related to the TGF-β pathway, highlighting the significance of TGF-β signaling in AD pathology.

TGF-β signaling has long been implicated in the pathogenesis of AD. A genetic polymorphism in TGFB1 is associated with the risk of developing AD [177]. TGF-β1 levels are increased in post-mortem AD brains [178]. However, the brains of AD patients have reduced levels of TGFBR2 [179], as well as decreased nuclear Smad2, Smad3, and Smad4 [180]. Nuclear p-Smad2 and p-Smad3 are also reduced in neurons [181–183]. Since the nuclear translocation of p-Smad2/3 is required for the activation of TGF-β signaling and transcription of TGF-β target genes (Figure 1), these observations suggest a defect of TGF-β/Smad signaling in neurons in AD. Such a defect is considered to compromise the neuroprotective effects of TGF-β/Smad signaling, as reducing TGF-β signaling in neurons resulted in age-dependent neurodegeneration and promoted Aβ accumulation and dendritic loss in a mouse model of AD [179]. It has therefore been proposed that impaired TGF-β signaling in neurons contributes to Aβ accumulation and neurodegeneration, and is a risk factor for AD [184].

On the contrary, TGF-β signaling in astrocytes seems to be detrimental. The enrichment of TGF-β signaling and upregulation of reactivity marker gene C3 in an AD-associated astrocyte subpopulation [85] indicate that TGF-β signaling may promote inflammatory astrocyte reactivity. The astrocyte-targeted overexpression of TGF-β1 promotes amyloid angiopathy in the frontal cortex and meninges [56,57] and increases the production of Aβ40/42 by astrocytes in GFAP-TGF-β1/APP (amyloid precursor protein) transgenic mice [59]. Interestingly, TGF-β1 drives APP production only in astrocytes and not in neurons [59], suggesting an astrocyte-specific mechanism of TGF-β contributing to AD pathology. More strikingly, the GFAP-TGF-β1 mice (without overexpressing mutant APP) develop an AD-like cerebrovascular pathology, including a reduction in CBF and increase in perivascular Aβ accumulation [58], further supporting the idea that astrocyte TGF-β1 can directly induce an AD-like vascular and amyloid pathology. Astrocyte TGF-β signaling also mediates APOE neurotoxicity and contributes to the risk of AD. A whole-body or astrocyte-specific deletion of ApoE significantly ameliorates spatial learning and memory deficits, reduces Aβ production, and inhibits astrogliosis in APP transgenic mice [185]. The overexpression of TGF-β in astrocytes abrogates the protective effects of ApoE knockout. In contrast, the inhibition of TGF-β in astrocytes of APP mice exerts therapeutic effects similar to ApoE knockout [185]. In summary, it seems like astrocytic TGF-β signaling plays a detrimental role in AD.

*5.5. Parkinson's Disease*

Parkinson's disease (PD) is the second most common neurodegenerative disease after AD [4,186,187]. Neuropathological hallmarks of PD are a loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and intracellular aggregates of α-synuclein (α-syn) [4,186,187]. A loss of DA neurons causes striatal dopamine deficiency, which has been identified as the main cause of the disease's movement symptoms [4,186,187]. The molecular mechanism of neurodegeneration in PD remains largely unknown, but likely involves multiple pathways and cell types. Neuroinflammation in the SNpc, including astrocyte reactivity, is a key feature of PD pathophysiology [4,186,187]. Accumulating evidence suggests that reactive astrocytes have a crucial role in initiating PD pathophysiology, rather than being merely a secondary phenomenon in response to the damage and death of DA neurons [4].

Genome-wide association studies have revealed numerous loci associated with the risk of development of PD [188]. Many of the genes identified in these studies are expressed in astrocytes at similar or greater levels than in neurons [189], supporting an important role of astrocytes in PD pathogenesis [4]. A systemic analysis indeed revealed that PD-associated genes are enriched in astrocytes of the cortex and substantia nigra [190]. The enrichment of PD heritability from PD GWAS datasets is observed in a lysosomal-related gene set that is highly expressed in astrocytes, microglia, and oligodendrocyte subpopulations [191]. Importantly, genes known to be causative in PD have important roles in astrocyte function [4]. For example, PARK7 (DJ-1), SNCA (α-syn), PLA2G6 (iPLA2), ATP13A2, PINK1, and PRKN (Parkin) are all involved in astrocyte-specific functions, including inflammatory responses, glutamate transport, and neurotrophic capacity [4]. Recent snRNA-seq studies have revealed neuroinflammatory signatures in astrocytes of idiopathic PD patients [192] and α-syn-A53T mice [193], supporting a pro-inflammatory and disease-promoting role of reactive astrocytes. The inflammatory ($C3^+$) astrocyte phenotype is induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [194] or β-sitosterol-β-D-glucoside (BSSG) [195], neurotoxins used to model PD. The genetic deletion of the potassium channel subunit Kir6.2 [196] or treatment with NLY01, a glucagon-peptide-1 receptor agonist [197], mitigates inflammatory astrocyte reactivity and prevents dopaminergic neurodegeneration and behavioral deficits. A direct demonstration of non-cell autonomous mechanisms during neurodegeneration was reported in a mouse model, where PD-related A53T α-syn mutation is specifically overexpressed in astrocytes [198]. These mice show widespread astrogliosis, dopaminergic neuronal loss, and movement disabilities [198], suggesting that α-syn in astrocytes is sufficient to impair astrocyte function and initiate neurodegeneration. In a *Drosophila* model of PD, where α-Syn and risk gene expression are manipulated in neurons and glia separately, several glial risk factors that modify neuronal α-Syn toxicity are identified [199]. The knockdown of these genes exacerbates dopaminergic neuron loss and increases α-syn oligomerization [199]. These results suggest that the PD risk genes exert their effects in glia, which influence neuronal α-Syn proteostasis in a non-cell-autonomous manner [199].

TGF-β has multiple associations with the nigrostriatal system and with pathological characteristics of PD, including DA neuron development and survival, dopaminergic degeneration, α-syn aggregation, and γ-Aminobutiryc acid (GABA) neurotransmission [200,201]. TGF-β is essential for the development and survival of embryonic DA neurons [201], promoting the survival of DA neurons in culture and protecting them against toxicity from the Parkinsonism-inducing toxin N-methyl pyridinium ion ($MPP^+$, a neurotoxic metabolite of MPTP) [202]. Genetic association studies suggest that a variation in the TGFB2 gene may influence susceptibility to idiopathic PD [203]. TGF-β2 haplodeficiency ($Tgfb2^{+/-}$) mice have fewer DA neurons in the SN and a significantly reduced dopamine concentration in the striatum in adulthood [204]. Smad3 and pSmad3 expression decreases with age in mouse substantia nigra [205]. Smad3-deficient mice develop a progressive loss of DA neurons and aggregation of α-synuclein [206]. These results suggest that deficiency in TGF-β signaling may increase the risk of developing PD. However, the continuous ad-

ministration [207] or overexpression [208] of TGF-β fails to show protective effects against MPTP in animal models of PD. The cause of the contradictory results from in vitro and animal studies is not known, but could be that the in vivo delivery of the TGF-β ligands may initiate TGF-β signaling in many cell types in vivo [209]. It is therefore important to study TGF-β signaling in a cell-type-specific manner. We used transgenic mice and viral-mediated gene transfer to drive the expression of mutant TGF-β receptors and achieve a neuron-specific manipulation of TGF-β signaling [209]. We generated mice with reduced TGF-β signaling in neurons by expressing a truncated kinase-defective Tgfbr2 under the control of a CamKII promoter [209]. These mice display age-related motor deficits and a mild degeneration of midbrain dopaminergic neurons [209]. Similarly, deleting Tgfbr2 in mature dopaminergic neurons with DAT-iCre caused a significant reduction in dopaminergic axons in the striatum in another study [210]. Moreover, we show that increasing TGF-β signaling by the overexpression of a constitutively active form of Tgfbr1 reduces MPTP-induced dopaminergic neurodegeneration and motor deficits [209]. In agreement with this finding, we recently show that C381 (formerly SRI-011381), a novel small molecule TGF-β/Smad activator, significantly reduces MPTP-induced dopaminergic neurodegeneration and improves motor function in mice [26]. Together, these studies suggest a protective role of neuronal TGF-β signaling in PD.

Compared with neuronal TGF-β signaling, astrocytic TGF-β signaling in PD is less studied. TGF-β signaling may be compromised in PD astrocytes, as the RNA sequencing of LRRK2 G2019S iPSC-derived astrocytes revealed a downregulation of TGFB1 [211]. The ICV injection of α-syn oligomers increases TGF-β1 secretion by reactive astrocytes [212,213]. The inhibition of TGF-β signaling reduces the density of striatal excitatory synapses and the expression of astrocyte glutamate transporters [212,213], supporting a protective role of TGF-β signaling in synucleinopathy. Astrocytes of aquaporin 4-deficient ($Aqp4^{-/-}$) mice fail to upregulate TGF-β1 in response to MPTP treatment and display significantly stronger inflammatory responses and greater losses of dopaminergic neurons than wild-type controls [50]. The stereotactic injection of TGF-β1 in the striatum significantly reduces neuronal damage and microglial activation in MPTP-treated $Aqp4^{-/-}$ mice [50]. These findings support the idea that astrocytic TGF-β1 is a potent inhibitor of neuroinflammation and mitigates dopaminergic neuron injury, and that AQP4 regulates the TGF-β pathway in astrocytes.

In addition to PD, atypical Parkinson disorders also exhibit parkinsonism, but with different clinical manifestations and pathologic features to PD [214]. Atypical parkinsonian disorders commonly include dementia with Lewy bodies (DLB), multiple system atrophy (MSA), progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD) [214]. The pathogenesis of atypical Parkinson disorders is not well understood, but current evidence suggests that the inflammatory mechanisms in PD and atypical parkinsonisms appear to differ [215]. Pathologically, PSP and CBD are tauopathies. PSP is characterized by tau-enriched tufted astrocytes and NFTs in subcortical nuclei [214,216]. The pathologic features for CBD are cortical and striatal tau-positive neuronal and glial lesions, especially astrocytic plaques and thread-like lesions, along with neuronal loss in focal cortical regions and in the substantia nigra [214,216]. The tau pathology in astrocytes does not correlate with neuron loss and is thus considered as an independent degenerative process rather than a reactive response [215]. It may be noted that PSP and CBD share multiple features in the clinical manifestations, pathology, biochemistry, and genetic risk factors [217–219]. The boundaries between PSP and CBD are thus often questionable and, currently, there is no definitive noninvasive antemortem diagnostic test [217,220]. DLB and MSA are synucleinopathies. Though α-syn is expressed predominantly in neurons, α-syn aggregates and inclusions in astrocytes are a common feature in these neurodegenerative diseases [221]. Astrocytes have been shown to interact with extracellular α-syn released by neurons and mediate neuroinflammation, cell-to-cell spread, and other aspects of pathogenesis [222]. Together, these pathological features indicate that astrocytes may be a key element in the pathogenesis of atypical Parkinson disorders [223]. The expression of TGF-β2 is observed

in both NFT-bearing neurons and tangle-bearing glial cells, and the immunoreactivity of TGFBR1 and TGFBR2 is increased in reactive glia in PSP [224,225], but the role of TGF-β has not been investigated.

### *5.6. Amyotrophic Lateral Sclerosis*

Amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) is a devastating neurodegenerative disease characterized by a progressive loss of motor neurons, with an incidence of 1–2/100,000 per year and mean survival of 3–5 years after diagnosis [226]. ALS occurs in two different forms, sporadic (>90% of cases) and familial (<10% of cases). To date, more than 120 genetic variants have been implicated in ALS (https://alsod.ac.uk accessed on 18 April 2022), and at least 25 genes have been shown to cause or significantly increase the risk of ALS. Among them, C9ORF72, SOD1, FUS, and TARDBP (TDP-43) are the most common causative genes [7,226]. The precise pathological mechanisms of ALS are not clear. Besides the main classical "neuron-centric" view, recent studies have substantiated that ALS could also be a non-cell-autonomous disease [7]. Gliosis is a pathological hallmark of ALS, and glial cells are all able to modulate the ALS pathology [7].

Experimental models of ALS have greatly improved our understanding of reactive astrocytes in ALS pathogenesis [7]. Most studies across ALS model systems support a neurotoxic role of reactive astrocytes in ALS [7]. $C3^+$ inflammatory astrocytes are abundant in familial and sporadic ALS patients and in *SOD1*$^{G93A}$ mice [39]. Knocking out C3-inducing factors (IL-1α, TNF-α, and C1q) markedly extends survival in the *SOD1*$^{G93A}$ ALS mouse model [39]. Direct evidence of a non-cell autonomous toxic mechanism came from models using cell-type-specific promoters. Whereas the selective deletion of SOD1$^{G85R}$ [227] or SOD1$^{G37R}$ [228] in astrocytes slows disease progression, the astrocyte-restricted expression of TDP43$^{M337V}$ leads to astrogliosis and progressive non-cell-autonomous motor neuron loss and paralysis [229]. Transplantation studies further support the neurotoxicity of reactive astrocytes. The transplantation of SOD1$^{G93A}$ astrocyte precursors induces host motor neuron death and motor dysfunction in wild-type rats [230], whereas the transplantation of wild-type glial precursors that have differentiated into astrocytes in the spinal cord of SOD1$^{G93A}$ rats prolongs survival and attenuates motor neuron loss and forelimb motor deficits [231]. In vitro, iPSC-derived astrocytes from patients with VCP and SOD1 mutations undergo cell-autonomous reactive transformation characterized by an increased expression of C3 [232]. Accordingly, astrocytes derived from adult NPCs isolated from post-mortem sporadic or familial ALS patients are toxic to healthy motor neurons in culture [233,234]. The neurotoxicity of reactive astrocytes seems to be mediated by neurotoxic factors and the failure of astrocytes to support motor neurons [7]. TGF-β is one of the neurotoxic factors mediating the neurotoxicity of ALS astrocytes [7,235].

A meta-analysis of human and mouse multi-omics reveals that ALS astrocytes are characterized by signatures of TGF-β signaling and inflammatory reactivity, i.e., the upregulation of genes involved in the extracellular matrix, endoplasmic reticulum stress and the immune response, and downregulation of neuronal support processes [236]. These features suggest that ALS astrocytes are pro-inflammatory and lose protective functions. Studies from both cell culture and animal models demonstrate that TGF-β signaling in ALS astrocytes plays a detrimental role and contributes to ALS pathology. In a co-culture model of human motor neurons and primary astrocytes, TGF-β1 secreted by reactive astrocytes disrupts autophagy and induces protein aggregation in motor neurons [237]. In the spinal cord of SOD1$^{G93A}$ mice, TGF-β1 is upregulated mainly in astrocytes, and Tgfb1 mRNA levels negatively correlate with the mouse lifespan [238]. The astrocyte-specific overproduction of TGF-β1 accelerates disease progression in the GFAP-TGF-β1/SOD1$^{G93A}$ mice [238]. Furthermore, the inhibition of TGF-β signaling by a selective Tgfbr1 inhibitor, SB-431542, extends the survival time of SOD1$^{G93A}$ mice [238]. In summary, these studies show that astrocyte TGF-β1 promotes the ALS pathology, and that astrocytes adopt neurotoxic properties in ALS.

*5.7. Multiple Sclerosis*

Multiple sclerosis (MS) is a chronic inflammatory and degenerative disease of the CNS characterized by focal neuroinflammatory lesions and demyelination [239,240]. MS is one of the most prevalent neurological disorders among young adults, with most cases diagnosed between 20 and 50 years of age [239,240]. MS patients show a variety of physical disabilities and cognitive impairments, with different disease progression patterns. Based on disease progression patterns, four types of MS have been identified: relapsing-remitting (RR), primary progressive (PP), secondary progressive (SP), and clinically isolated syndrome (CIS) [241]. Relapsing-remitting MS (RRMS) is the most common type, accounting for more than 80% of MS cases, and many RRMS further develop into secondary progressive MS (SPMS) [241,242].

MS is an autoimmune disease in which the immune system attacks the myelin sheath covering the axons, leading to inflammatory demyelinating lesions [240,243]. The immune attack is mediated by autoreactive T-cells, T-helper (Th)-1, and Th-17 [240,243]. Other immune cells, including cytotoxic T-cells, B-cells, monocytes/macrophages, and CNS glial cells, namely astrocytes and microglia, are also involved in the inflammatory attack and inflict myelin damage [240,243]. MS lesions can be classified into (i) acute lesion with numerous inflammatory cells and astroglial hypertrophy, (ii) chronic active lesion with edged demyelination, and (iii) chronic lesion with fewer leukocytes but profound demyelination, axonal loss, and astrogliosis [241] (based on [244]). Thus, astrogliosis is a key component of MS lesions, and reactive astrocytes play a critical role in lesion development [5].

The roles of astrocytes in MS are complex, and both beneficial and harmful roles have been attributed to reactive astrogliosis [242,245–247]. In both MS and its animal model, experimental autoimmune encephalomyelitis (EAE), astrocyte reactivity initiates during the early stage of lesion formation and persists into the chronic phases, even after the immune cell presence has receded [242,246,248]. C3-containing inflammatory astrocytes are abundant in MS lesions [35], as well as in EAE mice [249]. The conditional deletion of C3 in astrocytes attenuates EAE-induced axonal injury [250]. Using the GFAP-luc mice, we observed that astrocyte reactivity in EAE starts several days before the onset of paralysis, correlating with and predicting EAE clinical severity [22,251]. These findings suggest that reactive astrocytes promote inflammation and lesion formation and that astroglial reactivity is a reliable indicator of disease evolution [246,248]. In agreement with this view, the inhibition of astrocyte reactivity suppressed local CNS inflammation and neurodegeneration in EAE [252]. The underlying mechanisms of reactive astrocytes promoting inflammatory lesion include the recruitment of peripheral inflammatory cells and the activation of microglia and astrocyte intrinsic neurotoxic activities [5,32]. On the other hand, it has been shown that astrocytes have beneficial effects by restricting the infiltration of peripheral immune cells into the CNS and releasing neurotrophic factors to promote tissue repair [5,32]. The transcriptomic profile of astrocytes isolated from non-demyelinated normal-appearing white matter supports a neuroprotective role of reactive astrocytes [253].

The complex roles of astrogliosis in MS and EAE may be explained by the heterogeneity of reactive astrocytes [247], as demonstrated by recent transcriptomic studies. Astrocyte reactivity has been shown to differ between CNS regions in EAE [254] and in MS lesions [255]. Astrocyte heterogeneity is further demonstrated by the discovery of novel astrocyte subsets from recent scRNA-seq studies [256–258]. For example, a pro-inflammatory and neurotoxic astrocyte subset characterized by the downregulation of NRF2 and upregulation of MAFG has been discovered in EAE and MS [258]. NRF2 is a negative regulator of inflammation and oxidative stress. MAFG decreases NRF2 and interacts with MAT2$\alpha$ to block anti-inflammatory pathways. The downregulation of NRF2 and upregulation of MAFG likely promote inflammatory responses in EAE [258]. Similarly, a novel astroglial phenotype with neurodegenerative programming, "astrocytes inflamed in MS," is identified in another study using MRI-informed snRNA-seq [256]. Interestingly,

an anti-inflammatory subset of astrocytes characterized by the co-expression of LAMP1 and TRAIL has also been identified in EAE and MS [257]. This astrocyte population limits inflammation by inducing T-cell apoptosis and is driven by IFNγ [257]. Collectively, these studies suggest that reactive astrocytes display functional and phenotypic heterogeneity and can adopt either a pro-inflammatory or an anti-inflammatory phenotype under CNS autoimmune conditions [5]. A balance between the pro-inflammatory and anti-inflammatory subsets may be critical for the onset and progression of EAE and MS.

Given the potent immunomodulatory effects of TGF-β and the autoimmune etiology of MS and EAE, it is not surprising that TGF-β signaling has been extensively studied in the context of MS and EAE. TGF-β controls both innate and adaptive immune responses by regulating the generation and effector functions of many immune cell types [259]. TGF-β plays a major role in the development and function of both encephalitogenic and regulatory T-cells (Treg) [260]. It also regulates the complex behavior of natural killer cells, macrophages, and neutrophils [259]. These activities likely underlie the beneficial effects of the systemic administration of TGF-β in EAE models [261].

In both active demyelinating and chronic MS lesions, all three TGF-β isoforms and their receptors are strongly expressed in hypertrophic astrocytes [262]. It is therefore hypothesized that astrocyte TGF-β signaling participates in reactive processes and promotes the formation of chronic MS lesions. In the SBE-luc reporter mice, we observed EAE-induced early TGF-β1 production in glial cells and TGF-β signaling in the CNS several days before the onset of paralysis in EAE mice [22]. The astrocyte-targeted overexpression of TGF-β1 results in an earlier onset and more severe paralysis in the GFAP-TGFβ1 mice [22], and systemic treatment with pharmacological inhibitors of TGF-β signaling (Tgfbr1 antagonist or losartan) ameliorates the paralytic disease [22,63]. Therefore, the early production of TGF-β1 in astrocytes may create a permissive environment for the initiation of autoimmune inflammation in EAE. Recent findings from transcriptomic studies support this hypothesis. In patients with progressive MS, large areas of periplaque astrogliosis, partial demyelination, and low-grade inflammation in the spinal cord extend away from plaque borders; these areas are characterized by the up-regulation of TGF-β signaling [51,52]. Similarly, in a study of white matter from patients with progressive MS, RNA-seq analysis and de novo network enrichment based on shared DEGs discovered TGFBR2 as a central hub, which was most upregulated in remyelinating lesions [263]. RNAscope and immunohistochemistry demonstrated astrocytes as the cellular source of TGFBR2 [263]. Together, these findings support the idea that TGF-β signaling in astrocytes promotes astrogliosis, demyelination, and chronic inflammation, and that targeting TGF-β signaling might be a promising therapeutic strategy for MS.

### *5.8. Huntington's Disease*

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by motor disabilities, cognitive impairments, and psychiatric disturbances [264–266]. The genetic cause of HD is an expansion of a polyglutamine-encoding CAG repeat in exon 1 of the huntingtin gene (HTT), with longer repeat lengths leading to an earlier onset and more severe disease [264]. A neuropathological analysis of post-mortem HD brains reveals a dramatic degeneration of neurons prominent in the striatum and cerebral cortex [264]. Neuroinflammation, characterized by the presence of reactive microgliosis and astrogliosis, is frequently observed in HD patients before symptom onset [267]. The classical hypothesis postulates that HD pathogenesis stems from the toxicity of mutant HTT (mHTT) in neurons; however, increasing evidence has established that mHTT exerts toxic cell effects on other cell types of the CNS, which likely contribute to the pathogenesis of HD as well [6,264,268].

The aggregation of misfolded mHTT is a pathological hallmark of HD and is associated with neuronal loss [6,268]. Astrocytes express mHTT to the same extent as in neurons in HD and its preclinical models [6,268]. The selective overexpression of mHTT in mouse astrocytes is sufficient to recapitulate neurodegeneration and motor symptoms observed in HD and its animal models. Conversely, the selective deletion of reactive astrocytes

or astrocyte-specific rescue approaches attenuates neurodegeneration and neurological abnormalities observed in HD models [6,268,269]. In a Drosophila model expressing human mHTT, the downregulation of genes involved in synapse assembly in glial cells mitigates pathogenesis and behavioral deficits [270]. Strikingly, reducing dNRXN3 function in glia is sufficient to improve the phenotype of flies expressing mHTT in neurons [270]. It is now clear that the accumulation or overexpression of mHTT in astrocytes impairs the glutamate uptake and disrupts $K^+$ homeostasis and $Ca2^+$ signaling, leading to astrocyte dysfunction and neurodegeneration [6,268,269]. Brain cholesterol is produced mainly by astrocytes and is important for neuronal function [6,268]. The dysregulation of brain cholesterol homeostasis has been linked to HD [6,268]. The pathogenic impact of cholesterol pathways in HD is supported by a recent study showing that activating the cholesterol biosynthesis pathway in striatal glial cells restores synaptic transmission, clears mHTT aggregates, and attenuates behavioral deficits in an HD mouse model [271]. In addition, a recent study shows that region-specific neuronal toxicity in HD arises from the metabolic reprogramming of astrocytes [272]. Together, astrocyte dysfunction contributes to HD pathogenesis, and targeting astrocyte dysfunction may provide therapeutic potential in HD [6,268].

The role of astrocyte dysfunction in HD pathogenesis is further supported by transcriptomic studies. Transcriptional dysregulation is an early and progressive event that is hypothesized to play an important role in the pathogenesis of HD. Recent snRNA-seq/scRNA-seq studies of astrocytes isolated from post-mortem HD brains and mouse HD models reveal that HD astrocytes experience profound gene expression changes indicative of losing essential normal functions or the activation of inflammatory pathways [273,274]. Further analysis reveals that striatal HD astrocytes display context-specific molecular responses that are regulated by Gi-GPCR activation [275]. Importantly, the selective activation of Gi–GPCR signaling in astrocytes reverses the impairment of synaptic plasticity, $Ca2^+$, and GPCR signaling [275]. In another study, astrocyte-specific transcriptomic analysis shows that the activation of the JAK2-STAT3 pathway in astrocytes coordinates a transcriptional program associated with proteolytic capacity. Importantly, the selective activation of this cascade in astrocytes through viral gene transfer reduces the number and size of mHTT aggregates and improves neurological function in mouse models of HD [276]. These studies identify astrocyte GPCR and JAK2-STAT3 signaling as promising targets to control neurodegeneration in HD.

TGF-β signaling has been implicated in HD [277,278]. Early studies have reported changes in the serum or plasma levels of TGF-β1 in HD patients during disease progression and suggest that TGF-β1 could be used a potential biomarker [279–281]. However, a recent study found plasma levels of TGF-β1 in HD patients are not significantly different from the control group and do not change significantly with the progression of the disease [282]. Therefore, the usefulness of plasma TGF-β1 as a biomarker for the assessment of HD severity needs further investigation. In the brain, the expression of TGF-β1 is reduced in cortical neurons in post-mortem brains from HD patients and HD model mice [281], but whether the reduced TGF-β1 expression plays a role in HD pathogenesis is not clear. In the hippocampal stem cell niches of mouse and rat HD models, TGF-β/Smad signaling is elevated, which is considered to contribute to the induction of the quiescence of NSCs leading to reduced hippocampal neurogenesis [278]. The upregulation of the TGF-β pathway is also observed in a human iPSC model of HD, which can be corrected to normal levels by replacing the expanded HTT CAG repeat with a non-pathogenic normal length [283]. Further, the correction of TGF-β signaling pathways reverses disease phenotypes, such as susceptibility to cell death and altered mitochondrial bioenergetics, in iPSC [283]. These results suggest that mHTT activates the TGF-β signaling pathway and that TGF-β signaling plays a disease-promoting role in HD.

A transcriptome analysis of iPSCs derived from HD patients, neural stem cells (NSCs) from HD patients, or striatal cell lines expressing mHTT also reveals TGF-β signaling as the top dysregulated pathway [284,285]. A further network modeling of Smad3 target genes,

together with Smad3 expression and phosphorylation, and the Smad3 ChIP-seq of the striatum of HD knock-in mice, identified TGF-β/Smad signaling as a core regulator of early gene expression in HD that has therapeutic implications [286]. Systems-based genetic analyses performed on human transcriptomic datasets from post-mortem HD brains identify a novel astrocyte-specific transcriptional module most relevant to HD pathology [287]. This astrocyte HD module is regulated by TGF-β-FOXO3 signaling [287]. Together, these studies show that TGF-β signaling is an important regulator of HD-associated gene expression. In support of this view, TGF-β signaling has been shown to regulate HTT expression [284] and ameliorate mHTT-induced toxicity [285]. In summary, current evidence suggests that the TGF-β signaling pathway may play an important role in HD pathogenesis. However, it is important to note that no studies have directly tested whether manipulating TGF-β signaling alters the disease outcome in conventional animal models of HD. Therefore, further studies are needed to determine if TGF-β signaling is a potential therapeutic target for HD.

### *5.9. Epilepsy*

Epilepsy is a common neurological disorder characterized by an enduring predisposition to generate epileptic seizures [288,289]. Epilepsy is a major public health problem, affecting approximately 1% of the total population worldwide [288]. Temporal lobe epilepsy (TLE) is the most prevalent form of epilepsy and many patients with TLE develop drug resistance [290]. Epilepsy may occur as a result of brain injury, stroke, tumors, infections (meningitis or encephalitis), autoimmune diseases, or genetic mutations [288,289]. The underlying mechanism of epileptic seizures is excessive and abnormal neuronal activity in the cortex of the brain [291]. Recent research points towards the role of non-neuronal cells such as astrocytes in the genesis and spreading of seizures in the brain [289,292,293].

Reactive astrocytes are found both in animal models of epilepsy and in brain tissue from patients with seizures [288]. Astrocytes express ion channels, transmitter receptors, and transporters, and can thus sense and respond to neuronal activity [294]. Many molecular alterations during astrogliosis are causally linked to epileptogenesis, including the downregulation of gap junction connexins, glutamate transporters, potassium channels and aquaporin 4 channels, as well as the activation of inflammatory pathways [288]. Recent studies have shown that the C3-C3aR pathway is involved in epilepsy and epilepsy-associated neurodegeneration [295]. mRNA and protein levels of C1q and C3 are increased in the hippocampus of patients with TLE [296]. Similar results are also obtained from animal models. C3-positive astrocytes are significantly increased in the diisopropylfluorophosphate rat model of epilepsy [297]. C1q-C3 signaling activation in status epilepticus correlates with epileptic seizure frequency [296], and blocking C3 signaling reduces seizure activity and neuronal injury [298,299]. Since C3 is principally produced by activated astrocytes [45], these results further support the epileptogenic role of reactive astrocytes in epilepsy. In agreement with this view, it was recently suggested that astrocyte reactivity may be used as a biomarker for epilepsy [289].

TGF-β signaling is activated and components of the pathway are upregulated in human and experimental epilepsy. TGF-β1 levels are increased in the CSF of patients with drug-resistant epilepsy [300]. TGFBR1 expression is significantly upregulated in temporal neocortices of patients with TLE [301]. Single nucleotide polymorphisms in TGFBR1 are associated with a risk of epilepsy in a Chinese population [302]. More specifically, the TGFBR1 AT and TT genotypes emerge as a protective factor, whereas the TCTAT and TCCAA haplotypes emerge as a risk factor for epilepsy [302]. Further qRT-PCR analysis shows that TGFBR1 mRNA levels are significantly higher in epilepsy patients than in controls. Genotype–phenotype analysis show lower levels of TGFBR1 mRNA in carriers with the rs6478974 TT genotype, which is confirmed by eQTL data [302]. Biallelic loss-of-function mutations in TGFB1 result in very early-onset inflammatory bowel disease and CNS dysfunction associated with epilepsy, brain atrophy, and posterior leukoencephalopathy [303]. The expression of the Smad anchor for receptor activation (SARA) and the level of p-Smad3

are upregulated in the brain of an epileptic rat model, as well as in the temporal cortex of patients with TLE [304]. We have shown that the systemic administration of kainic acid, an epileptogenic and neuroexcitotoxic agent [305], results in a rapid and persistent activation of TGF-β signaling in the SBE-luc reporter mice [21]. Recently, microRNAs (miRNAs) have emerged as promising therapeutic targets for seizure control and disease modification. Veno et al. performed small RNA-seq of Ago2-loaded miRNAs from three different seizure models and identified dysregulated and functionally active miRNAs in seizure pathogenesis [306]. Combinatorial miRNA inhibition (by combining the three most effective antagomirs targeting miR-10a-5p, miR-21a-5p, and miR-142a-5p) reduced seizures in experimental TLE. Interestingly, target and pathway analysis revealed a role of the TGF-β signaling pathway in the anti-seizure effects of combinatorial miRNA inhibition [306]. This evidence suggests that TGF-β signaling is involved in the generation of seizure activity. In line with these observations, TGF-β pathway proteins are identified as key regulators driving epileptogenesis in TLE by a recent study of systems-level analysis [307].

TGF-β has been shown to increase neuronal excitability and trigger epileptogenesis and seizures [308]. Incubating cortical slices with TGF-β1 directly induces epileptiform activity and epileptogenic transcriptional responses, as well as astrocyte reactivity and inflammation [69]. This effect may be related to TGF-β's upregulation of IL-6, which causes neuronal hyperexcitability and epileptiform discharges in vitro and spontaneous seizures in mice [68]. TBI is a common cause of acquired epilepsy [308–310]. The role of TGF-β signaling in post-traumatic seizures is supported by the observation that the inhibition of TGF-β signaling with a Tgfbr1 inhibitor, LY-364947, significantly reduces the duration and severity of post-traumatic seizures [311]. Mechanistically, post-traumatic seizures are mediated via astrocytic TGF-β signaling activated by albumin [312,313]. Animal studies have demonstrated that albumin extravasation in epilepsy is due to BBB breakdown [312,314,315]. When the BBB function is compromised, albumin enters the brain's extracellular space, accumulates in perivascular astrocytes, and binds to TGFBR2, leading to the activation of astrocytic TGF-β signaling [314]. The activation of TGF-β signaling then causes astrocyte reactivity, with an impairment of potassium buffering and glutamate metabolism, and disruption of water homeostasis. All of these changes promote neuronal hyperexcitability and spontaneous seizures [289,312,313]. The role of albumin in epilepsy has been demonstrated in infusion studies. The intracerebroventricular infusion of albumin activates astrocytic TGF-β signaling and induces astrocyte reactivity and excitatory synaptogenesis that precedes the development of spontaneous seizures [64,316]. Similar results are also obtained for ex vivo studies. The arterial perfusion of albumin exacerbates bicuculline methiodide-induced epileptiform seizure-like events and astrogliosis in isolated guinea pig brain [317]. Strikingly, BBB breakdown, albumin, and TGF-β1 exposure provoke a similar hypersynchronous neuronal epileptiform activity and epileptogenic transcriptional response, including the activation of NF-κB, Jak-Stat, MAPKKK, and the complement signaling pathways [69]. Notably, blocking TGF-β signaling with Alk5 inhibitors SJN2511 or SB431542, or with angiotensin receptor 2 antagonist losartan, prevents the albumin-initiated gene response and epilepsy [69,314,316,318,319]. Therefore, astrocyte TGF-β signaling triggers hyperexcitability and seizures, and targeting TGF-β signaling could be a feasible strategy for the disease modification and prevention of epilepsy [315,320]. In addition to epilepsy, BBB breakdown contributes to neuronal hyperexcitability in aging and AD [66,319,321]. In patients with epilepsy or AD, BBB impairments are spatially associated with an electroencephalogram (EEG) signature of a transient slowing of the cortical network, termed paroxysmal slow wave events [321]. The infusion of albumin directly into the cerebral ventricles of naïve young rat results in a high incidence of this transient EEG abnormality, which is also observed in aged mice and models of AD and epilepsy [321].

In support of the critical role of TGF-β signaling in epilepsy, several studies have discovered the downstream target gene CDKN1A as a central hub in epileptogenesis. A transcriptional analysis of rat piriform cortex following sarin-induced seizures identified

several significant canonical pathways and de novo networks of genes most significantly modulated by seizure. Two of the five most significant networks identified are built around TGF-β and Cdkn1a [322]. An integrative analysis of epilepsy animal models and human epilepsy tissue found five key genes, including TGF-β and CDKN1A, as central nodes in the protein networks in epileptogenesis [323]. mRNA levels of TGF-β and CDKN1A are up-regulated in the cortex of patients with epilepsy [323]. Exosomes from epileptogenic tissue cause the induction of key pathways in cultured cells, including the inflammatory response and key signaling nodes SQSTM1 (p62) and CDKN1A (p21) [324]. Recently, dysregulated miRNA expression has been associated with epileptogenesis through inflammatory pathways, cell death, neuronal excitability, and synaptic reorganization [325]. TGF-β and CDKN1A-related pathways are common targets of epilepsy-associated miRNA [325]. These studies support a central role of CDKN1A in epileptogenesis. However, there has been no report on the exact role of CDKN1A in epilepsy, and CDKN1A has not been investigated in the context of TGF-β signaling.

## 6. Opportunities and Challenges of Targeting TGF-β Signaling in the Brain

As discussed above, the manipulation of the TGF-β signaling pathway in astrocytes alters the disease outcome. Findings from these studies not only help our understanding of disease pathogenesis but also have therapeutic implications. Depending on the role of TGF-β signaling in the disease (disease-promoting vs. mitigating), strategies of both the activation and inhibition of TGF-β signaling have been explored in brain injury and disease.

To harness the therapeutic potential of neuroprotective and anti-inflammatory effects, TGF-β must be delivered into the brain. However, TGF-β does not cross the intact BBB; thus, it requires intracerebral administration for TGF-β to be effective. The ICV delivery of TGF-β1 has been tested in animal models of TBI [114], stroke [131], AD [326], and PD [207]. The ICV route of drug delivery in general is a valuable option for achieving a high local drug concentration in a target brain region while minimizing systemic toxicity [327]. However, it requires an invasive procedure, with difficulties in providing long-term administration and risks of complications, such as infection [327], thereby limiting its clinical application. To overcome these shortcomings, several non-invasive strategies have been developed, including focused ultrasound [328] and nanoparticles [329]. It should be noted that it is almost impossible to achieve cell-type-specific effects when using the ICV delivery of TGF-β ligands, because the receptors TGFBR1 and TGFBR2 are widely expressed among many cell types in the brain. Adeno-associated virus (AAV) vectors have been shown to be safe and effective in targeting glial cells and have been used in clinical trials [330]. We have used AAV encoding a constitutively active form of Tgfbr1 to activate TGF-β signaling [209]. A number of studies have used different AAV serotypes with astrocyte-specific promoters to increase gene expression in astrocytes [163,276]. Excitingly, the newly developed AAV-PHP.B provides a non-invasive alternative for gene delivery to the brain through systemic injection [331]. AAV-PHP.B is able to transduce the majority of astrocytes in multiple brain regions. However, these approaches have not been used to manipulate TGF-β signaling, and true astrocyte-cell specific targeting remains elusive [330].

Based on the findings that the deficiency of TGF-β signaling in neurons promotes neurodegeneration in the context of AD and PD [179,209], we hypothesize that neuronal TGF-β signaling plays a protective role in AD and PD and that the rescue of neuronal TGF-β signaling has therapeutic potential. This hypothesis is supported by the findings that activating TGF-β signaling reduces MPTP-induced dopaminergic neurodegeneration and motor deficits [209]. These findings motivate us to screen and develop small molecule TGF-β/Smad activators. We have developed a novel small molecule drug, C381. C381 is orally bioavailable and brain-penetrant, and has exhibited great potential for clinical translation [26]. Meanwhile, other groups have proposed using drugs that cross the blood–brain barrier and are potentially able to activate TGF-β signaling [332]. Nutraceuticals, such as Hypericum perforatum (hypericin and hyperforin), flavonoids such as hesperidin, omega-3, and carnosine, can increase TGF-β1 production in the brain [332]. Some of these

may have synergism with currently approved cognition-enhancing medication, and thus represent a novel pharmacological approach in improving cognitive function [332].

In disease conditions where TGF-β signaling is activated and the outcome of signaling is diverted toward disease progression, blocking TGF-β signaling provides therapeutic benefits. The development of therapeutic approaches of blocking TGF-β signaling has mainly been driven by motivations to inhibit the progression of cancer and fibrosis [333–335]. Different strategies have been developed to block TGF-β signaling (Figure 1), including antisense oligonucleotides that abrogate the expression of the TGF-β ligand and its receptor, small molecules or antibodies that selectively interfere with TGF-β activation, monoclonal neutralizing antibodies against the TGF-β ligand and its receptor, ligand traps that sequester TGF-β and prevent its receptor binding, and small-molecule inhibitors of the TGF-β receptor kinases [333–335]. TGFBR1 (ALK5) is an attractive target due to its druggability, as well as specificity, in the pathway. A number of potent, selective inhibitors have been developed [333–335]. Many of them have advanced to clinical trials and demonstrated acceptable safety profiles and therapeutic effects in cancer treatment [336]. Some have been tested and shown beneficial effects in preclinical models of aging [66], ALS [238], and EAE [22]. Pirfenidone, an FDA-approved drug for treating fibrosis, inhibits TGF-β1 production and its downstream pathways [337] and has been shown to reduce neurodegeneration and neuroinflammation after kainic acid injury [338] and TBI [339]. These small molecule drugs have advantages, in that most can be administered orally, and some can pass BBB. However, none of the anti-TGF-β drugs have been tested in clinical trials for brain injury and neurodegeneration.

Losartan has gained great interest in targeting TGF-β's detrimental effects because it is an FDA-approved anti-hypertension drug. Losartan has been shown to ameliorate disease in TBI [118] and EAE [22,63]. For epilepsy, in addition to the epilepsy models involved in BBB breakdown and albumin extravasation [64,318,319], losartan has also been shown to attenuate seizure activity and neuronal damage in other models of epilepsy by different research groups [340–342]. A recent publication suggests that telmisartan, another angiotensin receptor 2 antagonist with anti-TGF-β effects, has the potential to reduce seizure frequency when administered as an add-on antiepileptic drug in dogs with refractory idiopathic epilepsy [343]. However, losartan fails to suppress seizure-like activity in cortical and hippocampal areas of human brain slices of patients with drug-resistant TLE, suggesting that further exploration may be required for losartan as an anticonvulsant drug in clinical trials [344]. The biggest hurdle for losartan clinical translation is the knowledge gap in dosing recommendations. It is not known whether dosing humans with the equivalent doses of losartan used in preclinical studies is necessary and feasible [345]. The dose range used in preclinical studies (1–100 mg/kg/day) is equivalent to 68–978 mg/day for a 60 kg person, whereas the FDA-allowed max dose is 100 mg/day [345]. Further dose–response studies are needed to determine whether human studies are feasible.

In summary, a number of strategies are now available to manipulate the TGF-β signaling pathway in the brain, and they have been demonstrated to able to improve the disease outcome in preclinical studies, but additional research is needed in order for them to be tested in clinical trials.

## 7. Conclusions and Future Directions

Astrocytes regulate multiple essential processes in the nervous system in normal and disease conditions. Astrocyte reactivity is not only a universal response of astrocytes to brain injury, aging, and age-related neurodegenerative diseases, but also a central mechanism driving the development and/or progression of these conditions. The response that astrocytes elicit is highly context- and disease-dependent. Reactive astrocytes are highly heterogeneous and constitute subpopulations with unique molecular signatures depending on the brain insult, disease state, and distance from primary lesions [1–8]. Furthermore, astrocyte reactivity is a dynamic process that could be normalized or even reversed. The modulation of the astrocyte reactivity state has emerged as an important

venue for new preventive and therapeutic strategies. However, attempts to block astrocyte reactivity globally have yielded inconsistent effects on functional outcomes. This is likely, at least in part, due to the heterogeneity of the astrocyte response to injury and disease. Different astrocyte subpopulations/subsets seemingly coexist in reactive astrogliosis; however, the source and regulation of such heterogeneity are not completely understood [346]. Therefore, it is of high importance to identify and target the astrocyte subsets that acquire maladaptive functions and are "harmful" for the diseased brain. Future research will aim to identify molecular pathways that drive beneficial and detrimental phenotypes, which will ultimately facilitate the development of pathway-specific therapeutic approaches to promote the beneficial effects while downregulating the harmful/maladaptive effects of reactive astrocytes [33]. The integration of advanced sequencing technologies (single-cell and spatial transcriptomics) with multi-omics approaches will facilitate the discovery of novel mechanisms for therapeutic intervention.

TGF-β signal transduction is overwhelmingly complex and diverse due to the large numbers of interacting components, as well as the complicated feedback and crosstalk with other pathways. The intensity and duration of TGF-β signaling are tightly regulated both spatially and temporally, at multiple levels (Figure 1), reflecting the remarkable delicacy, specificity, and context dependency of TGF-β functions. In the brain, TGF-β plays fundamental roles in development and homeostasis in normal conditions, as well as in inflammation and repair following injury and neurodegeneration. For astrocytes, TGF-β is a powerful regulator and effector of astrocyte reactivity. TGF-β regulates many aspects of astrocyte function and induces major changes in response to injury and aging. The genetic and pharmacological manipulation of the TGF-β signaling pathway in astrocytes alters disease outcomes in many preclinical models of CNS injury and disease, which present unique opportunities for the discovery and development of novel therapeutics. Despite an abundance of literature and significant progress to date, there are still outstanding questions remaining in the field. First, the precise mechanisms of TGF-β signaling in brain injury and neurological diseases remain to be elucidated. In particular, the downstream effectors and target genes have not been identified in most disease conditions. A thorough understanding of TGF-β's role at different disease stages is an urgent need for the development of effective, precise treatment regimens targeting TGF-β. Second, given the context- and cell-type-dependent nature of TGF-β function, it is critical to manipulate this signaling pathway in a cell-type-specific manner. However, it remains a challenge to achieve cell-type-specific manipulation with current TGF-β targeting strategies. This review wishes to provide a basis for future research aimed at gaining more mechanistic insights into astrocyte reactivity and TGF-β signaling, which are essential for the understanding and fine-tuning of the TGF-β signaling pathway in order to develop targeted therapies.

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## Abbreviations

| | |
|---|---|
| AD | Alzheimer's disease |
| ALS | amyotrophic lateral sclerosis |
| APP | amyloid precursor protein |
| AQP4 | aquaporin 4 |
| α-syn | α-synuclein |
| BBB | blood–brain barrier |
| BMP | bone morphogenetic proteins |
| C3 | third complement component |
| C9ORF72 | Chromosome 9 Open Reading Frame 72 |
| CBDCBF | corticobasal degenerationcerebral blood flow |
| CCL | chemokine (C-C motif) ligand |
| CNS | central nervous system |
| CTGF | connective tissue growth factors |
| CXCL | chemokine (C-X-C motif) ligand |
| DA | dopaminergic |
| DATDLB | dopamine transporterdementia with Lewy bodies |
| EAE | autoimmune encephalomyelitis |
| ECM | extracellular matrix |
| EEG | electroencephalogram |
| GABA | γ-aminobutiryc acid |
| GDF | growth differentiation factors |
| GFAP | glial fibrillary acidic protein |
| HDHTTICV | Huntington's diseasehuntingtinintracerebroventricular |
| IFN | interferon |
| IL | interleukin |
| iPSC | induced pluripotent stem cell |
| LPSmHTT | Lipopolysaccharidemutant huntingtin |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| MS | multiple sclerosis |
| MSAmTOR | multiple system atrophymechanistic/mammalian target of rapamycin |
| NFκB | nuclear factor kappa B |
| PAI-1 | plasminogen activator inhibitor-1 |
| PD | Parkinson's disease |
| PSPROS | progressive supranuclear palsyreactive oxygen species |
| SARA | Smad anchor for receptor activation |
| SBE | Smad binding element |
| scRNA-seq | single-cell RNA sequencing |
| SMAD | Sma- and Mad-related protein |
| SNpc | substantia nigra pars compacta |
| snRNA-seq | single-nucleus RNA sequencing |
| SOD1 | superoxide dismutase type 1 |
| STAT3 | signal transducer and activator of transcription 3 |
| TBI | traumatic brain injury |
| TGF-β | transforming growth factor-β |
| TGFBR1 | transforming growth factor-β receptor 1 |
| TGFBR2 | transforming growth factor-β receptor 2 |
| TLR | toll-like receptor |
| TLE | temporal lobe epilepsy |
| TNF | tumor necrosis factor |
| TSP-1 | thrombospondin-1 |

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*Systematic Review*

# Oral Microbiota, Its Equilibrium and Implications in the Pathophysiology of Human Diseases: A Systematic Review

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**Abstract:** Imbalances of the oral microbiota and dysbiosis have traditionally been linked to the occurrence of teeth and oral diseases. However, recent findings indicate that this microbiota exerts relevant influence in systemic health. Dysbiosis of the oral microbiota is implicated in the apparition and progression of cardiovascular, neurodegenerative and other major human diseases. In fact, the oral microbiota are the second most diverse and largely populated microbiota of the human body and its relationships with systemic health, although widely explored, they still lack of proper integration. The purpose of this systematic review is thus to widely examine the implications of oral microbiota in oral, cardiovascular and neurodegenerative diseases to offer integrative and up-to-date interpretations. To achieve that aim, we identified a total of 121 studies curated in PUBMED from the time interval January 2003–April 2022, which after careful screening resulted in 79 studies included. The reviewed scientific literature provides plausible vias of implication of dysbiotic oral microbiota in systemic human diseases, and encourages further research to continue elucidating the highly relevant and still poorly understood implications of this niche microbiota in systemic health. PROSPERO Registration Number: CRD42022299692. This systematic review follows relevant PRISMA guidelines.

**Keywords:** oral microbiota; gut microbiota; caries; periodontal disease; dysbiosis; cardiovascular disease; neurodegeneration; Alzheimer's disease

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## 1. Introduction

Commensal microorganisms, within the human body, have traditionally been regarded as foes on their way to abolition [1]. However, further and more recent research has proven these initial considerations to be wrong and novel essential role(s) attributable to human microbiota, in health and disease conditions, are appearing [2–4]. Currently, it is well known that we as humans are holobionts from our birth; thus, we should be considered as complex organisms composed of multiple cells and genomes of eukaryotic and microbial origins [5,6]. These cells, in turn, structure the different organs and systems of the human body and, most importantly, contribute to their proper and homeostatic functioning [1,7].

Therefore, the human microbiota richness and variety is an inseparable part of a dynamic organism and its functions, and its alterations are known to involve dysbiosis [1,8].

Dysbiosis in different organismic systems has in turn been proposed as a core contributor to the apparition of several diseases [9–11]. A clear example of the effects of dysbiotic microbiota in the human organism has been revealed in the gut [12]. This organ system contains the most extensive number of microbial cells, with more than 1000 different species [13]. Alterations in the compositions of the gut microbiota, as those caused by unhealthy diet and genetic predisposition [14,15], drive to microenvironment disequilibrium and apparition of disorders and diseases such as obesity, colorectal cancer, inflammatory bowel disease [3], heart failure, diabetes and neurodegeneration [14,16,17]. The gut microbiome is also known to influence the central nervous system (CNS), contributing to its proper functioning [18–20], and to the integrity of the blood–brain barrier (BBB) [21,22]. Induction of brain neuroinflammation as a consequence of alterations in gut bacteria populations has been reported and integrity of the BBB implicated; however, the mechanisms by which these pathological interactions may take place remain poorly defined [17]. Some studies indicate that bacteria metabolites [18,22,23] or the enzymatic effects of matrix metalloproteinases of bacterial origin [22] may alter the integrity of the BBB and contribute to increase the permeability of the BBB promoting pathologic leakage of diverse molecules and chronic neuroinflammation [22].

Although several studies have linked dysbiosis with the apparition of major diseases as indicated, it is not yet well understood how the gut microbiome can affect these distant organs and systems. Translocation of bacterial products by the gut endothelial barrier, however, is being considered as a plausible hypothesis [24,25]. Moreover, communication between the gut and the brain has been proposed to partially occur mediated by the vagus nerve. This nerve bundle directly connects the digestive and nervous systems in the mammal body; thus, bacterial species and/or metabolites may use it for bi-directional communication [17,18].

## 2. Oral Microbiome and Systemic Diseases

Following the gut microbiome, the second most diverse and largely populated microbiome in the mammal body is in the oral cavity [26–30]. Although gut and brain interactions have been explored, the interactions between the oral microbiome and the brain still remain mostly unexplored. The interest in the oral cavity microbiome, however, has increased in recent times, considering the proximity of this microbiota to the brain. Research on the oral microbiota has revealed that the oral microbiome is highly dynamic and becomes influenced by different lifestyle aspects, such as diet, stress, tobacco consumption and systemic conditions, which actively modify its composition and characteristics of this microbiome by even misleading it to dysbiosis [31–33]. The interaction of diet and microbiome can be a relevant risk factor for multiple systemic diseases, such as oral diseases, obesity, cardiovascular and neurodegenerative diseases, among others [34], and dietary modifications can prevent some of the symptoms related to these pathologies, treating or alleviating them, and thus, it may result in improved overall health [8].

### *2.1. The Microbiota of the Oral Cavity*

The microbiome compositions are distinct in each of the areas of the digestive tract, oral cavity, esophagus, stomach, intestine and colon, and become influenced by the ecosystem of each digestive organ [35,36]. Among the components that form these ecosystems, the oral cavity contains the exclusive presence of hard and soft tissues (e.g., teeth and oral mucosa, respectively) [26,37]. The oral mucosa is composed of the lining mucosa (floor of the mouth, buccal region, labial region and soft palate), masticatory mucosa (gingival region and hard palate) and the specialized mucosa (back of the tongue) (Figure 1). The mucosa of the lining is covered by a non-keratinized epithelium, while the masticatory mucosa is covered by a keratinized epithelium. The surface of the specialized mucosa presents a complex structure, containing different types of papillae [38,39]. These differences may be behind the

diversity in terms of microbiota niches that can be found within the oral cavity in normal conditions. Of note, the bacterial composition of dental surface plaque differs between the supragingival and subgingival region (Figure 1). In addition, the bacterial composition of saliva includes bacteria separated from various niches in the oral cavity and it is similar to that of the lining of the tongue [40], as detailed in Figure 1. For that reason, the importance of further defining the oral microbiota by embracing the complexity of the oral cavity should be highlighted, which in turn may contribute to improve our understanding on the basis of any potential occurring associations between the oral microbiota and general health [35].

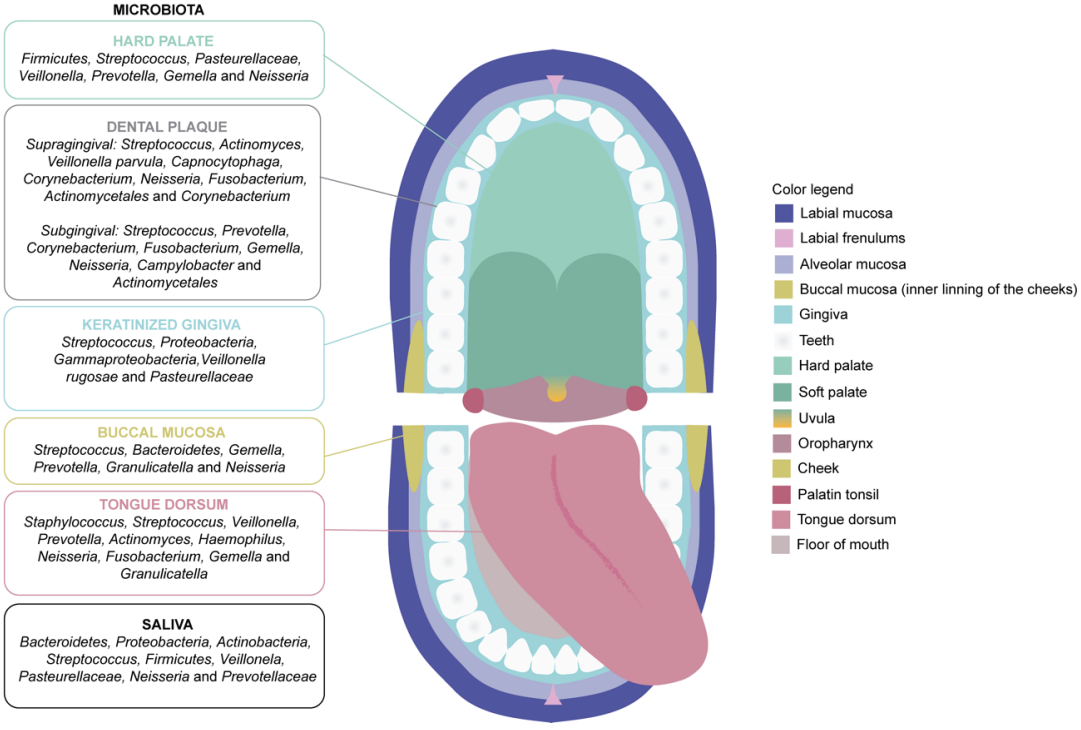


**Figure 1.** Illustrative diagram depicting the diverse microbiota populations that form the niche oral microbiota throughout the different oral cavity regions.

The oral microbiota is composed of various microorganisms, such as bacteria, fungi and archaea [26,40]. This symbiotic community, in homeostatic equilibrium, is supposed to contribute to a healthy oral environment that has a systemic impact, as previously reported [11,33,41]. However, alteration of the homeostatic equilibrium of the oral microbiota, such as in dysbiosis, has been implicated in the apparition and progression of several diseases of the oral cavity and has been suggested to have a severe and harmful imprint on overall health [32]. Interestingly, oral cavity-associated microbes have been found in many distant organ sites, such as small intestines, heart, lungs, placenta and brain [33,42]. Oral microbiota, thus, show an enormous interactive communication and influence over local and systemic responses, and play essential role(s) in host nutrition, immunity, metabolism and diseases, as detailed in Table 1. Although establishing an oral microbiota profile in oral and general health conditions is preferential and several efforts have been already made towards that aim, recent studies already focus on a step further, which implies the analysis and characterization of the oral microbiota in systemic disease conditions [10,43,44].

**Table 1.** Main factors influencing the compositions of the oral microbiota.

| Age | Host and Environment | Habitat | Biofilm Maturation |
|---|---|---|---|
| Changes in the host and its habits | Genetic factors | Surface [1] | Environment |
| Microevolution | Diet and lifestyle<br>Immune system | Oxygen | Probiotics |
| Horizontal transfer of microorganisms | Changes in host defenses | Nutritional status | Oral hygiene |
| Changes in diversity | Broad spectrum antibiotics<br>Hormonal balance<br>Environment | Oral hygiene<br>pH<br>Cell flaking in the mucosa<br>Salivary flow and gingival crevicular fluid | Microbial interactions<br>Immune response<br>Density |

[1] Tooth, mucosa, subgingival groove, tongue.

The oral bacterial ecosystem comprises more than 700 microbial species that form a vast network of interactions [35,39,45–47]. In the state of oral health, there is homeostasis in the bacterial ecosystem and it provides a dynamic balance with mutual benefits for the host and microorganisms [45]. The presence of the commensal microbiota in the oral cavity prevents colonization of the region by pathogenic microbes. For example, secretion of bacteriocins by the genus *Streptococcus* prevents the colonization of Gram-negative bacteria, conferring a fundamental healthy advantage to the host [48].

### *2.2. Diseases Related to the Oral Microbiota*

#### 2.2.1. Oral Diseases

##### Dental Caries and Tooth Decay

The number of studies suggesting that the composition of the oral microbiota and the metabolic potential of saliva and dental plaque vary significantly in health compared to disease conditions has tremendously increased in the last years [49]. A clear example of that research is tooth decay, which refers to an irreversible demineralization of the tooth hard tissues: enamel and dentin. Tooth decay is due to the production of organic acids by the bacteria that form the bacterial plaque. This process occurs through anaerobic metabolism of sugars ingested in the diet, especially following consumption of sucrose [50–52]. This fact accounts on the importance of the dietary patterns and the effects that these directly exert on the equilibrium of the oral microbiota. In a related vein, caries has been identified as the most prevalent non-communicable pathology at a global scale [53]. It is known that about 2.3 billion adults have cavities that affect their permanent teeth, and about 530 million children present cavities in their temporary teeth [54–56]. The current established mechanisms available to counteract the apparition and progression of that pathology are based on improved oral hygiene and fluoride administration [57]. However, in many of these cases, imbalances between the dynamics of enamel demineralization–remineralization and other protective factors have been identified [57]. Further research and appropriate dietary recommendations [58], thus, need to be implemented to manage the expanding global burden of tooth decay.

##### Periodontal Disease

Periodontal disease or periodontitis is a chronic immunoinflammatory pathology also considered a bacterial disease with a multifactorial cause. The pathogens that cause periodontitis are mainly anaerobic Gram-negative bacteria [17,59]. The high immune response that the presence of these bacteria causes withing the oral gums leads to high production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), as a response from the host immune system [60,61]. Periodontitis is also associated with increased serum levels of C-reactive protein (CRP) and decreased anti-inflammatory markers such as interleukin-10 [61]. This results in a first stage of severe gingival inflammation, followed by

an irreversible loss of the tooth supporting tissues (the periodontal ligament and alveolar bone) [62,63]. Aggravating factors for this pathology are poor oral hygiene, tobacco habits, stress and disease comorbidities, such as type 1 and type 2 diabetes mellitus, cardiovascular disease and osteoporosis [60,64]. The prevalence of periodontitis is globally high, affecting between 35 to 50% of the worldwide population, as recently informed by the World Health Organization [53,65,66].

#### 2.2.2. Systemic Diseases

Varying levels of tooth decay, gingivitis and periodontitis, as well as further comprehension achieved on the role(s) of bacteria in pathological conditions, reveals that oral microbiome possesses fascinating role(s) affecting human systemic health beyond the oral cavity [67,68]. In fact, it has been proposed that oral microbiome products, microorganisms and inflammatory molecules could reach distal organism systems and organs through two different ways, mainly by the bloodstream and the digestive tract [11,32]. Table 2 provides details of different oral microbiome-related disorders that influence the occurrence and progression of human systemic diseases [1,33,43,69–72].

**Table 2.** Systemic diseases and pathologies related to dysbiosis of the oral microbiome.

| Autoimmune Disorders | Metabolic and Inflammatory Diseases | Cancer Diseases | Neurodegenerative Diseases |
|---|---|---|---|
| Rheumatoid arthritis [42,73] | Non-alcoholic hepatic steatosis | Colorectal cancer (*F. nucleatum*) | Multiple sclerosis |
| Sjögren syndrome, systemic lupus erythematosus [32] | Insulin resistance, diabetes, atherosclerosis [74] | Pancreatic cancer (*P. gingivalis* and *A. actinomycetemcomitans*) | Dementia |
| Inflammatory bowel disease [29] | Chronic kidney disease | Gastrointestinal cancer [32] | Alzheimer's disease |
| | Hypertension, stroke, obesity | Head and neck tumors Oral cancer [75] | |

Cardiovascular Diseases

Arteriosclerotic cardiovascular disease (CVD) is a chronic condition that can lead to life-threatening clinical pathologies, such as acute myocardial infarction or stroke. These pathologies are responsible for a growing global burden of deaths and figure as the second global cause of mortality worldwide following sepsis [64,76]. Although the most well-known risk factors for the condition are hypertension, hypercholesterolemia and smoking, it is likely that other concomitant risk factors that remain poorly understood [77] may also be implicated. Numerous epidemiological studies have implicated oral dysbiosis and infection, specifically periodontal disease, as a significant risk factor associated with CVD [78–80]; the prevalence of CVD in patients diagnosed with periodontitis is 25–50% higher than in healthy individuals, although the mechanisms of this significant association are unclear [70]. Periodontal disease caused by dysbiosis and extended oral infection places a vast population of harmful bacterial strains and their secretions, which might involve the presence of extracellular vesicles [81], in direct contact with disrupted capillary terminals, which in turn introduces these harmful agents into the circulatory system facilitating a global systemic diffusion [60,79,82]. Thus, this systemic exposure to oral bacteria and their products probably exerts a significant impact on the onset and progression of CVD through activation of pro-inflammatory processes [42,83]. Although inflammation is induced by bacteria in the biofilms that comprise the microbiome formed on the teeth [79] and by the tongue-coating microbiota [84], these may not be the unique culprits. A genetically dependent effect on the immune mechanism or a modified immune reaction to the presence of pathogenic bacteria in the circulatory system may also be involved [70,78], a potential hypothesis that deserves further research.

Neurodegenerative Diseases

Neurodegenerative diseases involve a progressive loss of neurons in the central nervous system (CNS), resulting in impairment of several cognitive and motor domains and the apparition of dementia and movement disorders. Major neurodegenerative diseases include Alzheimer's Disease (AD), Parkinson's Disease, Huntington's Disease, Amyotrophic Lateral Sclerosis and Multiple Sclerosis, among others [85–87]. It should also be noted that vascular factors associated with cerebrovascular disease are also a major cause of cognitive decline in elders and relevant risk factors for neurodegeneration [88–91]. The implications that oral microbiota homeostasis and dysbiosis may exert on the pathological mechanisms of these diseases, and more specifically in neuroinflammation, will be specifically addressed in this work through a systematic review.

## 3. Materials and Methods

### *3.1. Study Design and Protocol Registration*

A systematic review protocol was designed and registered in PROSPERO (URL: https://www.crd.york.ac.uk/prospero/ (accessed on 30 May 2022), York, UK) with the identifier reference: CRD42022299692, to be carried out in PubMed. Only studies performed on humans and animals as well as review articles/metanalyses evaluating these populations were included.

#### 3.1.1. Inclusion and Exclusion Criteria

Studies meeting the following criteria were included for systematic revision: (a) articles and systematic reviews published in English; (b) articles and systematic reviews published in peer reviewed journals; (c) articles and systematic reviews published between January 2003 and April 2022; (d) Participants in the study: humans of all ages and mammalian animal models.

The exclusion criteria were established as follows: (a) in vitro and ex-vivo studies; (b) Grey literature considered as unpublished results, articles published in non-peer-reviewed sources, and studies published in indexed journals (Journal Citation Reports) with an impact factor lower than 1.7; (c) scientific literature falling out of the scope of the revision after consensual exclusion recommendation by two independent reviewer authors; (d) scientific literature published non-open access.

#### 3.1.2. Search Strategy, Risk of Bias and Limitations

Searches were performed by aleatory combination of the following keywords: "diet", "oral microbiome", "oral health", "oral disease", "nutrition", "oral dysbiosis", "cardiovascular disease", "Alzheimer disease" and "neurodegenerative disease"; the Boolean operator AND was used to establish combinations of keywords. Further assessment of the articles and review articles retrieved by the previous referred parameters was performed by careful screening of the title, abstract and keywords to confirm their relevance and suitability. When the title and abstract did not provide enough details to include/exclude the study, the full text was assessed.

The exclusive inclusion of scientific literature published in open access may create a risk of bias resulting from the exclusion of relevant scientific findings not published through this option. However, we strongly believe that open access is an option of scientific dissemination emphasized and prioritized by funders and authors worldwide, which has been exclusively considered here, as it allows easier management of the literature reviewed and avoids specific bias resulting from non-institutional allowed access to certain resources published under subscription. Finally, the authors declare that misleading interpretation of the reviewed scientific literature and inappropriate extrapolation of findings, as previously reported by Ducker et al. [92], have been specifically addressed and, to the best of our ability, avoided.

## 4. Results and Discussion

A total of 128 studies were initially obtained and pooled for further screening based on implementation of the above indicated inclusion and exclusion criteria as detailed in Figure 2. From these, 7 studies were removed during duplicate filtration, obtaining a remanent of 121 studies, from which 97 studies were published in the last 19 years. Additionally, 43 additionally studies were added manually. Thus, after careful screening, a total of 139 studies successfully passed the inclusion criteria and have been reviewed in this work, as detailed in Figure 2.

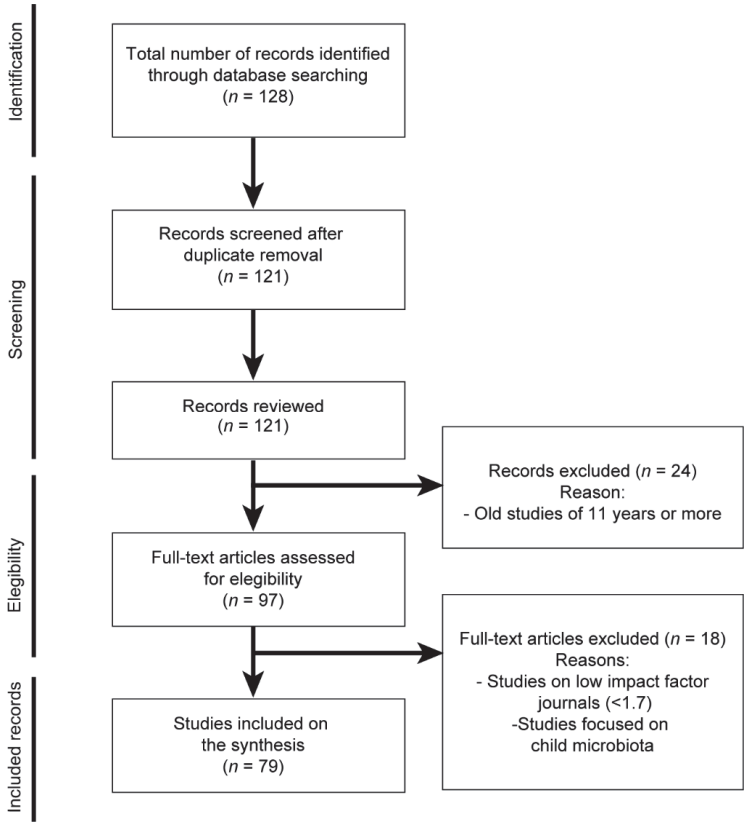


**Figure 2.** PRISMA flow diagram indicating the steps followed during the scientific literature review process of this work.

### *4.1. Oral Microbiota, Oral Diseases and Cancer*

The oral cavity and its microbiome colonies are openly exposed to the external environment, which differentiates this specific region from the rest of digestive tract regions, and which poses a challenge to the microbiota of preventing external pathogenic colonization [27,31,44]. Further than preventing external colonization, imbalanced oral microbiota based on the most recent studies has been directly implicated in the apparition of multiple oral pathologies [4,31,33,46]. Tooth decay and periodontitis are the most common and costliest chronic oral pathologies, while oral cancer has also been linked to the presence of buccal dysbiosis [29,44,93,94]. In line with these findings, several studies provide association between periodontal disease and increased risk of cancer affecting distant organs [95,96]. Furthermore, specific oral microbiome dysbiosis patterns have been related to several types of cancer. Of note, augmented colonization of the oral microbiome

by *T. forsythia* and *P. gingivalis* have been implicated in esophageal cancer [97], *P. gingivalis* and *A. actinomycetemcomitans* have been linked to pancreatic cancer [98] and genera *Fusobacterium* and *Porphyromonas* have been implicated in colorectal cancer [99], among others. Schwabe et al. proposed that the synergistic effects that eukaryotic and human cells take in human metabolism through the oral cavity, once imbalanced, could result in the apparition of carcinogenesis [100]. Abusleme and colleagues [101] investigated oral subgingival species and their association with periodontitis in a cohort of 22 subjects with chronic periodontitis and 10 periodontally healthy control subjects. Subgingival communities in this study were characterized using the 16S rRNA gene and quantitative PCR [101]. They observed that the increase in inflammation, linked to augmented bleeding, was not associated with a different microbiome, but corresponded to a higher community biomass in the bleeding areas. Moreover, oral microbiota showed clear differences in diversity and biomass; periodontic microbiota had an increase in the genus *Tannerella forsythia*, *Treponema denticola* and *P. gingivalis*, among others, compared to healthy microbiota.

Similarly, Yamashita and colleagues [35] studied the saliva microbiome of a Japanese population of 2343 adults using 16S rRNA gene and quantitative PCR. In this vast study, the authors found that high bacterial diversity in the saliva microbiome was significantly associated with poor oral hygiene, including presence of tooth decay and periodontitis. In addition, *Streptococcus mutans* (*S. mutans*) and *P. gingivalis* were identified in this study in higher frequency in subjects suffering from caries and periodontitis, which has also been previously demonstrated in vivo [41]. Yamashita and colleagues [35] also reported an association between the relative abundance of the predominant bacteria in saliva with conditions related to oral hygiene. Salivary microbiota organisms were present in the majority of individuals, including *Streptococcus*, *Rothia*, *Neisseria*, *Actinomyces*, *Prevotella*, *Granulicatella*, *Porphyromonas* and *Haemophilus*. Moreover, the information on the bacterial composition of the participants suggests that the predominant organisms encompass two groups of cohabiting bacteria, i.e., group I: *Prevotella histicola*, *Prevotella melaninogenica*, *Veillonella atypica*, *Veillonella parvula*, *Streptococcus salivarius* and *Streptococcus parasanguinis* and group II: *Neisseria flavescens*, *Porphyromonas pasteri*, *Haemophilus parainfluenzae*, *Granulicatella adiacens* and *Gemella sanguinis.* Based on this classification, predominance of species from group I in oral microbiome was associated with worsened health, such as presence of caries, periodontal disease and poor oral hygiene [35].

For a long time, the main bacteria strain considered cariogenic due to the production of acids and demineralization of the dental structure was *S. mutans* [74,94,102]. Instead, in recent decades, a more complex composition of the bacterial community associated with caries in its different stages of evolution has been considered and now includes the presence of *Streptococcus sobrinus*, *S. salivarius*, *S. parasanguinis*, *Actinomyces* and *Lactobacillus spp.* at the onset of caries [37,55,103], and *Veillonella*, *Propionibacterium*, *Bifidobacterium* and *Atopobium* in more advanced stages [55,103,104]. On the contrary, periodontitis-associated species include, but are not limited to, *P. gingivalis*, *Tennerella forsythia* and *Treponema denticola*, while *S. mutans*, *Lactobacillus spp.*, *Bifidobacterium spp.* and *Atopobium spp.* are associated with tooth decay [48,94].

Although one of the main bacteria associated with tooth decay is *S. mutans*, the genus Lactobacillus has been proposed as a possible probiotic agent against *S. mutans* [105]. Zhang et al. evaluated the presence of the genus Lactobacillus in a type of pickles to know their inhibitory properties on *S. mutans* both in vitro and in vivo. In that study, *L. plantarum* K41 strain showed the greatest inhibitory effect against *S. mutans* and also in the formation of exopolysaccharides and biofilm in vitro. In addition, the authors observed a significant reduction in the severity and incidence of tooth decay when treating rats with *L. plantarum* K41 strain [105].

It has also been demonstrated that different diet components can prevent or promote the development of caries [45]. In general, fiber-rich foods are known to stimulate the flow of saliva, buffering pH and protecting teeth [106,107]. On the other hand, plant phosphates (to a greater extent phytates) are able to reinforce teeth remineralization [45,108]. Some

plant components, such as flavonoids and other polyphenols in blueberries, help reduce the risk of tooth decay by reducing the adhesion of bacteria, inhibiting their growth, or by reducing the ability of bacteria to form biofilm [45]. There are also several studies on the relationship of vitamins and periodontal disease, both in animals and humans [109–113]. Among all the vitamins analyzed, those with antioxidant capacity and with effects on the immune system, as well as those involved in bone metabolism, seem useful for the prevention or improvement of periodontal periodontal disease, highlighting the action in oral health of the vitamins C and D [114].

In this line, Anderson et al. [55] investigated the modification of the composition of bacterial plaque during frequent carbohydrate consumption. Participants continued with their usual diet and suctioned 2 g of sucrose five times a day between meals for a period of 3 months. Samples of biofilms were collected from splint systems worn for 3 × 7 days with 7-day intervals. Changes in the microbiota, investigated using Illumina MiSeq amplicon sequencing (v1–v2 region), revealed that consumption of sucrose for three months significantly altered the oral microbiota. These alterations included a general decrease in the richness of bacteria, directly associated to the sucrose intake, and a significant proliferation of *Streptococcus gordonii*, *S. sanguinis* and *S. parasanguinis*. In addition, a proliferation of acidic and acidogenic species, which cause dental caries, and a decrease in Porphyromonas, associated with typical healthy oral microbiomes, were also detected [55].

### *4.2. Oral Microbiota and Cardiovascular Diseases*

It has been suggested that some bacteria, such as the aforementioned *P. gingivalis*, may systemically increase the risk to suffer certain cardiovascular diseases, being involved in the metabolic syndromes and autoimmunity, by causing alterations in the amino acid metabolism and the host's immune response [30,93]. The *Firmicutes/Bacteroidetes* ratio may be another of the multiple contributing factors to cardiovascular diseases development and progression, since it has been reported that the increased *Firmicutes/Bacteroidetes* ratio in the oral microbiota may indicate an upregulation in the systemic inflammatory response mediated by pro-inflammatory cytokines [28,48]. Epidemiological studies indicate that various types of bacterial infection (*Helicobacter pylori, Chlamydia pneumoniae, P. gingivalis*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia*) and the presence of metabolites derived from these bacteria in serum, such as lipopolysaccharides (LPS), contributes to the development of arteriosclerosis [70,115,116]. In addition, another study states that inflammatory risk factors for myocardial infarction have a systemic profile similar to those of periodontitis, suggesting a common final pathway of atherogenesis related to systemic inflammation [117]. The oral microbiome modulate oral immunity, but also the gut microbiome [46]; it can induce dysbiosis in the gut microbiota, leading to the disruption of the intestinal barrier and systemic inflammation [46,48,84,107]. Moreover, inorganic nitrate, found in high concentrations in meat, vegetables such as beets, lettuce and spinach and drinking water [118,119], has been studied as a potential prebiotic for oral microbiota [120]. In a clinical study focused on the cardiovascular benefits of dietary nitrates, oral bacterial profiles were measured [121]. Two nitrate-reducing species, *Rothia mucilaginosa* and *Neisseria flavescens*, were significantly increased in 65 hypercholesterolemic subjects who randomly daily received 250 mL of nitrate-rich beetroot juice or placebo juice daily during 6 weeks [121]. In addition, the presence of these bacterial strains is also associated, as a concomitant factor, with teeth and periodontal disorders [122]. Likewise, another study analyzed the microbiota present on the surface of the tongue of subjects who followed a diet with beetroot juice enriched in inorganic nitrate for 10 days by sequencing the bacterial genes 16S rRNA [123]. The results of this study showed that high prevalence of oral bacteria of the genus *Prevotella* and *Veillonella* should be considered harmful, while a high amount of the genus *Rothia* and *Neisseria* were beneficial for the maintenance of nitric oxide homeostasis and associated rates of cardiovascular disease and improved blood pressure [123].

*4.3. Oral Microbiota and AD Neurodegeneration*

AD is the main contributor to dementia worldwide and the fifth leading cause of death in people over 65 years [124]. Recently, a hypothesis has emerged that resident bacterial populations contribute to the development and progression of AD by promoting neuroinflammation, senile plaque formation and accumulation of toxic neurofibrillary tangles [17,125]. In a healthy brain, small amounts of soluble amyloid beta peptide (Aβ) are produced and degraded through enzymatic, proteasomal and lysosomal machineries [124,126]. In brains affected by AD, however, the brain performs insufficient degradation leading to an accumulation of Aβ fragments within proteinopathic brain plaques [17,124]. Furthermore, these peptides contain toxic proteoforms that impair the brain cell degradation machineries [124]. It has been recently proposed that accumulation of Aβ peptides in the brain performs a beneficial function as an antimicrobial peptide when the brain faces pathogen infections. However, if there is an overaccumulation of Aβ peptides, either due to prolonged colonization of pathogens or due to ineffectiveness in degrading them once they are no longer needed, it can lead to destruction of nearby tissue [124,127]. It has also been demonstrated that administration in mice of *P. gingivalis*, one of the main pathogenic bacteria present in periodontitis, increases intestinal permeability and, as a consequence, facilitates the transfer of LPS through the intestinal barrier [48,128]. This leakage of LPS leads to upregulated serum LPS levels, which in turn have been demonstrated to trigger systemic inflammation [48,128,129]. Repeated oral application of *P. gingivalis* induces neurodegeneration and the formation of extracellular Aβ42 in young adult wild type mice, strongly suggesting that low grade chronic periodontal infection with this pathogen can result in the development of neuropathology consistent with that of AD [130].

Oral pathogens, such as *P. gingivalis*, have also been investigated using post-mortem human brain tissues [63,127]. ApoE−/− [105,115] and pathogen-free BALB/c mice [127], and/or various *Spirochaetes*, have been reported to colocalize with Aβ plaques [124,127,131,132]. Moreover, dysbiosis of the oral and intestinal microbiotas can potentially initiate and accelerate the formation of Aβ plaques and neurofibrillary tangles [17]. As explained above, periodontitis is a dysbiotic immunoinflammatory disease that can directly mediate neuroinflammation [133–135]. Several studies claim that chronic periodontal inflammation can induce changes in the gut microbiota, increasing individual inflammatory responses. This is because periodontitis can cause a secretion of bacterial pathogens, such as LPS or peptidoglycans, which have been considered as modifiable risk factors for AD [136]. As previously indicated in this work, periodontitis has been associated with increased risk of dementia through the mechanisms of systemic inflammation [117,137,138]. Another study corroborates the fact that oral microbiota may influence the risk of AD through systemic access to the brain of imbalanced oral microbiota strains and hypothesizes the relationship that AD neuropathology may establish with periodontitis through this mechanism [139]. The former study takes into account that chronic periodontitis is significantly linked to increased risk of suffering AD and other age-related dementias [139]. It was also demonstrated that AD subjects present lower diversity of microorganisms in the oral microbiota compared to healthy controls, a fact that points to a particular AD-linked dysbiosis of the oral cavity [28]. Changes in the oral microbiome (e.g., due to predominant adoption of western diet) may result, according to this study [136], in intestinal dysbiosis, which in turn leads to low-grade inflammation in the intestine and increased permeability of biological barriers, including the blood–brain barrier (BBB). Consequently, neuroinflammation and cognitive impairment may appear as subsequent factors to oral dysbiosis based on the assumptions reported in these studies. Furthermore, oral pathogens such as *P. gingivalis* lead to an 'oralization' of the intestinal microbiota [134,140], a mechanism that produces intestinal inflammation, and may be linked to apparition and sustainment of neuroinflammation [128,141] through translocation of oral/intestinal toxic bacterial proteases to the brain [136]. Classical inflammatory mediators, such as eicosanoids and cytokines, may also contribute to neurodegeneration as detailed. Docosahexaenoic acid (DHA) is a fatty acid that plays a fundamental role in neural function and exhibits anti-inflammatory prop-

erties by inhibiting the production of pro-inflammatory mediators, such as eicosanoids and cytokines. It has been reported that high consumption of DHA-rich fish significantly reduces the probability of developing AD [142], and that a consumption of 900 mg/day of DHA can even provide neuroprotection while early-stage dementia-related cognitive deficits appear [143]. DHA deficiency occurs due to increased lipid peroxidation mediated by free radicals, dietary intake deficit and/or impaired hepatic DHA administration to the brain [144]. DHA has been assigned with several neuroprotective abilities, such as blocking the cascade between Toll-like/cytokine receptors and NF-κB activation. Toll-like receptors (TLRs) are transmembrane receptors that initiate signals in response to different stimuli, such as tissue injury and infection. It is now recognized that the lipid components of the diet can modulate transmembrane TLRs and the consequent immune and inflammatory responses. These receptors are expressed in a variety of cell types related to immunity in mammals and are also present in microglia, astrocytes, neurons and oligodendrocytes. Recently, there is evidence linking them to neurodegenerative conditions (53). In the same vein, various components of the Mediterranean diet, such as the fatty acids of long-chain w-3 fish derivatives, and plant polyphenols, including resveratrol, have been linked with positive effects on brain health [145,146].

In a related vein, recently, Ribeiro-Vidal et al. [147] investigated the in vitro antimicrobial properties of two w-3 fatty acids, DHA and eicosapentaenoic acid (EPA). The authors used a subgingival biofilm as model in their studies. The results showed that both DHA and EPA exerted significant reduction on the harmful bacterial strains studied, such as *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* and *Veillonella parvula*, among others [147]. All in all, more randomized controlled trials are needed to make better recommendations towards the acquisition of a healthy microbiota [146]. There are also multiple studies on the effect of various types of anthocyanins, which are an example of polyphenols, related to a positive impact on the prevention and amelioration of certain clinical manifestations of progressive AD. Among these, a literature review concluded that the intestinal microbiota has a significant impact on the pathogenesis of AD, which could be clinically delayed following administration of anthocyanins [148–150]. According to another study conducted in 2020, the neuroprotective ability of cyanidin-3-glucoside (C3G) was also reveled in a mice model of AD [151]. These studies indicate that is important to analyze the effects of these promising molecules on the oral microbiota to further understand if this type of microbiota is also implicated in the neuroprotective effects exerted by these compounds, as already demonstrated for gut microbiota.

It is known that oral status may be an important factor for general health and a diverse and well-developed microbiota has been associated with overall well-being. Thus, prevention of oral pathologies and inhibition of the protease of *P. gingivalis* or other bacteria associated with periodontitis and AD, such as *A. actinomycetemcomitans*, *Actinomycetales* and *Prevotella* [137,139], *T. forsythia*, *E. coli*, *Chlamydiapneumonia* and *F. nucleatum* may help to reduce the current neurodegenerative burden [152], an extremely compelling hypothesis that requires of further investigation. It has been presupposed that the oral microbiota remains in the oral cavity and does not have the ability to preferentially reach the intestine or other body sites. However, it has been already proven that the oral microbiota can easily reach the intestine or lungs of people who have a compromised immune system, being able to trigger inflammation and health issues, as reviewed here, at an organismic level [28,48].

### *4.4. Oral and Gut Microbiota in Chronic Human Diseases*

Although the general vision that oral bacteria, under normal conditions of ingestion, are unable to colonize a healthy intestine is the most accepted, high levels of oral microbes have been found in the gut microbiota of patients with different chronic human diseases, including colon cancer, inflammatory bowel disease and liver cirrhosis [153]. The former authors indicate that *Klebsiella spp.* can migrate from the saliva and colonize the gut when this former organ presents dysbiosis [153] In fact, more than 1000 mL of saliva is produced by adults daily and the majority of that amount enters the gastrointestinal tract.

Accordingly, oral microbiota, which is an important reservoir of intestinal microbes, should exert an important role in maintaining the stability of the intestinal microecosystem [153]. A clear example of the implications of both microbiota populations in human diseases is diabetes mellitus (DM), as it has been shown as Fusobacteria and Actinobacteria were more abundant in subjects with diabetes, while Proteobacteria were less abundant in the oral cavity of these subjects, indicating an orchestrated dysbiotic profile simultaneously affecting the oral and the gut microbiota [153]. However, the relationship between these two crucial organism microbiota is only incipiently uncovered and needs further efforts to be properly understood at the context of several other major diseases including cardiovascular and specifically AD.

## 5. Conclusions

This systematic review highlights the importance of the oral microbiota in health and pathological conditions and the diet's modulatory capacity over the oral microbiota. The relationship between the oral microbiota and oral diseases has been analyzed. Indeed, according to multiple recent studies, there is clear evidence of the relationship between dysbiosis of the oral microbiota and cardiovascular and neurodegenerative diseases. The recent development of molecular genetics is of paramount importance for the study of the oral microbiota and its association with oral and general health. Likewise, microbiomics and metagenomics are two areas of research that have emerged to identify the presence of specific bacteria in the body, and thus, understand the nature of microbial activity during health and disease [43].

The high sensitivity of oral microorganisms could predict subtle changes in health status and serve as a potential biomarker for early detection of disease [69]. Moreover, it may be possible to modulate the oral microbiota even in adults through changes in the dietary pattern, being able to make recommendations for the prevention of the appearance of oral pathologies [40]. It is very necessary to continue with more research, especially at the clinical level, to continue studying how to regulate, modify and improve the oral microbiota from nutrition, in order to improve general health in people. In addition, it is worth noting the importance of being able to treat and prevent possible disorders or pathologies especially through food and food supplements, in addition to following a healthy lifestyle.

The oral microbiota presents a crucial factor, since it is in contact with the external environment. This is fundamental since this factor is modifiable, and thus, could improve the individual health of the population. Since the broad spectrum of beneficial and potentially pathogenic bacteria are present in practically all microbiomes, a crucial path would be to favor the growth of beneficial species and moderate biofilm growth and metabolism to reduce dysbiosis. That is why it is of great relevance to know the optimal balance of the microbiota and also to know when there is a dysbiosis. Moreover, it may highlight the importance of more studies combining modifications in the diet and analyzing the impact that this factor has on the microbiota and in both oral and general people's health.

Another important aspect to consider is that a human safety assessment combined with health risk assessment is needed to better protect people from potential external hazards, whether the risks come from microbial pathogens, environmental chemicals or physical agents, such as radiation, medication or food additives. Tools are needed to identify hazards and assess exposure risks that may be problematic to individuals and populations [7]. Finally, it is not enough just to carry out an intervention in the diet and microbiota to improve the oral and general health of people, since it is understood that to avoid the disease, it is also necessary to take into consideration and improve other habits, such as increasing physical activity and reducing stress or improving the management of the latter. Maintaining a balance between all these pillars can be a good strategy to maintain a balanced microbiota, thus increasing the chances of maintaining proper systemic health.

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# Understanding Acquired Brain Injury: A Review

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**Abstract:** Any type of brain injury that transpires post-birth is referred to as Acquired Brain Injury (ABI). In general, ABI does not result from congenital disorders, degenerative diseases, or by brain trauma at birth. Although the human brain is protected from the external world by layers of tissues and bone, floating in nutrient-rich cerebrospinal fluid (CSF); it remains susceptible to harm and impairment. Brain damage resulting from ABI leads to changes in the normal neuronal tissue activity and/or structure in one or multiple areas of the brain, which can often affect normal brain functions. Impairment sustained from an ABI can last anywhere from days to a lifetime depending on the severity of the injury; however, many patients face trouble integrating themselves back into the community due to possible psychological and physiological outcomes. In this review, we discuss ABI pathologies, their types, and cellular mechanisms and summarize the therapeutic approaches for a better understanding of the subject and to create awareness among the public.

**Keywords:** Acquired Brain Injury (ABI); post-birth; brain impairment; brain functions; pathologies; cellular mechanisms; therapeutic approach

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## 1. Introduction

Acquired Brain Injury (ABI) is an umbrella term encapsulating its two main categories: Traumatic Brain Injury (TBI) or Non-Traumatic Brain Injury (Non-TBI) [1]. TBI is an external traumatic event in which injury to the brain is sustained, while Non-TBI occurs due to an internal disease process that also leads to damaged brain tissue. Causes of TBI include motor vehicle accidents, falls, sports-related injury, and violence, whereas Non-TBI could be triggered by a stroke, neoplasm, infection, and anoxia [1]. Clinical outcomes of both categories of ABI vary, depending on the specific disease process and the premorbid circumstances such as age, genetics, and socioeconomic background. Risk rates for TBI are the greatest in the elderly at and above 75 years, and male individuals are at greater odds of getting TBI [2–5]. The vectors of brain damage in both TBI and Non-TBI include

vascular abnormalities, broad axonal injury, focal or disseminated atrophy, and neuronal circuit disruption [6] (Figure 1).

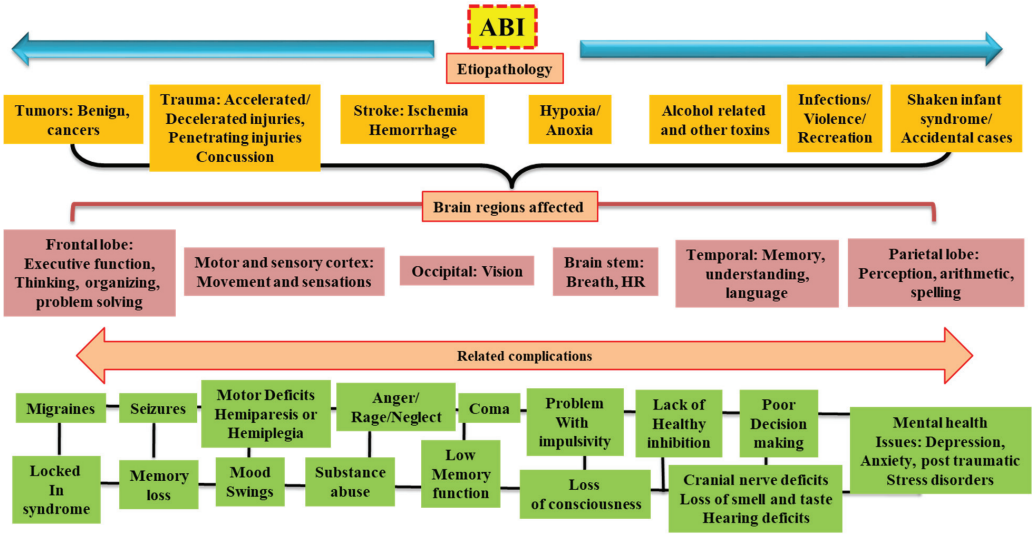


**Figure 1. ABI: Etiopathology, classifications, the brain region affected, and related complications.** The pictorial presentation of ABI describes its type (purple) and etiology of the disease (in orange texts). ABI is mainly divided into TBI and Non-TBI injuries. Non-TBI can arise from tumors, vessel occlusion, infection, or alcohol consumption. ABI can affect different regions of the brain depending on impact, insult, infection, or blockage (shown in pink) and may show related signs and symptoms (depicted in green). HR: Heart rate.

ABI describes a wide range of diseases, establishing it as a vastly important area in medicine and public health. According to the Centre for Disease Control and Prevention (CDC), TBI is one of the major groups of ABI and is a principal cause of mortality and lifelong disability [7,8]. As per CDC reports (2006–2014), the frequency of TBI-related hospitalizations, emergency department visits, and deaths had increased by 53 percent [9]. In 2013, roughly 2.8 million TBI cases occurred in the United States of America. Among the 2.5 million emergency department visits, there were approximately 300,000 TBI hospitalizations and about 60,000 deaths [2,10]. It is important to keep in mind that these numbers only refer to one-half of the diseases associated with ABI. Non-TBI also plays a large role in the number of individuals ending up in the hospital. The CDC reports that every year, approximately 800,000 people will have a stroke and, in 2018, one in every six cardiovascular-disease-related deaths was due to stroke [11,12]. These epidemiological data on ABIs elucidate the necessary interventions that hospitals and researchers need to accomplish to serve the large extent of individuals affected by ABI.

While both TBI and Non-TBI carry many different disease processes and medical problems (Figure 2), the patients usually receive treatment and rehabilitation in the same facilities in the hospital. This is important to mention because demographic characteristics of TBI and Non-TBI vary considerably. For example, the Toronto Rehabilitation Institute demonstrated that the patient population for TBI compared to Non-TBI were significantly younger, tended to be male, and lived in metropolitan areas [13]. In addition, the global population is aging, so leaders in the medical profession need to anticipate larger demand for units and specially trained staff to treat patients with TBI and Non-Traumatic Brain Injuries with possible comorbidities [14,15]. Nevertheless, to ensure exceptional clinical outcomes for patients with ABI, physicians, and nurses must be able to provide personalized and specific treatments to the patients. To achieve that, a good understanding of

ABI and its pathology in different categories is preferred. Therefore, the present review aims to give a comprehensive and clear description of ABI, its types, mechanism, and treatment strategy.

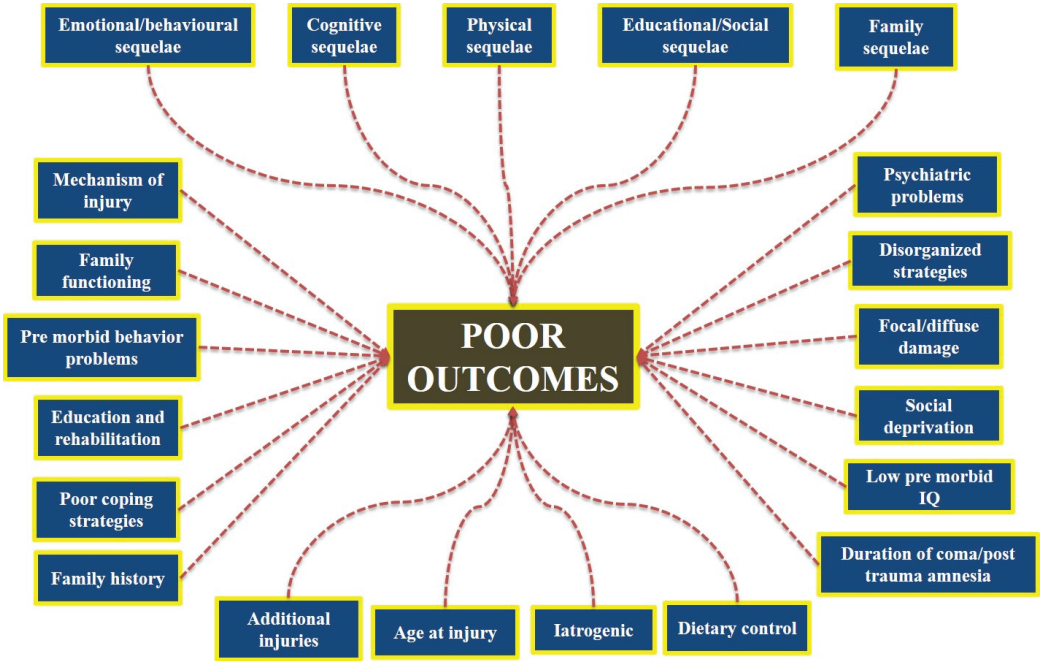


**Figure 2. Poor outcomes post-ABI.** A variety of parameters related to the ABI mechanism play a role in predicting the outcome. ABI can develop as a result of a stroke or disease or an iatrogenic cause, and there is some indication that those who suffer from a head injury are a self-selecting group, with poor attention, impulsivity, and overactivity being associated with poor road-crossing skills. These may interact with other premorbid characteristics that are predictors of poor post-injury prognosis.

## 2. Acquired Brain Injury and Its Types

As mentioned above, ABI is a broad classifying term encompassing any non-congenital brain injury; therefore, ABI is inherently diverse in the populations it affects, in the mechanisms by which brain injury ensues, and in prognosis. The following paragraphs break down the different types of TBI and Non-Traumatic Brain Injuries that make up ABIs (Figure 1).

### *2.1. Traumatic Brain Injuries (TBIs)*

TBI arises because of a hit or jolt to the brain and comprises mild to severe injury. TBI patients show symptoms such as unconsciousness, confusion, nausea, dizziness, headache, or incoordination and receive symptomatic and stabilizing treatment. Patients keep visiting clinics with chronic symptoms even after weeks or months post-initial traumatic experiences. If either symptoms continue or neurologic impairments appear, routine radiological re-imaging may be required to assess the situation [16]. There is a comprehensive review published by our group that might be of interest to the TBI audience to obtain more insight [17].

#### 2.1.1. Concussion

A concussion is one of the most widely recognized forms of TBI. It occurs due to a sudden strike or whip to the head that causes the brain to bounce or twist within the skull. Symptoms can range from minor confusion and disorientation to complete amnesia, nausea, vomiting, and loss of consciousness [18]. These symptoms occur due to abnormal brain movement upon impact, which at the molecular level disrupts neuronal cell membranes and axonal stretching. This, in turn, causes the extensive flux of ions across neuronal membranes resulting in diffuse waves of depolarization, which precipitate the classic concussion symptoms [18]. In 2006, a study on concussion epidemiology in Canada noted that 110 individuals per 100,000 had had a concussion within the previous year [19]. In 2014, 2.87 million cases of TBI in the United States were recorded by the CDC, and of those, 812,000 cases were children diagnosed with concussion alone or in combination with other injuries [2]. Similarly, according to a US study carried out in 2017, approximately 19.5% of adolescents (in grades 8–12) reported a minimum of one concussion, while 5.5% had more than one concussion in their lives [20].

#### 2.1.2. Skull Fractures

A skull fracture results from any impact to the head that surpasses the bone's capability to withstand the pressure. Although a fracture of the skull itself is not a brain injury, over 75% of all skull fractures are associated with some form of brain injuries, such as intracranial hemorrhages or subdural or epidural hematomas [21]. Fractures that occur at the basilar skull are more problematic as this area of the skull harbors essential areas of the brain that allow us to eat, breathe, and walk [22].

#### 2.1.3. Epidural or Subdural Hematomas and Subarachnoid Hemorrhage

In general, a hematoma is a bruise, and a hemorrhage is a bleeding blood vessel. In the case of TBI, it is called an epidural or subdural hematoma if the insult occurs above or below the dura matter. Hematomas mostly occur from blunt force trauma to the head and are typically found in the temporal brain; however, they may also occur from a penetrating TBI or spontaneously (the spontaneous type would not be considered TBI) [23]. A subarachnoid hemorrhage is divided into two groups: aneurysmal vs. non-aneurysmal. The non-aneurysmal hemorrhage most often occurs due to blunt force trauma to the brain or sudden acceleration changes. Epidural hematomas account for roughly 2% of injuries to the head and 5–15% of fatal head injuries. Subdural hematomas are more common, with an estimated rate of 5–25% in patients with head trauma [21].

#### 2.1.4. Penetrating Brain Injury

Penetrating brain injuries can be caused by assaults, collisions, or even suicide attempts [24] and may be defined from mild to severe TBI based on the Glasgow coma scale (GCS). Following a patient's preliminary examination, neurosurgical examination starts with a clinical exam and documentation of raised intracranial pressure (ICP). In the case of suspected arterial or venous injury, a CT scan is the first choice for diagnosis. As the possibility of post-TBI epilepsy is 45–53%, a prophylactic anticonvulsant is given to patients [25].

### *2.2. Non-Traumatic Brain Injuries (Non-TBI)*

#### 2.2.1. Infections

Due to the multiple layers of protection from the skull, meninges, and the blood-brain barrier (BBB), the brain is relatively repellent to any pathogenic invaders [26,27]. However, when bacteria and other pathogens breach the brain's defenses, the damage can be devastating. Two major types of brain infections are meningitis and encephalitis. Meningitis occurs when a bacterial agent infects the meninges and encephalitis is the infection of the brain tissue itself [28,29]. Approximately 1.2 million cases of meningitis occur globally each year [30], while the incidence of encephalitis infection tends to vary

between studies, but the 2019 census estimated 1.4 million cases with 89,900 deaths and 4.80 million DALYs [31].

#### 2.2.2. Anoxia

The brain needs a lot of oxygen and energy in the form of glucose. Anoxic brain injury results when the brain is completely denied of oxygen in incidences such as drowning, heart attack, carbon monoxide poisoning, and much more. As a result, the metabolic homeostasis of the brain is destroyed resulting in major neuronal injury and cell death. Since there are many different causes of anoxic brain injury, rates are hard to gauge [32].

#### 2.2.3. Stroke

There are two main types of stroke: ischemic and hemorrhagic. Ischemic strokes result from occluded cerebral arteries, which prevent nutrient-rich blood from supplying the surrounding brain tissue. This results in permanent tissue damage. Transient ischemic attack (TIA), also referred to as a mini ischemic stroke, only lasts for a short amount of time. Ischemia that affects more than two-thirds of the middle cerebral artery (MCA) territory is termed malignant cerebral infarction (MCI) and causes space-occupying edema and neurological deterioration [33]. Swelling and symptomatology peak in the first 48 h after a stroke. The first step in treatment is to reduce risk factors and keep ICP under control. Although there are no precise surgical recommendations, a hemicraniectomy is generally recommended [34]. Hemorrhagic strokes result from cerebral artery leakage into the brain, causing elevated ICP and cellular damage [35]. The incidence of stroke among adults aged between 35 to 44 years is roughly 30 to 120 per 100,000 per year. This number increases drastically for individuals aged between 65 to 74 years, where the yearly incidence is about 670–970 per 100,000 [36]. A detailed account of brain hemorrhage for further reading can be found here [37].

#### 2.2.4. Alcohol and Drug Use

The usage and abuse of alcohol and drugs are highly prevalent in modern-day lifestyles estimating a high lifetime risk for either drug or alcohol abuse and dependence [38]. There are many mechanisms through which drugs and alcohol can have negative effects on the normal functioning of the brain. These include disturbing nutrient distribution to brain tissue, direct cellular damage, altered chemical homeostasis of the brain, and hypoxia [38,39].

#### 2.2.5. Neoplasm

In a similar way to anoxia and infectious Non-TBI, brain cancers (neoplasm) are vastly diverse in pathophysiology and epidemiology. Gliomas are the most prevalent class of brain neoplasm, accounting for roughly 78% to 80% of all malignant brain tumors. These cancers stem from the supporting neuronal cells of the brain called glia. Gliomas include astrocytomas, ependymomas, glioblastoma multiforme, medulloblastomas, and oligodendrogliomas [40].

Meningiomas are the most prevalent primary tumors and are also classified as ABIs [41]. Patients with genetic predispositions to disorders, such as neurofibromatosis type 2 or multiple endocrine neoplasia type 1, are more likely to develop meningioma [42]. The preponderance is asymptomatic and histologically benign [43]. Initially, generalized symptoms (nausea, headache, or altered mental status) may be present, with localized neurological abnormalities developing later [44]. In situations with subtotal meningioma extraction, adjuvant therapy in combination with postoperative radiation may be recommended. Patients with meningioma have a good prognosis, albeit those with a higher WHO grade or partial resection have a higher chance of continuation [45].

## 3. Mechanism of ABI

A sort of physical trauma from an external entity may lead to a brain injury. The medical field has acquired tremendous success in the treatment of head injuries over the last few decades. A clearer understanding exists of the causes of tissue damage and the biophysical, biochemical, or physiological repercussions that culminate in a variety of clinical manifestations such as scalp laceration, syncope, and progression to a persistent vegetative state [46–51]. Various sorts of pathologies, such as skull fracture, hematoma (intracerebral, epidural, subdural, or intraventricular), as well as different types of contusion and brain injuries, could be recognized and their clinical and functional repercussions could be defined by contemplating the mechanisms of injury to the head [50].

### *3.1. Biophysical Mechanism of ABI*

The physical characteristics of the intruding substance, such as density, size, speed, and length of loading, determine how much energy is delivered to the cranium in ABIs [52]. When a harmful energy burden or mechanical response is exerted to the head, the length of the energy loading will be the first determinant to determine the severity of the injury [53]. This period has been set between 50 and 200 milliseconds. Static loads are defined as those lasting more than 200 milliseconds, while dynamic loads are defined as those lasting less than 200 milliseconds [54,55]. Static injuries are extremely rare and mainly occur when the head is ensnared between two hard objects. These enormous weights may cause distortion and injury to the skin and bones. The dynamic load could be caused by the passage of energy to cerebral tissue via impetuous loads, which are variable in speed. When the head does not receive direct impact but is put into motion as a result of an impulse generated by a force exerted on different parts of the body, this is known as impulsive loading [56]. In such cases, the injury is caused by inertial shifts in the head. In the next category, called impact loads, when an infringing object strikes the head, it may cause tissue injury in-depth, depending on the surface area, density, size, and speed of the object. It has the potential to alter the head's pace and induce acceleration or deceleration and may cause inertial shifts in the head too.

The influence of an object on the head can cause changes in the tissue's arrangement, including the skin, bone, and deep frameworks. If the adjustment is greater than the tissue's elasticity, it will lead to permanent disfigurement, skin laceration, or a bone fracture. With higher weights, the aggravating agent may produce depressed skull fractures and destruction of underlying tissue, such as the dura, brain, and arteries. This often results in an epidural hematoma, subdural hematoma, contusion, or intracerebral hemorrhage. Perforation and permeation may occur in more extreme situations, specifically at fast speeds, and with small agent sizes. The transmission of energy to the skull and cerebral tissue may be related to the collision. The brain tissues distort, and contusion could be because of this energy burden [57,58].

### *3.2. Injury to the Tissues*

Tissue distortion is caused by deformation, shock waves, and acceleration/deceleration, which all impose energy on the tissue. These can cause damage to the tissues in the skull, which include neural components, vessels, and bone. Injuries arise when the stress applied to the tissue exceeds the threshold. The physical properties of tissues, the amount of energy, the length of energy loading, and the magnitude of the load all influence their endurance. More intense activities, while still within the continuum, are at the brink of physiologic endurance and, if repeated, will cause progressive or even acute brain malfunction. In normal physiological conditions, the tissues' physical tolerance to injury is substantially lower, resulting in a variety of outcomes depending on the implicated components of pathological injury [59].

3.2.1. Primary and Secondary Injuries

The mechanism that causes the initial injury is a direct outcome of the delivered energy to the head [17,60]. They may cause additional injuries as a result of themselves, either as sequelae of the original event or by exacerbating it, resulting in secondary injuries, the most prevalent of which are hypotension and hypoxia. Secondary injury can include excitotoxicity, free radical formation, mitochondrial dysfunction, induction of damaging intracellular enzymes, and other pathways inside the wounded neural tissues, all of which can cause continued system dysfunction (Figure 3) [17,61–63]. Certain tertiary damages are also included, which are frequently secondary results of the head's energy loading. This includes electrolyte imbalance due to renal issues, various types of cardiac abnormalities, hepatic insufficiency, and so on.

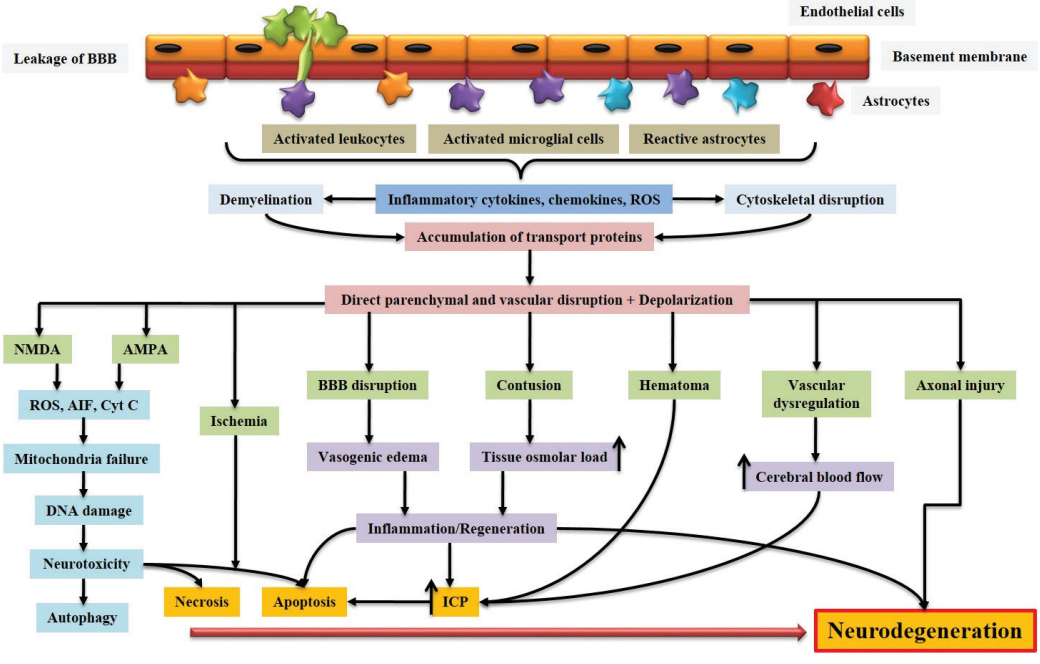


**Figure 3. Schematic representation of pathophysiology of ABI.** BBB dysfunction caused by injury allows the transmigration of activated leukocytes into the injured brain parenchyma, which is facilitated by the upregulation of cell adhesion molecules. Activated leukocytes, microglia, and astrocytes produce ROS and inflammatory molecules such as cytokines and chemokines that contribute to demyelination and disruption of the axonal cytoskeleton, leading to axonal swelling and accumulation of transport proteins at the terminals. On the other hand, excessive accumulation of glutamate and aspartate neurotransmitters in the synaptic space due to spillage from severed neurons activates NMDA and AMDA receptors located on post-synaptic membranes, which allow the production of ROS. As a result of mitochondrial dysfunction, molecules such as apoptosis-inducing factor (AIF) and cytochrome c are released into the cytosol. These cellular and molecular events including the interaction of Fas with its ligand Fas ligand (FasL) ultimately lead to caspase-dependent and -independent neuronal cell death. BBB: blood-brain barrier; NMDA: N-methyl-D-aspartate receptor; AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; ROS: Reactive oxygen species; Cyt c: Cytochrome c; ICP: Intracranial pressure; AIF: Apoptosis-inducing factor.

Various types of clinical cases can be distinguished based on the above-mentioned aspects in the formation of a head injury. It can begin with a bone injury, since prolonged static loading causes a change in the normal structure of the skull, ultimately leading to a fracture when the flexibility of the bone toleration is exceeded. The amount of force and the timing of the fracture determine the severity of the crack. When a large load is applied, the entire skull is severed into segments, and the brain tissue is ruptured, causing it to leak from the punctured nose, ear canals, and scalp. The sufferer may present with severe impairment of cerebral and brain stem function. Death is often the result [64–66].

If the injury occurs in an acoustic region, neurological deficits may occur as a result of damaged brain function. These could be the injury's direct or major consequences. There are certain further occurrences of the above-mentioned events, which are referred to as secondary traumatic effects. Various types of intracranial hematomas, as well as intraventricular hematomas and brain tissue contusions, can occur from injury to the cerebrovasculature in the affected areas. These particulate lesions can cause a mass effect, as well as an upsurge in ICP and brain herniation [17,65,67,68]. As a primary injury, brain laceration may predispose the sufferer to convulsions and epilepsy [69,70].

Infections of the bone and cerebral contents are another serious result of this type of injury, as the overlaying skin is punctured, allowing bacteria to enter the deeper structures. These later occurrences are also secondary impacts. Lesions can occur as a result of the expansion of a hematoma or contusion, or as a result of the mass effect caused by various subsequent effects of damage, such as edema around the lesions. Cerebral herniation, concussions, diffuse axonal damage, subdural hemorrhage, intracerebral hemorrhage, and intraventricular hemorrhages are some of the other problems [71–73]. Severe injuries can range from a short period of disorientation and cognitive impairment to concussion or loss of consciousness to a long-term coma or persistent vegetative state (PVS) due to extensive harm to brain neurons and axons [17,74].

#### 3.2.2. Prenatal and Birth Damage

Early prenatal injury can result in the embryo's mortality. On other hand, insufficient growth (agenesis of the corpus callosum or anencephaly) or abnormal development (lissencephaly or microcephaly) could be the possible outcomes of late injuries [75–78]. During a period of heightened development, trauma (such as fetal stroke in the womb or damage to the mother) will result in more structural issues than when progression has slowed or has ended. Intricate stent delivery or hypoxia may result in birth failure [79–82].

#### 3.2.3. Post-natal Injury

Pediatric patients may have acquired ABI from metabolic disturbances (phenylketonuria), systemic illness (sickle cell disease, diabetes), trauma, central nervous system tumors, infections (meningitis or encephalitis), toxins (use of alcohol or anticonvulsants during pregnancy), and clinical treatment such as radiotherapy or chemotherapy for leukemia [83–89].

#### 3.2.4. Injuries in Adulthood

According to recent CDC data, people aged 75 years or older had the highest rate of hospitalizations (325 of total TBI-related hospitalizations) and mortality (28% of TBI-related deaths) [90,91]. The CDC further stated that males are two times more likely to get TBI-related hospitalization and have three times higher mortality than females [90,91]. Alcohol intake is identified as a major risk factor for TBIs, with effects on assessment, intensity, and prognosis. It was discovered that 16 percent of brain injury patients aged 15 and above were intoxicated at the time of injury. The alcohol group had a fatality rate of 14.5 percent compared to 9 percent in the non-alcoholic group [92]. Individuals with an older ABI have a higher chance of poorer physical, intellectual, and psychosocial outcomes, as well as a lengthier recovery period and more comorbidities. The major cause of brain injuries that comes under this category is fall. More than half of all fatal falls and 8% of nonfatal

fall-related hospitalizations were caused by these brain injuries. ABIs have the highest incidence of death and hospital admittance among fall-related injuries in adults and older adults during the first year after the injury. Furthermore, even after controlling for age and gender, there are rising tendencies in the incidence and mortality of trauma-induced ABIs in older persons. According to several studies, those who take anti-arrhythmic medications are more prone to suffer from brain damage. Several studies show that men had a higher chance of serious brain injuries during a fall than women, despite the possibility of a reverse relationship with nonfatal brain injuries [92,93].

The number of elderly persons hospitalized for a fracture has decreased over the last decade, whereas the percentage of those with a TBI, subarachnoid hemorrhage, and/or, subdural, in particular, has risen dramatically [94]. TBIs are becoming more common, which appears to be linked to the increased use of anticoagulants and antiplatelet medicines like clopidogrel and warfarin. Chronic illnesses related to equilibrium disturbance (Stroke and Parkinson's disease), scenarios of falls likely to result in an ABI, and risky behaviors may happen more frequently in men, in contrast to the use of anticoagulants and antiplatelet drugs. There is a need for more investigation into the underlying principles [95,96]. It is reasonable to assume that elderly adults with chronic diseases that affect the joints, nervous system, cardiac system, and cognition are at a higher risk of falling and developing ABIs. These may also be exacerbated by a lack of visual perception and visuo-motor reflexes [97,98].

### *3.3. Physiological Mechanisms of ABI*

There are different mechanisms that arise from primary and secondary injury and contribute to ABI pathology (Figure 3). We briefly described the pathological events here to understand the pathology of ABI.

#### 3.3.1. Excitotoxicity

Glutamate, an excitatory amino acid neurotransmitter is primarily responsible for triggering cellular damage during brain ischemia. It has a multifaceted role in synaptic plasticity, brain development and maturation, axon guidance, and general neuronal growth [17,99]. In ABI, restricted blood flow to the brain diminishes energy reserves and causes membrane depolarization, thus leading to the reduced uptake of glutamate from the surroundings. Under stable conditions, glutamine activates multiple receptors such as N-methyl-D-aspartic acid (NMDA), kainic acid receptors, and alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), while its clearance is managed by active ATP-dependent transporters [100,101]. During ABI, glutamine triggers the activation of sodium channels (causes brain swelling), calcium channels (causes neuronal death), and intracellular catabolic enzyme activity via glutamate receptors thus leading to cell death, which further cascades into the generation of oxygen free radicals, membrane depolarization, and intracellular toxicity leading to brain injuries [102,103]. Preclinical studies suggest a protective effect of suppressing NMDA and AMPA receptors post-ABI but have undesirable side effects [104,105]. To overcome this, Memantine (partial NMDA antagonist) was tested, along with death-associated protein kinase and calcium-calmodulin-dependent protein kinase, and showed potential therapeutic efficacy without many side effects [100,106]. Another dopaminergic agonist, Amantadine was found to be promising in several brain injuries. It triggers the dopamine release in neurons and delays the reuptake of dopamine by neural cells and also inhibits the NMDA receptor signaling, thus proving its potential effect in the brain injuries [107–110]. A non-psychotropic cannabinoid (Dexanabinol), which acted as a potent NMDA receptor antagonist was also reported to have a potential effect in glutamate injury, but also showed unwanted side effects that impaired normal brain functioning [37,111]. Additionally, metabotropic glutamate receptors (mGluRs) were also reported to express a promising response in retarding excitability thus hindering excitotoxicity [112].

3.3.2. Oxidative Stress

A possible precursor to the pathogenesis of cerebral injury has been identified as oxidative stress. Several reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radicals, and per hydroxyls can be generated, followed by the development of several reactive nitrogen species (RNS), which can cause brain tissue damage through a variety of cellular and molecular pathways [17,37,102]. The reaction of nitric oxide along with superoxide forms peroxynitrite, which can also bind to DNA directly, altering its structural integrity and causing cell damage as well as apoptosis [17,113]. These highly reactive radicals can degrade nucleic acids, proteins, and lipids, leading to neuronal cell death. Edaravone and NXY-059, two promising antioxidants, were used to treat stroke but did not produce significant effects [102,114,115]. Polyethylene glycol (PEG)-conjugated SOD (PEG-SOD or pegorgotein) was reported to have promising effects in several studies but failed in a larger phase III clinical trial [17]. Another study with lecithinized superoxide dismutase (PC-SOD) showed that it inhibited secondary neuronal loss after brain injury and enhanced survival rates [116]. As a result, novel therapeutic strategies for minimizing the devastation caused by ROS are desired. Incipient interventions, such as modulation of transient receptor potential melastatin-2 channels or poly (ADP ribose) polymerase-1 control endogenous facilitators of oxidative stress [117,118]. More investigation into the neurological effects of oxidative stress could lead to new targeted therapies for the reduction of several brain injuries, especially ABIs [118].

3.3.3. Acidosis

When mitochondrial respiration is disrupted, acidosis may arise as a result of lactate buildup in the cells. Acid-sensing ion channels (AICs) are activated by protons and serve as pH sensors in the body. They are amiloride-sensitive cation ports that relate to the epithelial sodium group and enable calcium and sodium to enter neurons [37,119]. About six AIC domains have been reported, with AIC1a, AIC2a, and AIC2b being expressed in the brain and spinal cord. AIC1a and AIC2s are present in high-synaptic-density areas of the brain to help with excitatory signaling and are involved in several brain injuries [119]. With their activation, neuronal cell death occurs through sodium, zinc, and calcium influx into the cell. In experimental stroke models, the inhibition of AIC1a has a longer therapeutic window, which was much more effective than currently available drug therapies [100].

3.3.4. Inflammation

Inflammation may sometimes lead to brain injuries of several types including ABI [120]. Conversely, the pathogenesis of ABIs is further complicated by inflammation [121,122]. During brain injuries, there is an intense and long-lasting inflammatory response that includes the activation of microglia, development of pro-inflammatory mediators, and penetration of different kinds of immune cells into the brain tissue [17,123]. Cytokines such as interleukin IL-6, IL-1β, tumor necrosis factor-alpha (TNFα), transforming growth factor beta (TGFβ), and chemokines such as monocyte chemoattractant protein-1 (MCP-1) and cytokine-induced neutrophil chemoattractant play an important role in the pathogenesis of inflammation in neuronal cells. Depending on the type of inflammatory response and when it happens, the immune response in the brain might have a variety of outcomes [17,102]. While chronic inflammatory activities may contribute to secondary ABIs and more prolonged detrimental events, inflammation early point may be beneficial. However, elucidating the exact mechanisms of inflammatory responses is challenging, as it is a diverse set of perceptions involving inflammatory cellular components, all of which may be harmful or beneficial [124]. Broad-spectrum blockers of inflammation (AT1 receptor blockers, PPAR gamma blockers, beta-blockers, etc.), not shockingly, minimize neuronal cell damage [125–127]. The lack of systematic implementation progress highlights the need for a deeper knowledge of the numerous molecular and cellular pathways after inflammation. In addition, a better understanding of the different structural profiles of diverse inflammatory mediators is needed.

3.3.5. Tauopathies

Abnormal aggregation of tau proteins inside brain cells leads to several disorders including ABI [128–130]. The concentrations of a particular tau protein in brain tissue, CSF, and serum change in ABI pathogenesis (Figure 4) [131,132]. The events that lead to tau release can be numerous and complicated, as can the types of modified tau species. Tau's basic role is to promote microtubule flexion and saturation, which is dependent on its post-translational modifications [133–135]. When Tau attaches to microtubules with a poor or no phosphorylation state; microtubule flexion is hindered; phosphorylated tau has a low potential for microtubules (Figure 1) [136,137]. Alternative splicing, which results in different-sized tau isoforms, might be another significant tau modulation [138–140]. Tau's capacity to disperse amongst cells is also steered by its accumulation feature [141,142]. Oligomeric tau species disperse between cells, whereas integrated insoluble tau does not [143,144]. Tau spreads because of illness, and this property may express pathophysiological conditions triggered by ABI [145,146].

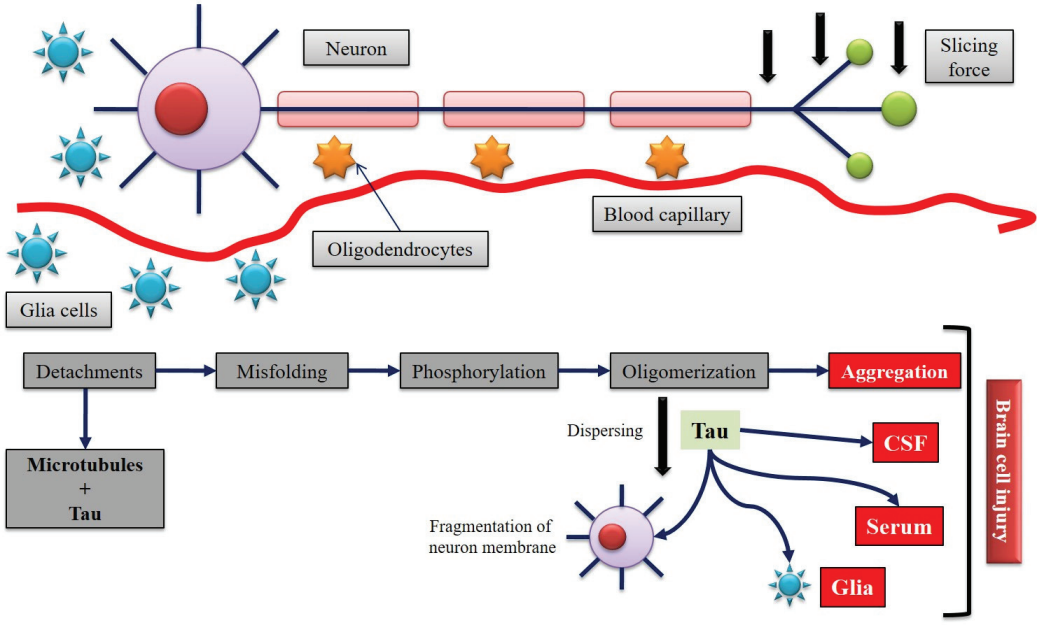


**Figure 4. Expected molecular mechanism of brain injury on tau in the nervous system.** Neurons, glia, oligodendrocytes, and blood vessels are damaged by the impact load that arises after a head injury. Injury to some or more of these cells causes intracellular unfolding, which causes the entire device to malfunction. Tau, which is highly correlated with microtubules, is abundant in axons. Impact forces devastate cell membrane integrity, as well as the microtubule framework in the axon. Tau disengages from the microtubule as it becomes unstable. Tau would then be misfolded, phosphorylated, develop a porous oligomeric conformation, accumulate, or disperse in a dysfunctional pathway. Tau may also invade other neighboring cells (glia, serum, or CSF) as it spreads.

Due to the extreme sudden TBI-induced protein abundance, protein catabolic pathways such as autophagy and proteasomal degradation may become exhausted [147–149]. When the plasma membrane of a compromised cell is disrupted, leftover cytoplasmic proteins such as tau which leave the cell can be absorbed by neighboring cells, confirming trophic rearrangement [149,150]. Tau can cross into the cerebrovasculature, and CSF, relying on where the weakened, tau-releasing cell is located, which further tends to contribute to brain injuries [151–153].

## 4. Injury and Outcome

Issues may occur as a result of an ABI in a variety of ways, either directly from the injured brain or implicitly from the response of an individual to the injury. Due to changing perceptions, family factors (pre-injury family functioning and managing), psychological background, as well as social exclusion lead to an abrupt cessation of current issues [154] (Figure 2).

The foreseen consequence is aided by a variety of factors related to the mechanism of insult. Given a severe brain injury, the duration and extent of coma with a duration of post-traumatic amnesia for less than 20–30 min and with a level of the coma of 12 or less on the Glasgow Coma Scale are common characteristics of an ABI [155]. The magnitude of the damage and the functional loss normally has a dose-response correlation. Many adults and infants with traumatic complications do better than expected, whereas those with relatively minor injury issues can encounter problems. There might be conflicts between parents and professionals. Even if the injuries are minor, it is crucial not to ignore the complaints [96]. Brain injuries can attribute to low concentration, impulsivity, and overactivity which can associate with other comorbid parameters that indicate poor post-injury performance. Learning difficulties that exist pre- and post-trauma can put many individuals of discrete ages at a heightened risk, exacerbating difficulties. The most critical thing to evaluate is the observed change in behavior or educational progression [156].

### *4.1. Physical Outcomes*

Aside from apparent gross motor problems, disabilities in the brain may have a significant effect on intellectual and behavioral performance. Sensory loss, weakness, tremors, seizures, excessive sweating, intermittent vision issues, ground abnormalities, and hearing problems are also possible side effects. All of these factors affect well-being, social interactions, and self-esteem [157]. Further longitudinal studies investigating these features of ABI are needed to uncover underlying mechanisms.

### *4.2. Cognitive Outcomes*

Mental manifestations are among the most obscure but recurrent issues which can lead to a variety of information-processing skills—thought, speed, as well as the ability to react to tasks—being slowed down. High levels of impulsivity and impaired judgment are normal, and they have important long-term consequences [158]. Verbal communication issues may not be apparent, but there may be impaired language abilities in word searching, interpretation, and comprehension [159]. Reading, painting, structural skills, and job performance can be challenging for patients with ABIs, as well as activities and knowing physical differences. Cognition and listening abilities are often harmed. The ability to prepare, specify objectives, coordinate, and implement a plan to achieve an intended goal are examples of executive skills. This also includes expertise in efficiency surveillance and planning. Any of these issues can affect individuals or some combination of them. The severity of these functional disabilities is determined not only by the extent of the injury but also by the age at the time of concussion [160,161].

### *4.3. Educational Outcomes*

Given the neuropsychological impact, the majority of rehabilitation emerges in the starting years, but developmental problems continue and can worsen. After the injury, issues about a lack of improvement in learning, speaking, and reading, as well as an inability to understand intelligent concepts and conceptualization [162]. More research is in demand to explore the underlying mechanisms.

### *4.4. Emotional and Behavioral Outcomes*

Unidentified cognitive episodes and a lowered self-image linked to an understanding of logical and technical difficulties can suggest behavioral and emotional issues [163]. Making rude remarks about others may be connected with impulsivity and this behavior

can be incredibly embarrassing and disconcerting for other people in society [164]. Elevated anxiety, rage, utterances of violence, fatigue, and inertia are the common and normal parts of the recovery stage and can last a significant amount of time. This is marked by a lack of enthusiasm and interest in everything, as well as difficulties maintaining focus and working at a fast pace, as well as passively carrying out recommendations rather than initiating activity. Both the extent of the incident and pre-existing symptoms can be linked to oppositional defiant disorder [165,166]. This is frequently linked to the realization of a lack of skill in certain things and being able to cope with everyday life less well [161]. The assumption by patients that they will be able to catch up with things soon can cause a lot of anxiety. Patients may be conscious of actual or potential losses, regardless of the circumstances of ABIs. Serious personal trauma can be humiliating and have a significant effect on one's self-esteem. Fear of failure might be really serious, and it could be the origin of depression and anxiety [161]. Post-traumatic stress disorder can occur even though there is no continuous recollection of head trauma [167].

**5. Pre-Existing Medications**

Nimodipine, triamcinolone, polyethylene glycol-conjugated superoxide dismutase, and mild hypothermia have all shown positive results in phase II clinical trials [162]. Excitatory amino acid inhibitors, calcium channel blockers, NMDA receptor antagonists, corticosteroids, free radical scavengers, magnesium sulfate, and growth factors have all been used in preclinical research to evaluate the therapeutic effects of drugs in various animal models [168,169]. Regretfully, none of the formulations or methods that have been examined in phase III trials have shown to be successful [167,170]. Mannitol has been shown to help reduce brain swelling after a brain injury [171]. However, its efficacy in the long-term treatment of serious TBI is unknown. Inordinate mannitol injection has been shown to be dangerous, as mannitol passes through the circulation and the brain, increasing pressure inside the skull and worsening internal brain injuries [171]. A new meta-analysis backs up earlier reports that hypothermic treatment is a good cure for brain injuries in some situations. Health professionals should continue to use vigilance when assessing hypothermia for TBI care before more data from well-conducted trials become clear [172]. After an extreme brain injury, elevated ICP is still the leading cause of disability and death. When estimated within any intracranial space, an accelerated ICP is usually characterized as 15–20 mmHg [173]. Raised ICP has been linked to increased mortality and morbidity after extreme brain injuries. A rise in brain size at the cost of one or more intracranial resources is the cause of high ICP [174]. In ABI, increased ICP is caused by mass lesions, edema, and increased cerebral blood flow. Fortunately, there is no proof to substantiate the regular use of decompressive craniotomy in any brain injuries in adults with high ICP to increase survival and the standard of living [175]. A decompressive craniotomy can be a valuable choice when optimum medical care has failed to stop ICP. One randomized trial of decompressive craniotomy (DECRA) with extreme brain injury is currently underway, which could provide more information on the procedure's effectiveness in adults [175].

**6. Plausible Drug Therapies**

*6.1. S100B*

The S100B protein is a member of a phenotypic family of low molecular weight calcium-binding S100 proteins that are primarily developed by glial cells. It also functions as a neurotrophic agent and a neuronal protection protein [176]. Excess supply of S100B by triggered glia, on the other hand, may exacerbate neuroinflammation and cause neuronal disruption. The brain and the serum S100B levels are scarcely associated, with serum concentrations largely determined by the blood-brain barrier's consistency contrary to the amount of S100B in the brain [177]. Cerebrospinal S100B can be valuable as an indicator of consequence in adults with serious brain injury. Long-term functional restoration after ABI was shown to be aided by intraventricular S100B implementation [178,179]. Five weeks after brain injury, it significantly boosted hippocampal neurogenesis. The Morris water

maze used to test spatial learning capacity on days 30–34 after injury, showed that an S100B injection improved cognitive efficiency [180,181]. S100B has not been used for the clinical care of any brain injury. S100B was used in a clinical trial called S100B as a Pre-Head CT Scan Screening Test After Mild TBI (NCT00717301) to see whether a serum can anticipate traumatic anomalies on a brain CT scan after a mild TBI. A change in serum S100B suggested whether the patient's neurological condition had improved or deteriorated [182]. Finally, surgical therapy resulted in lower levels of S100B. Serum S100B protein represented the seriousness of the injury and aided in the prediction of outcomes after a serious brain injury [183]. S100B was also useful in determining the effectiveness of treatment following a serious TBI [184].

### *6.2. Statins*

Statins, which are powerful inhibitors of cholesterol synthesis, can also help people with brain injuries [185]. Many of its effects, such as increased NO bioavailability, immunomodulatory activities, improved endothelial function, antioxidant properties, up-regulation of endothelial nitric oxide synthase, suppression of inflammatory responses, and platelet actin reduction are cholesterol-independent [186]. Simvastatin treatment significantly increased Akt, cAMP response element-binding proteins (CREB) phosphorylation, and GSK-3; amplified the production of BDNF and VEGF in the dentate gyrus (DG); enhanced tissue regeneration in the DG; and improved cognitive and memory restoration [187]. In rats with traumatic brain injury, atorvastatin injection decreased cognitive brain abnormalities, enhanced neuronal survival and synaptogenesis in the glioma parameter range and the CA3 areas of the hippocampus, and promoted angiogenesis in these areas [188]. Pre-treatment of rats with lovastatin enhanced mental outcomes and decreased the severity of brain injury, with a concurrent decline in serum concentrations of TNF-α and IL-1β mRNA and protein [189]. In addition, statin therapy increased cerebral hemodynamics in mice after a severe brain injury [190]. Statins helped animals regain their spatial memory quickly after a brain injury. A double-blind controlled clinical trial was conducted on 21 patients with TBI (aged 16 to 50 years) who had Glasgow Coma Scale scores of 9 to 13 and intracranial deposits as evidenced by a computed tomography (CT) scan [191]. Despite the overwhelming usefulness of statins, their desirable healthcare quality profile, and comprehensive preclinical research showing both neurorestoration and neuroprotection, further clinical trials are needed to assess statins' neuroprotective and neurorestorative properties after any type of brain injury [192].

### *6.3. Role of Phytochemicals in Brain Injury*

Plants develop metabolic systems that generate hazardous and/or antinociceptive bioactive molecules as a result of their static existence and exposure to herbivores and other pathogens [193,194]. Among phytochemicals, sulforaphane (isothiocyanato-4-(methylsulfinyl)-butane) has been demonstrated to have neuroprotective effects in several experimental paradigms. Sulforaphane has shown to have a protective effect on the neurological disorder and reduces Aβ1-42-induced inflammation via nuclear factor erythroid-2-related factor 2 (Nrf2) signaling [195,196]. The putrid phytonutrients have many other effects, particularly neuroprotective, anti-proliferative, neurogenerative, anti-microbial, and allelopathic properties, in addition to their bitter taste [197]. The majority of phytochemicals formed by plants to contend with environmental factors are classified as alkaloids, phenolics, or terpenoids. There are a variety of functions and ecological positions shared by these three classes [198]. Other phytonutrients, on the other hand, readily penetrate the brain when consumed (or inhaled), where they have the capability of altering brain functions, as psychoactive phytochemicals like cannabinoids, psilocybin, and mescaline have shown [199]. These behavior-altering phytonutrients are extremely potent, acting at subtherapeutic levels and connecting explicitly to particular neurotransmitter receptors. Neuroactive phytonutrients found in widely eaten fruits, vegetables, and nuts, on the other hand, contain a bitter or sour taste that is usually well-tolerated [200,201]. Nephrotoxic phytochemicals are found

in the body at concentrations much lower than their harmful level in the volume usually ingested, which explains their positive benefits [202]. Curiously, many phytochemicals are synthesized using cytochrome p450 (CYP450) enzymes [203]. It is worth noting that phytochemicals have emerged to stimulate all of the same cellular processes in mammalian cells as they have in plants. Signaling pathways involving Nrf2, SIRT1, and AMPK that developed in insects and other herbivores before humans in response to phytochemicals have been retained in human neurons [204–206]. Several of the compounds incorporated in the skins of fruits, according to new research, will boost cognitive function and safeguard against cognitive dysfunction in animal models of dementia, Alzheimer's disease, and many other neurodegenerative diseases [207]. Image recognition ability was improved in old rats fed a blueberry-augmented diet, and administering green tea catechins to mice alleviated age-related contextual memory formation decline [208]. By optimizing the expression of the transcription factor CREB, both blueberry and green tea phytochemicals can strengthen cognitive performance [209,210]. Caffeine, the most commonly consumed psychoactive phytochemical, has been shown to improve cognitive performance by enhancing intracellular calcium and cyclic AMP levels, which stimulate kinases that phosphorylate and thus activate the cAMP-response element binding protein (CREB) [211].

### *6.4. Magnesium*

Magnesium's impact on calcium channels, NMDA receptors, and neuron membranes makes it a potential clinical weapon [212]. In animal studies, magnesium has been shown to improve conditions such as intellectual and sensorimotor control after a brain injury [213]. Furthermore, because of the lack of side effects and proportional effectiveness to corticosteroids, the magnesium sulfate approach has proved to be the most appropriate move [214]. Clinical trials with patients with mild or extreme TBI who reported to a level-1 community trauma unit and were assigned randomly one of two magnesium doses or placebo within 8 h of injury continued for 5 days in a double-blind study. Consistent magnesium infusions for 5 days given to patients within 8 h of a mild or extreme TBI displayed less significant effects [215].

### *6.5. Barbiturates*

ICP is a risk factor for extreme ABI, and it is linked to a high risk of death. Barbiturates (pentobarbital and thiopental) are thought to lower ICP by preventing cerebral proliferation, which lowers cerebral physiological requirements and blood volume [174,216,217]. Barbiturates also lower blood pressure and can thus have an adverse impact on cerebral blood flow [218]. In one analysis, pentobarbital was considered less efficient than mannitol in lowering the ICP. In 25% of ABI patients, barbiturate therapy causes a drop in blood pressure. Any lowering ICP impact on cerebral blood flow would be compensated by this hypotensive effect [219]. Despite the fact that barbiturate coma is the secondary treatment for post-traumatic adjuvant ICP, and persistent hypotension is the most common side effect of it, recent studies indicate that low-dose corticosteroid therapy can be used in a fraction of patients to prevent hypotension [220]. ABI patients, who are plunged into a barbiturate coma, are more likely to experience adrenal insufficiency [221,222]. Some ABI patients who received barbiturates experienced adrenal dysfunction and needed higher concentrations of norepinephrine to manage cerebral blood flow than those who did not receive barbiturates [169,223].

### *6.6. Glutamate Receptor Antagonist*

Neuronal cells, as a result of TBI, may become excitotoxic, which is when there is a buildup of the excitatory neurotransmitter, glutamate. This results in the overactivation of glutamate receptors causing brain damage to occur at multiple levels such as loss of the blood-brain barrier (BBB), neuron cell membrane integrity, cerebral edema, and cell death [224]. To quell the excitotoxic effects of glutamate, researchers have tried introducing a glutamate receptor antagonist (Dizoclipine), in rats with TBI [225] or topiramate in an

epilepsy model in rodents [226]. The receptor antagonist was shown to alleviate continued brain damage in rodents; however, the drug was associated with an inadequate therapeutic window [227,228]. This research, although in its infancy, shows that glutamate receptors could be a viable target for TBI therapy.

### *6.7. Antioxidants*

Another prominent process of secondary brain injury in TBI is through the presence of free radicals in the cerebral tissue. There are many complex mechanisms through which the injured brain produces free radicals; however, in TBI, the balance of oxidants and antioxidants is shifted [229]. The shifted balance toward oxidant production results in increased membranous lipid peroxidation, oxidized proteins, DNA damage, and mitochondrial respiration leading to neuronal cell death [17,37,230]. Researchers have demonstrated that melatonin and N-acetylserotonin have anti-inflammatory, antioxidant, and anti-apoptotic effects [231]. The administration of melatonin was shown to up-regulate antioxidant enzymes in rodent studies, which could provide a possible neuroprotective effect in humans [232].

### *6.8. Targeting Inflammation*

Upon receiving a TBI, the immune system is already at work. Drugs such as dexamethasone, pioglitazone, indomethacin, or ibuprofen have shown to have prominent effects on ABI-induced inflammation [233,234]. Damage-associated molecular patterns (DAMPs), chemokines, and cytokines flush through the neural tissue, recruiting armies of white blood cells to help clean up the injury. While the intent of the WBCs is to save the brain from further disastrous damage, activated microglia (macrophages in the brain) release reactive oxidative species and excitatory neurotransmitters that contribute to further cell death [235]. Researchers are targeting inflammatory cytokines such as IL-1β and TNF-α. These studies show some promise as they were able to reduce neurologic damage and even improve cognition and motor ability [236]. Another avenue of research of neurologic immunology in TBI is through the endocannabinoid system, which has been shown to play an essential role in the homeostasis of the cell and may play a prominent role in cellular repair mechanisms after or during disease processes [237]. Endocannabinoid clinical trials failed to show significant protective effects; however, tests of synthetic endocannabinoid receptors showed some therapeutic promise in rodent studies [17,238].

### *6.9. Programmed Cell Death Inhibitors*

Apoptosis of neuronal cells is another result of TBI and is considered a poor prognostic factor. Studies are targeting different areas of the apoptotic cascade. The design of drugs that inhibit cyclin-dependent kinases (regulators of the cell cycle) showed potential therapeutic outcomes as they slowed the progression of neuron death and improved health outcomes in TBI mice [239]. Another area of the programmed cell death pathway researchers are targeting for therapies is that of caspase-dependent apoptosis. Caspase-3 and 12 inhibitors (peptide-based inhibitors such as z-VAD-fmk, and qVD-oph and the small molecule inhibitors, IDN-6556 and p35) have improved health outcomes in a hemorrhagic and TBI model of rodents and can be used as an effective therapy due to its wide therapeutic window [17,240].

## 7. Future Prospective

Because of the breadth of diseases and pathophysiological mechanisms, ABI encompasses there is a range of different therapeutic options specified for each disease process. These treatments range from chemotherapies to surgical interventions. However, when it comes to TBI, contemporary treatment options are limited due to the innate complexity of TBI pathophysiology [241]. Due to this complicated nature of TBI care, modern-day intervention plans are generalized approaches that may be able to address the primary brain damage (occurring due to direct brain damage after immediate impact) but customarily

fail to impede secondary neuronal tissue damage (damage that continues months to years after the traumatic episode) from the brain's response to the traumatic event. The following covers the current treatments for TBI and their strengths and weaknesses, in addition to identifying promising future therapies.

*7.1. ICP Monitoring and Management*

ICP is the pressure that results from the closed system of the craniospinal compartment. Increasing pressure can produce disastrous amounts of stress on the brain tissue causing neuronal damage and possible brain herniation. Within the skull, there is a precious balance in secretion, composition, and volume of CSF [242]. Increased ICP is a pervasive result of TBI and is a significant result of secondary brain injury; therefore, monitoring and managing its levels is established as a critical aspect of TBI treatment. There are multiple methods healthcare providers use to monitor ICP. Computed tomography (CT) scans are often used to visualize the increase in pressure while intraventricular catheters are the "gold standard" for ICP monitoring. The catheter is usually surgically placed into the lateral ventricle where a standard pressure transducer will monitor pressure changes [243]. The catheter can also double as a drain, which can be used for the therapeutic draining of the intracranial space or, as mentioned above, for diagnostic objectives [244]. In addition to catheter use as a means of ICP therapy, head elevation is used to displace much of the CSF from the cranium and encourage venous return to the heart. With head elevation, ICP may be reduced without the disruption of cerebral blood flow [245]. Hyperventilation is another means through which ICP can be reduced medically. Hyperventilation lowers the ICP by increasing the intraarterial $CO_2$ partial pressure which signals the sympathetic nervous system to vasoconstriction; however, it is only used for brief periods, when the brain tissue is under stress [246].

*7.2. Medically Induced Coma*

The brain is the single largest organ in the form of glucose consumer in the body. Accounting for roughly 2% of the body's weight, it nonetheless consumes 20% of the body's glucose [247]. This shows how active the human brain is. In traumatic episodes, it is important to preserve its function by reducing the metabolic demand of the brain. To do this, physicians often administer benzodiazepines or infuse barbiturates to induce a coma in the patient. This can save brain tissue from excitotoxic events and seizures, saving a great amount of neuronal tissue [246,248].

*7.3. Surgical Intervention*

As mentioned above, hematomas and hemorrhages are often associated with TBI. If the significant mass effect from a hematoma or bleeding is appreciated in imaging, then surgery is warranted. A hematoma may continue to grow, which could apply large amounts of pressure to the brain tissue resulting in neuronal death. If the hematoma begins to expand rapidly this is considered a neurosurgical emergency and pressure must be relieved via decompressive craniotomy [244].

ICP monitoring and management, medically induced comas, and surgical interventions are adequate means of immediate therapy; however, their limitation is that they fail to address the secondary effects of TBI. These effects can last from months to years and can result in neuronal cell dysfunction and neurodegeneration. There are currently little to no therapies that adequately address the main pathophysiologic mechanisms of secondary brain damage; nevertheless, many researchers are working to address this aspect of TBI treatment by addressing the different aspects of disease phenomenon such as excitotoxicity, oxidative stress, inflammation, and programmed cell death [17].

*7.4. Remote Ischemic Conditioning as an Adjuvant Therapy*

Remote Ischemic Conditioning (RIC) is a non-invasive adjuvant therapy particularly useful in treating ischemic and hemorrhagic injuries [249,250]. Recent studies val-

idated the therapeutic effect of RIC on the treatment of several brain disorders such as focal ischemia [251,252], acute ischemic stroke [253,254], aneurysmal sub-arachnoid hemorrhage [255–257], and intracranial arterial stenosis [258] and in the prevention of stroke-associated pneumonia [259]. It consists of repeated cycles of temporary ischemia-reperfusion in the arms or legs. The procedure involves a manual or electronic tourniquet, which applies a pressure of 30 mm of Hg above the systolic blood pressure to establish repeated cycles of occlusion and reperfusion [249,250].

The principle of RIC, as both pre-and post-conditioning, has been validated in different in vitro, preclinical, and clinical studies in distinct disease models such as myocardial, pulmonary, and endothelial injury [260,261]. Multiple mechanisms have been put forward to explain the therapeutic effect of RIC and might involve the release of humoral factors such as nitric oxide or biogenic amines such as ornithine, glycine, kynurenine, spermine, carnosine, and serotonin [262]. These factors modulate the systemic immune response by regulation neutrophils activation [263,264], macrophage polarization [265], and/or T cell activation [266]. These mediators are also transported through the bloodstream towards the site of injury, where they attenuate disease progression by regulating multiple pathways at cellular and molecular levels [267]. This involves changes in mitochondrial metabolism characterized by a reduction in the levels of glycerol, a decrease in the lactate/pyruvate ratio, and a reduction in the rate of ATP depletion through the regulation of KATP channels [268]. At the molecular levels, it regulates distinct pathways such as AMPK [269], opioid pathway [270], Notch signaling [271], and peroxisome proliferator-activated receptor (PPAR) gamma [272]. RIC also regulates gene expression at the site of injury at both genetic and epigenetic levels. It downregulates the expression of genes associated with the regulation of metabolism, molecular transport, oxidative stress, and cell cycle regulation [273]. In the case of brain injury, its clinical relevance produced results in several clinical trials and was discussed in detail in the recently published review article by Baig S et al. [249]. While the research on the effect of the RIC on ABI is still in its infancy, further progress in this area requires investigating which patient groups respond best to RIC, identifying the optimal protocol such as dose and duration of therapy, and establishing biological and radiological biomarkers of the conditioning response.

### *7.5. Elastin Derived Peptides in Acquired Brain Injury*

Elastin-derived peptides (EDPs), the fragmented product of elastin protein, have been implicated in the progression of neurological degeneration during acquired brain injury. EDPs are detectable in CSF and blood in healthy people and patients after ischemic injury [274,275]. Elastin is the major structural matrix protein found on the surface of arteries, lung tissue, cartilage, elastic ligaments, brain vessels, and skin. Due to extensive crosslinking, elastin is highly insoluble and has a long half-life [276,277]. However, under normal and disease conditions, it is degraded by serine protease (also known as elastase) [278], cathepsins [279], and matrix metalloproteases (MMPs) particularly MMP-2, -3, -7, -9, -10, and -12 [280,281].

During brain injury, elastase is released from interstitial and inflammatory cells, and together with cathepsins and MMPs degrades elastin to release EDPs [276,282]. EDPs bind to the cell-surface protein complex consisting of elastin-binding protein (EBPs), cathepsin A, and neuraminidase (Neu1) [283,284]. Another EDP receptor, Galactin-3, is expressed in inflammatory cells and is associated with tumor progression and metastasis [285–287]. Other less characterized EDP-binding proteins are integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ [288,289]. The immune cells recognize EDPs as a foreign antigen and produce anti-elastin antibodies that result in an autoimmune reaction as seen during psychiatric diseases and other neurodegenerative disorders such as Alzheimer's disease [290–292].

The binding of EDPs to their receptors in astrocytes activates various intracellular signaling pathways such as peroxisome proliferator-activated receptor gamma (PPAR$\gamma$) [293] and AHR [294]. Together, these pathways regulate cellular activities such as cytotoxicity, apoptosis, cell proliferation, and metabolism [295]. Limited studies have investigated the

effect of EDPs on nervous system cells, particularly in astrocytes. An in vitro study suggested that astrocytes express EBPs, which might be involved in the process of astrocytoma invasion [296]. In the mouse cortical glial cells, EDPs peptide decreased the expression of *Mmp-2* and *Mmp-9*, whereas it increased the expression of *Timp-2*, *Timp-3*, and *Timp-4* mRNA indicating its inhibitory effect on neovascularization [297]. The EDPs also decreased NO production and increased ROS production in astrocytes [298]. Further studies suggested that EDPs reduce the proliferation of undifferentiated neuroblastoma cells, thereby promoting aging which may underlie several neurodegenerative diseases [299].

Taken together, the studies so far indicate pro-inflammatory and anti-angiogenic effects of EDPs in brain injury. How EDPs levels in different pre-clinical and clinical ABI models affect the outcome of the diseases is worth further investigation.

### *7.6. Stem Cell—Contemporary Clinical Trial*

Early studies of stem cell therapies in brain injury focus on the restoration and rehabilitation of damaged neuronal connections. Recently, the paradigm of stem cell therapy has shifted from the reconstruction of neural networks to the reduction of the secondary immune response, responsible for secondary brain injury and neurodegeneration [300]. Studies showed that hematopoietic stem cells attenuated proinflammatory markers like TNF-α and IL-β. In turn, this resulted in lower immune cell infiltration of the brain and decreased activation of microglia [301]. In cellular therapy studies, researchers found that the infusion of multipotent adult progenitor cells (MAPCs) after TBI in rodents achieved some level of neuroprotection from the secondary immune response. The results showed that MAPCs led to the efflux of IL-4 and IL-10 cytokines which stimulated the M2-microglial phenotype and MAPCs led to modulated microglial activity in neural tissue [302]. Currently, the same researchers are carrying out clinical trials with the use of mononuclear cells derived from bone marrow as a treatment for TBI. In phase 1, the bone marrow mononuclear cells were delivered intravenously to pediatric patients with a severe traumatic brain injury which showed some central nervous system preservation. Phase 2 clinical trials were completed in adults with the same functional, structural, and neurocognitive outcomes measured as in phase 1 [302].

The new developments of different therapies, targeting different aspects of the complex nature of TBI are important as they will help save the lives of millions worldwide. These possible treatment options will also give physicians more options in treatment which may be personalized to the patient's specific injury and bodily response to said damage.

## 8. Conclusions

The established medical treatment of ABI patients consists primarily of advanced prehospital treatment, comprehensive clinical care, and long-term recovery; however, there is no scientifically validated successful management of neuroprotective agents to prevent subsequent injury or improve healing. The massive impact of ABI, on the other hand, clearly demonstrates the need for certain neuroprotective and/or neurorestorative strategies or therapies.

Combining therapies can lead to improved results. Several agents and cells or even other strategies may be used in these possible permutations. Inadequacies in clinical trial designs and analyses can affect the outcome. In new clinical trials, a more responsive interpretation of the outcome is needed with substitute performance indicators and new forms of outcome analyses. Certain phytochemicals' potential to stimulate certain beneficial stress response mechanisms that exercise and energy restriction suggests that they can enhance brain performance and reduce the risk of neurodegenerative diseases. These neurodegenerative diseases, including Alzheimer's and Parkinson's disease, show that oxidative stress, reduced immune bioenergetics, mitochondrial function, and the aggregation of protein aggregates all play a role in the malfunction and continued degeneration of the brain. There are mounting results showing that neurohormetic phytochemicals have the capability to stimulate mechanisms that resist or restore oxidative damage, boost

bioenergetics, and improve the elimination of proteopathic proteins like amyloid peptide and synuclein, which is helpful in improving the patient's condition. Additionally, stem cell therapy and non-invasive RIC showed promise in improving ABI. However, extensive and robust research is needed to investigate a number of significant, unanswered questions about the underlying neural mechanisms of action of particular phytochemicals, as well as their therapeutic effectiveness in animal experimental models and human beings. Further advancement of scientific proof therapies and application of these recommendations is likely to increase the likelihood of quantitatively effective agents showing promising effects in potential clinical trials.

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# Behavioral and Psychiatric Symptoms in Patients with Severe Traumatic Brain Injury: A Comprehensive Overview

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**Abstract:** Traumatic brain injury (TBI) is defined as an altered brain structure or function produced by an external force. Adults surviving moderate and severe TBI often experience long-lasting neuropsychological and neuropsychiatric disorders (NPS). NPS can occur as primary psychiatric complications or could be an exacerbation of pre-existing compensated conditions. It has been shown that changes in behavior following moderate to severe TBI have a prevalence rate of 25–88%, depending on the methodology used by the different studies. Most of current literature has found that cognitive behavioral and emotional deficit following TBI occurs within the first six months whereas after 1–2 years the condition becomes stable. Identifying the risk factors for poor outcome is the first step to reduce the sequelae. Patients with TBI have an adjusted relative risk of developing any NPS several-fold higher than in the general population after six months of moderate–severe TBI. All NPS features of an individual's life, including social, working, and familiar relationships, may be affected by the injury, with negative consequences on quality of life. This overview aims to investigate the most frequent psychiatric, behavioral, and emotional symptoms in patients suffering from TBI as to improve the clinical practice and tailor a more specific rehabilitation training.

**Keywords:** psychiatric symptoms; severe traumatic brain injury; neuropsychiatric disorders; behavior

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## 1. Introduction

Approximately 150–200 per million people annually die because of traumatic brain injury (TBI) representing a core health problem [1].

TBI is defined as an altered brain structure or function produced by an external force. Besides the neuroradiological evidence of the acute intracranial injury, the clinical signs are neurologic impairments, loss of consciousness, amnesia for peritraumatic events, and compromised mental status. Severity of TBI has been categorized based on "the patient's level of consciousness (assessed by the Glasgow Coma Scale [GCS]), and the duration of unconsciousness and posttraumatic amnesia (if present)" [2].

Neuropsychiatric symptoms such as delirium may be transient in the acute phase of the TBI, whereas mood, personality, and sleep changes, as well as psychosis and behavior impairment, are chronic symptoms that persist over time and may be seriously disabling [3,4]. Indeed, these prevent TBI patients from returning to normal activities, work, or maintaining meaningful social relationships.

A prevalence rate of 25–88% in moderate or severe TBI has been reported [5].

Even though extensive research is available, the disorders onset, evolution, and risk factors for NPS after TBI is still indeterminate.

In this overview, we sought to describe the most common neuropsychiatric and behavioral problems following TBI, indicating the prevalence, main manifestations, comorbidities, and clinical indications to better manage these important but often underestimated symptoms that may negatively affect both patients and caregivers' quality of life.

**2. TBI and Risk Factors of NPS**

Identifying the risk factors of poor outcome is the first step to reduce the sequelae. Recently, TBI has been considered as a disease process instead of an isolated event, with acute and chronic consequences [6]. After the injury, neuropsychiatric disturbances (NPS) can occur as primary psychiatric complications or could be an exacerbation of pre-existing compensated conditions. Lauterbach et al. have showed that NPS were higher in patients with a pre-injury history (83.2%) than those without (63.6%). Moreover, 59% showed one or more Axis I disorder before TBI and substance-use was the most common pre-injury disorder (38.5%), whereas 56.5% were diagnosed as a new diagnostic class. [7]. In all probability, premorbid factors of the psychological status, personality, contingencies, environmental reinforcements play an important role in determining the clinical picture of the "frontal personality" [8,9]. The symptoms could appear acutely or develop more gradually and insidiously influencing the grade of disability. Patients with TBI have an adjusted relative risk of developing any NPS several-fold higher than in the general population after six months of moderate-to-severe TBI [10]. A review by Babbage et al. [11] reported a rate of 39% of impairment in emotion recognition and reduced levels of empathy as well as impairment of the so-called theory of mind (ToM) [12], with detrimental effect on life satisfaction of their relatives and caregivers. [13].

The most common psychiatric complication associated with TBI is depression, with prevalence rates ranging between 6% and 77%, while 2–50% of TBI patients affect from generalized anxiety disorders [14]. It seems that rates of mood and anxiety disorders increase throughout the first year and that anxiety disorders emerges earlier post-injury than mood disorders [15–18]. A peak of NPS was recorded in the first year, with a subsequent significantly declined over time of anxiety disorders by 27% with each year post-injury, although mood and substance-use disorder rates persisted steady. [7].

Factors associated with depressive disorders in TBI patients include age, lifestyle, young adult, premorbid substance misuse, especially in male patients [19]. Other risk factors are lower education level [20], previous psychiatric symptoms, including anxiety, intellectual deficits and left prefrontal cortical lesions [9]. Some studies reported a frequency of NPS of 77% in males and of 71.4% in females ($\chi^2 = 0.46$, df = 1, $p = 0.50$). Depression in TBI individuals has been linked to decreased community integration, overall functioning, a decrease in quality of life, aggression, poor recovery, and higher rates of suicidal ideation and suicide attempts. Likewise, anxiety disorders in TBI patients are associated with poor social interpersonal functioning, decline in independent living, and acts as a positive predictive factor for the development of depression in TBI peoples. Moreover, delirium [16] and status epilepticus [21] in neurointensive care unit are very well-known risk factors for developing behavioral disorders at long term follow up.

**3. Neuropsychiatric Disorders in Severe TBI: Neuroanatomical Basis**

The pathophysiology usually comprises volume of large brain areas, because of the high velocity impact of trauma that causes extreme motion, torsion, tensile effects, and diffuse shearing and tearing to brain structures [22]. Studies with functional magnetic resonance imaging have shown changes in task-mediated activation in the dorsolateral prefrontal cortex, ventrolateral PFC, and basal ganglia in mild TBI. In the same way, Electroencephalography has also been used to characterize and localize brain–behavior relationships with neurophysiological findings in TBI [21].

Pathologically, chronic traumatic encephalopathy (CTE) is characterized by an accumulation of neuronal phosphorylated tau (p-tau) in the perivascular regions and p-tau fibrils as in Alzheimer's disease, or tauopathy [23].

TBI patients with a distinguishable structural lesion on neuroimaging have shown behavioral and emotional dyscontrol as compared to patient without brain lesions [24]. Numerous studies have documented behavioral changes in patients with very extensive unilateral-bilateral lesions of the frontal lobe [25]. A reduction in spontaneous activity and speech was the main finding, although apathy, loss of initiative, and reduction of work efficiency, as well as hyperactivity and an inability to inhibit impulsive and emotional responses, infantilism, scurrility, and fatuous euphoria were also demonstrated [25]. However, even temporal lobe structures have been commonly associated with dyscontrol syndromes, regularly in combination with the frontal lobe, as in frontotemporal degenerative illnesses [25]. Moreover, cerebellar lesions have been associated with post-TBI dyscontrol disorders [26], leading to the so-called cerebellar cognitive affective syndrome [27] The variability of symptoms and the frontal, cerebellar lesions correlation have been described in Table 1.

**Table 1.** The wide spectrum of "Frontal Personality" and other syndromes.

| Clinical Syndrome | Brain Lesions | Personality Changes, Prevailing Signs |
|---|---|---|
| Prefrontal syndrome | extensive unilateral-bilateral lesions of the frontal lobe | uninhibited, attitudes of swagger, variable mood, euphoric, with lack of worries or with excessive worries, with self-centeredness, obstinacy apathy, indifference, distractibility, neglect in clothing, intolerance to frustrations, eating disorders, drowsiness, lethargy |
| Pseudo-psychopathic | Lesions of the fronto-orbital cortex | euphoria, restlessness, sexual disinhibition, childishness, inappropriate social behaviour, lack of interest |
| Psychomotor slowdown | Lesions of the medial frontal cortex: dorsal region (cingulate gyrus and supplementary motor area), | psychomotor slowdown (the patient only answers when he is asked direct questions) abulia |
| Cognitive-behavior syndrome | Ventro-medial frontal lesions (limbic cortex and diencephalic structures) | severe memory impairment, socially inappropriate behavior, disorientation, changes in consciousness, apathy and uncontrolled aggression. |
| Cognitive impairment | dorsolateral lesions (the orbital and medial frontal areas) | neurocognitive deficits spare many aspects of mental functioning. |
| Cerebellar cognitive affective syndrome | Cerebellum | emotional dyscontrol, aggression, Cognitive dysfunction (executive, visuospatial, and language function |

### *3.1. Behavioral Dyscontrol in TBI Patients*

Behavioral dyscontrol (BD) indicates a propensity to instinctively response to overall provocations. Moreover, BD have been defined as "behaviors that are noxious for the health or physical integrity of the person" [28]. It has been reported that approximately 62% of TBI patients present BD at one year from trauma, [29] described as "are not the way they used to be" and 74% at five years [30]. Brooks et al. found that the most frequent BD were irritability (64%), bad behavior (64%), drowsiness (62%), depression (57%), rapid mood change (57%), anxiety (57%), and aggression (54%) [30].

In the five years following TBI, the most common BD were irritability and lack of initiative (44%) with inappropriate social behavior (26%) [31].

In 2015, according to experts of the direction-finding group and based on the prevalence and incidence of BD reported in the literature, the symptoms of BD were classified into four subgroups [32]:

1. Disruptive primary behaviors by excess (i.e., aggression, disinhibition, agitation)
2. Disruptive primary behaviors by default (poor initiative, isolation, withdrawal)
3. Affective disorders (anxiety, depression, somatization).
4. Psychosis, suicide attempts and suicide.

However, classifying BD is still complex, and the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) not adequately explain the complexity of neuropsychiatric symptoms of patients with TBI. In fact, DSM-V includes these symptoms as "mild" or "major" neurocognitive disorder due to TBI history diagnosis. Usually, the differential diagnosis between idiopathic and secondary symptoms is controversial. TBI patients frequently present similar symptoms of general population with NPS but without TBI history, without exactly achieving the required DSM criteria. For example, psychosis after TBI manifests with positive symptoms than negative symptoms of the general population [7].

Moreover, some symptoms could be due to either major depression or be consequences of frontal lobe lesion; and depression could be expressed as irritability, frustration, anger, hostility, and aggression instead of sadness [33]. Therefore, the diagnosis is usually a difficult matter.

Behavioral dyscontrol may be distinguished in (i) disinhibited type, positively associated with MRI brain volume loss, (ii) aggressive type, positively associated with more left, than right, hemispheric lesions, or (iii) combined type (if more than one of these disturbances predominate the clinical presentation) [34], characterized by global traumatic axonal injury [35–37].

#### 3.1.1. Disinhibition Syndromes after TBI

Disinhibition refers to socially or contextually inappropriate non-aggressive verbal, physical, and sexual acts involving a reduction or loss of inhibitions with inability to follow social or cultural behavioral norms and rules. In patients affected by TBI, this behavior usually coexists with impulsivity, as well as deficits in tolerance and social cognition [38], but its prevalence is not well established, and the frequencies range from 12% to 32% [38]. The severity of disinhibition has been associated to poor outcomes, decreased functional independence, as well as social disability. Then the symptoms should be considered during both the assessment and the training.

The assessment of disinhibition syndromes included a self-report scale, i.e., the Barratt Impulsiveness Scale (BSI). This is a 15-item short form tool providing a framework to investigate and monitor disinhibited behavior among individuals with TBI. However, the scale mainly measures impulsivity and related symptoms, and the differential diagnosis must rule out many neurologic and psychiatric disorders [39]. Then, it could not be used in the case of patients with post-traumatic self-awareness deficits. In these cases, the disinhibition subscale of the Neuropsychiatric Inventory (NPI) may provide useful data.

The treatment of posttraumatic disinhibition usually needs psychobehavioral, environmental and pharmacologic approaches. In fact, applied behavioral analysis (ABA) and treatment is a useful element of the treatment plan for disinhibited behaviors. This approach entails careful characterization of the disinhibited behaviors, their internal emotional and behavioral dyscontrol [39]. Social skills training, including individual and group interventions, with social communication skills training also may be useful. Even the telehealth group was more efficient than the in-patient group on some variables [40]. Moreover, it was demonstrated that patients with TBI had significant improvements in both goal attainment scaling (GAS) scores and qualitative evidence of enhanced social communication skills after the training with the INSIGHT program, a client-centered contextualized approach that promotes positive identity construction with consideration of efficacy of treatment [41,42]. On the other hand, there is no specific therapy for disinhibition. Pharmacological treatment of impulsivity and sexual disinhibition is variable and depend on the cause of symptom. For

example, impulsivity, may be caused by executive (frontal lobe) dysfunction, mood lability, or be associated with attentional deficits. Therefore, a serotonin-reuptake inhibitor could treat frontal impulsivity but is contraindicated for impulsivity due to mood cycling [7].

#### 3.1.2. Aggression in Patients with TBI

Aggression includes irritability, anger, agitation, and disinhibition, and represents the most challenging consequences of TBI to manage, interfering with rehabilitation and social support networks. Aggression was found to have a frequency of 5–8% in a sample of military servicemen, who were assessed within the first six months following TBI [43]. Among persons with more severe injuries, the early phase of posttraumatic confusional state is related with a rate of 30–80%, with as many as 20% demonstrating violent behavior. In the late phase of post-injury period following non-penetrating severe TBI, rates of aggression range from 15% to 55% [44,45]. Aggression is influenced by preinjury/post-injury comorbidities [46,47], especially depression and anxiety [48].

In animal models, it has been shown that serotonin, dopamine, and adrenaline are neurotransmitters involved in behavioral disorders. In particular, the level of serotonin is negatively correlated with aggressiveness [3], influencing personality changes, agitation, and aggressiveness [49]. Adrenaline and dopamine are involved in attention, memory, and executive functions [50].

Neuroimaging studies, assessed by the Buss-Perry Aggression Questionnaire, have revealed that increased diffusivity in the splenium correlated with physical aggression whereas raised diffusivity in the body of the corpus callosum was related to aggressive attitude. Moreover, functional magnetic resonance imaging studies showed a positive association between aggression and increased right hippocampus to midcingulate cortex connectivity, and between the right cerebellar lobule VII region and the default mode network [51,52]. Studies with EEG exclusively showed a positive association between frontal lobe abnormalities and aggression [53,54].

Assessment of the severity and frequency of posttraumatic aggression in individuals with preserved capacity for self-report may be performed by the administration of the Aggression Questionnaire (AQ) [55] and the Personality Assessment Inventory (PAI), investigating posttraumatic aggression and psychiatric symptoms. Among patients with cognitive deficits or BD, the assessment of aggression requires more specific tools. The Modified Overt Aggression Scale could be used for this purpose [56], although when multidimensional neuropsychiatric assessment is needed, the NPI could be administered.

To treat posttraumatic aggression in an effective way, a multimodal approach involving both non-pharmacologic and pharmacologic interventions, is often necessary. The former includes either replacement or decelerative techniques. Replacement strategies are based on assertiveness training (involving patients who are angry when they fail to get their goals) and several reinforcement scheduling (to reduce the rate of pre-violent behaviors) [57]. Decelerative methods involve contingent observation, social extinction, self-controlled time-out, and contingent restraint [58]. Pharmacologically, frequently aggression in patients with TBI could be treated with carbamazepine, tricyclic antidepressants, trazodone, amantadine; beta-blockers (propranolol, nadolol), valproate, lorazepam, with good tolerability in TBI patients (less frequently with haloperidol, and benzodiazepines). An excessive use of sedative may interfere with rehabilitation project. However, these drugs are limited in terms of use because of the complexity of aggression and the comorbidity with mood disorders or asthma. Therefore, usually, the treatment of aggression is conducted with mood stabilizers [59,60] (see Table 2).

**Table 2.** Aggression therapy with only Expert opinion and guidelines [59–61].

| Class of Therapy | Indication | Mechanism of Action | Dosage | Expert Opinion |
|---|---|---|---|---|
| Beta-blockers (propanolol) | Agitation management | / | 60−80 mg per day, initially 20 mg/day, then gradually increased up to a maximum of 420 mg per day (60 mg raises every 3 days) and a follow-up of 8-weeks (if no effects after 8 weeks, stop it) | Propranolol is the Gold Standard when a TBI patient suffers from both high-blood pressure and agitation. Recommended to perform an electrocardiogram before beginning the treatment |
| Antiepileptics | Mood-regulating | Act on neurotransmitters: glutamatergic GABAergic agents limbic system | Oxcarbazepine 1200 mg/day, (max 2400 mg over a 10-week period) carbamazepine 400–800 mg/day for 8-weeks lamotrigine 50 mg twice a day | Gold Standard for agitation or aggressiveness, after TBI, when associated with epilepsy or bipolar disorders |
| Antidepressants | Agitation and aggressiveness | Act on neurotransmitters involved in behavioral disorders: serotonin, dopamine and adrenaline. | Sertraline 100 mg/day (max 200 mg/day, first line) Amitriptyline 25 mg/day (second-line treatment) | Gold standard when agitation or aggressiveness crisis is associated with depression |
| Neuroleptics | Agitation crisis | Sedative | Olanzapine, clozapine, quetiapine 25–300 mg/day ziprasidone 20–80 mg/day | Neuroleptics can be used in the treatment of agitation associated with delirious symptoms |
| Benzodiazepines | Agitation crisis | / | Clorazepate dipotassium | Indicated when anxiety is the predominant symptom (symptomatic prescription) |

#### 3.1.3. Irritability: An Affect and Mood Disorder

Irritability has been defined as a disproportionate response with unjustified anger fits. Eames [61] suggested that in the early post injury period, irritability is transient because it is arising in response to nearly any stressor or frustration. Instead, irritability in the late postinjury period is marked by regular, cyclic, ego-dystonic outbursts modifying the premorbid affective responding.

It has been reported an incidence of 29–71% in individuals with severe TBI, being male gender, age range 15–34, unemployment, social isolation and depression the main risk factors [62].

To assess irritability, the self-report Neurobehavioral Symptom Inventory (NSI), a multi-symptom assessment, may be used as screening tool for diagnosing irritability and frustration. However, this tool is limited with regard to its characterization of these specific problems. In patients with limited self-awareness after TBI, the NPI appears to provide functionally important levels of irritability among TBI patients [63].

### *3.2. Affective-Emotional Dysregulation in TBI*

Affective-emotional dyscontrol following the brain injury may be related to the disruption at the emotion regulation neural networks [64]. This includes several disorders of affect, such as pathological uncontrollable laughing and crying (PLC), and affective lability, triggered by stimuli that commonly do not cause such emotional feeling state, also known as "pseudobulbar affect" or "emotional incontinence" represent a prototypical form of affective-emotional dyscontrol [45]. These events must cause subjective distress and/or interfere with everyday life activities. The prevalence of PLC during the first year after injury is 5–11% [65] and tends to decrease in the late post-injury period.

The pathophysiology is still unknown. However, it seems that PLC is the result from the relief of cortical inhibition of upper brainstem centers that incorporate the motor activation patterns involved in laughing and crying. Tateno et al. showed that lesions of the lateral aspect of the left frontal lobe were significantly linked to the presence of PLC [45]. In particular, the prefrontal cortex modulates the emotional motor and autonomic responses of sensory and limbic information involved in emotional expression. Therefore, lesions of prefrontal control of hypothalamic, pontine, and medullary centers that mediate these responses lead to PLC. Thus, a correlation between PLC and the dorsolateral/anterior frontal cortices, internal capsule, prefrontal cortex, amygdala, hippocampus, basal ganglia, and thalamus has been show, leading to a chronic condition (cortico-limbic–subcortico-thalamic–ponto-cerebellar network), beyond the cortical inhibition of upper brainstem centers involved in the integration of motor activation patterns [66]. It has been shown that PLC is associated with psychiatric disorders, especially anxiety and mood disorders (depression in particular) and personality changes [45,67]. The quantitative scale that could measure and quantify the severity of PLC is the Pathological Laughter and Crying Scale [68].

The first-line treatment of PLC is pharmacotherapy with serotonin reuptake inhibitors (SSRI) and/or selective noradrenaline reuptake inhibitor (NaRI) active antidepressants [45,68–70]. Moreover, in a recent case report, a patient with TBI and PLC was treated with dextromethorphan/Quinidine (DM/Q 20 mg/10 mg, Nuedexta) showing an improvement in PLC. Dextromethorphan is a non-competitive antagonist of the NMDA receptors and a sigma receptor agonist, having a neuroprotective effect on injured brain [71].

On the other hand, quinidine is a potent cytochrome P450 2 D6 inhibitor and reduces the metabolism of DM [72].

Affective lability (also known as emotional lability) refers to a tendency to be easily overcome with intense emotions in response to personally, socially meaningful stimuli or events that ordinarily would induce modest emotional responses [58]. It manifests as brief, non-stereotyped episodes of congruent emotional expression and experience that are not discretely paroxysmal, of variable intensity, and partially amenable to voluntary control or interruption by external events. Affective lability is observed in association with a broad range of psychiatric and medical conditions [73] with a prevalence ranging from 33% to 46% in the early post-injury period, and to 14–62% in the late period [74]. The Affective Lability Scale or the CNS-LS is a self-report scale to evaluate affective lability [75].

### *3.3. Empathy and Alexithymia Following TBI*

The presence of alexithymic behaviours in people who had an acquired brain injury as defined as 'organic alexithymia' [76,77] and may considered as a deficit in affective-cognitive handling [78]. Respectively, people with alexithymia reveal impairment in emotional orientation, consciousness, and communication of their emotions [79]. The prevalence of organic alexithymia has been estimated at approximately 15% in the acquired brain injury population. Consequences of alexithymia are verbal explosions, maladaptive coping, impulsivity, and aggression with a lack of consideration for other's perspectives [79,80]. The limit in awareness of emotion impedes the efficiently application of coping strategies inhibiting an adequately expression and communication of emotions, leading to increased risk of suicidal ideation [38].

Higgins and Endler [81] identified the main strategies for coping with psychological distress: (1) task-oriented coping, (2) social-emotional coping, and (3) avoidance-oriented coping [82]. In contrast, social-emotional coping has proven to be a significant risk factor in the development of anxiety and depression and has been linked to poor quality of life. Avoidance-oriented coping seems to create the highest risk of psychological distress. On the contrary, those who apply task-oriented coping over a long period are more likely to report a better quality of life. Recent research on personality traits suggests that alexithymia may influence the coping styles following severe TBI [83]. Krpan et al. [84] found that

individuals with executive dysfunction and difficulty identifying feelings showed a greater use of avoidance coping and loss of empathy [50], in agreement with other studies that have demonstrated alexithymia to be more associated with avoidant than task oriented.

Alexithymia has been associated with dysfunction in the right hemisphere cortex, the corpus callosum, anterior cingulate cortex, front striatal networks, and amygdala [77,85]. A relation between brain–injury and high alexithymia and psychiatric disorders has been shown [45]. [86,87]. This has been confirmed in a number of studies using the 20-item Toronto Alexithymia Scale (TAS-20) [86,87]. The scale is a multi–dimensional self–report inventory constructed to assess the three dimensions of alexithymic traits: (1) difficulty identifying one's feelings (DIF); (2) difficulty describing one's feelings (DDF); and (3) externally orientated thinking (EOT) [79]. The cut off for diagnosis of alexithymia is $\geq$61 [88]. In a recent meta-analysis on the estimation of the prevalence of alexithymia in patients with acquired brain injury, [89] it was found that the magnitude of the effect size of global alexithymia was approximately 25–30%, larger with respect to others meta-analysis on the effect of cognitive ability. Additionally, moderate–to–large effect sizes were reliable through the TAS–20 subscales, i.e., DIF, DDF, and EOT. Moreover, comparing the impact brain lesions on the emotion disorders between patients with TBI with patients with stroke or tumors (i.e., non–TBI), the authors found that patients with a TBI evidenced statistically significantly greater degrees of alexithymia, respect to non–TBI patients. Therefore, the lesions in TBI occur in the brain area responsible for identifying emotions, describing emotions, and externally oriented thinking. Thus, it is important to assess TBI patients for emotional deficits [88].

The risks of alexithymia after TBI are important to identify. It has been shown that one risk is represented by gender. A meta-analysis in the general population reported the same discrepancy, explained by the "normative male hypothesis", as males during childhood are dejected to feel or exhibit or transfer any kind of emotions [89]. A recent study on gender differences in terms of alexithymia in TBI patients did not found dissimilarities between women and men in the TAS score, but the authors found a higher proportion of alexithymia in TBI patients respect to uninjured controls [90]. An association between alexithymia and empathy in the TBI population has been showed [91,92]. Moreover, there has not been shown to be a sex difference in empathy between men and women with TBI. However, 44% of women with TBI fell below emotional empathy norms than men with TBI [93]. Another study showed that men with TBI were more impaired in empathy than women [91]. An inverse relationship between alexithymia and empathy in clinical and nonclinical populations has been shown [94–96].

As a construct, empathy can be divided into emotional and cognitive components [97]. The emotional problems exhibited by many TBI people often leads to relationships failure. Emotional empathy refers to the capacity to feel the emotions of others and has been associated with the insula and inferior frontal gyrus [98]. On the other hand, cognitive empathy refers to the aptitude to understand another's viewpoint, and it has been linked to the ventromedial prefrontal cortex [97–99]. Wood et al. [87] suggested the evaluation of emotional empathy by the Balanced Emotional Empathy Scale (BEES).

**4. Depression, Anxiety and Post-Traumatic Stress Disorder (PTSD)**

Although during their first year after injury, approximately 14–61% of patients with TBI may experience depressed mood, the prevalence of depression, as well as anxiety, reduced progressively over five years, with rates similar to general population. The most common diagnosis was Major Depressive Disorder and Anxiety Disorder NOS (usually PTSD or generalized anxiety disorder) [100]. Recently, the American Psychological Association (APA) has developed a website with the overall assessments and complete guideline treatment of PTSD for a better diagnosis and treatment of the disorder [101].

The events that cause TBI might be severely emotionally traumatic. Even a psychiatric disorder before TBI was a strong predictor. However, most of the studies showed that mood and anxiety disorders were developed de novo [17,90,102]. Symptoms of PTSD are

re-experiencing symptoms, escaping, and impairment of arousal, cognition, and mood. Risk factors of PTSD are TBI, less education, being black, and the kid of injury. It has been shown that mild TBI raised predicted PTSD symptoms by a factor of 1.23, while moderate or severe TBI amplified predicted symptoms by a factor of 1.71 [19,103]. Moreover, the risk of developing panic disorder (odds ratio = 2.01), social phobia (odds ratio = 2.07), and agoraphobia (odds ratio = 1.94) in mild TBI was twice doubling [104]. Anxiety is often related to injury to the mesial temporal lobe and can present as generalized anxiety disorder (GAD), panic disorder, obsessive–compulsive disorder (OCD), posttraumatic stress disorder (PTSD), or different phobias (as per DSM-V) [105,106]. Nonetheless, there is overlap between the cognitive deficits of PTSD and those due to TBI itself, with a rate of 3–23% in more severely affected patients. PTSD is a complex disorder that develops after a traumatic situation [107,108]. This risk is very greater in severe TBI patients [109]. PTSD and TBI are usually linked with neuropsychological impairments of executive functions, attention and working memory. In TBI PTSD patient populations, there has been hypothesized to be an effect on the amygdala and an endocrine malfunction that may underlie a vulnerability to the development of PTSD following subsequent injury and/or stressor exposure [110]. Actually, a clear neurobiological basis for understanding the complexity of PTSD is still unknown. Three theories of pathophysiology are valid: one depends on the negative effect of TBI on neural circuits regulating fear responses [111].

In TBI patients, as well as in the general population, functional neuroimaging studies have shown impaired brain activity in the dorsolateral and ventrolateral prefrontal cortices as well as anterior cingulate, which are known to subtend emotional regulation self-control and [112]. The second is based on the consequently cognitive impairment after TBI that could negatively influenced the coping capacity using adaptive cognitive strategies [113]. Lastly, the inflammatory theory after TBI that might contribute to aggravated mental health outcomes [114]. Also, because of similar symptoms of affective and cognitive deficits in depressive patients without TBI respect to TBI patients, a clear distinction needs to be made determining if the depressed mood is situational and/or reactive to the trauma, primary mood disorder, or secondary to either a structural lesion or a specific medical condition [115]. Patients with major depression or depressive disorder not otherwise specified (NOS) might be treated with antidepressants, such as selective serotonin reuptake inhibitors, sertraline, or citalopram, as a recommended first-line treatment [116]. Even if less studied, selective serotonin-epinephrine reuptake inhibitors have been recommended [117]. On the other hand, due to the effect of lowering the seizure threshold in patients with moderate–severe TBI, tricyclic antidepressants are not suggested [118,119].

Nonpharmacological treatment with psychotherapy has to be proposed as an alternative or adjunctive treatment [7]. Telerehabilitation is a valid and reproducible therapy as effective as in-person therapy [120].

**5. Suicide Attempts and Suicide Ideation**

Patients with TBI have a rate of suicide attempts and suicide ideation (SI) as well as suicide higher than the general population, even after checking for psychiatric problems. The suicide relative risk in TBI severe was 3–4 times higher than the general population whereas SI was found in 21–22% in individuals with TBI, and it has been associated to a higher risk of suicide attempts. Suicide attempts indeed could reach 18% [121]. Findings on the causes of post-injury SI are heavily limited. Some clinicians have suggested that the dysregulation of serotonergic and noradrenergic neural pathways that results from TBI may be implicated in suicidality. No evidence, however, is available to support this hypothesis. Lately, suicidal behavior has been related with decreased brain-derived neurotrophic factor functioning.

A relationship between neuropsychological impairment and SI has been argued as well. Executive dysfunction functioning has been associated with suicidality in non-TBI samples [122]. Patients with depression and a history of attempted suicide or ongoing SI tend to perform significantly worse on measures of executive functioning, problem

solving and mental flexibility, as compared to depressed peoples with no history of suicide attempt/ideation. Depression and history of emotional/psychiatric disturbance are associated with completed and attempted suicide in severe TBI patients. Indeed, mood, personality, and substance abuse disorders are identified as significant risk factors for suicidal behavior in the general population. As would be expected, depression has been identified as the most significant correlate of post-TBI SI, although no data exist demonstrating the amount of variance in SI explained by depression. It has been shown that patients with TBI and depression were six times more likely to report SI compared with people with minimal or borderline depression [123]. Few systematic investigations have explored the relationship between postinjury psychosocial functioning and suicide ideation (SI). Among the others, employment seems to play a role in SI, whereas adverse life events postinjury, social isolation, and relationship breakdown have been shown not to be related to the presence of postinjury suicide plans.

## 6. Discussion and Clinical Advice

TBI represents a main cause of death and disability worldwide. Indeed, many severe TBI survivors present with long-term physical, neuropsychiatric, and cognitive disorders with a need of long-term rehabilitation [124]. Notably, some TBI patients may recover motor function, but neuropsychological and behavioral problems (the so-called "invisible disability") persist months or years after the brain injury [125]. Given that NPS may affect every aspect of an individual's life, an adequate assessment of TBI must include the investigation of cognitive (including memory and executive function abnormalities) and neuropsychiatric problems (such as anxiety, depression, agitation, and substance abuse) [125]. Nonetheless, only a few studies have dealt with the effect of NPS on patients' and caregivers' social and occupational reintegration [126].

Personality changes, intolerance to frustration, anxiety, depressive mood, anger, or aggression negatively affects both patient and family quality of life [127]. For a better management of this disabling problem, the first recommendation concerns the need for all healthcare professionals to use common definitions and nomenclature for neuropsychiatric and behavioral abnormalities. When consensus lacks, the disorders should be classified in "disruptive primary behaviors, affective disorders, anxiety-psychosis and suicide attempts/suicide". Older and more recent psychiatric guidelines, i.e., ICD-10 and DSM-V, report limits in the description and classification of TBI-related disorders. However, the psychiatric aspects of TBI have expanded and improved through the last several editions of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM) [34,127], encouraging clinicians to consider risk factors that may modify TBI outcomes and treatment [128–130].

Secondly, the use of specific scales tailored to the patients with TBI is fundamental for an accurate assessment of these frail individuals. This is why we have pointed out the most commonly used scales to investigate these still often disregarded post-TBI problems (Table 3).

**Table 3.** Behavior and personality assessment in TBI.

| Cognitive Domain | Authors | Test | Short Description of Test | Time to Administer |
|---|---|---|---|---|
| Personality Emotional Conditions Social cognition | Schroeder et al. (2019) [131] | Minnesota Multiphasic Personality Inventory-2 (MMPI-2) | The Minnesota Multiphasic Personality Inventory (MMPI) is a psychological instrument used to identify signs of psychopathology. The MMPI-2 contains around 567 items. | 60–90 min |

**Table 3.** *Cont.*

| Cognitive Domain | Authors | Test | Short Description of Test | Time to Administer |
|---|---|---|---|---|
| | Schroeder et al. (2019) [131] | Personality Assessment Inventory (PAI) | The Personality Assessment Inventory (PAI) is a self-report measure of personality and psychopathology consisting of four Validity, eleven clinical, five Treatment Consideration, and two Interpersonal scales, as well as conceptually derived subscales and risk indexes | Requires 50–60 min |
| | Westerhof-Evers et al. (2014) [132] | The Awareness of Social Inference Test (TASIT) | The The Awareness of Social Inference Test (TASIT) is used to evaluate the patients' ability to understand emotional states, thoughts, intentions and conversational meaning in everyday exchanges. | 1 h |
| | Gorgoraptis et al. (2019) [133] | Beck Depression Inventory–Second Edition (BDI-II) | The Beck Depression Inventory (BDI-II) is a 21-item self-report tool assessing the severity of depression symptoms. | 10–15 min |
| | Bertisch et al. (2013) [134] | Beck Anxiety Inventory (BAI) | The Beck Anxiety Inventory (BAI) is a brief, self report assessment for anxiety. | 5–10 min |
| | Quaranta et al. (2008) [135] | Poststroke Depression Rating Scale (PSDRS) | The Poststroke Depression Rating Scale (PSDRS) is a clinical tool specifically devised to assess depression after stroke. | 10 min |
| | Benaim et al. (2004) [136] | Aphasic Depression Rating Scale (ADRS) | The Aphasic Depression Rating Scale (ADRS) was developed to detect and measure depression in aphasic patients during the subacute stage of stroke. | 8 min |
| | Kieffer-Kristensen and Teasdale (2011) [137] | Symptom checklist -90 R questions (SCL-90 R) | The Symptom checklist -90 R questions (SCL-90-R) is a largely-used questionnaire for self-report of psychological distress and multiple aspects of psychopathology, as part of the evaluation of chronic pain patients | It takes 15–20 min to administer |
| | Diaz et al. (2012) [17] | Brief psychiatric rating scale (BPRS) | The Brief Psychiatric Rating Scale (BPRS) is one of several tools used to evaluate patients with schizophrenia and related psychotic disorders. They measure it to track changes in symptoms over time. | 15 min |
| | Malec et al. (2018) [138] | Neuropsychiatric Inventory (NPI) | The Neuropsychiatric Inventory (NPI) is a clinical interview with a family member or friend who knows the patient well and can evaluate 12 behavioral areas commonly affected in patients with dementia. | Taking roughly 20 min |

The assessment of personality and psychopathology following TBI is an important aspect of clinical care and rehabilitation. Although the most overt symptoms of moderate-to-severe TBI are cognitive and physical disorders, personality and psychiatric disturbances are important as they persist even after cognitive and physical symptoms resolve [139]. The

use of a comprehensive and multi-modal approach is then fundamental in the evaluation of behavioral symptoms and personality disorders in severe TBI patients. Among the others, the Personality Assessment Inventory (PAI) is a self-report measure of personality and psychopathology that has demonstrated good convergent validity for a small subset of items among TBI persons [140].

The Neuropsychiatric Inventory (NPI) is a clinical scale that evaluates psychiatric symptoms in patients with neurological disorders and severe traumatic brain injury (TBI) patients to explore neurobehavioral impairments.

Third, to reduce and better manage the psychological and behavioral consequences of TBI, clinicians should be aware of the available pharmacological and psychotherapeutic approaches.

Two important reviews have concluded that there is limited evidence for effective pharmacological treatment of aggression after TBI, lacking in available treatment guidelines [141,142]. However, both reviews showed the potential utility of beta blockers to decrease the intensity of agitated episodes and the frequency of assault attempts, although there is often a need for high doses to achieve the clinical outcomes with risk of hypotension and bradycardia. The use of SSRI antidepressants could be of help in the treatment of impulsive and episodic aggression [143]. Although neuroleptics, such as chlorpromazine and haloperidol, can cause severe sedation, they can be used to control aggression. Atypical antipsychotics, such as quetiapine, are believed to be less harmful [143].

Cognitive behavioral therapy (CBT) has been successfully employed to manage behavioral and neuropsychiatric symptoms, such as impulsive aggression in a variety of settings [144]. In clinical settings, CBT interventions have also demonstrated efficacy in treating intermittent explosive disorder, leading to a reduction in aggressive acts, hostile thinking, and associated depressive symptoms, by adaptive self-regulating behaviors or simply avoidance behaviors that will minimize the risk of violence [145]. Some authors [58,146] have presented guidelines on how to apply CBT methods in the management of aggression after TBI but, so far, no clinical research has been published to examine the effectiveness of CBT in the treatment of these conditions. In contrast, there is a considerable body of literature on behavior management techniques, especially in the clinical management of irritability, disinhibition, and impulsive aggression [58]. By now, the psychological methods that have been shown useful in a variety of rehabilitation and community settings, are those that utilize operant learning theory, often combined with cognitive methods such as verbal mediation [58]. Another efficacious therapy is the replacement strategy, which includes assertiveness training (intended for patients who become angry when they fail to get their needs met) and differential reinforcement scheduling (to decrease the rate of pre violent behaviors) [147]. Effective treatment of behavioral symptoms and posttraumatic aggression focuses on a multidisciplinary approach involving both non-pharmacologic and pharmacologic interventions. Patients diagnosed with disorders who present with mood lability might be treated with mood stabilizers over 95% of the time. Because of their efficacy in the treatment of agitation and mood lability, mood stabilizers might also be effective in the treatment of personality change or cognitive dysfunction, such as impulsivity and distractibility [148].

## 7. Conclusions

Behavioral dyscontrol, neuropsychiatric and emotional symptoms are relatively common and disabling consequences of TBI, especially in the case of severe injury. To better understand the phenomenology and epidemiology and effectively manage these problems, a multidisciplinary approach is required. Valid and reliable symptom-specific and multidimensional neuropsychological metrics are useful to assess these disorders. Emotional, neurocognitive, and BD frequently co-occur with other posttraumatic neuropsychiatric disturbances, and this should be considered when dealing with these frail patients and their caregivers. Moreover, a symptom-specific treatment characterized by a combination of non-pharmacologic, i.e., psychological, behavioral, and environmental, and pharmacologic approaches is usually required. When properly administered, these interventions may

provide severe TBI patients and their families with substantial relief from posttraumatic emotional and behavioral impairments.

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*Review*

# Neuropsychological Assessment in Patients with Traumatic Brain Injury: A Comprehensive Review with Clinical Recommendations

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**Abstract:** Traumatic brain injury is damage to the brain occurring after birth, often resulting in the deterioration of cognitive, behavioural, and emotional functions. Neuropsychological evaluation can assist clinicians to better assess the patient's clinical condition, reach differential diagnoses, and develop interventional strategies. However, considering the multiple rating scales available, it is not easy to establish which tool is most suitable for the different brain injury conditions. The aim of this review is to investigate and describe the most used neurocognitive assessment tools in patients with traumatic brain injury to provide clinicians with clear indications on their use in clinical practice. Indeed, during the acute phase, after the head trauma, alertness and wakefulness of the patients affected by a disorder of consciousness can be assessed using different scales, such as the Coma Recovery Scale-Revised. In both postacute and chronic phases after traumatic brain injury, general cognitive assessment tools (such as the Mini Mental State Examination) or more specific cognitive tests (e.g., Wisconsin Card Sorting Test and Trail Making Test) could be administered according to the patient's functional status. In this way, clinicians may be aware of the patient's neuropsychological and cognitive level, so they can guarantee a personalized and tailored rehabilitation approach in this frail patient population.

**Keywords:** neuropsychology; traumatic brain injury; disorder of consciousness; cognitive assessment

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## 1. Introduction

Traumatic brain injury (TBI) is an acquired insult to the brain from an external mechanical force that may result in a temporary or permanent impairment of motor and cognitive functions [1]. It represents the leading cause of mortality and disability among young individuals in western countries [2]. TBI is classified as mild, moderate, or severe depending on the level of consciousness and the duration of coma and post-traumatic amnesia (PTA) [3]. TBI is considered as severe (sTBI) when it causes a condition of coma protracted over time (GCS $\leq$ 8 lasting more than 24 h). Following sTBI, patients often show long-term alterations in their state of consciousness. Briefly, the state of coma is the condition that occurs after a head injury or from the temporary absence of oxygen to the brain, where the patient lacks any residual consciousness. From this state, it is possible to achieve a nearly complete recovery or to evolve into a disorder of consciousness (DOC), like a vegetative state (more recently named unresponsive wakefulness syndrome, UWS), in which the patient regains a sleep–wake cycle although he or she is not aware of him/herself or of the surrounding environment [4]. Sometimes, such patients can partially reacquire a state of awareness and this state is called a Minimally Conscious State (MCS).

The awakening process, with an improvement in awareness and consciousness, is related to the location and extent of the brain lesion [5]. Notably, within the UWS group, the traumatic causes have a better prognosis than nontraumatic ones, given that recent studies show long-term recovery over a year after injury [6]. On the contrary, cognitive recovery tends to be rapid in patients with mild to moderate TBI [7,8]. Even after the recovery of consciousness, the main dysfunctions following TBI are (i) sensory–motor impairments, (ii) cognitive deficits involving attentional processes, executive functions, memory abilities, reasoning and problem-solving, and linguistic and visual–spatial cognition; and (iii) behavioural alterations, such as apathy, irritability, aggression, disinhibition, inertia, and mood disorders [9,10]. These symptoms are also frequently associated with anosognosia [11] and alexithymia, negatively affecting emotional status and awareness of the condition [12].

A careful and constant clinical observation, including a cognitive assessment, is critical in guiding clinicians in the assessment of the level of consciousness (UWS vs. MCS), as well as in determining the prognosis and the likelihood to recover cognitive abilities [13]. In particular, in the acute phase of sTBI, only observational scales, such as the Glasgow coma scale (GCS), the Disability Rating Scale (DRS), the Coma Recovery Scale-Revised (CRS-R), and Level of Cognitive Functioning (LCF), can be administered, since the patient can only be subjected to very simple requests or observed in spontaneous behaviours. Further neurophysiological investigation of the specific cognitive domains may be carried out only if the patient passes the state of MCS, as it needs the active participation of the subject [14]. Neuropsychological assessment in TBI patients requires a comprehensive approach to investigate the main cognitive abilities, psychiatric symptoms [15], as well as psychological factors [16]. This issue has already been investigated in previous reviews. In 2010, Podell et al. [17] described both cognitive and psychiatric symptoms of TBI (mild, moderate, and severe) globally, reporting also the neuropsychological and psychiatric evaluation tools. In a similar way, Soble et al. [18] reported the neuropsychological assessment routinely used in clinical settings in TBI patients, but without specifying the severity level of brain injury. Despite the variety in the literature in this field, research into sTBI and its neuropsychological assessment is still poor. In fact, our aim was to investigate and update the most used neuropsychological tools for cognitive assessment in patients with moderate-to-severe TBI, including those with DOC, to provide clinicians with indications on their use in current clinical practice.

In detail, the major contributions of our research consists of

- Individuating the most-used assessment tools for neuropsychological impairments after severe TBI as well as the screening tools for early cognitive evaluation during the acute phase, when the level of consciousness is altered.
- Highlighting the importance of assessment in clinical practice to achieve the most personalized and tailored rehabilitation intervention, both conventional and/or advanced.
- Providing a rationale for clinical advice about the choice of neuropsychological tools and the makeup of the clinical setting, for those who are new to this topic, based on the latest literature.

## 2. Materials and Methods

*Search Strategy and Study Selection*

Our scoping review followed the acronym of PCC (population/problem, concept, context). We considered adult patients affected by moderate and severe TBI as the population/problem, the concept was the use of neuropsychological assessment tools, while the context was the neurorehabilitation setting. The studies included in our review were obtained through a search on PubMed/Medline, Scopus, Cochrane Library, and Web of Science databases. We considered the period from 2000 to 2022, and we used the following word combinations: "cognitive evaluation" AND "severe brain injury" OR "severe traumatic brain injury" OR "neuropsychological evaluation in traumatic brain injury"

OR "traumatic brain injury" OR "neurocognitive evaluation" OR "assessment scales". To obtain a complete search, we also analysed the references of the selected articles. Two reviewers (WT and MT) screened 300 studies, according to title, abstracts, and text; among these, 166 papers were initially selected and eventually 146 were included as they met the inclusion criteria (Figure 1).

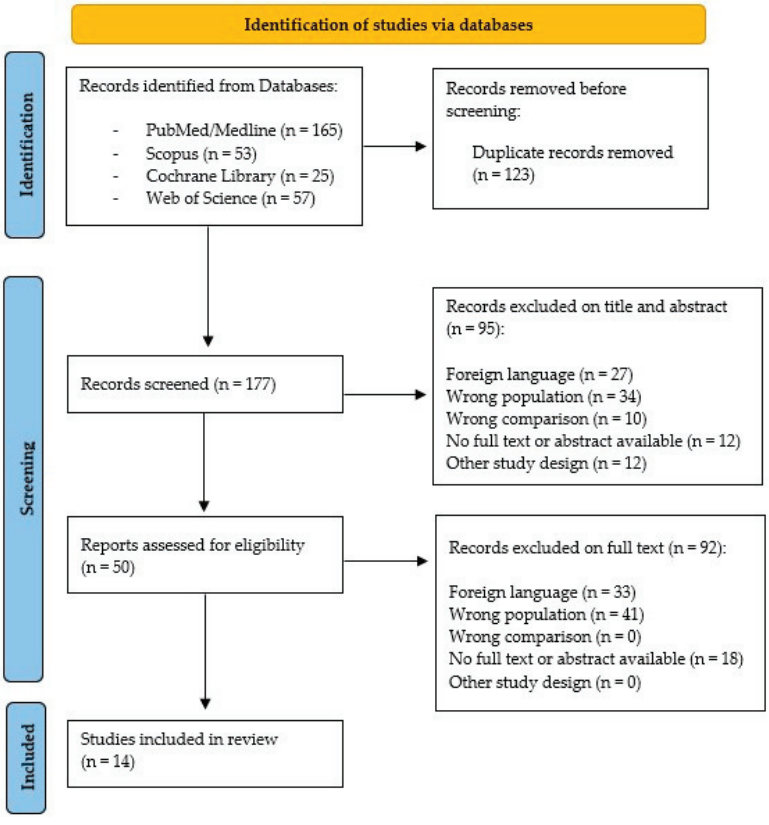


**Figure 1.** PRISMA flow-chart for the study selection.

Inclusion criteria were (i) adult patients (age $\geq$ 18), (ii) patients with severe TBI, (iii) other severe brain damages that may cause a state of coma for at least 24 h, and (iv) a GCS score between 3 and 8. The exclusion criteria were (i) individuals with mild to moderate stroke and TBI, (ii) patients with previous psychiatric disorders (depression, anxiety, psychosis, delirium, and (iii) previous neuropsychological impairments.

## 3. Results

Our review included articles dealing with the neurocognitive rating scales most used by clinicians and researchers, according to the cognitive domain to investigate and the level of disability (Tables 1–3). In fact, it is necessary to distinguish between scales administered in acute/postacute and chronic phases as well as in patients with moderate-to-severe disability.

**Table 1.** Memory assessment in TBI patients.

| Reference n° | Sample Size | Assessment Description | Major Findings |
|---|---|---|---|
| | | Learning memory | |
| [19] | 37 patients with severe TBI | The Rey Auditory Verbal Learning Test (RAVLT) is used to measure delayed recall and recognition memory, while the Rey–Osterrieth Complex Figure Test (ROCF) is a neuropsychological assessment tool commonly used to measure the visuo-constructional and visual memory abilities of neuropsychiatric disorders, including copying and recall tests. By drawing the complex figure, the dysfunctional decline of an individual in multiple cognitive dimensions can be evaluated, such as attention and concentration, fine-motor coordination, visuospatial perception, nonverbal memory, planning and organization, and spatial orientation. | ROCF Recognition Hits and MEP displayed at least acceptable discriminant strength with 35% sensitivity and at least 90% specificity. |
| | | Visual memory | |
| [20] | 100 patients with TBI ($n$ = 35 complicated mild or moderate TBI; $n$ = 65 severe TBI) | The Wechsler Memory Scale (WMS-IV) is a neuropsychological test built to assess different memory functions. The last version is the fourth edition (WMS-IV) which was published in 2009 and which was designed to be used with the WAIS-IV. Performance is reported as five index scores: Auditory Memory, Visual Memory, Visual Working Memory, Immediate Memory, and Delayed Memory. | WMS- IV also showed good sensitivity and specificity for classifying individuals with severe TBI versus controls, but not for classifying individuals with memory impairments relative to those without (there was adequate specificity but poor sensitivity). |
| | | Short-term memory/working memory | |
| [21] | 64 patients with moderate-to-severe TBI (TBI) | The Digit Span Forward/Backward (DSF/DSB) is a measure of verbal short-term and working memory that can be used in two formats, DSF and DSB. It is a verbal task, with stimuli presented auditorily, and responses spoken by the patients. | TBI patients are more likely to perseverate on prior instructions during DS sequencing. |
| [22] | 30 patients with severe chronic TBI | Digit Span (see description above) | Severe TBI patients are associated with an impairment of executive aspects of working memory. The anatomic substrate of this impairment remains to be elucidated. It might be related to a defective activation of a distributed network, including the dorsolateral prefrontal cortex. |

Legend: RAVLT (Rey Auditory Verbal Learning Test), ROCF (Rey–Osterrieth Complex Figure Test), WMS-IV (Wechsler Memory Scale), DSF (Digit Span Forward), DSB (Digit Span Backward).

**Table 2.** Assessment of attention and executive functions in TBI.

| Reference n° | Sample Size | Assessment Description | Major Finding |
|---|---|---|---|
| | | Attention | |
| [23] | 30 moderate-to-severe TBI patients | The continuous performance test (CPT) is a valuable way to measure different aspects of attention. During a CPT, the user is instructed to respond only when the target stimulus is presented and to withhold responses to other stimuli. Stimuli may be visual, auditory, or both simultaneously. | The CPT-II has validity for use as an attentional measure among patients with TBI. |
| [24] | Twenty-five patients with complicated mild-to-severe BI | The Symbol Digit Modalities Test (SDMT) is a screening tool usually used in clinical and research settings to evaluate neurological dysfunction. Like other substitution tasks, performance on the SDMT is underpinned by attention, perceptual speed, motor speed, and visual scanning. | The present data strongly support the pervasive influence of reduced speed of information processing on attentional performance after TBI. |
| | | Executive functions | |
| [25] | 29 severe TBI patients (9 females and 20 males) | The Wisconsin Card Sorting Test (WCST) is a neuropsychological test that is usually used to assess such higher-level cognitive processes as attention, perseverance, working memory, abstract thinking, and set shifting. The Delis–Kaplan Executive Function System (D- KEFS) is a neuropsychological clinical battery used to assess high-level cognitive functions such as verbal and nonverbal executive domains in nine different areas. | Inter-rater reliability showed the translation to be reliable and effective. The D-KEFS ST can effectively distinguish TBI patients from control subjects, with the TBI group consistently demonstrating difficulties in category/concept formation and in flexibility of thought. |
| [26] | 176 patients with TBI | WCST (see the description above) | Results suggested a dose–response relationship between TBI severity and deficits on the WCST in patients providing good effort during testing. |
| [27] | NA | WCST (see description above) | Although there are shorter and/or automated variations, the classic WCST with 128 cards is still the most popular. The WCST is a helpful tool for clinical and research applications; however, it is usual practice to report only one or a small number of potential values, preventing further accurate comparisons between studies. |
| [28] | 56 patients with complicated mild-to-severe TBI | The Tower of London (TOL) task has been used largely as a test of planning ability in neuropsychological patients. Patients are asked to preplan mentally a sequence of moves to match a start set of discs to a goal, and then to execute the moves one by one. | Poor sensitivity of this measure limits its use in isolation; the TOLDX may provide a complementary measurement of aspects of problem-solving deficit in TBI that may not be captured by other tests. |
| [29] | Sixty-three patients (and relatives) were included within 63.4 months ($\pm$20.7) after sTBI | The dysexecutive questionnaire (DEX) was designed to assess different domains of executive functioning in daily life. | Executive function, episodic memory, attention (phasic alert sustained and divided attention), the GOSE, and the volume of the corpus callosum (an MRI marker) were all substantially linked with DEX-O. The mean diffusivity measurement was associated with the anosognosia score (DEX-O minus DEX-S). These findings demonstrate DEX-O's clinical use in determining long-term impairment. |

Legend: (CPT) continuous performance test, (SDMT) Symbol Digit Modalities Test, (WCST) Wisconsin Card Sorting Test, (D-KEFS) Delis–Kaplan Executive Function System, (TOL) Tower of London, (DEX) Dysexecutive questionnaire.

**Table 3.** Assessment of language/speech in TBI patients.

| Reference n° | Sample Size | Assessment Description | Major Findings |
|---|---|---|---|
| | | Language–Speech | |
| [30] | 169 TBI patients | The Western Aphasia Battery-Revised (WAB-R) measures linguistic skills most frequently affected by aphasia, plus key nonlinguistic skills, and provides differential diagnosis information. Flexible to various administration settings from hospital room to clinic, it provides a baseline level of performance to measure change over time. | WAB-R alone is insufficient to detect or fully characterize aphasia and the motor speech deficits that may accompany speech impairment; it should be considered for use only as one component of a larger communication assessment battery. |
| [31] | Twenty patients with moderate-to-severe TBI | The Neuropsychological Assessment Battery (NAB) is a comprehensive test battery that assesses five cognitive domains (Attention, Language, Memory, Spatial, and Executive Functions). | NAB demonstrated by the current sample is consistent with the neuropsychological profile observed in postacute patients with moderate-to-severe TBI without focal deficits (e.g., aphasia), demonstrating its relative sensitivity in this patient population. |
| [32] | 355 TBI patients with aphasia | A well-known test battery that looks at several aspects of language functioning is the BDAE-3 version. The BDAE-3 is a more thorough aphasia battery as compared to other aphasia batteries since it includes more than 50 subtests and may be interpreted using the Boston Process Approach [33,34]. BDAE-3 offers strong validity and reliability for assessing patients with severe TBI. | Across different degrees of aphasia, the BDAE-3 shows high construct validity, and specific language functions (such as receptive vs. expressive language) continue to be important, separate language domains. |

Legend: The Western Aphasia Battery-Revised (WAB-R), The Neuropsychological Assessment Battery (NAB), Boston Diagnostic Aphasia Examination (BDAE).

We divided our results into two main sections. The first, Section 3.1, deals with the screening tools used to test global cognition in the acute (like CSR-R or LCF) and postacute phase (like Mini Mental State Examination—MMSE and Montrel Cognitive Assessment—MoCA). In this section we reported five studies [35–39]. The second, Section 3.2, reports the neuropsychological assessment investigating specific cognitive domains. Notably, in this section we found (i) four articles related to the assessment of memory functions [19–22]; (ii) two articles assessing attention functions [23,24]; (iii) five articles that evaluated executive functions [26–29]; (iv) three articles dealing with social cognition [40–42]; (v) three studies about the evaluation of language disorders [30–32]; and (vi) only one study [43] assessing limb apraxia.

### *3.1. Global Cognitive Assessment*

During the acute period after sTBI, there is often a great deal of uncertainty regarding the extent of cognitive and physical recovery as well as the long-term functional outcome. In this condition, only a few observational scales can be administered, and these are based on the evaluation of the patient's spontaneous behaviours (eye opening, reflex responses, visual pursuit) or response to simple commands. For instance, by using the CRS-R, the examiner obtains a score that allows them to classify the patient within two main categories: VS/UWS or MCS (plus or minus). Conversely, using the LCF, it is possible to frame the patients in one of eight levels, from unconsciousness to normal consciousness. During the acute period, the most reliable prognostic clinical indicators are vegetative state duration,

the duration of post-traumatic amnesia, and the CRS-R score [44–46]. The score of CRS-R reaches a sensitivity of 93% and a specificity of 96%. In fact, the CSR-R allows patients with DOC to be accurately differentiated from those that do not manifest alterations in conscious awareness. Additionally, clinicians should adopt a cut-off score of 8 to perform an appropriate differential diagnosis, since it offers the best odds of concurrently avoiding false-positive and -negative errors. However, the most accurate diagnosis is performed when the full subscale profile is available [35].

The Galveston Orientation and Amnesia Test (GOAT) investigates the presence of post-traumatic amnesia (PTA) following an sTBI. Some studies have identified the GOAT scores as a good predictor of long-term outcomes [36,37]. Specifically, when sTBI patients recover from an agitation state, clinicians should consider the use of screening tools to investigate cognitive functions globally with the administration of the MMSE and the MoCA, which are the most widely used means to this end.

Generally, these global assessment tests allow complete and fast administration, maximizing patients' cooperation and reducing fatigue effects. Tay et al. [38] showed that MoCA has good reliability in both the inpatient and outpatient settings after TBI. It can be considered a valid tool to determine cognitive impairments in postacute traumatic events. It seems that MoCA presents both a sensitivity and specificity, respectively, of 79.4% and 74.1%. However, it still lacks sensitivity for estimating mild cognitive impairments. The Repeatable Battery for the Assessment of Neuropsychological Status (RBANS) has proven a very useful tool for screening a variety of cognitive domains in a relatively brief amount of time, and the current results suggest that the RBANS has a good sensitivity and specificity in assessing the neurobehavioural sequelae in TBI patients [39].

### *3.2. Specific Cognitive Domains in Neuropsychological Assessment*

The different cognitive domains may be investigated using a wide range of psychodiagnostics tools that the neuropsychologist can choose based on the clinical picture and the patient's needs [47]. Neuropsychological evaluation in TBI patients is of extreme importance, since, in addition to motor symptoms, cognitive deficits are also very disabling [48]. Most of the instruments listed below require the active participation of the subject, so they are suggested in those TBI patients who have recovered consciousness.

#### 3.2.1. Evaluation of Memory Deficits

TBI patients present a wide range of memory impairments, including working, prospective, semantic, and episodic as well as the encoding, storage, and retrieval of words. For these reasons, it is useful to consider an assessment battery that includes all subtypes of memory or to choose the appropriate tests for the domain that is most severely impaired in the patient.

In detail, Ashendorf et al. [19] administered the Rey–Osterrieth Auditory Verbal Learning Test (RAVLT), revealing its usefulness in assessing learning memory, by means of consolidation of new verbal information. Otherwise, the Rey–Osterrieth Complex Figure Test (RCFT) provides an assessment of visuospatial memory, associated with praxis skills. Carlozzi et al. [20] performed a memory evaluation in subjects with TBI (mild, moderate, and severe) using the Wechsler Memory Scale (WMS-IV), showing great capacities in distinguishing sTBI subjects from controls. On the other side, other authors [21,22] used the Digit Span to assess memory skills in TBI patients, finding a strong correlation between reduced working memory function and executive dysfunction (see Table 1).

#### 3.2.2. Assessment of Attention Deficits

Attention is the behavioural and cognitive process of selectively concentrating on a discrete stimulus while ignoring other perceivable stimuli [49]. Attention components include divided (carrying out several tasks at the same time), selective (ability to focus on a single stimulus in the presence of distractors), and sustained (ability to remain focused on a stimulus for a long period of time), which are often associated with mental slowness [50].

Attention deficits (ADs) are one of the most common cognitive impairments after a brain injury. The prevalence of ADs in the acute phase after sTBI ranges between 45% and 88%, while in the postacute phase, i.e., at discharge from hospital, the prevalence of these complaints is between 25% and 51% [49]. Patients with ADs often present with diminished concentration, distractibility, reduced error control, mental slowness, and fatigability. In addition, an AD is often associated with memory and language impairments [50]. Jourdan et al. [23] found that ADs were reported by patients at a 4 year-follow-up after brain injury and included mental slowness and concentration difficulties (56.7% of the patients) as well as dual-task difficulties (51.7%). Different tests may be used in the assessment of attentional functions in sTBI patients (see Table 2).

Neuropsychological tools that can detect ADs in this patient population include the continuous performance test (CPT), which measures different aspects of attention, although focusing on selective attention processes, and the Symbol Digit Modalities Test (SDMT), an instrument evaluating attention, perceptual speed, motor speed, and visual scanning [23,24]. To differentiate the components of attention, [51] the Paced Auditory Serial Addition Task (PASAT) can also be used, which is a neuropsychological test measuring auditory information, processing speed and flexibility, as well as calculation ability. Indeed, mental slowness is significantly correlated with injury severity and task complexity. Although it is a common opinion that mental slowness affects test scores, this is not particularly true in TBI patients, as shown by some studies [52]. According to the literature, sustained, divided, and selective attention in sTBI patients could be assessed with the popular Stroop test, as confirmed by some authors [53]. In fact, Stroop interference was significantly greater in TBI groups and scores were influenced by mental slowness and changes in sensory-speed processing. Ben-David et al. [53] reported that the presence of specific impairments in attentional functioning, such as divided attention, could depend on the complexity of the task.

#### 3.2.3. Evaluation of Executive Dysfunctions

The frontal lobes are known to play a key role in cognition, with regards to executive functions, i.e., the ability to engage in intentional, planned, and goal-directed behaviour. Executive dysfunction (ED) following sTBI is a well-documented problem, and it is often the most disabling aspect [54]. Some authors [55] compared the sensitivity of conventional tests for executive functions with a new one, called the Behavioral Dysexecutive Syndrome Inventory (BDSI). They found that 87% of patients showed ED, but 49% also presented a combination of behavioural and cognitive impairments. sTBI patients with ED also showed greater difficulties in community reintegration post injury, because of their decreased socialization, vocational loss, and increased family burden [56]. In particular, patients following sTBI frequently lost the ability to sort items into a specific category as demonstrated using the Wisconsin Card Sorting Test (WCST) [25–27].

Another tool frequently used in these patients is the Tower of London, which targets planning abilities [28]. During the assessment, clinicians should also consider "inhibition" (that is, the ability to control interference stimulus), which could be evaluated with the Stroop test [57]. Other authors investigated the role of using the Dysexecutive Questionnaire (DEX) in sTBI patients, finding that the DEX score was strongly correlated with cognitive alterations, mood disorders, and the ability to carry out simple activities of daily living [29] (see Table 2).

#### 3.2.4. Social Cognition and TBI Assessment

Social cognition refers to the ability to figure out people's behaviour and to respond appropriately according to social situations [58,59]. Some authors found that sTBI patients tend to manifest alterations in recognizing facial expressions and emotions. However, the relationship between social cognition and neurocognitive deficits is still under debate [60].

The most used tests for the evaluation of social cognition in TBI patients are The Awareness of Social Inference Test (TASIT) [40] and The Social Decision-Making Task

(SDMT) [41]. The TASIT is an audio–visual tool designed for the clinical assessment of social perception with alternate forms for retesting, and it is used to evaluate the ability of patients to understand emotional states, thoughts, intentions, and conversational meaning in everyday exchanges [42]. Notably, the TASIT in sTBI should be administered with caution because this patient population shows deficits in working memory, learning new tasks, and ED [40]. In this context, memory tasks related to social information, like remembering faces or stories, were considered more related to social perception. Otherwise, the SDMT offers a new way of examining decision making within a social context, especially following TBI. Kelly et al. [41] assessed patients affected by sTBI, comparing them with healthy controls using the Iowa Gambling Task (IGT) and a battery of neuropsychological tests and social cognition tasks. To summarize, the SDMT was found to be more sensitive in distinguishing people with TBI and healthy controls. However, performances on SDMT and IGT are not associated since they measure different constructs [61].

#### 3.2.5. Language Impairments and Assessment

Commonly, the most used assessment tools for language impairment in TBI patients include the Western Aphasia Battery-Revised (WAB-R) [30], the Aachener Aphasia Test (AAT) [62], the Boston Diagnostic Aphasia Examination (BDAE) [32], and the Boston Naming Test (BNT) [31]. In specific situations, clinicians can administer the BDAE test, which is considered the most representative to tailor assessment process and reduce testing time (especially in patients with TBI that often have AD), while preserving diagnostic sensitivity [32]. Rather than aphasia, about 80% of individuals with TBI have disorders in the interactional use of language and at discourse level [62]. These impairments are subsumed under the term "Cognitive Communication Disorders (CCD) [63] that demonstrates how "discourses are more than just a string of sentences", with verbal macrostructures that are realized as either monologues or interactive dialogues. Research on discourse production in TBI has shown that working memory, attention, and ED are crucial for the construction of coherent verbal macrostructures [64,65].

A proper assessment of post-sTBI language disorders should therefore not exclude an analysis of discourse and narrative skills, and should evaluate macro-components (quantity of concepts expressed) and micro-components (total number of words, density, words per minute) [66]. A tool that provides an accurate investigation of this aspect is the "Protocole Montreal d'évaluation de la communication" (MEC) consisting of a 10 min conversation on two different topics with the examiner that is then rated on a 17-point checklist [67]. However, it is important for a clinician to carefully choose the tool to be adopted according to the severity and evolution of the TBI. It is also important to evaluate the long-term outcomes since communication disorders also have a negative impact on social relationships [63]. The most used tools to investigate language functions in TBI patients are shown in Table 3.

#### 3.2.6. Visuospatial and Praxis Assessment

People with TBI can present limb apraxia, which is a disorder in performing purposeful skill movements. The evaluation of these specific apraxia components has not been fully investigated, since the literature about the psychometric issues in testing limb apraxia is lacking. However, some authors identified some scales that can be useful in assessing apraxia in TBI patients, like the Western Aphasia Battery, the Boston Diagnostic Aphasia Exam, the Apraxia Battery for Adults-II (ABA), and the Florida Apraxia Screening Test-Revised (FAST-R) of the Florida Apraxia Battery (FAB) [43]. It should be considered that sTBI patients with limb apraxia are more likely to show concomitant cognitive dysfunctions in multiple domains, such as language, planning, imagery, inhibition of distraction, declarative memory, working memory, initiation, and overcoming fatigue [43]. This issue should be considered when dealing with apraxia assessment in sTBI.

## 4. Discussion

Neuropsychological assessment is a useful process able to detect and monitor global neuropsychological deficits or impairments in specific cognitive domains, especially in sTBI patients where cognitive dysfunctions are present in almost all affected subjects [68]. The novelty of this review consists of having comprehensively introduced a wide set of test batteries that evaluate and fully explore the patient's functioning from the state of coma to the possible functional recovery following sTBI. In fact, the severity of the injury is decisive for the development of cognitive sequelae, as confirmed by Tsai et al. [69], who highlighted a prevalence of 40 to 60% of memory and attention deficits, in which 1–3 still had memory impairments years after the brain injury, and they may depend on coma length. In addition, it was found [70,71] that TBI patients are more likely to develop verbal or visual memory tasks, especially in the retrieval of both semantic and autobiographical information. As regards working memory, it has been demonstrated that task accuracy is poorer in subjects with TBI [72]. According to Dunning et al. [73], patients affected by TBI manifested significant deficits in verbal short-term memory and visuospatial and verbal working memory. On the other hand, Mioni et al. [74] assessed memory functions in sTBI patients, showing that their performances were related to executive functions. Several studies support the idea that cognitive impairment affects long-term functional status after TBI [33]. Notably, adequate assessment in TBI patients allows the implementation of their care path and establishes specific therapeutic and rehabilitative programs [16]. Indeed, sTBI patients may recover gross motor functions although they still maintain neuropsychological/behavioural problems months or years after the brain injury, affecting social and family relationships. Lesions of the frontal lobes seem to cause the most severe form of cognitive deficit, as these involve executive function. Executive dysfunction represents the most disabling cognitive deficit in patients with TBI, since it affects self-regulation, planning skills, and affective lability. In this context, clinicians should consider the differential diagnosis between ED and limb apraxia. It is not uncommon that motor and executive components often overlap with spatial aspects in construction tasks [34]. In fact, visuospatial assessment usually does not involve directly executive components, such as in the ROFC, in which the patient must copy a complex project and evaluate the respondent's ability to use signals to retrieve information [19]. Benton's spatial orientation test (BSOT) does not have a strong executive control component, like the Benton visual retention test (BVRT) in which the patient is briefly shown cards containing two or three geometric designs and then asked to reproduce them by heart. This test evaluates visual perception, short-term visual memory, and visuo-constructive ability [75]. On the other hand, other tests, i.e., the clock drawing test (CDT) [76] and the judgement of line guidance (JOL) [77], have a strong visuospatial and executive control component. Although these tests have been tailored to patients with neurodegenerative diseases, they could probably also be used in TBI patients.

What is more, language disorders and aphasia evaluation should be addressed by clinicians, since the incidence is quite frequent, ranging from 2% to 32% [78,79], and involves difficulties in naming and word-finding, sentence comprehension, or sentence production [79]. For this reason, in many cases, the use of aphasia batteries, which evaluate vocabulary and grammatical abilities at the single-word and sentence levels, may not be sensitive to the evaluation of these impairments [30,32,62].

Lastly, the importance of the method of test administration should not be underestimated. Face-to-face interaction within the paper–pencil modality is the most used. However, the psychometric properties of these conventional neuropsychological tests, especially those to investigate ED, have been widely discussed. In fact, TBI patients may have good scores in executive tests, although they manifest serious ED in daily life activities [55,80]. This is why some researchers have suggested using tasks with an open-ended structure, such as going shopping, finding one's way in natural surroundings, preparing a meal, and dealing with a complex multitasking situation [81]. To overcome this concern, clinicians can consider using a PC-based/telecognitive virtual assessment for providing

healthcare services at distance, which remotely connect patients and health professionals. Currently, these advanced assessment and treatment tools have proven essential during the pandemic to ensure continuity of care, increasing motivation and the patient's positive mood [82,83].

Several tests and batteries are available to date, and it is important to understand how they are used and can be interpreted appropriately to properly interpret the results to maximize patient outcomes. In this vein, the International Mission for Prognosis and Analysis of Clinical Trials in TBI study group and the development of uniform data standards named common data elements have supported a broad list of tests and batteries. Notably, this protocol can be administered in TBI patients, divided into sub-categories: "concussion/mild TBI", "acute hospitalized", and "moderate/severe TBI: Rehabilitation" [83,84]. To this end, TBI is one of the most heterogeneous neurological disorders which makes the assessment standardization process really challenging, but also points out the need for a better definition of clinical assessment of TBI subgroup severity level.

The main limitation of this scoping review that needs to be addressed is the absence of quality and risk-of-bias assessments in the selected papers. However, our aim was to investigate the available literature about the neuropsychological tests/scales used to evaluate sTBI patients, also providing some clinical advice. In the near future, a systematic review should be considered to better point out the validity of studies and the reliability of the scales, since the literature is still lacking on this issue. Moreover, a quality assessment of the studies should be performed (e.g., by using the pyramid of evidence) to provide the reader with clearer information on the quality of the manuscripts selected.

## 5. Author's Clinical Advice

Herein we present some clinical advice/suggestions for performing neuropsychological assessment, for both moderate and sTBI, according to the updated literature. In fact, the clinical neuropsychological assessment in this patient population is poorly standardized, especially concerning the distinction between moderate and severe TBI.

During the first phase following coma (acute phase 0–1 month), responsiveness monitoring can be carried out using some scales. In detail, the CRS-R is considered as the gold-standard in this phase for its psychometric parameters. The reliability of the scale is improved when there is the presence of caregiver during the assessment, as this represents an important emotional stimulus for patients [85].

After the acute phase, the difference between moderate and severe TBI is more pronounced. In fact, patients with moderate TBI tend to show improvements in the first months after the acute phase. In the postacute (1–3 months) phase, when and if consciousness and cognitive activity improve, clinicians should monitor the patients' cognitive status through observation of behaviours, vigilance, and/or spontaneous speech [86]. After that, the possible administration of a general examination through MMSE [87,88] and/or MoCA [89] can be considered to evaluate the patient's global neurocognitive and functional status. Additionally, the assessment of visuospatial praxis can be addressed through CDT [76]; however, the moderate and/or severe TBI patient must not have aphasia or severe ideomotor praxis.

In the chronic phase (6 months), both moderate and severe TBI patients could be tested only when they regain sufficient cognitive status. It should be considered that the in-depth cognitive evaluation is based on results achieved for MMSE and/or MOCA; otherwise, the domain-specific diagnostic process might be incoherent [87,88]. In clinical practice, the administration of RAVLT [19], CPT [23], SDMT [24], PASAT [51], and Stroop [18] to evaluate frontal abilities can be very challenging, due to the lack of compliance of patients and the presence of language disturbances. This is why those tests should be administered in TBI patients with a moderate cognitive impairment level. Similarly, for the assessment of praxis skills, some clinicians use the FAST-R. However, the drawing and copying tasks can be easily administered to assess constructive apraxia [90], while the gesture-imitation test is used to evaluate ideomotor apraxia [43]. In cases in which the patient presents the optimal condition (active participation), it is possible to proceed using

second-level batteries to investigate the "deeper cognitive functions" for specific domains. It is noteworthy that first-level test batteries, administered in sTBI, can be divided into two or more evaluation sessions; alternatively, it is possible to choose the tests to be performed based on the specific cognitive domain that is going to be explored. It is advisable to use shorter clinical evaluation batteries to avoid the fatigue, distractibility, and flooring effects typical of these patients.

In the end, clinicians should consider some tips in order to perform the most valid neuropsychological assessment in sTBI. In detail, the testing needs to be administered in a lab or clinic office setting, with minimal noise and distraction (because of the high prevalence of attention deficits in TBI), where all testing is performed one-on-one. As many specific neuropsychological tests are available, they should be administered only in TBI patients who have recovered sufficiently to cooperate with testing [91]. In fact, DOC as well as potentially severe motor [92] and language impairments make some sTBI individuals inappropriate for neuropsychological referral because of a lack of testability. In this vein, clinicians should be aware of the demotivating effect and the patient's effort to avoid test invalidity. This is why neuropsychological tests can be marked up as "invalid" in two cases: (1) individuals not motivated to perform and thus not "trying hard" and (2) individuals attempting to exaggerate impairment by answering incorrectly or feigning deficits. Poor effort can be caused by factors other than TBI.

Lastly, clinicians should monitor and evaluate patients after eight months or one-year post injury, since this is the period in which natural brain recovery can still occur. In this vein, sustained and persistent symptoms in TBI patients should be screened for anxiety and depression, as they are the most powerful predictors of prolonged recovery [93,94]. Specifically, brief standardised self-report measures such as the Generalised Anxiety Disorder and Patient Health Questionnaire can facilitate screening [93,94].

## 6. Conclusions

In summary, neuropsychological evaluation in TBI provides a comprehensive assessment of patients' cognitive strengths and weaknesses, and this should be administered by using different tools at the different stages of the disorder. However, since all cognitive tests require that patients give their best effort when completing them and patients with sTBI present a complex clinical picture, more methodologically rigorous research is needed to demonstrate the sensitivity/specificity of the tools to identify which patients are most likely to respond. A systematic review with a metanalysis overcoming this important consideration is needed to better point out the validity of the papers, and the reliability of the scales, in order to guarantee a global holistic approach to these very frail individuals.

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## Abbreviations

| | |
|---|---|
| AAT | Aachener Aphasia Test |
| ABA | Apraxia Battery for Adults-II |
| AD | Attention Dysfunction |
| BDAE | Boston Diagnostic Aphasia Examination |
| BNT | Boston Naming Test |
| BSOT | Benton's spatial orientation test |
| BVRT | Benton's visual retention test |
| CCD | Cognitive Communication Disorders |
| CDT | Clock drawing test |
| CPT | Continuous performance test |
| CRS-R | Coma recovery scale-revised |
| DEX | Dysexecutive questionnaire |
| D-KEFS | Delis–Kaplan Executive Function System |
| DoC | Disorder of Consciousness |
| DRS | Disability Rating Scale |
| ED | Executive Dysfunction |
| FAB | Florida Apraxia Battery |
| FAB | Frontal Assessment Battery |
| FAST-R | Florida Apraxia Screening Test-Revised |
| GCS | Glasgow coma scale |
| GOAT | Galveston Orientation and Amnesia Test |
| IGT | Iowa Gambling Task |
| JOL | Judgement of line guidance |
| LCF | Level of Cognitive Functioning |
| MCS | Minimally Conscious State |
| MEC | Protocole Montreal d'évaluation de la communication |
| NAB | Neuropsychological Assessment Battery |
| PASAT | Paced Auditory Serial Addition Task |
| PTA | Post-traumatic amnesia |
| SDMT | Symbol Digit Modalities Test |
| SDMT | Social Decision-Making Task |
| TASIT | The Awareness of Social Inference Test |
| TBI | Traumatic brain injury |
| TOL | Tower of London |
| UWS | Unresponsive wakefulness syndrome |
| VS | Vegetative state |
| WAB-R | Western Aphasia Battery-Revised |
| WCST | Wisconsin Card Sorting Test |
| WMS-IV | Wechsler Memory Scale |

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# A Pilot Study of Whole-Blood Transcriptomic Analysis to Identify Genes Associated with Repetitive Low-Level Blast Exposure in Career Breachers

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**Abstract:** Repetitive low-level blast exposure is one of the major occupational health concerns among US military service members and law enforcement. This study seeks to identify gene expression using microRNA and RNA sequencing in whole-blood samples from experienced breachers and unexposed controls. We performed experimental RNA sequencing using Illumina's HiSeq 2500 Sequencing System, and microRNA analysis using NanoString Technology nCounter miRNA expression panel in whole-blood total RNA samples from 15 experienced breachers and 14 age-, sex-, and race-matched unexposed controls. We identified 10 significantly dysregulated genes between experienced breachers and unexposed controls, with FDR corrected <0.05: One upregulated gene, *LINC00996* (*long intergenic non-protein coding RNA 996*); and nine downregulated genes, *IGLV3-16* (*immunoglobulin lambda variable 3-16*), *CD200* (*CD200 molecule*), *LILRB5* (*leukocyte immunoglobulin-like receptor B5*), *ZNF667-AS1* (*ZNF667 antisense RNA 1*), *LMOD1* (*leiomodin 1*), *CNTNAP2* (*contactin-associated protein 2*), *EVPL* (*envoplakin*), *DPF3* (*double PHD fingers 3*), and *IGHV4-34* (*immunoglobulin heavy variable 4-34*). The dysregulated gene expressions reported here have been associated with chronic inflammation and immune response, suggesting that these pathways may relate to the risk of lasting neurological symptoms following high exposures to blast over a career.

**Keywords:** repetitive low-level blast; experienced breacher; traumatic brain injury

## 1. Introduction

Blast exposure is a prominent feature of the Iraq and Afghanistan conflicts due to the use of improvised explosive devices [1]. The prevalence of blast-exposure injury dramatically increased from 60% (2008) to 74% (2009) in the US military, which accounts for most combat-related casualties [2]. Repetitive high-blast exposure has been associated with neuronal changes as well as cognitive and affective symptoms [3,4]. In addition to these concerns from high-pressure blast exposure, repetitive low-level blast exposure is a major occupational health concern for military and law-enforcement training. Experienced breachers in military and law-enforcement training encounter more than 100 occurrences of repetitive low-level blast overpressure throughout their careers [5]. However, the underlying biological mechanism of repetitive low-level blast exposure and related neurological effects are not well-understood. The lack of understanding of repetitive low-level blast exposure on neurological functioning makes its identification and potential health intervention challenging in a military setting. Previously repetitive low levels of blast exposure have been associated with neurocognitive and neurosensory decline [6–8], which positively correlated with blood-based levels of tau, amyloid β (Aβ)40, and Aβ42 proteins [9,10], suggesting that there may be biological changes in the blood that result from these high levels of blast exposure over a career.

To date, a growing number of investigations have been conducted to identify biomarkers following repetitive blast exposure [5,9–13]. Previously we reported the effects of acute blast exposure during military training, which include acute changes in amyloid precursor protein [12], inflammatory markers [interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α)] [13], and longitudinal changes in DNA methylation [14]. It is important to understand the biological mechanism following repetitive low-level blast exposure to develop interventions in preventing short- or long-term associated symptoms that influence the health-related quality of life of US military service members. To address this critical issue, we performed transcriptome-wide analysis in whole-blood RNA sequencing to identify potential gene-expression activity across the genome in an experienced breacher population with a high number of repetitive low-level blast exposures and an unexposed control group. In addition, we used multiplexed miRNA assays to quantify the levels of microRNA expression in the whole blood of the experienced breacher population and unexposed control group.

## 2. Materials and Methods

### *2.1. Study Protocol*

This study was reviewed and approved by the Naval Medical Research Center (NMRC) and the National Institutes of Health (NIH) Institutional Review Boards. All the study procedures were performed at NIH Clinical Center after obtaining written informed consent. The detailed procedure of this study protocol has been published elsewhere [5,15].

### *2.2. Demographic, Clinical History, and Psychometric Testing*

All participants were administered demographic and clinical information. Psychometric tests were conducted to assess cognitive domains and symptomology of the participants. The groups are well-matched on age, sex, race, and ethnicity. The Immediate Post-Concussion Assessment and Cognitive Test (ImPACT 2.0) was utilized to evaluate verbal-memory composite, visual-memory composite, reaction-time composite, impulse-control composite, and total symptom composite score [16]. The Brief Symptom Inventory (BSI)-18 is an 18-item scale of psychological distress classified into somatization, depression, anxiety, and global severity index subscales [17]. The Combat Exposure Checklist (CEC) is a self-report scale used to measure the frequency of stressful events experienced during deployments. The Neurobehavioral Symptom Inventory (NSI) was used to assess post concussive symptoms. The NSI is a 22-item self-report scale and has shown both excellent internal consistency ($\alpha = 0.95$) as well as the ability to differentiate veterans with TBIs from those without [18]. Post-Traumatic Stress Disorder (PTSD) Checklist-Military (PCL-M) was

used to assess PTSD symptoms. The PCL-M is a 17-item self-report PTSD-symptom scale with scores ranging from 17 to 85. It has been shown to have high test–retest reliability ($r = 0.96$) and internal consistency ($\alpha = 0.96$) in Vietnam veterans [19].

### *2.3. RNA Sequencing and Bioinformatic Analysis*

Peripheral blood samples were collected in PAXgene tubes and stored at −80 °C until analyzed. Samples from 29 participants were analyzed using RNA-seq with Illumina's HiSeq 2500 system, using paired-end sequencing. Each sample has at least 30 million reads—15 million reads for read 1 and 15 million reads for read 2. Each read has 101 bp for its read length. For bioinformatics analysis, we first performed bioinformatics quality control (QC) using FastQC, version 0.11.9. Then, we trimmed 15 bp from 5′-end, and 10 bp from 3′-end, to remove adapter contamination as well as low-quality base calls in 3′-end. We aligned to GRCh38 reference genome using STAR, version 2.7.6a. We counted number of reads mapped to genes using htseq, version 0.11.4. Finally, we found differentially expressed genes using DESeq2, version 1.30.1 with the cutoff of 0.05 on false discovery rate (FDR) adjusted by independent hypothesis weighting. R, version 4.0.3 (10 October 2020) and Bioconductor, version 3.11 was used for analysis.

### *2.4. MicroRNA Profiling and Bioinformatic Analysis*

Analysis was performed with nCounter® Human v3 miRNA Expression Panels (NanoString Technologies, Seattle, WA, USA). The expression panel contained 798 miRNA probes; this was the maximum number of probes available for analysis in human samples. The probes were incorporated in the code sets and used for analysis along with positive and negative controls. All hybridizations took place at 18 h ± 30 min, and all counts were obtained from nCounter® Digital Analyzer. Raw miRNA data were subtracted from the geometric means of the negative control incorporated in the code sets, and top-100 normalization was performed using the nSolver analysis software (version 4.0, NanoString technologies). Normalized data were analyzed by ROSALIND® (with a HyperScale architecture developed by ROSALIND, Inc. (San Diego, CA, USA). Read-distribution violin plots, identity heatmaps, and sample MDS plots were generated as part of the QC step. The limma R library [20] was used to calculate fold changes and *p*-values and perform optional covariate correction.

### *2.5. Statistical Analysis*

Statistical analysis was conducted with SPSS version 28.0 (IBM Corp., Armonk, NY, USA). Demographic and clinical characteristics were compared between the experienced breacher and control groups using chi-square and independent-samples *t*-test. Pearson correlation coefficient was performed to assess the association of the interested study variables. Statistical tests were two-tailed and $p < 0.05$ was considered a significant difference.

## 3. Results

### *3.1. Demographic and Clinical Characteristic*

The participants recruited for this study were well-matched on demographic characteristics between the unexposed control (N = 14) and experienced breacher (N = 15) groups (Table 1). The majority of participants were white, military personnel, and had a mean age of 40 years. There were no differences in demographics including age, sex, and ethnicity between experienced breachers and unexposed controls. The mean values of self-reported career breachers were 4659.20 breaching blast exposures in the experienced breacher group and 5.86 breaching blast exposures in the unexposed control group over their careers.

Self-reports of several symptoms were different between the experienced breacher and unexposed control groups (Table 2). A total of 10 out of 15 experienced breachers reported having memory problems and ringing in the ears, whereas only 4 out of 14 reported these symptoms in the unexposed control group ($p = 0.04$). A total of 8 out of 15 experienced breachers reported having irritability problems, and only 2 out of 14 unexposed controls

reported irritability ($p = 0.027$). A total of 9 out of 15 experienced breachers reported having concentration problems, whereas only 2 were reported in unexposed controls ($p = 0.011$). Although it did not reach significance, sleep problems were reported in 9 experienced breachers as compared to 5 unexposed controls. Self-reports of headaches and depression were not different between experienced breachers and unexposed controls.

**Table 1.** Demographic characteristics of study participants.

| | **Unexposed Control (N = 14)** | **Experienced Breacher (N = 15)** | **Significance** |
|---|---|---|---|
| Age, mean (SD) | 38.86 (7.81) | 41.60 (8.42) | $t = 0.907$, $p = 0.372$ |
| Sex (Male), N (%) | 14 (100) | 15 (100) | N/A |
| Race, N (%) | | | |
| White | 12 (85.7) | 13 (86.7) | |
| Black | 1 (7.1) | 0 (0.0) | $\chi^2 = 2.008$, $p = 0.571$ |
| Asian | 1 (7.1) | 1 (6.7) | |
| American Indian/Alaskan | 0 (0) | 1 (6.7) | |
| Ethnicity (Non-Hispanic), N (%) | 13 (92.9) | 15 (100) | $\chi^2 = 1.110$, $p = 0.483$ |
| Type of Service, N (%) | | | |
| Military | 10 (71.4) | 10 (66.7) | N/A |
| Civilian Law Enforcement | 4 (28.6) | 5 (33.3) | |
| Duration of service, mean (SD) | 13.71 (7.12) | 18.40 (6.82) | $t = -1.809$, $p = 0.082$ |
| Total blast exposures, mean (SD) | 5.86 (10.42) | 5659.20 (9649.52) | $\mathbf{t = -2.269, p = 0.040}$ |
| Breaches in career, N (%) | | | |
| 0 | 13 (92.9) | 0 (0.0) | N/A |
| 10–39 | 1 (7.1) | 0 (0.0) | |
| 100–199 | 0 (0.0) | 1 (6.7) | |
| 200–399 | 0 (0.0) | 1 (6.7) | |
| 400+ | 0 (0.0) | 13 (86.7) | |
| Breaches in past year, N (%) | | | |
| 0 | 14 (100) | 2 (13.3) | N/A |
| 1–9 | 0 (0.0) | 2 (13.3) | |
| 10–39 | 0 (0.0) | 1 (6.7) | |
| 40–99 | 0 (0.0) | 1 (6.7) | |
| 100–199 | 0 (0.0) | 3 (20.0) | |
| 200–399 | 0 (0.0) | 3 (20.0) | |
| 400+ | 0 (0.0) | 3 (20.0) | |

N/A: Not Applicable.

PCL-M scores were higher in experienced breachers when compared with unexposed controls ($p = 0.029$), indicating increased PTSD-related symptoms, although these levels do not meet clinical criteria for PTSD diagnosis (>44 PTSD cutoff) (Table 2). There was no difference in BSI subscale score, including somatization, depression, anxiety, and global severity index scores in experienced breachers when compared with unexposed controls. There was a significant difference between the groups in visual memory ($p = 0.009$) and reaction time ($p = 0.034$).

The results of Pearson correlation coefficients between behavioral symptoms and number of blast exposures are shown in Table 3. The number of blast exposures was positively correlated with BSI subscale, including somatization ($\rho = 0.399$, $p = 0.032$), depression ($\rho = 0.430$, $p = 0.020$), anxiety ($\rho = 0.496$, $p = 0.006$), and global severity index ($\rho = 0.413$, $p = 0.026$).

**Table 2.** Clinical symptoms of study participants.

| | Unexposed Control (N = 14) | Experienced Breacher (N = 15) | Significance |
|---|---|---|---|
| Headaches, Yes, N (%) | 2 (14.3) | 2 (13.3) | $\chi^2 = 0.006$, $p = 0.941$ |
| Memory problem, Yes, N (%) | 4 (28.6) | 10 (66.7) | **$\chi^2 = 4.209$, $p = 0.040$** |
| Ringing in ears, Yes, N (%) | 4 (28.6) | 10 (66.7) | **$\chi^2 = 4.209$, $p = 0.040$** |
| Sleep problems, Yes, N (%) | 5 (35.7) | 9 (60.0) | $\chi^2 = 1.710$, $p = 0.191$ |
| Irritability, Yes, N (%) | 2 (14.3) | 8 (53.3) | **$\chi^2 = 4.887$, $p = 0.027$** |
| Depression, Yes, N (%) | 3 (21.4) | 3 (20.0) | $\chi^2 = 0.009$, $p = 0.924$ |
| Concentration problems, Yes, N (%) | 2 (14.3) | 9 (60.0) | **$\chi^2 = 6.428$, $p = 0.011$** |
| PCL-M, mean (SD) | 20.64 (4.48) | 26.07 (7.69) | **$t = -2.338$, $p = 0.029$** |
| NSI, mean (SD) | 16.86 (5.29) | 16.80 (6.70) | $t = -0.025$, $p = 0.980$ |
| BSI subscale, mean (SD) | | | |
| Somatization | 45.29 (4.41) | 49.33 (7.46) | $t = -1.762$, $p = 0.089$ |
| Depression | 45.21 (6.40) | 46.00 (7.05) | $t = -0.313$, $p = 0.756$ |
| Anxiety | 44.07 (5.81) | 44.60 (8.95) | $t = -0.187$, $p = 0.853$ |
| Global severity index | 43.50 (6.40) | 46.40 (9.15) | $t = -0.986$, $p = 0.333$ |
| ImPACT, mean (SD) | | | |
| Verbal memory | 92.21 (6.33) | 92.13 (6.53) | $t = 0.034$, $p = 0.973$ |
| Visual memory | 68.00 (9.12) | 78.60 (11.06) | **$t = -2.804$, $p = 0.009$** |
| Visual motor speed | 27.83 (3.25) | 27.33 (4.57) | $t = 0.337$, $p = 0.738$ |
| Reaction time | 0.57 (0.07) | 0.64 (0.10) | **$t = -2.213$, $p = 0.034$** |
| Impulse control | 0.07 (0.28) | 0.33 (0.62) | $t = -1.500$, $p = 0.150$ |
| Total symptom | 4.71 (6.75) | 12.40 (17.59) | $t = -1.573$, $p = 0.133$ |

PCL-M, Post-Traumatic Stress Disorder Checklist-Military; NSI, Neurobehavioral Symptom Inventory; BSI, Brief Symptom Inventory.

**Table 3.** Pearson Correlation Coefficients of Study Variables.

| | Number of Blast Exposures | | CEC Total Score | |
|---|---|---|---|---|
| | $\rho$ | $p$ | $\rho$ | $p$ |
| BSI-somatization | **0.399** | **0.032** | **0.377** | **0.044** |
| BSI-depression | **0.430** | **0.020** | −0.003 | 0.990 |
| BSI-anxiety | **0.496** | **0.006** | −0.056 | 0.773 |
| BSI-global severity index | **0.413** | **0.026** | 0.162 | 0.402 |
| ImPACT-verbal memory | −0.055 | 0.776 | −0.114 | 0.557 |
| ImPACT-visual memory | 0.053 | 0.784 | **0.508** | **0.005** |
| ImPACT-visual motor speed | −0.324 | 0.087 | 0.133 | 0.491 |
| ImPACT-reaction time | **0.448** | **0.015** | 0.264 | 0.166 |
| ImPACT-impulse control | 0.202 | 0.293 | −0.069 | 0.722 |
| ImPACT-total symptom | 0.243 | 0.204 | 0.223 | 0.244 |

BSI, Brief Symptom Inventory; CEC, Combat Exposure Checklist; ImPACT, Immediate Post-Concussion Assessment and Cognitive Test.

### *3.2. Differential microRNA Expression Differences between Experienced Breacher vs. Unexposed Control*

We identified 14 miRNAs differentially expressed in experienced breachers compared to unexposed controls ($p < 0.05$). Among them, eight miRNAs were upregulated and six miRNAs were downregulated in the experienced breachers compared with the unexposed controls. These microRNAs were not significantly different after FDR correction. The volcano plot of differentially expressed miRNAs is shown in Figure 1 and the fold change of each probe is presented in Table 4.

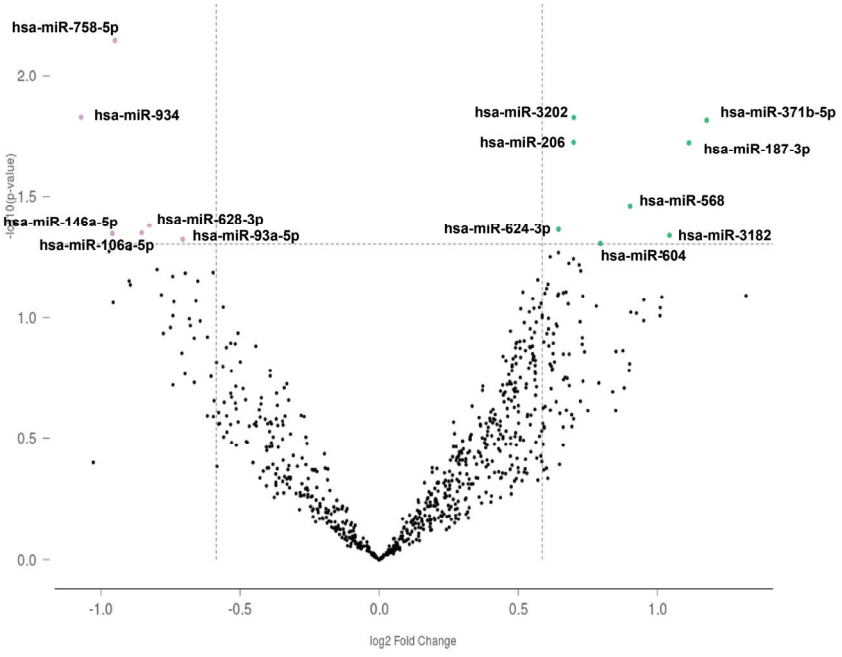


**Figure 1.** Volcano plot for differentially expressed microRNAs between experienced breacher vs. unexposed control. Green dots indicate genes that are upregulated, and purple dots indicate genes that are downregulated.

**Table 4.** MicroRNA differential expression between experienced breacher vs unexposed control.

| Probe Name | Log2FC | *p*-Value | FDR |
|---|---|---|---|
| hsa-miR-371b-5p | 1.177 | 0.015 | 0.848 |
| hsa-miR-187-3p | 1.113 | 0.019 | 0.848 |
| hsa-miR-3182 | 1.043 | 0.046 | 0.848 |
| hsa-miR-568 | 0.901 | 0.035 | 0.848 |
| hsa-miR-604 | 0.795 | 0.049 | 0.848 |
| hsa-miR-3202 | 0.699 | 0.015 | 0.848 |
| hsa-miR-206 | 0.699 | 0.019 | 0.848 |
| hsa-miR-624-3p | 0.644 | 0.043 | 0.848 |
| hsa-miR-93-5p | −0.706 | 0.048 | 0.848 |
| hsa-miR-628-3p | −0.826 | 0.042 | 0.848 |
| hsa-miR-106a-5p | −0.853 | 0.045 | 0.848 |
| hsa-miR-758-5p | −0.950 | 0.007 | 0.848 |
| hsa-miR-146a-5p | −0.959 | 0.045 | 0.848 |
| hsa-miR-934 | −1.071 | 0.015 | 0.848 |

### *3.3. Differential Gene Expression between Experienced Breacher vs. Unexposed Control*

We performed whole-blood RNA-seq in experienced breacher and unexposed control individuals. The comparison between experienced breacher vs. control shows one upregulated gene and nine downregulated genes. We identified one upregulated gene, *long intergenic non-protein coding RNA 996 (LINC00996)*, and 9 downregulated genes, namely *immunoglobulin lambda variable 3-16 (IGLV3-16)*, *CD200 molecule (CD200)*, *Leukocyte immunoglobulin-like receptor B5 (LILRB5)*, *ZNF667 antisense RNA 1 (ZNF667-AS1)*, *leiomodin 1 (LMOD1)*, *contactin-associated protein 2 (CNTNAP2)*, *envoplakin (EVPL)*, *double PHD fingers 3 (DPF3)*, and *immunoglobulin heavy variable 4-34 (IGHV4-34)* in the experienced breacher

group compared to control with the multiple corrected threshold of FDR < 0.05. Differential gene expression with fold changes and adjusted *p*-values are shown in Table 5.

**Table 5.** Gene-expression differences between experienced breacher vs unexposed control.

| Gene Symbol | Gene Name | Log2FC | FDR |
|---|---|---|---|
| *IGLV3-16* | *Immunoglobulin lambda variable 3-16* | −1.880 | 0.002 |
| *CD200* | *CD200 molecule* | −0.879 | 0.024 |
| *LILRB5* | *Leukocyte immunoglobulin-like receptor B5* | −1.689 | 0.024 |
| *ZNF667-AS1* | *ZNF667 antisense RNA 1* | −0.974 | 0.024 |
| *LMOD1* | *Leiomodin 1* | −2.688 | 0.025 |
| *CNTNAP2* | *Contactin-associated protein 2* | −1.715 | 0.030 |
| *EVPL* | *Envoplakin* | −2.263 | 0.030 |
| *DPF3* | *Double PHD fingers 3* | −1.542 | 0.039 |
| *LINC00996* | *Long intergenic non-protein coding RNA 996* | 0.866 | 0.039 |
| *IGHV4-34* | *Immunoglobulin heavy variable 4-34* | −1.250 | 0.043 |

## 4. Discussion

In this study, we report significant transcriptome differences in whole-blood associated with repetitive low-level blast exposures compared to unexposed controls. Differentially expressed genes reported are related to inflammation and immune-response process. In addition, the number of blast exposures are strongly correlated with clinical symptoms of BSI-somatization, BSI-anxiety, BSI-depression, and BSI-global severity scores. Dysregulation of these genes may associate with persistent clinical symptoms following repetitive blast exposures. These findings provide some initial insights into the biological changes related to repetitive low-level blast exposure.

Our finding of downregulated *CD200* may play a role in chronic inflammation within the CNS by releasing inflammatory cytokines after exposure to blast. *CD200* is an immune inhibitory molecule which is highly expressed in neurons and plays a critical role in inhibiting microglia activation [21]. The downregulation of *CD200* expression has been observed in both chronic active and inactive multiple sclerosis lesions from postmortem brains in patients [22]. Downregulation of *CD200* in our study may reflect the chronic neuroinflammation activity by microglia activation in individuals exposed to repetitive low levels of blast. Activated microglia is a neuroinflammatory process that affects the astrocytes, leading to astrogliosis, which was observed in the elevation of glial fibrillary acidic protein reported in the preclinical model of repetitive low-level blast exposure [11]. The ongoing inflammatory activity in the CNS can be detected in the peripheral circulation. In support of this, we also report the downregulation of *IGLV3-16*, *IGHV4-34*, and *LILRB5* genes, which are linked to immune-system response and may play an important role in proinflammatory cytokine production [23,24]. Previously, we observed the elevation of plasma IL-6 and TNF-α proteins in a military training population with >5 psi blast exposure compared with low-level <2 psi blast exposure [13]. More recently, analysis of inflammatory proteins in this population of experienced breachers and control subjects showed increases in brain-derived extracellular vesicles (EVs) for IL-6 and TNF-a with a corresponding decrease in IL-10 EVs (unpublished data). These findings further support the important role of *CD200* in the peripheral and CNS activity in response to inflammation after exposure to a high number of blasts during a career.

In addition, we reported a downregulation of *CNTNAP2* gene expression in this cohort. *CNTNAP2* encodes CASPR2, a transmembrane protein associated with voltage potassium channels and a neurexin superfamily protein that plays a critical role in neurodevelopment [25,26]. The *CNTNAP2* gene, located on chromosome 7q35, is one of the largest genes in the human genome [27,28]. Multiple mutations within the gene have been identified and are characterized with a set of neurologically related phenotypes that include intellectual disability, seizures, and language impairment. Additionally, mutations have been clinically associated with neurological disorders such as autism spectrum disorder and Pitt Hopkins-

like Syndrome 1 [29–31]. Most identified mutations within *CNTNAP2* were heterozygous, indicating that a single allele disruption could be sufficient to cause disorder and deficit. Preclinical models examining homozygous disruption and complete loss of function in the *CNTNAP2* gene have demonstrated an exacerbated and severe neurodevelopmental and neurocognitive deficit outcome [32].

Currently, the majority of investigations revolve around *CNTNAP2* gene mutations with large deletions, which likely lead to a nonfunctional protein product. However, our results demonstrate that in our cohort of experienced breachers, who have repeated low-level blast exposure, *CNTNAP2* gene expression is downregulated. Interestingly, studies have identified individuals with deletions predicted to not interfere with the protein product, or with a deletion to an upstream promoter [33–35]. Unlike the majority of deletions that result in a loss of function, these mutations resulted in decreased expression [36], reduced protein function, and displayed more moderate phenotypes including epilepsy, schizophrenia, obsessive compulsive disorder, Tourette syndrome, and attention deficit hyperactivity disorder [33,35,37,38]. Furthermore, downregulation of *CNTNAP2* has been clinically implicated in neurodegeneration, and found significantly downregulated in a cohort of Alzheimer's patients [39]. Thus, our findings suggest that dysregulation of this gene may be implicated in neurocognitive declines in repetitive low-level blast exposure. Longitudinal follow-up and further analysis of our cohort's psychiatric health could potentially elucidate an association of blast-related *CNTNAP2* downregulation with other psychological disorders. Additionally, future investigations should examine if differential *CNTNAP2* expression is acutely impacted by blast exposures and possible roles in symptom development in a more lasting manner.

In summary, this preliminary study suggests that occupational exposure to repetitive low-level blasts is associated with dysregulation of gene expression in whole-blood samples. The differentially expressed genes are largely associated with the chronic inflammation process, and linked to various neurological disorders. A major strength of this study is that it is the first to analyze differential gene expression in the whole blood of a unique population with similar occupational factors between experienced breacher and unexposed control groups. Although this is the first study to provide molecular insight into repetitive low level blast exposure, it was constrained by a small sample size and only one timepoint. In addition, the experienced breachers group had significantly higher PCL-M scores compared to unexposed controls, indicating increased PTSD-related symptoms. Higher scores of PTSD-related symptoms may have a significant role on these genes' expression levels, although these levels do not meet clinical criteria for PTSD diagnosis. Our findings suggest that need for future studies to be undertaken in larger cohorts over time.

Despite these limitations, this is the first study to the best of our knowledge to quantify microRNAs and mRNAs, and this provides initial insights into the pathophysiological mechanism of repetitive low-level blast exposure. Replicating these findings in a larger cohort may provide potential biomarkers and therapeutic targets for experienced breacher populations.

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**Data Availability Statement:** The datasets presented in this study can be found in online repositories at Gene Expression Omnibus with the access number GSE193952. The anonymized clinical data that support the findings of this study are available upon reasonable request from any qualified investigator to the corresponding author.

**Conflicts of Interest:** The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of the Department of the Army, Department of the Navy, Department of Defense, or the U.S. Government. Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The investigators have adhered to the policies for protection of human subjects as prescribed in AR 70-25. Several of the authors are military service members or federal civil service employees. This work was prepared as a part of their official duties. Title 17 U.S.C. § 105 provides that "Copyright protection under this title is not available for any work of the United States Government." Title 17 U.S.C. § 101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person's official duties.

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# Cognitive and Cellular Effects of Combined Organophosphate Toxicity and Mild Traumatic Brain Injury

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**Abstract:** Traumatic brain injury (TBI) is considered the most common neurological disorder among people under the age of 50. In modern combat zones, a combination of TBI and organophosphates (OP) can cause both fatal and long-term effects on the brain. We utilized a mouse closed-head TBI model induced by a weight drop device, along with OP exposure to paraoxon. Spatial and visual memory as well as neuron loss and reactive astrocytosis were measured 30 days after exposure to mild TBI (mTBI) and/or paraoxon. Molecular and cellular changes were assessed in the temporal cortex and hippocampus. Cognitive and behavioral deficits were most pronounced in animals that received a combination of paraoxon exposure and mTBI, suggesting an additive effect of the insults. Neuron survival was reduced in proximity to the injury site after exposure to paraoxon with or without mTBI, whereas in the dentate gyrus hilus, cell survival was only reduced in mice exposed to paraoxon prior to sustaining a mTBI. Neuroinflammation was increased in the dentate gyrus in all groups exposed to mTBI and/or to paraoxon. Astrocyte morphology was significantly changed in mice exposed to paraoxon prior to sustaining an mTBI. These results provide further support for assumptions concerning the effects of OP exposure following the Gulf War. This study reveals additional insights into the potentially additive effects of OP exposure and mTBI, which may result in more severe brain damage on the modern battlefield.

**Keywords:** mTBI; organophosphates; cognitive and behavioral tests; neuronal loss; neuroinflammation

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## 1. Introduction

Traumatic brain injury (TBI) occurs when the head is compacted by an object or external force. TBIs are primarily caused by road accidents, falls, wars, assaults, and sports injuries [1]. Several mechanisms of damage may cause TBIs, including blunt injury (when there is significant acceleration or malformation of the brain tissue), penetrating injury (when the invasion of the skull causes damage), blast injury, and concussion [2]. The pathophysiology of TBI can be divided into primary and secondary injuries. Primary injury occurs when an outside force is applied to the brain, directly affecting neural tissue, glial

cells, and the vascular system based on their physical characteristics. Secondary injuries result from the progression of biological events triggered by the primary injury. They can include ischemia, glutamate toxicity, neuroinflammation, edema, increased permeability of the blood–brain barrier, oxidative stress, and cellular dysfunction leading to apoptosis [3].

Currently, there is a dearth of effective interventions designed to address the secondary injury cascades that occur following an initial traumatic event. However, prior research has suggested that a multifaceted approach incorporating a combination of therapeutic modalities, supplementation strategies, and pharmaceutical agents may yield promising results in mitigating the effects of these secondary injuries [4].

Head injuries are commonly categorized by the severity of injury according to the Glasgow Coma Scale (GCS), which assesses a patient's condition based on their eye, motor, and verbal responses. This test distinguishes mild, moderate, and severe injuries [5].

Severe TBI can be diagnosed relatively easily by characterizing brain tissue damage. In contrast, blood–brain barrier disruption and the development of edema following a mild traumatic brain injury (mTBI) are more challenging to assess because routine tests, including imaging, fail to show changes in brain structure [6]. Additionally, many patients do not lose consciousness after the injury [7]. More than 80% of head injury cases are classified as mild TBIs. In most cases of mild neurotrauma, immediate symptoms gradually disappear within a year following the trauma. However, patients sometimes suffer from persistent and long-lasting neurocognitive impairments, including various cognitive, emotional, and behavioral disorders [8]. mTBI is a common injury in active combat zones and areas subject to frequent terrorist attacks, typically caused by proximity to breaching devices, heavy weaponry, and improvised explosive devices (IEDs), affecting both civilians and military personnel [9,10].

Organophosphates (OPs) are a group of toxic, broad-impacting chemicals with various uses ranging from chemical warfare agents (e.g., Soman, Sarin, Tabun, Cyclosarin, VX) to pesticides. The human body can absorb OPs through inhalation, digestion, or cutaneous penetration. Exposure to OP-based pesticides accounts for 3 million cases per year, rendering insecticide poisonings common in developing countries. Previous studies have demonstrated that acute poisoning from exposure to OP-based pesticides can cause adverse health effects, including vasomotor and verbal memory deficits [11,12]. The effects of OP poisoning are dependent on several factors, including the specific type of OP, the amount of OP to which an individual is exposed, the route of exposure, the duration of exposure, and the age of the individual [13].

OP poisoning poses a life-threatening primary effect, primarily affecting the peripheral nervous system. OP inhibits the enzyme acetylcholinesterase (AChE) in an irreversible manner, which can lead to damage to the cholinergic system. Immediate clinical signs of OP poisoning include tremors, paralysis, and even death due to respiratory failure. Furthermore, OP poisoning may also cause immediate and long-term damage to the central nervous system [14]. Within the brain, the cholinergic system is essential for learning, memory, and consciousness [15–17]. Consequently, exposure to OPs may impair memory, consciousness, and motor and emotional abilities [18–20].

In recent years, conventional injuries (e.g., explosives) and unconventional exposures, such as chemical weapons (e.g., civil war in Syria), have occurred more frequently in combat zones and terrorist-stricken areas. As such, the likelihood that civilians, military, and law enforcement agents are exposed to either insult or experience a combined exposure to both OPs and mTBI has significantly increased. This combined insult makes it challenging to determine the etiology of the injury and to provide appropriate treatment. In addition, there is currently no research-based medical–therapeutic protocol to treat this combined injury.

In the present study, we utilized an animal model to quantify the extent of damage from co-exposure to OP poisoning and mTBI and examined their combined effects on cerebral functioning over time. These effects were assessed using a series of accepted behavioral and immunohistochemical analyses.

## 2. Materials and Methods

### *2.1. Animals*

Male ICR mice at 6–8 weeks of age (30–40 g) were purchased from Invigo RMS Inc. (Ein Karem Jerusalem, Israel). The mice were kept at room temperature (22 ± 1°C) on a 12-h light/dark cycle with five mice per cage (32 × 21.5 × 12 $cm^3$). Access to standard rodent chow (Purina, Neenah, WI, USA) and water was unrestricted (ad libitum). All experimental procedures were conducted during the light phase. The cage bedding was sawdust replaced twice a week simultaneously for all the cages. Upon arrival at the veterinary service center, the animals were provided with three days of recovery and acclimatization to the new location. Two days prior to the experiment, all cages were moved to the experimental room for habituation and anxiety reduction. The Ethics Committee of the Sackler Faculty of Medicine approved the experimental protocol (01–16-058) in compliance with the guidelines for animal experimentation of the National Institutes of Health (DHEW publication 85–23, revised, 1995).

### *2.2. Experimental Groups*

The study consisted of five experimental groups. Each group included 10–12 mice for the behavioral and cognitive tests and 5 mice for the immunohistochemistry analysis (total of $n = 85$). To avoid behavioral confounds, each group of animals was tested once. Previously, a variance analysis was conducted to determine how many animals were required in each assessment group and how long the measurements lasted. Several previous studies related to the subject were considered when determining sample size [21,22].

The animals were divided into TBI experimental groups receiving either mTBI or sham insults, as well as paraoxon groups receiving either paraoxon or a vehicle. The experimental design was as follows:

A. Control group (sham, vehicle): The treatment conditions were identical, including anesthetization by inhalation of isoflurane vapor for several minutes and an intraperitoneal (IP) injection of 1 mL of saline solution.
B. mTBI group (TBI, vehicle): The treatment conditions were identical. The mice were exposed to mild traumatic brain injury and IP injection of 1 mL of saline solution.
C. Paraoxon group (sham, paraoxon): The treatment conditions were identical, including anesthetization by inhalation of isoflurane vapor for several minutes. Paraoxon was diluted in 0.9% saline and absolute ethanol (dehydrated, 99%) and administered by IP injection at a dose of 0.3 mg/kg.
D. mTBI + paraoxon group (TBI, post-paraoxon): The mice were exposed to mTBI followed by paraoxon administration after 1 h. Paraoxon was diluted in 0.9% saline and absolute ethanol (dehydrated, 99%) and administered by IP injection at a dose of 0.3 mg/kg.
E. Paraoxon + mTBI (TBI, pre-paraoxon): The mice were exposed to paraoxon followed by mTBI after 1 h. Paraoxon was diluted in 0.9% saline and absolute ethanol (dehydrated, 99%) and administered by IP injection at a dose of 0.3 mg/kg.

### *2.3. Mouse Closed-Head Mild Traumatic Brain Injury*

Mild traumatic brain injury (mTBI) was implemented in accordance with past protocols conducted by our group [21]. The head injury was induced by a concussive head trauma device, which involves a fixed weight freefalling along a defined trajectory. The device consisted of a hollow aluminum tube (80 cm in length and 13 mm in diameter). The weight (10 g) and height from which the metal weight was dropped determined the severity of the injury. At the time of injury, mice were placed on a spongy surface with the tube vertically above their heads; this allowed the head to move parallel to the plane of injury during the weight drop, thus simulating a head injury condition. Deliberate trauma was caused specifically to the fronto-lateral area on the right side of the head (midway between the ear and the right eye) [23]. This model was chosen to simulates diffuse traumatic brain injury, which is characteristic of road accidents or falls. After the injury, the mice were

assessed using the Neurological Severity Score (NSS) scale to confirm the absence of any severe acute neurological injuries [21].

*2.4. Paraoxon Administration*

Mice received IP injections of a single dose of paraoxon (N-12816, Sigma-Aldrich, Rehovot, Israel, 0.3 mg/kg). The paraoxon dilution was performed in a chemical hood. Paraoxon was first diluted with propylene glycol to 50 mg/mL, and then further diluted with saline to 1.36 mg/mL. Paraoxon was chosen as a representative substance for the organophosphate group due to our familiarity with this substance through previous work, and because paraoxon is easy to handle and administer with low collateral damage. The dosage given to the animals followed the protocol described by Golderman et al., who treated the animals at a dose of 0.5 mg/kg. Our protocol reduced the dosage to 0.3 mg/kg to ensure that seizures would not be induced in combination with the onset of mTBI [24].

*2.5. Behavioral and Cognitive Tests*

All behavioral and cognitive tests were performed in succession 30 days post-injury. Figure 1).

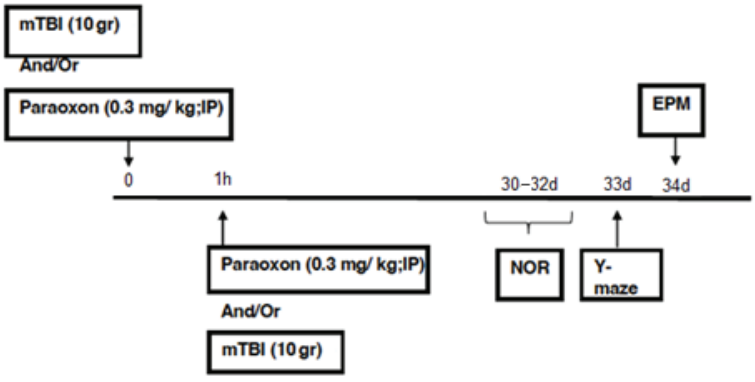


**Figure 1.** Timeline.

The test arenas used for the EPM, NOR, and Y-maze were manufactured to meet the specific size requirements of our group and have been extensively validated throughout our previous studies. The time spent in each part of the arena was manually measured by a double-blinded researcher.

2.5.1. Elevated Plus Maze (EPM)

The EPM test is used to estimate anxiety behavior in rodents. The test capitalizes on the conflict between the innate fear of rodents in open spaces and their curiosity and desire to explore a new environment [25,26]. The maze consisted of two open arms ($30 \times 5 \times 0.25$ cm$^3$) and two closed arms ($30 \times 5 \times 15$ cm$^3$) made of Polymethyl Methacrylate (PMMA). Each pair of arms faced each other on a 50 cm-high surface, forming a "+" shape. At the beginning of the test, we placed the animal in the center of the platform facing one of the open arms and allowed the animal to explore the maze for 5 min. This test measured the time the animal spent in the open arms and the number of times it entered the open space.

2.5.2. Novel Object Recognition (NOR) Test

The NOR test is based on the natural curiosity of rodents to explore new objects and is intended to test the visual memory of the animals [27]. The arena was a square surface ($60 \times 60$ cm) with high walls (20 cm). The NOR test consisted of three steps, with a 24-h interval between each step: (A) Acclimatization step: The tested mouse was placed into the arena for five minutes to become acclimated to the arena itself. (B) Learning step:

The mouse was placed into the arena with two identical "old" objects for five minutes to familiarize the mouse with the objects. (C) Test step: The mouse was placed into the arena with one "old" object from the learning phase and one "new" object for five minutes. Between each animal, the surface and objects were cleaned with ethanol to minimize the odors left by the previous animals. The Aggelton index [23] was calculated to assess the degree of learning and visual memory of the animals according to the following formula:

$$\text{Time exploring new object} - \text{time exploring old object/time exploring new object} + \text{time exploring old object} = \text{preference index.} \quad (1)$$

$$\frac{\text{time A object} - \text{time B object}}{\text{time A object} + \text{time B object}} = \text{Preference index} \quad (2)$$

A higher preference index indicated better recall. Animals that explored the objects for less than 10% of the total time spent in the arena (i.e., less than 30 s with the two objects together) were excluded from the statistical calculations because it is not possible to estimate the visual memory level of a mouse that does not engage with the objects at all.

2.5.3. Y-Maze

The Y-maze test is used to evaluate short-term spatial memory and relies on the animal's preference for exploring a new place [23]. The maze was made of black Perspex and had 3 arms ($8 \times 15 \times 30$ cm) arranged at a 120° angle. One arm was randomly selected as the "start arm". First, each animal was placed on the outer edge of the "start arm" with one of the remaining two arms blocked. The blocked arm was defined as the "new arm", while the accessible arm was defined as the "old arm". The animal was given five minutes to freely explore the two open arms. At the end of the allotted time, the animal was returned to its cage for two minutes. During this time, the maze was cleaned with ethanol to remove any traces left by the animal. After two minutes, the animal was returned to the maze and allowed to freely roam in all three arms for two additional minutes. The time that the animal spent in each arm was measured during these two minutes to assess the animal's ability to distinguish between the "new" and the "old" arms. To avoid any bias due to individual preferences for a specific arm, we changed the place of the "new" arm between animals. During the test phase of the experiment, naïve (untreated) animals were expected to prefer the "new" arm over the "old" arm due to their natural curiosity to explore a new area. The ability to distinguish between the "new" and "old" arms depends on the spatial memory of the animal. The Aggleton index [28] was calculated to assess spatial memory according to the following formula:

$$\frac{\text{time in new arm} - \text{time in old arm}}{\text{time in new arm} + \text{time in old arm}} = \text{Preference index} \quad (3)$$

An animal with intact spatial memory will display a high preference index, whereas an animal with impaired spatial memory will have a low preference index.

*2.6. Immunohistochemistry*

Immunohistochemical studies were performed on hippocampal (dentate gyrus hilus—DGH) and temporal cortex (Cx) tissue sections obtained from animals euthanized 30 days post-injury. The mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and underwent transcardiac perfusion with 10 mL of phosphate-buffered saline (PBS) followed by 20 mL of 4% paraformaldehyde (PFA) in 0.1 M PBS at pH 7.4. The brains were removed, fixed overnight in 4% PFA, and then placed in 1% PFA. The brains were prepared in a multiblock orientation by Neuroscience Associates (Knoxville, TN, USA), and 35 μm sections were collected successively through the brains. Floating section staining and mounting were performed using the antibodies detailed in Table 1. Microscopy was performed using a Fluoview 3000 laser scanning confocal microscope (Olympus, Waltham, MA, USA). The marked locations were determined on a stitched map with only Hoechst 33342 (ThermoFisher, Asheville, NC, USA) staining captured. For all analyses, regions of

interest were selected on the stitched map, which depicted only the nuclear staining and was blinded with respect to groupings. These regions were then collected by multi-area routines and sequenced with Fluoview 3000 version 2.5.1 software (Olympus, Waltham, MA, USA) without intervention. The images were created by combining as Z stacks and maximum Z projections of coronal sections centered around approximately −2.9 mm from Bregma. These sections were identically illuminated (405, 488, 561, and 640 nm diode lasers) and detected. Automated analysis of cell morphology, intensities, and cells counts was conducted using cellSens version 18.0 (Olympus, Waltham, MA, USA) and ImageJ version 1.52a (NIH, Bethesda, MD, USA) [29] software. Macros were employed to automate image analyses. Autothresholding was used to prevent bias using the Li Dark algorithm for NeuN counting and the RenyiEntropy algorithm for astrocyte morphology. Counting was accomplished by converting NeuN images to binary and running the Watershed tool to separate any cells in contact, then executing the Analyze Particles tool. For cell morphology analysis, skeletons were produced with the Skelotonize tool after thresholding, and process characteristics were determined using the Analyze Skeleton (2D/3D) tool.

**Table 1.** Immunohistochemistry reagents.

| Target/Fluorochrome | Primary/Secondary | Probe | Manufacturer | Catalog | Dilution |
|---|---|---|---|---|---|
| Nuclei | | Hoechst 33342 | ThermoFisher (Asheville, NC, USA) | | |
| Astrocytes | Primary | Chicken anti-GFAP | Encor (Gainesville, FL, USA) | CPCA-GFA | 1:1500 |
| Alexa488 | Secondary | Donkey anti-chicken | Jackson (West Grove, PA, USA) | 703–545-155 | 1:500 |
| NeuN + Neurons | Primary | Rabbit anti-NeuN | Abcam (Cambridge, MA, USA) | Ab104225 | 1:5000 |
| Alexa647 | Secondary | Donkey anti-rabbit | ThermoFisher (Asheville, NC, USA) | A31573 | 1:500 |

### *2.7. Statistics*

All values are presented as mean values ± standard deviation. Statistical calculations were performed using IBM SPSS version 24 (Genius Systems, Petah Tikva, Israel). The behavioral data were analyzed using ANOVA tests for continuous variables. For more detailed data, LSD post hoc tests were used. Statistically significant differences between the averages were indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

## 3. Results

### *3.1. Behavioral Effects of Closed-Head mTBI Caused by 10-g Weight Drop*

#### 3.1.1. Anxiety Measured with the Elevated Plus Maze (EPM)

The EPM test was applied to assess anxiety-like behavior. A one-way ANOVA demonstrated no significant main effect of group [$F(4, 50) = 2.008$, NS = 0.108].

Abnormal anxiety behavior was ruled out in all groups. mTBI exposure did not affect anxiety.

#### 3.1.2. Recognition Memory Evaluated by Novel Object Recognition (NOR)

The NOR test was used to assess visual recognition memory (Figure 2A). A one-way ANOVA using LSD post-hoc analysis [$F(4, 50) = 9.886$, $p = 0.000$] did not show a significant difference between the control and mTBI groups ($p = 0.620$), indicating that the mTBI induced by the 10-g ("light") weight had a negligible effect when compared with the uninjured mice. The control group performed significantly better on this task than the paraoxon ($p = 0.002$), paraoxon + mTBI ($p < 0.001$), and mTBI + paraoxon groups ($p < 0.001$). We found a significant difference between the mTBI and the mTBI + paraoxon ($p < 0.001$) groups, as well as the mTBI and paraoxon + mTBI ($p = 0.003$) groups; however, no difference

was observed between mTBI and paraoxon alone. In addition, we found that there was a significant difference between the paraoxon and mTBI + paraoxon groups ($p = 0.042$). There were no significant differences in preference index between the paraoxon + mTBI and the mTBI + paraoxon groups ($p = 0.887$).

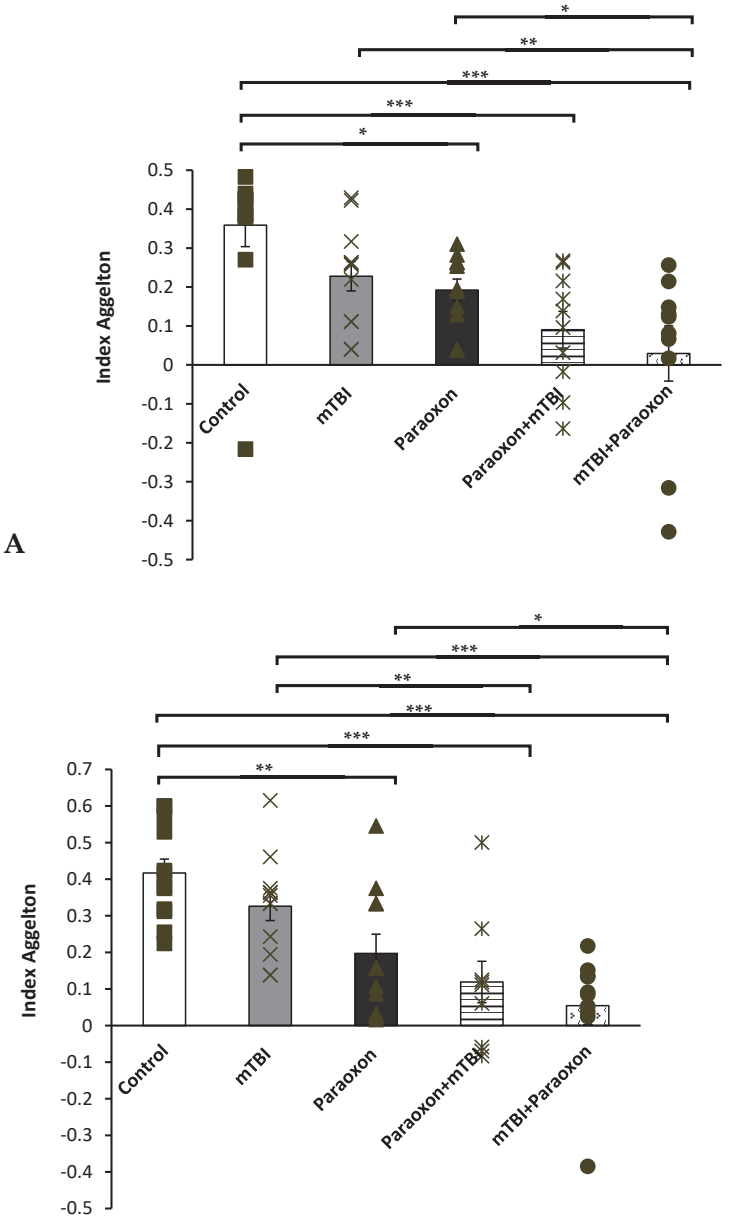


**Figure 2.** Behavioral test scores. (**A**) NOR test: differences in visual recognition memory performance between mice in the control ($n = 12$), mTBI ($n = 12$), paraoxon ($n = 11$), paraoxon + mTBI ($n = 10$), and mTBI + paraoxon ($n = 10$) groups. (**B**) Y-maze test: differences in spatial memory performance between mice in the control ($n = 12$), mTBI ($n = 12$), paraoxon ($n = 11$), paraoxon + mTBI ($n = 10$), and mTBI + paraoxon ($n = 10$) groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.1.3. Spatial Memory Tested with the Y-Maze

The Y-maze test was used to assess spatial memory (Figure 2B). A one-way ANOVA using LSD post hoc analysis [$F(4, 50) = 6.729$, $p = 0.000$] showed that mice in the mTBI + paraoxon ($p < 0.001$) and paraoxon + mTBI ($p < 0.001$) groups performed significantly worse than mice in the control group.

Similar to our findings with the NOR test, we also found that the mTBI group performed significantly better than the mTBI + paraoxon group ($p = 0.007$). We found that the paraoxon group performed significantly worse during Y-maze testing when compared to the control group ($p = 0.018$). Moreover, a significant difference was found between the paraoxon and mTBI + paraoxon groups ($p = 0.027$). No significant difference was found between either the control and mTBI groups or between the mTBI and paraoxon groups.

*3.2. Combined Insult with mTBI and Paraoxon Induces Neuronal Loss*

The number of $NeuN^+$ neurons in the temporal cortex (Figure 3A) was analyzed using a one-way ANOVA with an LSD post hoc test: $F(4, 20) = 5.618$, $p = 0.003$]. The total number of $NeuN^+$ neurons within the temporal cortex was significantly lower in the paraoxon + mTBI and mTBI + paraoxon groups compared to the control group ($p = 0.008$, $p = 0.028$). The mTBI mice had significantly more neurons than paraoxon + mTBI and mTBI + paraoxon ($p = 0.001$, $p = 0.003$). The combined groups of paraoxon + mTBI and mTBI + paraoxon showed significantly more neuronal damage than paraoxon alone ($p = 0.009$, $p = 0.03$).

The number of $NeuN^+$ neurons in the dentate gyrus (Figure 3B) was analyzed using a one-way ANOVA with an LSD post hoc analysis: [$F(4, 20) = 1.945$, NS = 0.142]. Significant differences were found in the DGH between the mTBI and paraoxon + mTBI groups ($p = 0.049$). No significant difference was found between mTBI and mTBI + paraoxon groups.

Images of immunohistochemistry staining in the temporal cortex and DGH with $NeuN^+$ cells shown in red are presented in Figure 3C.

*3.3. Paraoxon Elevates Neuroinflammatory Responses, as Indicated by Reactive Astrocytosis*

Astrocyte reactivity was examined in the dentate gyrus (Figure 4B). A one-way ANOVA with an LSD post-hoc test was used to analyze GFAP intensity [$F(4, 20) = 3.438$, $p = 0.027$], $GFAP^+$ astrocyte counts [$F(4, 20) = 2.755$, $p = 0.056$], and astrocyte morphology [$F(4, 20) = 3.294$, $p = 0.032$]. The results indicated that the GFAP intensity in the DGH was higher in the mTBI, paraoxon, paraoxon + mTBI, and mTBI + paraoxon groups than in the control group ($p = 0.013$, $p = 0.002$, $p = 0.038$, and $p = 0.027$, respectively).

GFAP counts in the DGH (Figure 4D) revealed significant changes in astrocyte counts in the dentate gyrus between the paraoxon and control groups compared with mTBI animals ($p = 0.018$, $p = 0.019$, respectively). Astrocyte morphology in the DGH (Figure 4F) showed marked alterations in astrocyte morphology in the dentate gyrus of paraoxon + mTBI mice vs. controls ($p = 0.003$). Astrocyte morphology was also significantly different between the mTBI and paraoxon + mTBI groups, and between the paraoxon and paraoxon + mTBI groups ($p = 0.019$, $p = 0.017$, respectively).

Images of immunohistochemistry staining in the temporal cortex and DGH with $GFAP^+$ cells shown in green are presented in Figure 4G. No statistically significant differences in neuroinflammatory responses were found in the temporal cortex as measured by GFAP intensity [$F(4, 20) = 1.001$, NS = 0.430], counts [$F(4, 20) = 1.288$, NS = 0.308], or morphology [$F(4, 20) = 1.319$, NS = 0.297] (Figure 4A,C,E).

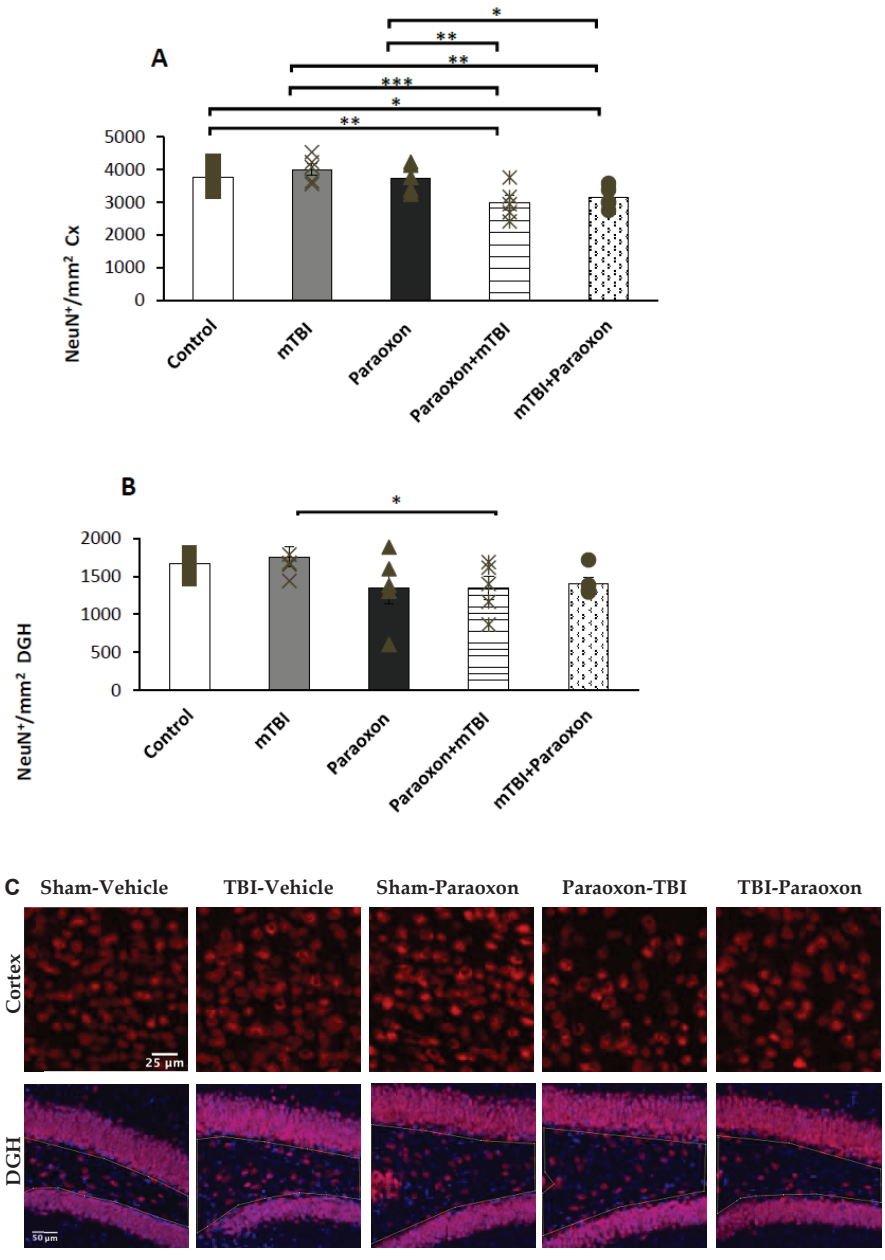


**Figure 3.** NeuN$^+$ counts in the cortex and hippocampus of control ($n$ = 5), mTBI ($n$ = 5), paraoxon ($n$ = 5), paraoxon + mTBI ($n$ = 5), and mTBI + paraoxon ($n$ = 5) mice. Paraoxon exposure before or after mTBI led to a significant decrease in the density of NeuN$^+$ neurons compared to control and mTBI tissues in the cortex. (**A**) Quantification of total surface area labeled with NeuN in the temporal cortex. (**B**) Quantification of total surface area labeled with NeuN in the DGH. (**C**) Representative images of immunohistochemical staining in the temporal cortex (upper panel) and DGH (lower panel). NeuN+ cells are shown in red, and nuclei are shown in blue. Yellow lines outline the hilus region of the DG. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

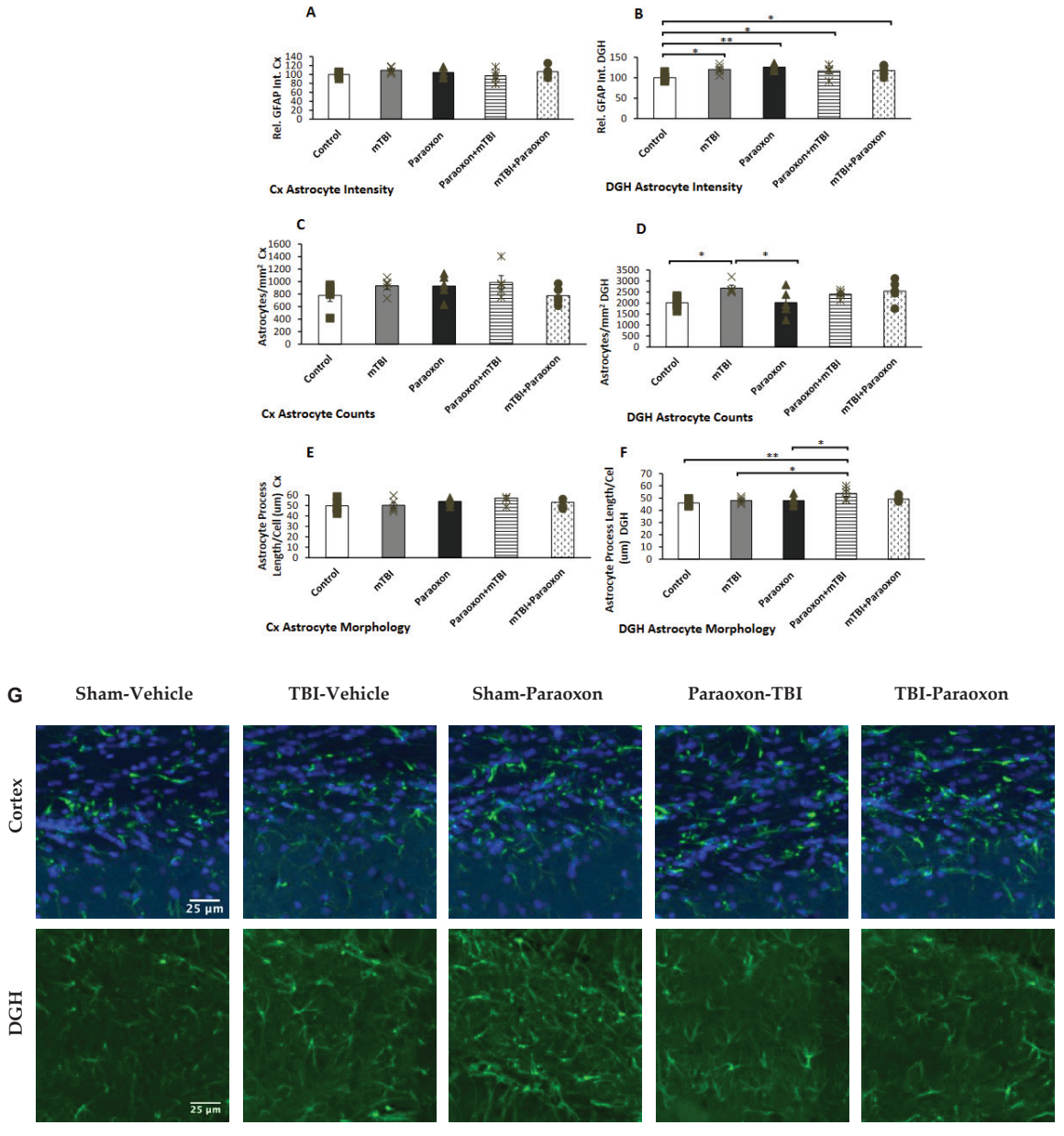


**Figure 4.** Astrocyte changes in the cortex and hippocampus of control ($n$ = 5), mTBI ($n$ = 5), paraoxon ($n$ = 5), paraoxon + mTBI ($n$ = 5), and mTBI + paraoxon ($n$ = 5) mice. Paraoxon increases active astrocyte expression in the DGH only and changes astrocyte morphology before mTBI. The graphs present quantifications of: (**A**) GFAP intensity in the temporal cortex; (**B**) GFAP intensity in the DGH; (**C**) astrocyte counts in the temporal cortex; (**D**) astrocyte counts in the DGH; (**E**) astrocyte morphology in the temporal cortex; and (**F**) astrocyte morphology in the DG. Representative images of immunohistochemical staining in the DGH and the temporal cortex are presented in (**G**). GFAP-positive cells are shown in green, and nuclei are shown in blue. Scale bars are 25 µm. * $p < 0.05$, ** $p < 0.01$.

## 4. Discussion

Recently, we have witnessed an increase in military conflicts. The resurgence of countries on the battlefield and the hazards related to the use of chemical or biological weapons highlight the importance of understanding the combined damage caused by OPs and mTBI. In this study, we traced patterns of damage that characterize these combined injuries. Using our mouse model, we tested spatial and visual recognition memory (NOR and Y-maze behavioral tests, Figure 2) at 30 days post-injury. We found that mTBI caused by a relatively minor weight drop (10 g) alone did not have any observable effect compared with the sham injury. In contrast, all groups exposed to paraoxon in combination with mTBI exhibited cumulative damage.

These results provide several valuable insights. First, the use of a 10-g weight to induce mTBI resulted in minimal to non-existent damage, confirming that the trauma given was indeed mild, as previously demonstrated by Tashlykov et al. (2009) [6]. This finding aligns with results from prior experiments in our laboratory which utilized weights up to 70 g [22,30,31]. Previous protocols that included weights from 30–70 g induced significant cognitive damage in the groups that received only the mTBI intervention compared to the control groups. In this study, no significant cognitive dysfunction was observed following a 10-g weight drop. However, mice exposed to paraoxon demonstrated significant spatial and visual memory impairments. These results indicate that isolated paraoxon exposure had a significant effect on memory, and that combined mTBI and paraoxon exposure resulted in additive repercussions. This was reflected both in the results of the behavioral tests and according to the immunohistochemistry tests in the cortex using NeuN antibody and DGH. In addition, immunohistochemical analysis (Figures 3 and 4) revealed that paraoxon exposure induced neuronal loss in the temporal cortex and neuroinflammatory reactions in the hippocampus at 30 days post-injury. These results are supported by literature showing that paraoxon exposure in a rat model reduced the survival rate of neurons and astrocytes in the cortex [32]. There was a modest reduction in NeuN+ cell density in the cortex after paraoxon + mTBI and after mTBI + paraoxon exposure relative to the control, while a similar trend was only found in the dentate gyrus hilus. Further research is warranted to investigate the underlying mechanisms responsible for the responses that we observed, such as whether the loss of neurons and astrocytes involves apoptosis or other mechanisms.

Research conducted after the Gulf War, during which Sarin (OP) or other toxicant exposures were likely combined with mTBI, supports our findings [33–35]. Approximately 25–32% of U.S. Veterans of the Gulf War suffered from a disorder known as Gulf War illness (GWI), which is characterized by multiple symptoms including fatigue, headaches, cognitive impairment, and musculoskeletal pain [33]. These studies examined the combined effect of mTBI + CBW (chemical–biological weapon) and demonstrated that such damage is associated with chronic morbidity, similar to the etiology of OP-induced damage combined with mTBI. Our observations significantly reinforce these findings by confirming tissue damage, which was not possible in early studies based on imaging and clinical evaluations of Veterans. The hypothesis that emerged from early studies was that GWI might be a disorder that is also expressed neuropathologically. More recently, animal studies have indicated that the effects of toxicant exposure in combination with TBI can lead to more severe consequences in the brain [36]. Both our behavioral and immunohistochemistry results support the additive effect of the combined insults. The significance of such a combined effect is important, given that the battlefield may expose individuals to unusual combinations of insults.

This study found no difference between paraoxon exposure before or after mTBI, but pronounced differences were observed when comparing both groups to the controls. This finding may support previous studies suggesting isoflurane administration as a treatment for neurotoxicity, as isoflurane was administered in proximity to paraoxon exposure as anesthesia prior to injury, potentially reducing brain damage [37,38]. However, this effect was not supported by immunohistochemistry, as we observed greater neuronal damage

after paraoxon exposure than after TBI, and more neuroinflammation was present when paraoxon exposure occurred after TBI.

The hippocampus (DGH) was selected for immunohistochemical analysis due to its role in memory and learning to understand and further investigate the deficits observed in the cognitive tests. Additionally, the temporal cortex was examined as the region of impact.

Spatial and visual memory deficits were more pronounced when paraoxon was administered after mTBI compared with exposure to mTBI alone. This finding supports the existing hypothesis that mTBI can cause blood–brain barrier dysfunction. Therefore, the combination of mTBI and paraoxon is more detrimental to spatial and visual memory [39].

Analysis of GFAP intensity in astrocytes showed that mTBI caused diffuse damage to the DGH, but no mechanical damage was found in the temporal cortex. In particular, in the dentate gyrus region, neuroinflammatory responses may account for the paraoxon-induced loss of neurons while GFAP immunoreactivity was elevated. Astrocyte numbers were similar between controls and paraoxon-treated animals. As shown in Figure 4B, paraoxon exposure alone resulted in greater damage than control, paraoxon + mTBI, or mTBI + paraoxon insults. The effects of paraoxon may have been mitigated by exposure to isoflurane, but this result is somewhat unclear.

In summary, these results suggest that paraoxon exposure, particularly when combined with mild TBI, may significantly affect neurological functions, including both spatial and visual memory. Animals that received both paraoxon and mTBI insults exhibited the most significant cognitive and behavioral deficits, suggesting an additive effect. Mice exposed to paraoxon with or without mTBI experienced a decrease in cortical neuronal survival, while those exposed to paraoxon and then mTBI exhibited a decrease only in the dentate gyrus hilus. All groups exposed to mTBI and/or paraoxon demonstrated increased neuroinflammation in the dentate gyrus. Animals exposed to paraoxon followed by mTBI showed significant changes in astrocyte morphology. Additional inflammatory markers, e.g., CD68 and gene expression changes, could be examined in future studies. These findings validate assumptions about the effects of paraoxon exposure in both war settings and pesticide exposure, while shedding light on the potentially more severe, additive damage resulting from a combination of OP and mTBI in modern warfare and exposure to pesticides.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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*Review*

# Brain Trauma, Glucocorticoids and Neuroinflammation: Dangerous Liaisons for the Hippocampus

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**Abstract:** Glucocorticoid-dependent mechanisms of inflammation-mediated distant hippocampal damage are discussed with a focus on the consequences of traumatic brain injury. The effects of glucocorticoids on specific neuronal populations in the hippocampus depend on their concentration, duration of exposure and cell type. Previous stress and elevated level of glucocorticoids prior to pro-inflammatory impact, as well as long-term though moderate elevation of glucocorticoids, may inflate pro-inflammatory effects. Glucocorticoid-mediated long-lasting neuronal circuit changes in the hippocampus after brain trauma are involved in late post-traumatic pathology development, such as epilepsy, depression and cognitive impairment. Complex and diverse actions of the hypothalamic–pituitary–adrenal axis on neuroinflammation may be essential for late post-traumatic pathology. These mechanisms are applicable to remote hippocampal damage occurring after other types of focal brain damage (stroke, epilepsy) or central nervous system diseases without obvious focal injury. Thus, the liaisons of excessive glucocorticoids/dysfunctional hypothalamic–pituitary–adrenal axis with neuroinflammation, dangerous to the hippocampus, may be crucial to distant hippocampal damage in many brain diseases. Taking into account that the hippocampus controls both the cognitive functions and the emotional state, further research on potential links between glucocorticoid signaling and inflammatory processes in the brain and respective mechanisms is vital.

**Keywords:** hippocampus; brain trauma; glucocorticoids; corticosterone; cortisol; stress; neuroinflammation; neurodegeneration; remote damage

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## 1. Introduction

Brain injury is a common cause of death and disability for people of all ages worldwide [1–3]. Depending on the biomechanics, brain lesions may occur both in areas of the brain directly adjacent to the place of force application and in remote areas [4]. The mechanisms of hippocampal damage are of particular importance, since they underlie late complications of traumatic brain injury (TBI), such as epilepsy, depression and cognitive impairment. The mechanisms of reorganization of neuronal networks in the hippocampus include long-lasting chronic neuroinflammation and secondary damage to the nervous tissue [5]. Responses and disturbances of the hypothalamic–pituitary–adrenal (HPA) axis may play a critical role in late post-traumatic pathology, in particular by modulation of synaptic activity and neuroinflammation in the hippocampus.

Even though stress-induced neuroinflammation and neurodegeneration in the hippocampus is fairly well described, and secondary hippocampal damage after TBI is studied by several groups, so far there have been no reviews on the glucocorticoid-dependent mechanisms of inflammation-mediated distant hippocampal damage with a focus on the consequences of TBI. PubMed search for the combination of words "TBI" (or "traumatic brain injury" or "brain trauma") and "corticosterone" shows no review papers. A search query for "TBI" and "glucocorticoids" results in 15 relevant reviews, though none deals

with the hippocampus. Ten of them are focused precisely on the clinical aspects of TBI: four reviews are related to pituitary disfunction after TBI [6–9]; three reviews are devoted to treatment [10–12], including usage of glucocorticoids (GCs) as medication; two reviews are focused on post-traumatic stress disorder [13,14]. Only five reviews include experimental studies; three of them are focused on the mechanisms of progesterone, dexanabinol and dexamethasone effects [15], TNF inhibitors [15] and mesenchymal stem cells in TBI [16]. One review describes the blood–brain barrier disfunction and the effects of GCs on its permeability [16]. The last and most relevant review was published in 2019 and was devoted to pro- and anti-inflammatory action of GCs after TBI [17]. Nevertheless, it lacks several important aspects of GC action on synaptic transmission and excitotoxicity in the hippocampus.

The aim of the present review was an analysis of the glucocorticoid-dependent mechanisms of inflammation-mediated distant hippocampal damage with a focus on the consequences of traumatic brain injury. In addition, we have systematically analyzed relevant papers resulting from a PubMed search for the combination of "TBI" and "corticosterone".

## 2. TBI, Its Late Consequences and the Hippocampus

Post-traumatic epilepsy (PTE) is a severe complication of traumatic brain injury (TBI). It occurs in 10–20% of patients after TBI [18]. About 57% of patients with PTE suffer from medial temporal lobe epilepsy diagnosed by the semiology of epileptic seizures, signs on the EEG and MRI [19]. Histological analysis reveals a specific epilepsy-related pathology of the hippocampus, hippocampal sclerosis, in at least half of these patients [20]. The main risk factors for PTE include the severity of injury, subdural, intracerebral hematomas and early seizures [18,21–24].

Major depressive disorder development after TBI is quite frequent. Over 50% of patients met major depression disorder criteria at least once, the disease being associated with poorer health-related quality of life [25]. Pathological changes in the hippocampus may represent a basis for post-traumatic depression [26], and, taking into account the common pathophysiological mechanisms, depression is considered the main comorbid pathology for epilepsy [27]. Interestingly, anxiety and depression in patients can be diagnosed before the diagnosis of epilepsy [28], thus confirming common bases of disease mechanisms but not a unidirectional causal relationship between depression and epilepsy.

The mechanisms of late TBI complications and reorganization of neuronal networks in the hippocampus include long-lasting chronic neuroinflammation and secondary damage to the nervous tissue [5]. The causes of chronic neuroinflammation development and circuit reorganization are obviously complicated and, so far, remain obscure. Recent data suggest that disturbance in the HPA axis function plays a critical role in late post-traumatic pathology.

## 3. HPA Axis in Patients with TBI

HPA axis (Figure 1) is the main neuroendocrine system of the organism implementing stress response and controlling adaptive mechanisms at different levels, from subcellular to the whole organism [29–31]. Normally, physiological stress is realized due to HPA action and release of glucocorticoids (GCs). Clinical studies of cortisol-dependent damage in TBI are limited, and the data are scarce and contradictory. Different groups report that cortisol level after TBI is decreased [32] or increased [33,34]. TBI is an acute physiological stress and is expected to increase cortisol levels, at least in TBI patients with preserved HPA axis function. However, some patients with TBI develop dysfunction of the anterior or posterior pituitary gland, which, in turn, leads to secondary hypocorticism (a decrease in cortisol levels due to a decrease in the production of pituitary adrenocorticotropic hormone, ACTH). Agha et al. [35] showed that ACTH and cortisol production after stimulation by glucagon in patients with TBI may be normal or reduced. In patients with a reduced response, the basal cortisol level after TBI was also decreased, but in patients with a normal response, it was increased. The risk factors for adrenal insufficiency and a decreased

cortisol level in the acute period of TBI are basal skull fractures, hypotension and the use of propofol [36]. Hydrocortisone replacement therapy may be associated with a favorable neurologic outcome after TBI, suggesting the involvement of corticosteroids in the consequences of brain trauma [37]. In general, signs of mild TBI, including absence of amnesia and a higher Glasgow coma scale score, are associated with higher cortisol levels [38], while the severity of coma positively correlates with acute cortisol level (within 6 h after TBI) [34]. On the contrary, during the first 3 days after TBI, the cortisol level is higher in patients with lower Glasgow coma scale score and predicts mortality [39].

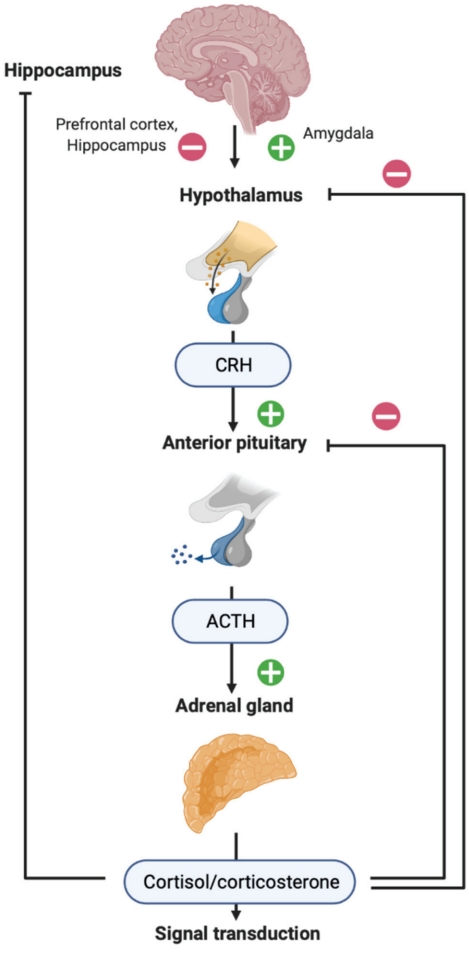


**Figure 1.** Hypothalamic–pituitary–adrenal (HPA) axis. Neuroendocrine response to stress includes the reaction of HPA axis: the release of hypothalamic corticotropin-releasing hormone (CRH), which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland and, finally, the release of glucocorticoids (GCs) from the adrenal glands (corticosterone in most rodents; cortisol in humans). GCs enter the blood circulation, implementing both peripheral and central action via specific receptors in almost all organs and tissues, including the brain. The prefrontal cortex, hippocampus and amygdala control the activity of the hypothalamus, thus regulating the HPA axis [29–31].

The time course of cortisol level during the first weeks after TBI also depends on the initial HPA axis state. In patients with stressful events prior to brain injury, cortisol levels

were significantly decreased, as compared with patients without stress before TBI [40], indicating stress-induced HPA dysfunction. In patients without stressful events before TBI, HPA function was preserved, and GC levels increased. Chronic HPA disturbances in patients with TBI are studied even less. In mild TBI, hair cortisol did not diverge before and months after TBI, its level reflecting individual coping with stress in general [41]. However, HPA dysregulation was shown two years after TBI, when hypocortisolemia and low diurnal GCs variability were detected [42]. Another study reported normal cortisol and circadian variations for two years after mild-to-moderate TBI, even with the presence of depression [43].

According to the data of basic experimental research, it can be assumed that an altered physiological response to acute stress may underlie some long-term effects of TBI. However, convincing clinical studies in this area are still lacking. Corticoid-related primary and secondary mechanisms of TBI, studied in animal models, are discussed below.

## 4. Distant Hippocampal Damage in Rodent TBI Models

Lateral fluid percussion brain injury model in rats [44,45] or mice [46,47] is the most conventional TBI model. A "golden standard" of TBA modeling, it allows studying the mechanisms of primary and secondary brain damage induced by TBI, though a few other models are also used [48].

Primary damage includes direct impact to brain tissue, which is accompanied by rupture of cell membranes, mechanical disruption of the blood–brain barrier, release of albumin and other blood components into the extracellular space. Acute damage causes severe metabolic disturbances inducing deficits in ATP production, energy deficiency and subsequent impairment of $Na^+/K^+$ ATPase, as well as an increase in the concentration of extracellular $K^+$. Changes in the extracellular $K^+$ cause depolarization of neuronal membranes and additional opening of voltage-gated calcium channels (VGCC), neurotransmitter release and a fast increase in the level of excitatory amino acids in the extracellular space [49]. Continuous changes in the concentration of extracellular ions further reduce the threshold of neuronal excitability and are aggravated by their repeated excitation. In addition, energy deficiency leads to the generation of free radicals and reactive oxygen species involved in oxidative stress and secondary damage to the nervous tissue [49]. Primary damage results in continued metabolic changes, excitotoxicity and the edema formation, inflammation, apoptosis and necrosis, representing the mechanisms of secondary brain damage.

TBI applied to the neocortex induces secondary, distant damage to the hippocampus. Neuronal death and glial activation are detected in the CA3 field and the dentate gyrus (DG) of the hippocampus [50]. Less pronounced changes are detected in the contralateral hippocampus [51,52]. Bilateral changes in the hippocampus after repeated TBI were also shown [53]. GABAergic neurons (parvalbumin (PV), calretinin, somatostatin and neuropeptide Y-immunoreactive) in the polymorphic layer of the DG are among the most vulnerable populations of hippocampal neuronal cells. Previously, we described the development of distant hippocampal damage after lateral fluid percussion brain injury in rats [54,55]. Selective neuronal cell loss in the polymorph layer of the hippocampal DG was demonstrated bilaterally; in the ipsilateral hippocampus, it was evident on day 3, but in the contralateral hippocampus, these changes were delayed and detected on day 7. Microglial activation was evident in the hippocampus bilaterally on day 7 after TBI, while pro-inflammatory cytokines mRNA levels increased bilaterally from day 1 after TBI.

It is worth noting that distant damage to the hippocampus has been reported to be a result of different extremal factors, including brain ischemia [31]. Remote hippocampal damage is a well-documented consequence of chemoconvulsant injection (kainate [56], dendrotoxin [57], pentylentetrazole [58,59]). Neuronal death [60], excitotoxicity and the involvement of glutamate receptors in distant hippocampal damage were shown after TBI in rats [61,62], indicating the similarity of damage mechanisms, irrespective of primary injury nature. In general, many epileptogenic lesions are characterized by secondary neuronal death in the DG, both in experimental and clinical settings [63]. The involvement of both

NMDA and non-NMDA receptors [53,56], excitotoxicity and spreading of epileptiform activity are [64,65] discussed as mechanisms of the damage. Acute and excessive release of glutamate and aspartate leads to the activation of glutamate receptors (primarily AMPA receptors) and depolarization of neuronal membrane. Activation of glutamate, kainate and calcium-permeable AMPA receptors contributes to an increase in intracellular levels of calcium, a universal secondary messenger [49]. Excessive intracellular calcium concentration activates phospholipases, endonucleases and proteases (calpains), accelerating neuronal death as a result of excitotoxicity by apoptotic and necrotic mechanisms [66–68].

Thus, excessive glutamate level after TBI is a trigger for secondary neurodegeneration, which involves death of GABAergic neurons. Based on (1) the relative selectivity and distant character of hippocampal damage; (2) lack of specificity regarding the type of primary impact inducing distant hippocampal damage and (3) the involvement of both ipsilateral and contralateral hippocampus, it can be assumed that there are common ***systemic*** mechanisms underlying selective death of hippocampal neurons and, possibly, the development of chronic neuroinflammation. This selectivity may be explained by the effects of GCs, affecting the hippocampus and functional properties of the hippocampal networks [26,31].

**5. Glucocorticoid Signaling, Hippocampus and Neuronal Death**

GCs (corticosterone, CS, in most animals, and cortisol in humans) are essential hormones in all vertebrates, regulating two basic systems: glucose metabolism and immune response. In general, GCs suppress inflammation and increase blood glucose level by stimulating gluconeogenesis and inhibition of glucose uptake by cells [69]. Specific receptors mediating signals of GCs are present in most cells of the organism. GCs regulate the behavioral response to stress, and their receptors are widely expressed in the brain. The effects of GCs are critically determined by the specific aspects of their action [70]. During the stress response, GCs modulate the hippocampal function, affecting numerous signaling and metabolic systems [31]. It is also important that, unlike other brain structures, the basal membrane covers only 30% of the vascular surface in the hippocampus [71], which facilitates the penetration of hormones into hippocampal neurons.

The functions of the GCs in the hippocampus are mediated by high-affinity mineralocorticoid (MR) and low-affinity glucocorticoid receptors (GR). MRs bind GCs at low hormone levels, while the affinity of GRs to GCs is much lower, and the activation of these receptors occurs when GC levels increase, for example, during stress response. Each type of corticosteroid receptor is presented by two forms: intracellular cytoplasmic/nuclear receptors, exerting primarily slow genomic action (iMR, iGR), and non-genomic membrane-bound receptors (mMR, mGR), rapidly altering excitatory neurotransmission [29,72,73]. The affinity of membrane-bound and intracellular receptors decreases in the order: iMR > iGR = mMR > mGR [30] (Figure 2). The genomic binding loci of GR and MR comprise hundreds of partially overlapping DNA sites changing during the circadian cycle and stress [74]. Intracellular MRs and GRs translocate to the nucleus, where they act as nuclear transcription factors and modify gene expression, affecting protein synthesis. The genomic effects are realized within hours and may persist for many days, underlying adaptation, synaptic and cellular plasticity. Membrane-associated GRs and MRs act through G-proteins and affect the ion channels, rapidly modulating cell excitability. In general, the effects of GCs on specific cell populations depend on: (1) their concentration, (2) duration of exposure, (3) cell type with definite balance of specific intracellular and extracellular GRs and MRs.

MRs are expressed mainly in the brain regions that are crucial to the formation of memory and emotions, such as the hippocampus, amygdala, frontal, enthorinal and insular cortex. GCs trigger rapid non-genomic effects on excitability of neurons in brain through mMRs, thus influencing the cognitive and emotional functions and adaptive behavior within minutes. Besides limbic structures, GRs are also expressed in the prefrontal cortex and are involved in cognitive and executive functions, such as reasoning and attention [30,75]. GRs are involved in negative feedback on HPA axis; the amygdala stimu-

lates the HPA axis, whereas both the hippocampus and prefrontal cortex have inhibitory effects [76,77]. It is noteworthy that after TBI in rats, CRH may increase the excitability of the amygdala and hippocampus [78].

The group of R. Sapolsky studied the effects of stress in animals for many years and showed that sustained exposure to stress induces neuronal loss in the hippocampus [79,80]. Chronic stress resulted in about 20% loss of neurons in CA3 field of the hippocampus of rats, and GCs also worsened other types of damage produced by ischemia, seizures or excitatory amino acids in CA1 and CA3 fields. The authors explained these effects by suppression of glucose transport, changes in calcium metabolism and suppression of neurotrophic factors expression [79,81,82]. Since neuronal energy metabolism is almost exclusively dependent on oxidative phosphorylation, and neurons have almost no glucose storage, they are the cells most vulnerable to energy restriction. Adaptive processes are highly energy dependent, and GCs may worsen neuronal survival.

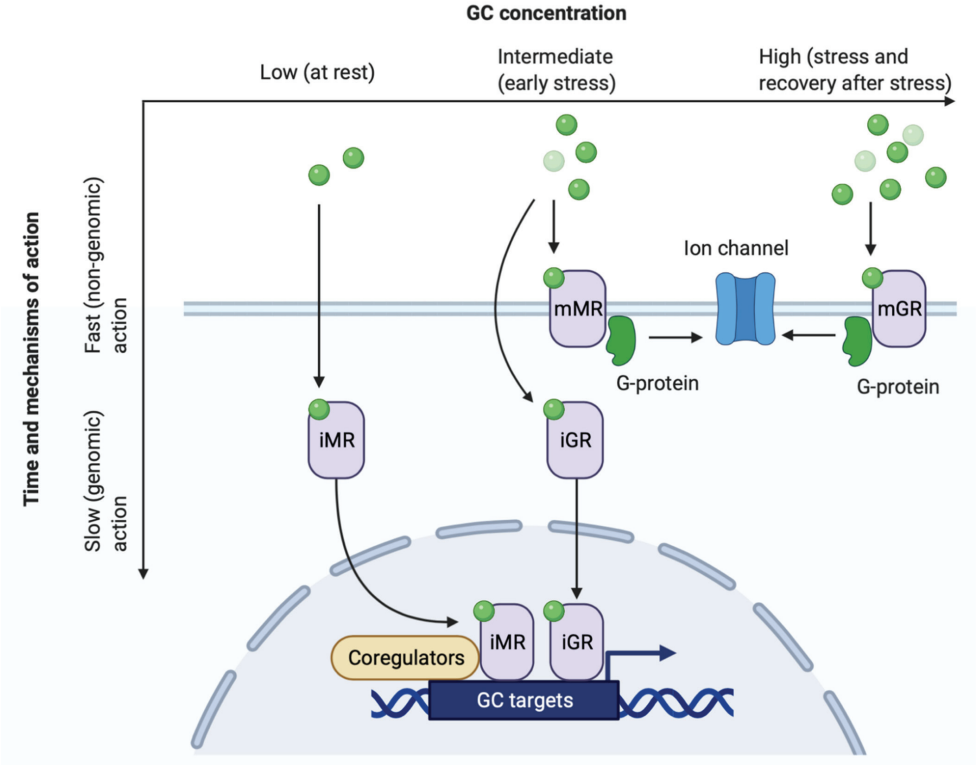


**Figure 2.** Receptors of glucocorticoids [29–31,83]. See details in Chapter 5. Green circles—glucocorticoid molecules.

Another mechanism of GCs-dependent neurodegeneration may be the elevation of intracellular calcium and glutamate excitotoxicity. GCs dose-dependently affect the excitability of neurons: glutamatergic synaptic transmission is enhanced by intermediate doses of GCs acting on mMR (rapid response to stress) and is reduced by higher doses acting on GR in addition to the already activated MRs (recovery after a stressful situation) [72,84] (Figure 2).

The influence of GCs on neuronal excitability was extensively studied by the M. Jöels group. The effects of GCs on CA1 pyramidal neurons are explained by a U-shaped curve: very low (not physiological, e.g., after adrenalectomy), as well as very high CS levels suppress neuronal activity, but intermediate doses increase the amplitude of excitatory

postsynaptic potentials (EPSPs) [83,85,86] (Figure 3). In animals subjected to chronic stress, changes of pyramidal neurons are rarely found. In pyramidal neurons of CA3 field, EPSP amplitude increased, likely due to NMDA action in chronically stressed animals [87] (Figure 4).

In granular neurons of the DG, MR-dependent effects on field potentials caused by the activation of AMPA receptors were demonstrated [83,85]; however, they are almost insensitive to physiological GS changes, including acute stress. In adrenalectomized rats, extremely low level of GCs reduced neuronal activity of granular cells [88] (Figure 3). In contrast, in animals exposed to chronic stress, GCs enhanced glutamatergic AMPA-mediated signaling in granular cells [89] (Figure 4).

Voltage-gated calcium channels (VGCC) are among the principal players involved in the control of calcium homeostasis. They are activated at depolarized membrane potentials and become permeable for calcium. Amplitudes of VGCC currents increased after 1–4 h of exposure to CSs, more likely through iGR signaling [90]. In chronic stress, GCs also increased calcium currents through VGCC, both in granular cells of the DG [91] and in pyramidal cells of CA1 field [92]. Thus, VGCC activation increases risk of cell death at glutamate excess, especially in chronic stress.

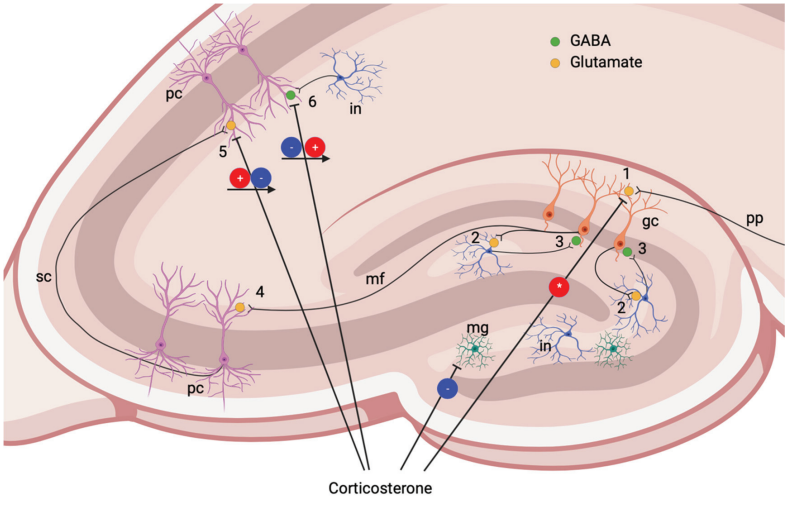


**Figure 3.** Corticosterone effects in the hippocampus at rest and during acute stress. 1, Glutamatergic synapse on granular cell. In normal conditions, granular cells are almost insensitive to physiological GSs changes, including acute stress, but extremely low levels of GCs reduce neuronal activity of granular cells [88]. 2, Glutamatergic collateral to an interneuron. 3, GABAergic synapse on granular neuron. Though little data on the modulation by GCs of collateral inhibition in the DG are available, the effects of GCs on pyramidal cells of CA1 field may be similar. 4, Glutamatergic synapse on pyramidal neuron of CA3 field. 5, Glutamatergic synapse on pyramidal neuron of CA1 field. Pyramidal neurons demonstrate U-shaped modulation by GCs: very low (not physiological), as well as very high CS levels suppress neuronal activity, but intermediate doses increase EPSP amplitude [83,85,86]. 6, GABAergic synapse on pyramidal neuron. GCs may temporarily reduce IPSP with subsequent rapid or slow elevation of inhibitory postsynaptic potential (IPSP) amplitude [93,94]. pp, perforant path; gc, granular cell; mf, mossy fibers; pc, pyramidal cells (CA3 and CA1 fields), sc, Schaffer collateral; in, interneuron. Red circle (+)—activating action; red circle (*)—activation by very low GC levels; blue circle (−)—inhibiting action; arrows show changes in GC action with increasing concentration or over time.

Thus, GC excess plays an essential role in the selective vulnerability of the hippocampus, promoting calcium overload, energy deficits and secondary death of neurons [95]

by increasing the susceptibility of neurons to glutamate excitotoxicity [96]. Importantly, glutamatergic axons terminate on interneurons (Figure 3), and excitotoxic damage affects primarily GABAergic interneurons, the most vulnerable population of cells in the hippocampus.

On the other hand, GCs impair the protective activation of inhibitory neurotransmitter systems during insults [79]. Patch-clamp recordings show that GCs, through the activation of MRs, reduced the frequency of spontaneous inhibitory postsynaptic potentials (IPSP) in pyramidal cells of CA1 field of the ventral hippocampus (more likely due to membrane-associated receptors) but not in the dorsal part. The effect of a GR agonist was different: it slowly increased IPSP magnitude in the hippocampus, more likely through iGRs [93]. Another group demonstrated rapid increase in spontaneous inhibitory postsynaptic currents (IPSCs) via mGR in CA1 pyramidal cells [94] (Figure 3). The authors explained that the controversial result of rapid GC action is likely due to the difference in experimental conditions.

In chronic stress, rhythmic IPSCs originating from the PV-positive GABAergic neurons was impaired due to selective PV-positive cell loss, demonstrating lack of inhibition in CA1 [94] (Figure 4). The authors explained the selective loss of PV-positive neurons by the sustained activation of interneurons and imbalance in perisomatic inhibition.

Thus, GCs modulate the excitability of the hippocampus in acute and chronic stress and enhance glutamate excitotoxicity, potentially causing selective neurodegeneration in the hippocampus. Since TBI increases GC levels in humans and in animal models [54,55], the consequences of stress can be considered as one of the secondary brain injury mechanisms.

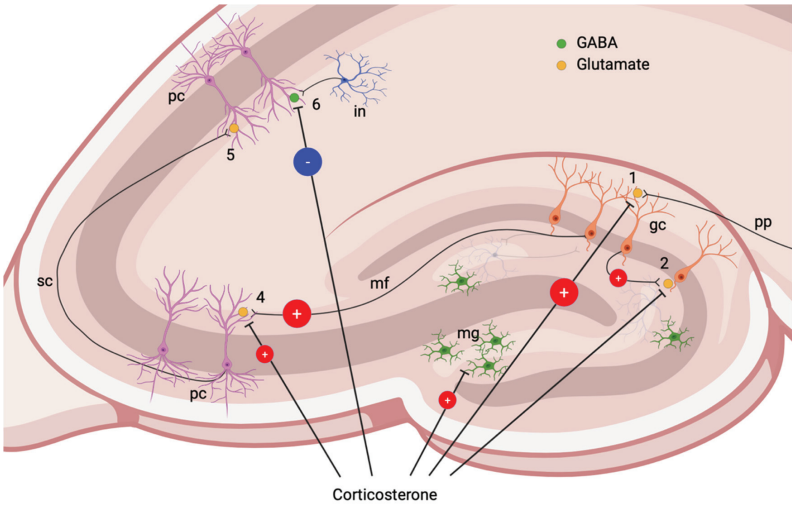


**Figure 4.** Effects of GCs in chronic stress and structural post-traumatic changes in the hippocampus. GABAergic neuronal loss in the DG and neuroinflammation are histological hallmarks of late post-traumatic changes. 1, Glutamatergic synapse on granular cell. GCs enhance glutamatergic AMPA-mediated signaling [89]. 2, Glutamatergic collateral on granular neuron (mossy fiber sprouting) enhances DG excitability [97]. 4, Glutamatergic synapse on pyramidal neuron of CA3 field. Chronic stress increases EPSP amplitude via NMDA-dependent signaling [87]. Failure of inhibition due to GABAergic neuronal loss is demonstrated [50,98]. 5, Glutamatergic synapse on pyramidal neuron of CA1 field. Though little data on the modulation by chronically elevated GCs of glutamatergic synapses in the CA1 are available, the effects of GCs on pyramidal cells of CA3 field may be similar. 6, GABAergic synapse on pyramidal neuron. Rhythmic IPSCs due to loss of interneurons are demonstrated [94]. pp, perforant path; gc, granular cell; mf, mossy fibers; pc, pyramidal cells (CA3 and CA1 fields); sc, Schaffer collateral; in, interneuron. Red circle (+)—activating action; blue circle (−)—inhibiting action.

**6. Neuroinflammation and TBI**

Both primary and secondary mechanisms of brain damage discussed above are associated with neuroinflammatory response. Neuroinflammation is one of the essential mechanisms of brain damage modulated by GCs. Under normal conditions, microglial activity and cytokine release are intimately associated with neuroplasticity and memory; however, uncontrolled excessive neuroinflammation leads to cell death and further progression of brain pathology [99]. In pathological conditions, microglia play a crucial role in the expression of both pro-inflammatory (IL-1ß, IL-6, TNFα) and anti-inflammatory (IL-4, IL-10) cytokines, chemokines, as well as molecular fragments associated with damage (DAMP, damage-associated molecular patterns, such as HMGB1, ATP, S100ß). These substances enable microglial modulation of cyclooxygenase-2 and the components of the complement system [49]. Cytokines produced by inflammatory cells are released within minutes after TBI and alter the functioning of glutamate and GABAergic receptors, as well as potential-dependent ion channels, inhibit the reuptake of glutamate by astrocytes and provoke an increase in extracellular $K^+$. Thus, cytokines can participate in hypersynchronization of neurons and the occurrence of epileptiform activity [100], as well as contribute to further neurodegeneration [101]. Microglial activation also occurs in remote areas of the brain, microglial properties and cytokine profile changing over time [102].

In the area of TBI, neutrophils and other cells of the immune system are recruited as well. These cells, along with glia, take part in the production of cytokines, chemokines, free radicals, prostaglandins and components of the complement system. The profile of peripheral immune system cells changes over time. Neutrophils first appear in the focus of injury; after 3–5 days they are replaced by mononuclear leukocytes and, to a lesser degree, by T cells, dendritic cells and natural killers [49]. The peripheral immune system is also activated. It has been shown that 1 day after TBI, the number of CD4+ and CD8+ T cells in rat spleen increases, indicating an activation of adaptive immunity. Suppression of adaptive immunity improves TBI outcomes [103]. Thus, autoimmune mechanisms are involved in the development of post-traumatic pathology [49], though their role has not been studied in detail yet.

Astrocytic gliosis in the neocortex develops about 1 week after TBI and, in the long-term period of injury astrogliosis, serves as an important histopathological marker of hippocampal sclerosis [104–106]. Astrocyte dysfunction may be involved in increasing the excitability of neurons and circuit reorganization via several mechanisms. Astrocytes normally participate in the utilization of extracellular $K^+$ (due to active transport into the cell and distribution through the astrocyte system) and utilization/metabolism of glutamate. Changes in $K^+$ homeostasis and an increase in its concentration lead to a decrease in neuronal excitability threshold, while impairment of glutamate utilization results in an increase in its toxic effects. In addition, astrocytes play an important role in water homeostasis of the brain [107] and form the brain glymphatic system involved in the development and resorption of edema, transport of metabolites and immune cells [108].

The transition from acute activation of the brain immune system to chronic neuroinflammation in TBI is the subject of quite a few studies [5,17,109]. Chronic neuroinflammation caused by TBI induces progressive edema and neurodegeneration associated with cognitive and emotional disorders [110]. The first week after TBI is an important time interval, day 7 being considered a borderline between acute and chronic post-traumatic changes. It is noteworthy that edema resorption and the early development of astrogliosis in the focus of direct impact to the neocortex was shown 7 days after TBI [104–106].

**7. Neuroinflammation and GCs**

Chronic neuroinflammation is a recognized consequence of chronic stress; its definitive association with GCs is rigorously discussed but still remains obscure [111]. The available data indicate dual effects of GCs, both anti- and pro-inflammatory. Suppression of inflammation is among the well-established systemic effects of GCs. This ability of GCs is widely used in clinical practice for treatment of inflammatory and autoimmune diseases.

The activation of GRs and MRs in peripheral tissues results in inhibition of immune cell activity and induction of apoptosis in lymphocytes [112]. GCs also inhibit inflammation via several other mechanisms, including inhibition of tissue infiltration by cells from the blood, inhibition of cytokine expression, changes of lymphocyte functioning and others [111]. In the brain, GCs realize either pro- or anti-inflammatory properties depending on the degree and duration of exposure, external factors preceding injury, injury characteristics and the specific brain region [31,70,111].

The order and time period between GC increase and immune challenge may be important for the effects of GCs on neuroinflammation (Figure 5). This was confirmed in a study with administration of GCs and lipopolysaccharide (LPS, immunogenic component of Gram-negative bacteria) in a different order [113]. If GCs were injected prior to LPS (2 and 24 h), they potentiated pro-neuroinflammatory effects (TNFa, IL-1b, IL-6 expression). In contrast, GCs injected 1 h after LPS had an anti-inflammatory action in the brain. LPS injection directly into the hippocampus of the stressed animals also increased the number of reactive microglial cells and expression of pro-inflammatory cytokines [114] as compared to non-stressed animals. The second factor affecting GCs action is the duration of their exposure (Figure 5). Many groups have demonstrated that chronic stress is definitely a pro-inflammatory condition [111]. Chronic stress potentiated LPS-induced activation of several pro-inflammatory pathways, including nuclear factor kappa B (NF-κB) [115], and increased basal activation of other intracellular pathways, such as ERK1/2, p38, SAPK/JNK and AKT [116].

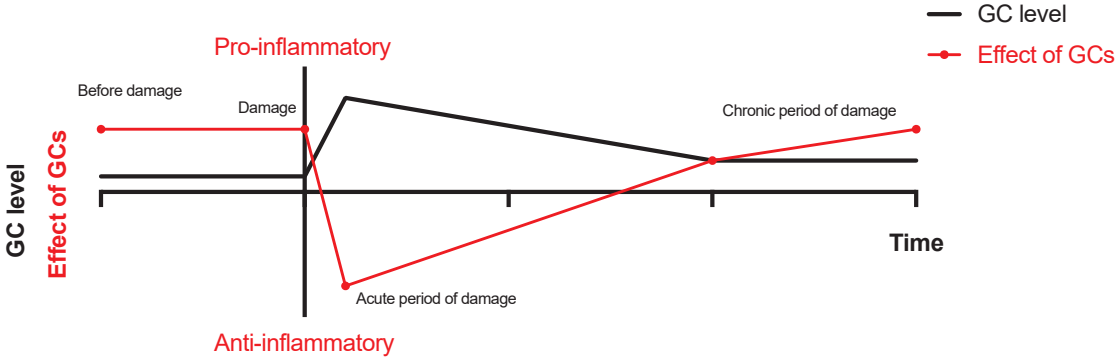


**Figure 5.** Effects of GCs on neuroinflammation depend on time of damage [54,55,111,113]. Timing of GCs exposure is critical for its pro- or anti-inflammatory action in the brain.

The interaction between GCs and the inflammatory mechanisms seems really intricate. Dexamethasone injected directly into the rat hippocampus was able to induce weak neuroinflammation but, when applied during LPS-induced neuroinflammation, evoked differential effects on pro-inflammatory cytokines expression [117]. Systemic administration of dexamethasone for 3 weeks in mice, mimicking chronic stress, induced depressive-like behavior and glucocorticoid resistance, a potential priming factor enhancing inflammatory response [118]. After ten days of corticosterone exposure in adrenalectomized rats, GCs, in a dose-dependent manner, primed microglia to pro-inflammatory stimuli by gene expression associated with inflammation (NLRP3, Iba-1, MHCII and NF-κB), thus potentiating microglial pro-inflammatory response to LPS [119]. Interestingly, diffuse TBI also primes microglia and promotes depressive-like behavior after secondary LPS-induced inflammatory challenge 1 month after trauma [120].

Recent information about relationships between inflammation, GCs and TBI is scarce. CS increased 1 hour after TBI, and its level negatively correlated with the number of peripheral T cells, confirming the anti-inflammatory effect of GCs [121]. The number of circulating T cells positively correlated with TBI core infiltration and destructive neuroinflammatory response in the brain.

Using the lateral fluid percussion model of TBI, we showed CS elevation in the blood and the hippocampus on day 3 after TBI [54,55]. The correlations between CS and neuroinflammatory response in the hippocampus were time dependent and vague. On day 3, the blood CS level negatively correlated with microglial cell count in the hippocampus. In contrast, on day 7 after TBI, when CS almost returned to baseline, noticeable and bilateral microglial activation was detected. The levels of IL-1β in the contralateral hippocampus positively correlated with CS in the same region. These results may reflect an early anti-inflammatory and latter pro-inflammatory effect of CS in TBI (Figure 6).

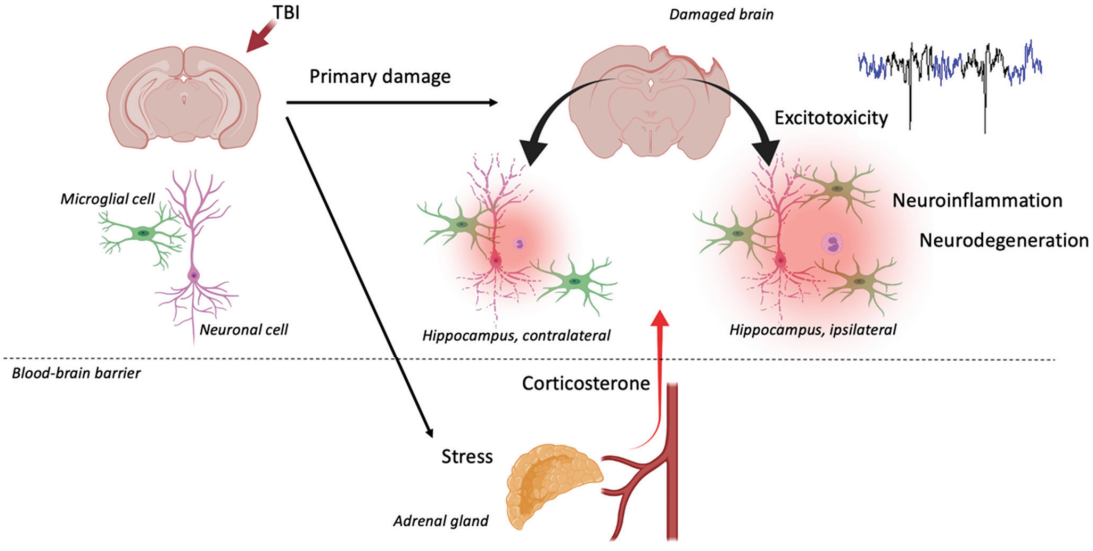


**Figure 6.** Local and systemic effects of TBI ([54,55]). Based on (1) selectivity and distant character of hippocampal damage, (2) lack of specificity to the type of primary impact leading to distant hippocampal damage and (3) involvement of both ipsilateral and contralateral hippocampus in models of unilateral primary neocortical injury, it can be assumed that there are common CS-dependent mechanisms underlying selective death of hippocampal neurons and chronic neuroinflammation.

Since GCs modulate the secondary mechanisms of damage, the HPA axis state during trauma is also an important factor for GC action. On the one hand, the time course of cortisol levels after TBI depends on the initial HPA state; in patients experiencing stressful events before brain injury, acute cortisol levels significantly decreased during the acute period of TBI [40]. Thus, patients with previously activated HPA demonstrate impaired stress reactivity. This may defeat the positive effects of GCs (early anti-inflammatory action) and enhance negative ones (e.g., enhancement of excitotoxicity). On the other hand, moderate stress may increase the resistance of neurons to brain insults and protect from further excitotoxic damage; the expression of cytokines and neurotrophic factors may underlie the protective effects of mild stress [122].

## 8. CS Changes and Associated Events in Animal Models of TBI: Summary Table

Additionally, we have summarized the data generated from systematic analysis of all 48 relevant papers resulting from the PubMed search for the combination of "TBI" and "corticosterone" (21 of them combined with "hippocampus" and 7 of them with "neuroinflammation"). The results on CS changes are presented in Table S1 (Supplementary Material). In addition to CS alterations, the cellular, molecular and behavioral changes revealed in these papers are shown in the last column. The data are different and sometimes appear contradictory; however, the main reasons for discrepancies seem to be significant

differences in the aims of the studies and, hence, in the experimental designs used by different groups.

## 9. Conclusions: TBI and Beyond

In this review, we discussed GC-dependent common mechanisms of stress- and inflammation-mediated distant hippocampal damage, focusing on the consequences of TBI. The effects of GCs on specific neuronal populations in the hippocampus depend on GC levels, duration of GC exposure and cell type (in particular, the balance of specific intracellular and extracellular GR and MR). Pro- or anti-inflammatory effects of GCs also depend on their concentration and exposure duration. Previous stress and elevated GC level prior to pro-inflammatory impact may inflate pro-inflammatory effects. Long-term and moderate elevation of GCs may also enhance neuroinflammatory response. GC-mediated long-lasting neuronal circuit changes in the hippocampus after TBI are involved in late post-traumatic pathology development, such as epilepsy, depression and cognitive impairment. Complex and diverse actions of HPA axis on neuroinflammation may be essential for late post-traumatic pathology.

Importantly, these mechanisms are applicable to remote hippocampal damage occurring after other types of focal brain damage (stroke, epilepsy) or central nervous system diseases without obvious focal injury (e.g., infections). Secondary damage to the hippocampus is shown in the middle cerebral artery (MCAO) model in rats [123,124]. MCAO induces accumulation of the pro-inflammatory cytokine IL-1β accompanied by elevated CS at the early and delayed stages of stroke [124]. High initial level of GCs and previous stress exacerbate damage to the hippocampus after brain strokes in humans [125] and rats [126].

Thus, the liaisons of excessive GCs /dysfunctional HPA axis with neuroinflammation, dangerous to the hippocampus, may be crucial for distant hippocampal damage in many brain diseases. Taking into account that the hippocampus controls both the cognitive functions and the emotional state, further research of potential links between GC signaling and the inflammatory processes in the brain and respective mechanisms is vital.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines10051139/s1, Table S1: Corticosterone changes in TBI models [127–171].

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## Abbreviations

| | |
|---|---|
| ACTH | adrenocorticotropic hormone |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| BDNF | brain-derived neurotrophic factor |
| CRH | corticotropin-releasing hormone |
| CS | corticosterone |
| DAMP | damage-associated molecular patterns |
| DG | dentate gyrus, hippocampal field |
| EPSP | excitatory postsynaptic potential |
| GABA | gamma-Aminobutyric acid |
| GCs | glucocorticoids |

| | |
|---|---|
| GR | glucocorticoid receptor |
| HPA | hypothalamo-pituitary axis |
| IL-1ß | interleukin 1 beta |
| IL-6 | interleukin 6 |
| iMR, iGR | intracellular cytoplasmic/nuclear receptors subtype |
| IPSC | inhibitory postsynaptic current |
| IPSP | inhibitory postsynaptic potential |
| LPS | lipopolysaccharide |
| MCAO | middle cerebral artery |
| mMR, mGR | membrane-associated receptors subtype |
| MR | mineralocorticoid receptor |
| NF-κB | nuclear factor kappa B |
| NMDA | N-methyl-D-aspartate |
| PTE | post-traumatic epilepsy |
| PV | parvalbumin |
| TBI | traumatic brain injury |
| TNFα | tumor necrosis factor alpha |
| VGCC | voltage-gated calcium channels |

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# Alcohol-Induced Alterations in the Vascular Basement Membrane in the *Substantia Nigra* of the Adult Human Brain

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**Abstract:** The blood–brain barrier (BBB) represents a highly specialized interface that acts as the first line of defense against toxins. Herein, we investigated the structural and ultrastructural changes in the basement membrane (BM), which is responsible for maintaining the integrity of the BBB, in the context of chronic alcoholism. Human post-mortem tissues from the *Substantia Nigra* (SN) region were obtained from 44 individuals, then grouped into controls, age-matched alcoholics, and non-age-matched alcoholics and assessed using light and electron microscopy. We found significantly less CD31+ vessels in alcoholic groups compared to controls in both gray and white matter samples. Alcoholics showed increased expression levels of collagen-IV, laminin-111, and fibronectin, which were coupled with a loss of BM integrity in comparison with controls. The BM of the gray matter was found to be more disintegrated than the white matter in alcoholics, as demonstrated by the expression of both collagen-IV and laminin-111, thereby indicating a breakdown in the BM's structural composition. Furthermore, we observed that the expression of fibronectin was upregulated in the BM of the white matter vasculature in both alcoholic groups compared to controls. Taken together, our findings highlight some sort of aggregation or clumping of BM proteins that occurs in response to chronic alcohol consumption.

**Keywords:** vascular basement membrane; collagen-IV; laminin-111; fibronectin; substantia nigra; alcoholism; light microscopy; electron microscopy

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## 1. Introduction

The blood–brain barrier (BBB) represents a highly specialized, organized, and dynamic interface that is involved in the regulation, homeostasis, and protection of the central nervous system (CNS). The barrier is composed of three different cell types, namely endotheliocytes, pericytes, and astrocytes, all of which interact with each other to maintain the function and integrity of the barrier [1,2]. However, it has been demonstrated that the BBB, in fact, is a part of a larger functional structure called the neurovascular unit (NVU), first described in 2001 by the Stroke Progress Review Group [3]. The different components of the NVU, including neurons and perivascular cells such as microglia and the basement membrane (BM), together with astrocytes, pericytes, and specialized unfenestrated endothelium, share complex and intimate associations that allow them to regulate blood flow along with the permeability of the BBB [3–5]. Under physiological conditions, the NVU prevents the entry of neurotoxic substances, blood cells, and pathogens into the brain parenchyma, allowing restricted transport of water, certain gases, ions, and molecules in and out of the CNS to maintain proper neuronal functioning [6–9].

However, in states of disease, trauma, or stress, the structural integrity of the BBB can be compromised, leading to barrier breakdown and dysfunction. Ultimately, this dysregulated BBB allows unrestricted movement of neurotoxins into the brain parenchyma,

thereby attenuating and disrupting the signaling pathways involved in ensuring brain connectivity [10,11]. One such causative agent for BBB stress is alcohol (ethanol), a commonly abused psychoactive neurotoxin known to be associated with neuroinflammation and multiple neurodegenerative diseases, including Parkinson's disease (PD), Alzheimer's disease (AD), and epilepsy [12–15]. Previous studies have shown that alcohol and other similar drugs-of-abuse can cause a dose- and distribution-dependent dysfunction of the NVU by causing oxidative stress, altering protein expression, or dysregulating the endothelial tight junctions that bind the barrier [5,16–19].

The first line of contact, the endothelial cells in the BBB, are surrounded by a complex BM matrix comprising a three-dimensional network of a range of glycoproteins that include collagen-IV, laminin, and fibronectin [20]. These extracellular matrix (ECM) glycoproteins are mainly synthesized by the perivascular cells [21–23]. Collagen-IV is the most abundant fibrous protein in the BM and is secreted by endothelial cells, astrocytes, and pericytes. It plays an essential role in providing structural strength and integrity to the ECM by retaining laminin and other ECM proteins [24,25]. Laminins are the most abundant non-collagenous BM proteins. They are trimeric molecules consisting of $\alpha$, $\beta$, and $\gamma$ subunits. Out of the 16 possible different laminin isoforms, two isoforms, laminin-111 and laminin-211 ($\alpha1\beta1\gamma1$ and $\alpha2\beta1\gamma1$, respectively), are unique to the BBB and are produced by perivascular astrocytic cells [23]. These astrocytic laminins play a crucial role in maintaining BBB integrity and regulating pericyte function and astrocytic polarity [23]. Fibronectin is a disulfide-linked dimer secreted by endothelial cells, astrocytes, and pericytes [26]. Along with collagen-IV, fibronectin provides a structural support to the BBB. It mediates cell–cell attachment and functions to provide the organization of the ECM. Additionally, it stimulates the proliferation and survival of brain capillary endothelial cells in vitro [27].

In previous studies, it has been shown that the thicknesses of the glycoproteins containing BM ranges from 20 to 200 nm [21,28]. However, under pathological conditions such as AD and cerebral amyloid angiopathies, the BM thickness has been reported to increase, as evident from studies involving various animal models, human stroke tissue, and aging brain tissue [24,29–33]. In the AD brain, the degenerating endothelium has been postulated to cause disintegration of the cerebral BM, whilst an imbalance in lipid metabolism has been postulated as the cause of age-related lipid accumulation in the nearby end-feet of astrocytes, which leads to structural alterations in the BM [34,35]. In addition, various NVU-related factors such as activated matrix metalloproteinases, secreted inflammatory cytokines, extravasation of immune cells, and pathogens can play significant roles in the alteration and disruption of the BBB [34].

As part of the midbrain, the *Substantia Nigra* (SN) is located posteriorly to the *crus cerebri* fibers and morphologically divided into the dopaminergic *Pars Compacta* (SNpc) and inhibitory gamma-aminobutyric acid-containing (or GABAergic) *Pars Reticulata* (SNpr) [36–38]. The SN region plays an important role in a wide range of physiological processes, including movement control, learning, developing substance dependence, reward-seeking, and cognitive functioning [39–42]. Previous studies have shown the critical role of the SN region in addiction development or alcohol consumption processes, especially in correlation with other neurodegenerative disorders, including AD and PD [14,43]. Alcoholic beverages are rich in $\beta$-carbolines and their derivatives, which upon in vivo metabolization, form compounds resembling 1-methyl-4-phenylpyridinium ions (MPP+), neurotoxicants involved in the pathogenesis of idiopathic PD [44]. Additionally, in a number of aging-related and neurodegenerative diseases, it has been shown that there are significant changes in the microvascular length, tortuosity, and diameter in a region-specific manner [45]. At the same time, very limited data have been reported regarding the alcohol-specific effects on the network and density of the microvasculature, as well as the effects on the structural integrity and thickness of the BM and expression of ECM proteins in the human brain.

All of the above-mentioned findings highlight the role of the BBB in the SN in maintaining and preserving the SN microarchitecture. Since little is known about the disruptive role that alcohol can play in altering the BBB composition of the SN region, in the present study,

we investigated the structural and ultrastructural changes in the BM matrix glycoproteins in the SN region, which are responsible for maintaining the integrity of the BBB in the context of chronic alcoholism.

## 2. Materials and Methods

### *2.1. Human Autopsy Brain Tissue Collection*

The present study included 44 individual brain autopsies as described previously (Supplementary Table S1) [46]. Briefly, 44 individuals were subdivided into three groups—controls (group A; 13 individuals, median age 31 ± 6.79 years), age-matched young alcoholics (group B; 13 individuals, median age 31 ± 4.85 years), and non-age-matched chronic alcoholics (group C; 18 individuals, median age 49.5 ± 8.66 years). The inclusion of the age-matched young alcoholics (group B) allowed us to exclude age-related effects in the present study.

The brain tissue was collected retrospectively (2007–2012) from the Latvian State Center for Forensic Medical Examination and preserved in paraffin blocks using standard laboratory techniques [46]. The protocol for the present study was approved by the Research Ethics Committee of Rīga Stradiņš University (Decision No. 6-1/12/9), dated 26 November 2020, per the provisions of the Declaration of Helsinki.

### *2.2. Immunohistochemistry Reactions*

Formalin-fixed paraffin-embedded (FFPE) brain tissue samples from the *Substantia Nigra* (SN) region were used in the present study. Consecutive sections of 4–5 µm were used for histopathological and immunohistochemical (IHC) evaluation. FPPE samples were deparaffinized and dehydrated, followed by blocking of endogenous peroxidase activity using 3% $H_2O_2$ in methanol [46]. Sections were boiled in citrate buffer (pH 6) for retrieval of the antigens followed by incubation with primary antibodies (Table 1).

**Table 1.** Primary antibodies used in the present study.

| Primary Antibody * | Antibody Properties ** | Clone | Working Dilutions | Manufacturer | Catalogue No. |
|---|---|---|---|---|---|
| CD31 | Mouse monoclonal AB against human AG | JC70A | 1/30 | DakoCytomation (Glostrup, Denmark) | M0823 |
| Collagen-IV | Mouse monoclonal AB against human AG | PHM-12 | 1/100 | Novocastra (Deer Park, IL, USA) | NCL-COLL-IV |
| Laminin-111 | Rabbit polyclonal AB against human AG | - | 1/1000 | Arigo Biolaboratories (Hsinchu City, Taiwan) | ARG10736 |
| Fibronectin | Rabbit Polyclonal AB against human AG | - | 1/400 | DakoCytomation (Glostrup, Denmark) | A0245 |

* CD31—cluster of differentiation 31; ** AB, antibody; AG, antigen.

Anti-CD31 antibody, a commonly used immunomarker, was used for the detection of endothelial cells [47]. Collagen-IV, a major constituent of the BM, was detected using anti-collagen type IV antibody [48]. Anti-laminin-111 antibody was used to determine the alpha 1, beta 1, and gamma 1 subunits found in the BM of CNS blood vessels [48]. Anti-fibronectin antibody was used for the determination of fibronectin in the BM [30].

Primary antibody amplification and visualization were performed via the HiDef Detection™ HRP Polymer system (catalogue no: 954D-30; Cell Marque, Rocklin, CA, USA) and 3,3′ diaminobenzidine (DAB) tetrahydrochloride kit (catalogue no: 957D-30; DAB+ Chromogen and DAB+ Substrate buffer, Cell Marque, Rocklin, CA, USA) or using the HiDef Detection™ Alkaline Phosphatase Mouse/Rabbit Polymer System (catalogue no: 962D-30; Cell Marque, Rocklin, CA, USA) and Permanent Red Chromogen Kit (catalogue no: 960D-2; Cell Marque, Rocklin, CA, USA). Counterstaining of the sections was performed with Mayer's hematoxylin, followed by series of washing, dehydration, clearing, and mounting in polystyrene. Visualization of brown-stained (DAB chromogen) or red-stained (red

chromogen) structures was considered a positive reaction for respective IHC antibodies. For negative controls, IHC samples were stained with PBS (phosphate-buffered saline) solution. Microphotographs were collected using a Leitz bright-field microscope and DFC 450C digital camera (Leica-Leitz DMRB; Wetzlar, Germany), along with a Glissando Slide Scanner (Objective Imaging Ltd., Cambridge, UK). Additional measurements of tissue markers and their spatial distribution were obtained using Aperio ImageScope program v12.2.2.5015, Leica Biosystems, Chicago, IL, USA.

### *2.3. Immunofluorescence Reactions*

Immunofluorescence (IF) was performed by immunostaining samples with primary antibodies, followed by washing with PBS buffer. Fluorescent secondary antibodies (goat anti-mouse IgG (H + L) antibody, Alexa Fluor® 488 conjugate (catalogue no. A1100; Thermo Fisher Scientific, Invitrogen, Waltham, MA USA, 1:300)) were used. Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI) followed by co-labeling with Prolong Gold and DAPI. Visualization of green-stained (fluorescent dyed) structures was considered a positive reaction. Confocal microscope Eclipse Ti-E (Nikon, Brighton, MI, USA) was used to capture digital images.

The immunofluorescence intensity was measured for each of the three basement membrane proteins (collagen-IV, laminin-111, and fibronectin) using ImageJ software (ImageJ version 1.53p, U. S. National Institutes of Health, Bethesda, ML, USA). The corrected total cell fluorescence (CTCF) method was used to adjust the IF intensity for background noise correction. For CTCF calculations, thirty microvessels per protein were randomly chosen (ten per grade) and visualized at 1000× magnification (Supplementary Figure S1). Negative controls for IHC and IF are shown in Supplementary Figure S2.

### *2.4. Transmission (TEM) and Scanning (SEM) Electron Microscopy*

TEM and SEM were used for the ultrastructural examination of the tissue samples. Tissue processing was performed in accordance with routine laboratory protocols by fixing samples in 2.5% glutaraldehyde. Post-fixation was performed using osmium tetroxide ($OsO_4$). This was followed by dehydration and embedment in epoxy resin (Sigma-Aldrich, Buchs, Switzerland). LKB ultramicrotome was used to obtain semi-thin sections measuring 1 μm, which were then stained with 1% toluidine blue for structural analysis in the light microscope. Next, ultra-thin (60 nm) sections were obtained, collected on formvar-coated 200-mesh nickel grids, and stained with 2% uranyl acetate and lead citrate. Sections were examined by JEM 1011 (JEOL, Akishima, Tokyo, Japan). For the ultrastructural analysis, thirty microvessels with a transverse profile were randomly chosen and visualized at 12,000× magnification equally from each group. The thickness of the BM was determined by taking 10 measurements per vessel in the ImageJ program.

Tissue samples underwent dehydration using a series of graded solutions of acetone for SEM analysis, and then dried with liquid $CO_2$ using the critical point method (E3000 drying device, Agar Scientific, Stansted, UK). Samples were then covered with a gold layer and examined using JSM-6490LV (JEOL, Akishima, Tokyo, Japan) at an accelerating voltage of 25 kV and at magnification range of 5000–10,000×.

### *2.5. Scoring System and Statistical Analysis*

A quantitative scoring system was used to assess the positively stained microvessels (using CD31 antibody) by two independent observers at 400× magnification in 10 visual fields per region, per sample. For the analysis of the endothelial BM architecture, a semi-quantitative grading scale was developed (Figure 1). Two different parameters were registered for each immunohistochemically positive protein (collagen IV, laminin-111, and fibronectin) in the BM microvessels, namely thickness and integrity. The scale was designed based on the color signal intensity with low to high expression of BM proteins. For BM thickness: Grade I—normal thickness (defined as a visually detectable baseline or minimal quantity of immunoreaction products); grade II—moderately increased thickness; grade

III—highly increased thickness. For BM integrity: Grade I—no visible changes; grade II—mildly to moderately disrupted BM; grade III—highly disrupted BM. All vessels in five random visual fields per region, per sample were evaluated by two independent observers to assess thickness and integrity. Approximately 8000–10,000 microvessels were investigated per immunoreaction for thickness and integrity.

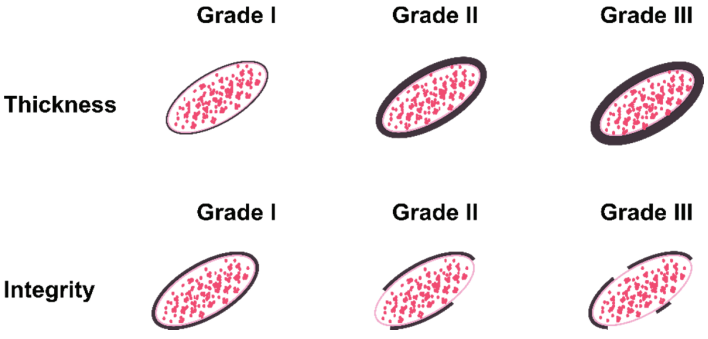


**Figure 1.** Semi-quantitative grading scale used for analysis of the thickness and integrity of the vascular BM. Red dots symbolize erythrocytes. Each vessel was analyzed for both these parameters individually in five random visual fields per region, per sample for both gray and white matter. Grade I represents normal vessels. Grade II represents moderately damaged vessels. Grade III represents severely damaged vessels.

The collected data was stored and digitalized in MS Excel (Microsoft Office 365). For CD31, the data distribution was checked using the Shapiro–Wilk test for normality ($p < 0.05$ indicates a violation of the normality). Non-parametric tests were used due to violation of normality. Kruskal–Wallis ANOVA was used for inter-group analysis with appropriate post hoc tests and Bonferroni correction. Intra-group analysis was performed using related-samples Wilcoxon signed rank test [46].

Due to the Likert-type nature of the semi-quantitative scoring system, for other immunohistochemical markers (collagen-IV, laminin-111, and fibronectin), Kruskal–Wallis ANOVA was used for inter-group analysis and related-samples Wilcoxon signed rank test was used for intra-group analysis for a location shift of distribution. For the purpose of visualization, the weighted average of the grades assigned was calculated and used. Correlation analysis was performed using Spearman's Rho. Statistical significance was set as $p < 0.05$. All analyses were perfomed using SPSS (IBM Corp. Released 2020; IBM SPSS Statistics for Windows 10, Version 27.0; Armonk, NY, USA: IBM Corp). Graphical representations were performed in R studio and MS Excel.

## 3. Results

### *3.1. Alcoholics Showed Significantly Less CD31+ Vessels Than Controls in Both Gray and White Matter*

We found significantly more CD31+ vessels in the gray matter than in the white matter for both the SNpc and SNpr in all three groups ($p < 0.001$; Table 2). Further, a significant decrease in the number of vessels was noted in age-matched alcoholics (group B) when compared with controls (group A; Figure 2). There was further a significant decrease between age-matched and non-age-matched alcoholics (group B vs. C).

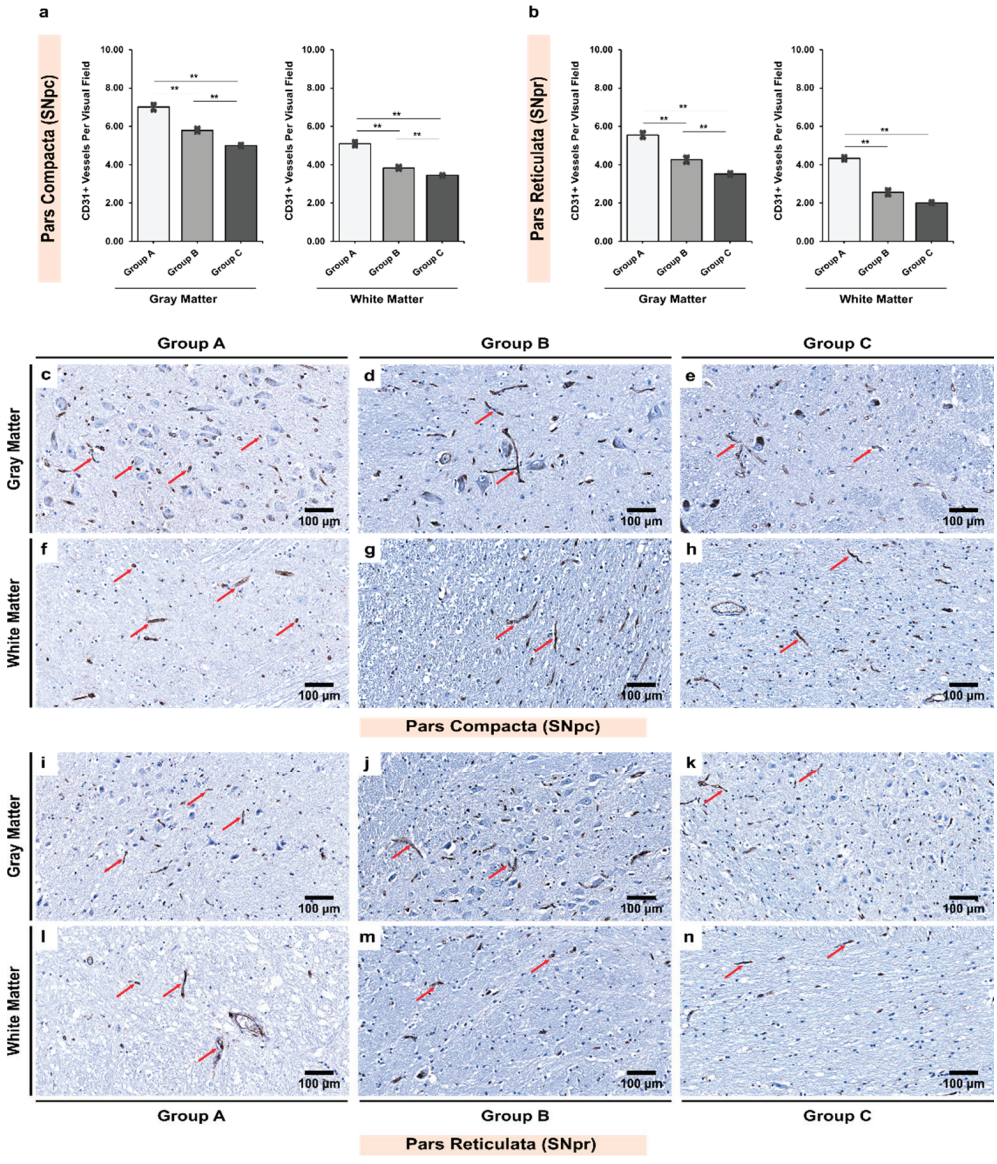


**Figure 2.** Inter-group analysis of CD31+ vessels per visual field in the (**a**) *Pars Compacta* (SNpc) and (**b**) *Pars Reticulata* (SNpr) for the three studied groups in both gray and white matter. The bar plots indicate the average number of CD31+ vessels ± S.E. (Standard Error) seen per visual field; ** indicates a significant difference between the groups ($p < 0.05$ with Bonferroni correction is considered as significant). Distribution of CD31+ vessels per visual field in (**c**,**f**,**i**,**l**) group A (controls), (**d**,**g**,**j**,**m**) group B (age-matched alcoholics), and (**e**,**h**,**k**,**n**) group C (non-age-matched alcoholics). Red arrows indicate CD31+ vessels. Original magnification, 200×. Scale bars, 100 µm.

**Table 2.** Distribution of CD31+ vessels per visual field in different regions of SN.

| | Controls (Group A) | Young Alcoholics (Group B) | Chronic Alcoholics (Group C) | *p* Value † |
|---|---|---|---|---|
| | | ***Pars Compacta* (SNpc)** | | |
| Gray Matter | 07.00 ± 0.17 | 05.80 ± 0.14 | 05.03 ± 0.09 | <0.001 ** |
| White Matter | 05.09 ± 0.14 | 03.83 ± 0.11 | 03.34 ± 0.07 | <0.001 ** |
| ***p* Value ‡** | <0.001 ** | <0.001 ** | <0.001 ** | - |
| | | ***Pars Reticulata* (SNpr)** | | |
| Gray Matter | 05.55 ± 0.18 | 04.26 ± 0.16 | 03.52 ± 0.10 | <0.001 ** |
| White Matter | 04.34 ± 0.12 | 02.56 ± 0.17 | 02.02 ± 0.08 | <0.001 ** |
| ***p* Value ‡** | <0.001 ** | <0.001 ** | <0.001 ** | - |

Note: † *p* value was calculated for Kruskal–Wallis ANOVA (inter-group analysis); ‡ *p* value was calculated for related-samples Wilcoxon signed rank test (intra-group analysis). The numbers represent the average number of CD31+ vessels per visual field ± S.E. (standard error); ** indicates a significant difference between groups ($p < 0.05$ is considered as significant with Bonferroni correction for Kruskal–Wallis ANOVA and without correction for related-samples Wilcoxon signed rank test).

### *3.2. Alcoholics Showed Significant Increases in Collagen-IV Expression Coupled with Significant Losses of Vessel Integrity in Both Gray and White Matter*

The expression of collagen-IV in the BM of white matter microvessels appeared to be more abundant when compared with gray matter microvessels in both the SNpc (insignificant; $p = 0.072$; Table 3) and SNpr (significant; $p = 0.002$; Table 3) in the control group. Overall, we observed that alcohol exposure led to a significant increase in the BM thickening due to the over-expression of collagen-IV (Figure 3). In the SNpc, there was a significant increase in the collagen-IV expression between chronic alcoholics and controls in both gray and white matter (groups A–C; $p < 0.001$ and 0.009, respectively). Similar difference was noted in the SNpr gray and white matter (groups A–C; $p < 0.001$ and 0.001, respectively). Additionally, the SNpc seemed to be more affected by alcohol-mediated collagen-IV over-expression (Supplementary Table S2).

**Table 3.** Inter-group and intra-group comparisons of the differences in the grades of thickness based on the expression of collagen-IV in the BM of microvessels in the SN region (shown by weighted average).

| | Controls (Group A) | Young Alcoholics (Group B) | Chronic Alcoholics (Group C) | *p* Value † |
|---|---|---|---|---|
| | | ***Pars Compacta* (SNpc)** | | |
| Gray Matter | 1.084 | 1.102 | 1.195 | <0.001 ** |
| White Matter | 1.121 | 1.143 | 1.187 | 0.009 ** |
| ***p* Value ‡** | 0.072 | 0.031 ** | 0.355 | - |
| | | ***Pars Reticulata* (SNpr)** | | |
| Gray Matter | 1.063 | 1.130 | 1.158 | <0.001 ** |
| White Matter | 1.115 | 1.160 | 1.201 | 0.002 ** |
| ***p* Value ‡** | 0.002 ** | 0.991 | 0.187 | - |

Note: † *p* value was calculated for Kruskal–Wallis ANOVA (inter-group analysis); ‡ *p* value was calculated for related-samples Wilcoxon signed rank test (intra-group analysis); ** indicates a significant difference between groups ($p < 0.05$ is considered as significant with Bonferroni correction for Kruskal–Wallis ANOVA and without correction for related-samples Wilcoxon signed rank test).

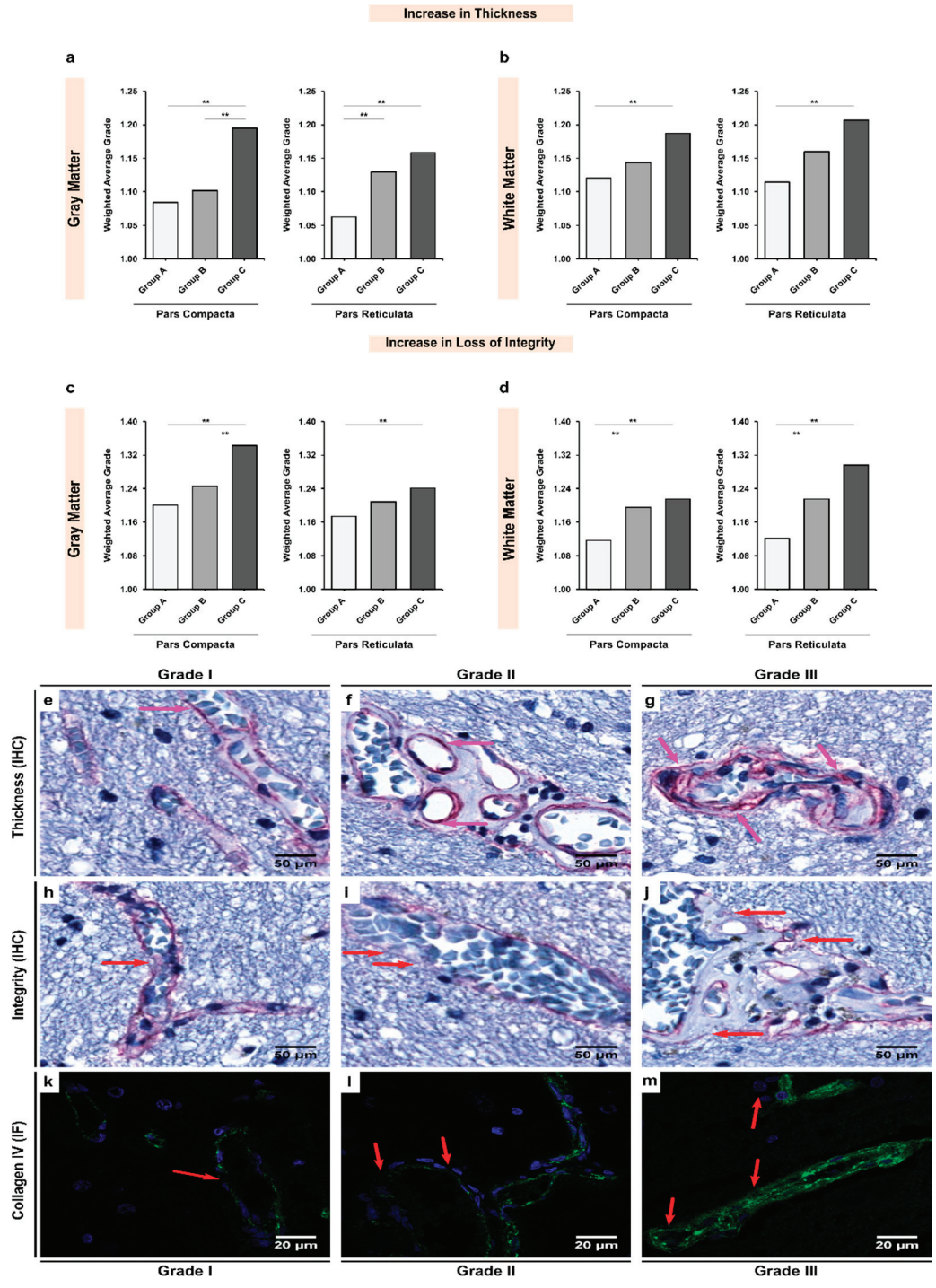


**Figure 3.** Inter-group analysis of the increase in (**a**,**b**) thickness and (**c**,**d**) loss of integrity of the BM based on the expression of collagen-IV in the microvessels in the (**a**,**c**) gray matter and (**b**,**d**) white matter of the SN region. Group A represent controls, group B represents young alcoholics, whilst

group C represents chronic alcoholics. The bar plots indicate the weighted average grading of all the visualized blood vessels in each group; ** indicates a significant difference between the groups ($p < 0.05$ with Bonferroni correction is considered as significant). Representative photomicrographs showing the different grades of BM thickness and integrity based on the expression of collagen-IV as visualized in the brain tissue material using immunohistochemistry (IHC) and immunofluorescence (IF) as follows: (**e**,**h**,**k**) grade I vessel with normal (baseline) thickness and unchanged integrity; (**f**,**i**,**l**) grade II vessel with moderate thickness and damaged integrity; (**g**,**j**,**m**) grade III vessel with extremely thickened and split BM. The pink and red arrows show the extent of thickening and loss of integrity as visualized using IHC and IF, respectively. In IF, green color shows the collagen-IV protein, whilst blue color shows DAPI-stained nuclei. Original magnification (IHC), 400×. Scale bars, 50 μm. Original magnification (IF), 1000×. Scale bars, 20 μm.

In terms of the integrity of the collagen-IV in the BM of vasculature, gray matter presented with more fragile BM than white matter in both the SNpc and SNpr of controls ($p < 0.001$; Table 4). In line with our expectations, alcoholism led to a significant disruption of the BM's integrity, especially more in the white matter (Supplementary Table S3 and Figure 3). In both the SNpc and SNpr white matter, there were significant differences noted between controls and age-matched alcoholics (groups A and B; $p = 0.008$ and 0.029, respectively) and controls and chronic alcoholics (groups A–C; $p < 0.001$ and 0.002, respectively). In the gray matter, chronic alcoholics showed a significantly more disrupted BM in both the SNpc and SNpr (groups A–C; $p = 0.012$ and $p < 0.001$, respectively).

**Table 4.** Inter-group and intra-group comparisons of the differences in the grades of integrity based on the expression of collagen-IV in the BM of microvessels in the SN region (shown by weighted average).

| | **Controls (Group A)** | **Young Alcoholics (Group B)** | **Chronic Alcoholics (Group C)** | ***p* Value** † |
|---|---|---|---|---|
| | | ***Pars Compacta* (SNpc)** | | |
| Gray Matter | 1.201 | 1.245 | 1.343 | <0.001 ** |
| White Matter | 1.116 | 1.195 | 1.216 | <0.001 ** |
| ***p* Value** ‡ | <0.001 ** | 0.004 ** | <0.001 ** | - |
| | | ***Pars Reticulata* (SNpr)** | | |
| Gray Matter | 1.173 | 1.209 | 1.241 | 0.012 ** |
| White Matter | 1.121 | 1.216 | 1.296 | <0.001 ** |
| ***p* Value** ‡ | <0.001 ** | 0.939 | 0.452 | - |

Note: † *p* value was calculated for Kruskal–Wallis ANOVA (inter-group analysis); ‡ *p* value was calculated for related-samples Wilcoxon signed rank test (intra-group analysis); ** indicates a significant difference between groups ($p < 0.05$ is considered as significant with Bonferroni correction for Kruskal–Wallis ANOVA and without correction for related-samples Wilcoxon signed rank test).

### *3.3. Expression of Laminin-111 Showed Significant Increases in Alcoholics Coupled with Significant Changes in the Vessel Integrity in Both Gray and White Matter*

Although a stronger expression of laminin-111 was observed in the BM of white matter microvessels, the difference when compared with gray matter microvessels remained insignificant across all three groups ($p > 0.05$; Table 5). Inter-group analysis revealed that alcohol exposure led to a significant increase in laminin-111-mediated BM thickening, in line with the changes observed for collagen-IV (Figure 4). A significant increase in laminin-111 expression was noted between chronic alcoholics and controls in both gray and white matter of the SNpc (groups A–C; $p < 0.001$ and 0.005, respectively) and SNpr (groups A–C; $p = 0.001$ for both regions). However, unlike collagen-IV, it was the SNpr that seemed to be more affected by alcohol-mediated laminin-111 overexpression (Supplementary Table S2).

**Table 5.** Inter-group and intra-group comparisons of the differences in the grades of thickness of the BM based on the expression of laminin-111 in microvessels in the SN region (shown by weighted average).

| | **Controls (Group A)** | **Young Alcoholics (Group B)** | **Chronic Alcoholics (Group C)** | ***p* Value** † |
|---|---|---|---|---|
| | | ***Pars Compacta* (SNpc)** | | |
| Gray Matter | 1.132 | 1.192 | 1.249 | <0.001 ** |
| White Matter | 1.179 | 1.213 | 1.280 | 0.005 ** |
| ***p* Value** ‡ | 0.146 | 0.949 | 0.611 | - |
| | | ***Pars Reticulata* (SNpr)** | | |
| Gray Matter | 1.163 | 1.226 | 1.295 | <0.001 ** |
| White Matter | 1.188 | 1.257 | 1.344 | <0.001 ** |
| ***p* Value** ‡ | 0.333 | 0.786 | 0.279 | - |

Note: † $p$ value was calculated for Kruskal–Wallis ANOVA (inter-group analysis); ‡ $p$ value was calculated for related-samples Wilcoxon signed rank test (intra-group analysis); ** indicates a significant difference between groups ($p < 0.05$ is considered as significant with Bonferroni correction for Kruskal–Wallis ANOVA and without correction for related-samples Wilcoxon signed rank test).

Similar to the results for collagen-IV expression, the BM of the gray matter vasculature was found to be more fragile and disintegrated, as also demonstrated by the expression of laminin-111, potentially indicating a complete breakdown in the BM's structural composition due to chronic exposure to alcohol (Table 4). These changes were more prominent in the gray matter than white matter. This shows that possibly the gray matter vasculature is more sensitive to laminin-111-mediated changes whilst the white matter vasculature is more sensitive to collagen-IV mediated changes (Tables 3–6; Supplementary Tables S2 and S3). In both the SNpc and SNpr gray matter, we observed significant laminin-111 disruption in chronic alcoholics when compared with controls (groups A–C; $p < 0.001$ and 0.014, respectively). Additionally, there were significant differences noted between age-matched alcoholics and chronic alcoholics (Groups B and C; $p = 0.001$ and 0.013, respectively), indicating that laminin-111 integrity may be linked to consumption patterns of alcohol.

**Table 6.** Inter-group and intra-group comparisons of the differences in the grades of integrity of the BM based on the expression of laminin-111 in microvessels in the SN region (shown by weighted average).

| | **Controls (Group A)** | **Young Alcoholics (Group B)** | **Chronic Alcoholics (Group C)** | ***p* Value** † |
|---|---|---|---|---|
| | | ***Pars Compacta* (SNpc)** | | |
| Gray Matter | 1.214 | 1.219 | 1.396 | <0.001 ** |
| White Matter | 1.169 | 1.213 | 1.360 | 0.003 ** |
| ***p* Value** ‡ | 0.011 ** | 0.936 | 0.830 | - |
| | | ***Pars Reticulata* (SNpr)** | | |
| Gray Matter | 1.176 | 1.251 | 1.326 | <0.001 ** |
| White Matter | 1.174 | 1.227 | 1.318 | <0.001 ** |
| ***p* Value** ‡ | 0.428 | 0.482 | 0.526 | - |

Note: † $p$ value was calculated for Kruskal–Wallis ANOVA (inter-group analysis); ‡ $p$ value was calculated for related-samples Wilcoxon signed rank test (intra-group analysis); ** indicates a significant difference between groups ($p < 0.05$ is considered as significant with Bonferroni correction for Kruskal–Wallis ANOVA and without correction for related-samples Wilcoxon signed rank test).

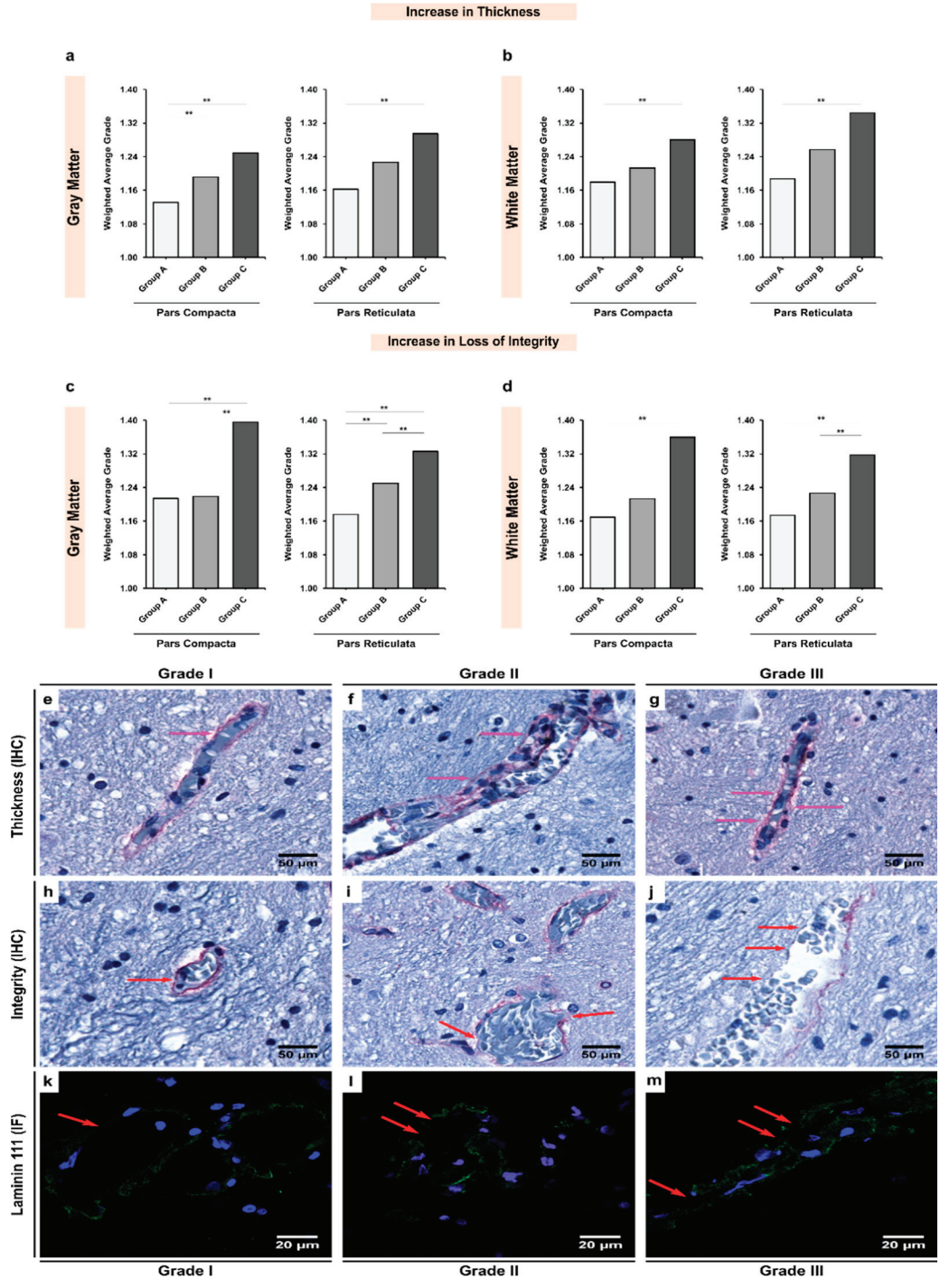


**Figure 4.** Inter-group analysis of the increase in (**a**,**b**) thickness and (**c**,**d**) loss of integrity of the BM based on expression of laminin-111 in the microvessels in the (**a**,**c**) gray matter and (**b**,**d**) white matter of the SN region. Group A represent controls, group B represents young alcoholics, whilst group C

represents chronic alcoholics. The bar plots indicate the weighted average grading of all the visualized blood vessels in each group; ** indicates a significant difference between the groups ($p < 0.05$ with Bonferroni correction is considered as significant). Representative photomicrographs showing the different grades of thickness and integrity of BM based on expression of laminin-111 as visualized in the brain tissue material using immunohistochemistry (IHC) and immunofluorescence (IF) as follows: (**e**,**h**,**k**) grade I vessel with normal thickness and unchanged integrity; (**f**,**i**,**l**) grade II vessel with moderate thickness and damaged integrity; (**g**,**j**,**m**) grade III vessel with extremely thickened and split BM. The pink and red arrows show the extent of thickening and loss of integrity as visualized using IHC and IF, respectively. In IF, green color shows the laminin-111 protein, whilst blue color shows DAPI-stained nuclei. Original magnification (IHC), 400×. Scale bars, 50 μm. Original magnification (IF), 1000×. Scale bars, 20 μm.

### *3.4. Expression of Fibronectin Was Significantly Upregulated in Alcoholics, Which Was Coupled with Significant Loss of Structural Integrity in Both Gray and White Matter*

We observed that fibronectin was significantly more expressed in the BM of the white matter vasculature in both the SNpc and SNpr across all three groups ($p < 0.05$; Table 7), except in the SNpr of the controls, where the difference was found to be insignificant ($p = 0.923$; Table 7). Furthermore, like the other two BM glycoproteins, expression of fibronectin was also upregulated due to alcohol exposure (Figure 5), thereby contributing to the thickening of the BM. In the SNpc, a significant increase in the expression of fibronectin was noted in both gray and white matter in chronic alcoholics when compared with controls (groups A–C; $p < 0.001$ and 0.036, respectively). In SNpr gray and white matter, a similar observation was made (groups A–C; $p = 0.001$ and 0.041, respectively). Similar to laminin-111, the SNpr seemed to be more affected by alcohol-mediated overexpression of fibronectin (Supplementary Table S2).

**Table 7.** Inter-group and intra-group comparisons of the differences in the grades of thickness based on the expression of fibronectin in the BM of the microvessels in the SN region (shown by weighted average).

| | **Controls (Group A)** | **Young Alcoholics (Group B)** | **Chronic Alcoholics (Group C)** | ***p* Value** † |
|---|---|---|---|---|
| | | ***Pars Compacta* (SNpc)** | | |
| Gray Matter | 1.145 | 1.158 | 1.217 | <0.001 ** |
| White Matter | 1.185 | 1.317 | 1.380 | <0.001 ** |
| ***p* Value** ‡ | 0.010 ** | <0.001 ** | 0.033 ** | - |
| | | ***Pars Reticulata* (SNpr)** | | |
| Gray Matter | 1.122 | 1.159 | 1.210 | <0.001 ** |
| White Matter | 1.162 | 1.240 | 1.339 | <0.001 ** |
| ***p* Value** ‡ | 0.923 | 0.002 ** | 0.026 ** | - |

Note: † $p$ value was calculated for Kruskal–Wallis ANOVA (inter-group analysis); ‡ $p$ value was calculated for related-samples Wilcoxon signed rank test (intra-group analysis); ** indicates a significant difference between groups ($p < 0.05$ is considered as significant with Bonferroni correction for Kruskal–Wallis ANOVA and without correction for related-samples Wilcoxon signed rank test).

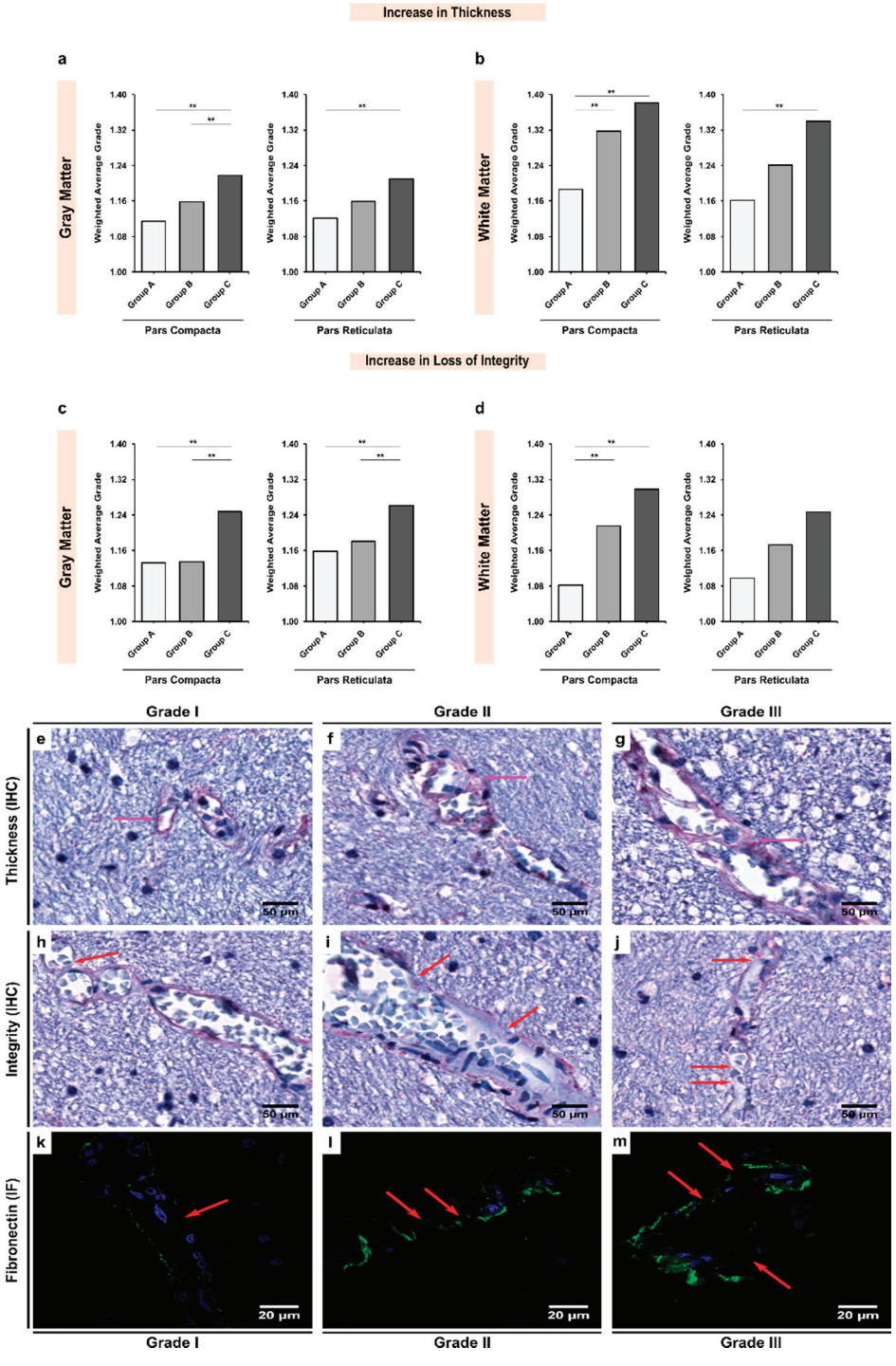


**Figure 5.** Inter-group analysis of the increase in (**a**,**b**) thickness and (**c**,**d**) loss of integrity of the BM based on the expression of fibronectin in the microvessels in the (**a**,**c**) gray matter and (**b**,**d**) white matter of the SN region. Group A represent controls, group B represents young alcoholics, whilst group C represents chronic alcoholics. The bar plots indicate the weighted average grading of all the

visualized blood vessels in each group; ** indicates a significant difference between the groups ($p < 0.05$ with Bonferroni correction is considered as significant). Representative photomicrographs showing the different grades of BM thickness and integrity based on expression of fibronectin as visualized in the brain tissue material using immunohistochemistry (IHC) and immunofluorescence (IF) as follows: (**e**,**h**,**k**) grade I vessel with normal (baseline) thickness and unchanged integrity; (**f**,**i**,**l**) grade II vessel with a moderate thickness and damaged integrity; (**g**,**j**,**m**) grade III vessel with extremely thickened and split BM. The pink and red arrows show the extent of thickening and loss of integrity as visualized using IHC and IF, respectively. In IF, green color shows the fibronectin protein whilst blue color shows DAPI-stained nuclei. Original magnification (IHC), 400×. Scale bars, 50 μm. Original magnification (IF), 1000×. Scale bars, 20 μm.

It is noteworthy that for fibronectin, it appeared that the BM of the vasculature was affected differently based on the region. In the SNpc the white matter vasculature was more affected, whilst in the SNpr it was the gray matter vasculature that was more affected in terms of the fibronectin structural integrity (Table 8). The underlying mechanisms and an explanation for such observations would be essential to investigate in future studies. In SNpc gray and white matter, there were significant differences between chronic alcoholics and controls (groups A-C; $p < 0.001$ and 0.006, respectively) and age-matched alcoholics (groups B and C; $p = 0.001$ and 0.003, respectively). In the SNpr, no significant changes were observed in the expression of fibronectin in the white matter vasculature, but a significant increase was noted in the gray matter vasculature (Table 8 and Supplementary Table S3). A significant disruption in fibronectin expression was noted in chronic alcoholics when compared with controls (groups A–C; $p = 0.004$) and age-matched alcoholics (groups B and C; $p = 0.001$).

**Table 8.** Inter-group and intra-group comparisons of the differences in the grades of integrity based on expression of fibronectin in the BM of the microvessels in the SN region (shown by weighted average).

| | **Controls (Group A)** | **Young Alcoholics (Group B)** | **Chronic Alcoholics (Group C)** | ***p* Value** † |
|---|---|---|---|---|
| | | ***Pars Compacta* (SNpc)** | | |
| Gray Matter | 1.132 | 1.135 | 1.248 | <0.001 ** |
| White Matter | 1.082 | 1.215 | 1.298 | <0.001 ** |
| ***p* Value** ‡ | <0.001 ** | 0.063 | <0.001 ** | - |
| | | ***Pars Reticulata* (SNpr)** | | |
| Gray Matter | 1.158 | 1.181 | 1.261 | <0.001 ** |
| White Matter | 1.097 | 1.173 | 1.247 | 0.273 |
| ***p* Value** ‡ | <0.001 ** | 0.086 | <0.001 ** | - |

Note: † $p$ value was calculated for Kruskal–Wallis ANOVA (inter-group analysis); ‡ $p$ value was calculated for related-samples Wilcoxon signed rank test (intra-group analysis); ** indicates a significant difference between groups ($p < 0.05$ is considered as significant with Bonferroni correction for Kruskal–Wallis ANOVA and without correction for related-samples Wilcoxon signed rank test).

### *3.5. Increases in the Thickness or Expression of BM Glycoproteins Were Negatively Correlated with the Integrity of the BM*

Although the correlation analysis revealed significant associations, the strengths of the correlation factors between the expression levels of the three BM glycoproteins were negligible in all cases. This indicates that although though the expression patterns of glycoproteins are inter-related, the expression remains independent of the changes in the expression of the other two glycoproteins. In terms of the integrity of the gray matter vasculature, the expression of fibronectin was significantly correlated with the expression of collagen-IV and laminin-111 ($\rho = 0.03$ and 0.04, respectively; $p = 0.040$ and 0.006, respectively). In the BM of the white matter vasculature, only the expression of laminin-111 and fibronectin was significantly correlated ($\rho = 0.06$; $p = 0.021$). In terms of the thickness of the BM (overexpression of glycoproteins), the fibronectin expression in the gray matter

vasculature correlated negatively and significantly with that of collagen-IV ($\rho = -0.04$; $p = 0.005$).

In terms of individual protein analysis, collagen-IV expression in both gray and white matter was found to be significantly and negatively correlated in terms of thickness and integrity of the glycoprotein ($\rho = -0.08$ and $-0.07$, respectively; $p = 0.001$). Similar observations were made for laminin-111 in both the gray ($\rho = -0.11$; $p = 0.001$) and white matter vasculature ($\rho = -0.09$; $p = 0.001$). Fibronectin also showed a similar trend in both the gray ($\rho = -0.07$; $p = 0.038$) and white matter vasculature ($\rho = -0.06$; $p = 0.008$). These findings indicate that the integrity of the BM decreases whilst the thickness or expression of the glycoproteins increases, thereby implying some sort of aggregation or clumping of the glycoproteins. Such structural alterations in the BM of the vasculature in the SN region require further investigation.

### *3.6. Ultrastructural Analysis of the Vascular Basement Membrane and BBB*

The integrity of the BM varied from a homogeneous appearance to pronounced multilamellar aspects. Using perpendicular measurements for the distance between the inner and outer edges of the BM, the detected thickness varied from 29.8 nm to 2406.7 nm, with an average thickness of the separate lamella of around 256 nm (Supplementary Table S4). Grade I vessels showed an average thickness of the BM of $107.2 \pm 43.4$ nm, whilst for grade II vessels the average thickness was $205.4 \pm 115.9$ nm. Grade III vessels showed an average thickness of around $433.9 \pm 402.1$ nm.

Apart from the various shapes, BM lamellae encircled the cytoplasm of the pericytes and the endothelial cells (Figure 6). The endothelial cells were characterized by a variably flattened shape and showed an electron-dense cytoplasm. Large lipid-containing lysosomes were frequently found inside the endotheliocytes. In some vessels, the tight junction complexes appeared swollen. Whilst a wavy nuclear envelope appearance was detected in both the endothelial cells and pericytes, nuclei showed peripheral and homogeneous clusters of heterochromatin. Pericytes showed an electron-dense cytoplasm with an expanded rough endoplasmic reticulum cisternae and variable mitochondria. Perivascular astrocytic foot processes often were swollen, showing an electron-lucid "empty" cytoplasm. At the same time, astrocyte processes that were diffused from the microvessels demonstrated large, swollen mitochondria and well-preserved cytoskeleton elements.

Interestingly, we also observed fenestrae on the surfaces of the endothelial cells. The fenestrae showed different shapes, ranging from an elongated shape to a more roundish shape, with an average size of around 500 nm in diameter. Additionally, we found large paracellular pores between the neighboring endothelial cells, which varied in size from around 500 nm to 2 μm (Figure 7).

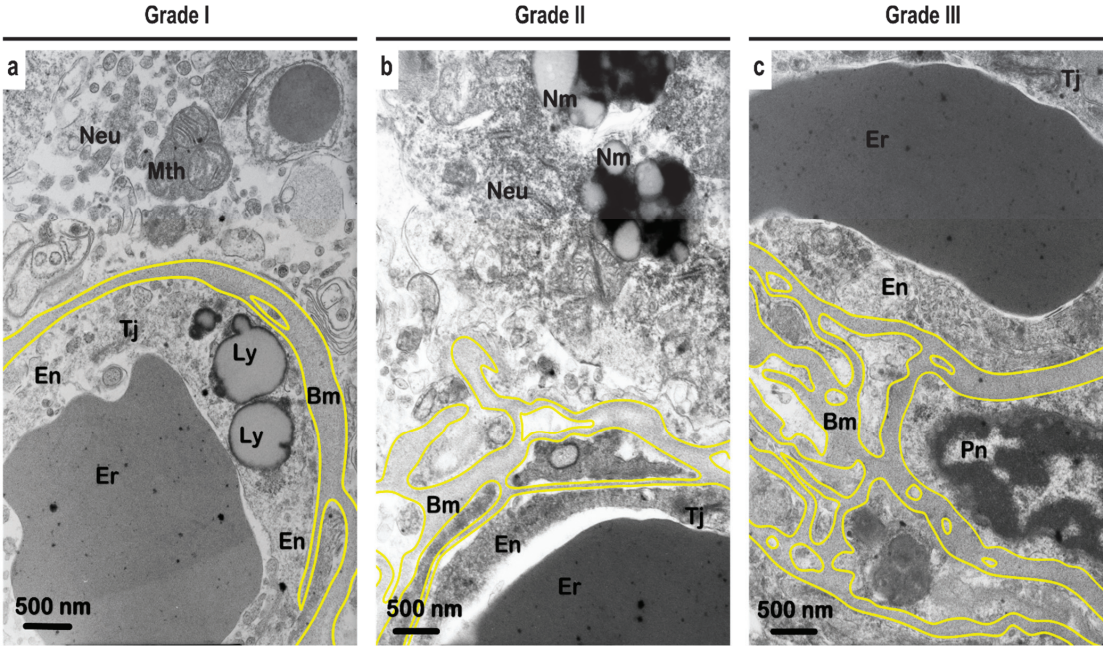


**Figure 6.** Representative transmission electron microscopy (TEM) micrographs of ultrastructural changes observed in different grades of blood vessels in the gray matter of predominantly (**a**) controls and (**b**,**c**) alcoholics. The yellow lines indicate the outer borderline of the basement membrane (Bm). (**a**) Accumulation of lipolysosomes in the cytoplasm of the endothelial cell. Homogenous and smooth BM can be seen. (**b**) Endothelial cells with tight junctions and nearby neuron containing neuromelanin is seen. The vessel has a lamellar BM. (**c**) Endothelial cells with tight junctions and pericyte with nucleus can be seen. The vessel shows splitting of the BM. Abbreviations: Neu, neuron; Er, erythrocyte; Pn, nucleus of pericyte; En, endothelial cell; Tj, tight junction; Mth, mitochondria; Nm, neuromelanin; Ly, lipolysosomes. Original magnification, 12,000×. Scale bars, 500 nm.

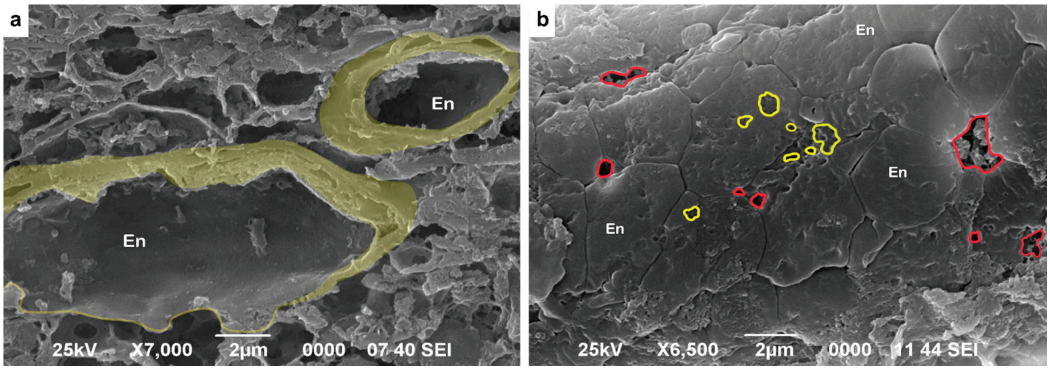


**Figure 7.** Representative scanning electron microscopy (SEM) micrographs of the vascular endothelium in the gray matter of predominantly (**a**) controls and (**b**) alcoholics. (**a**) Characteristic continuous endothelium (En) seen in the walls of SN microvessels in the lateral view. Original magnification, 7000×. (**b**) Ultrastructural changes observed in between neighboring endothelial cells on the luminal surface. Large paracellular pores (shown by a red line) and fenestrae (shown by a yellow line) can be seen on the luminal surfaces of endothelial cells. Original magnification, 6500×. Scale bars, 2 μm.

## 4. Discussion

### *4.1. White Matter Has Significantly Fewer CD31+ Microvessels Than Gray Matter in Physiological Conditions*

The SN region receives its blood supply from both the paramedian branches of the basilar artery and posterior cerebral artery providing a blood supply to the medial–caudal portion, as well as the anterior choroid artery providing blood supply to the most medial–superior part [49]. The rich blood supply to the region makes it extremely sensitive and more exposed to the alcohol in circulation [50]. The unique tree-like branching geometry of the vasculature in the SN region extends from the white matter to the gray matter, thereby causing the white matter to have less branched and more dilated microvessels whilst the gray matter possess more branched but narrower microvessels, in line with our findings from CD31+ observations (Table 2 and Figure 2). Similar distribution patterns of microvessels have been reported in other subcortical regions [51–53].

In a human brain autopsy study, the authors showed that the intraparenchymal vessels in the basal ganglia resembled long arterioles and long muscular arteries with no interdigitating arteriolar fields, and originated from a single source, thereby putting the region at increased risk of hypoperfusion and anoxia [54]. In fact, it is no coincidence that this region is the most frequent site for small lacunar infarcts and other degenerative vessel wall conditions [55] and is extremely sensitive to hypertensive and aging-related changes, with arteries forming twists, spirals, and loops [56].

A study in human fetuses by Ballabh et al. showed that both the % of blood vessel area and number of vessels per $mm^2$ were higher in gray matter than the white matter in the frontal cortex, and the trend remained the same from as early as 16–20 gestational weeks [57]. In fact, the difference in vasculature density increased with increasing gestational weeks, with the authors finding that the gray matter vasculature started to expand in density and % from the 16th gestational week, whilst the white matter vasculature showed a similar phenomenon only close to gestational maturity after the 32nd gestational week [57]. It has been demonstrated that in comparison to the white matter, the basal ganglia and frontotemporal cortex of premature infants had more cerebral blood flow [58]. Similar observations have been reported using susceptibility contrast enhancement MRI techniques in mature infants and adult brains [59]. The results obtained in the present study confirm these previous findings, especially since these previous studies have used different antibodies to report their findings (we used the endothelial anti-CD31 antibody); whilst one study relied on anti-laminin antibody [57], the other study used anti-laminin, anti-collagen-IV, and anti-fibronectin antibodies [58].

The coherence of our anti-CD31 antibody results, which is a more specific endothelial marker, will enhance our understanding of the differences in vasculature distribution across different regions in the SN region. In a study in the mouse cerebellum, authors reported that the gray matter (cerebellar cortex) had a relatively short but dense network of microvessels (stained using anti-laminin antibody) with a short diffusion distance, whilst the white matter had a longer, less dense microvasculature with a greater diffusion distance [60], thereby highlighting that the character of the vascular bed corresponds with the demand for nutrients and blood supply. Similar findings were reported by Schnieder et al., who reported approximately 60% more vascular surface area density in both ventral and dorsal gray matter than white matter in the human brain (stained using anti-GLUT-1 antibody) [61]. Additionally, we found a higher CD31+ vessel density in the SNpc than the SNpr (Table 2), which depicts the regional vulnerability to vessel loss in aging, as reviewed by Pandya and Patani [45].

### *4.2. Alcohol Use Aggravates Decreased Microvascular Density in Both Gray and White Matter*

Aging has long been considered as a potent factor influencing the density of the microvessels in brain and other tissues [62,63]. In a study in adult rats, Villar-Cheda et al. demonstrated that aged and sedentary low-exercising rats showed significant decreases in SN vascular density, indicating an age-related progressive decline in the functional and

structural integrity of the SN region coupled with increased vulnerability to injury [64]. In our study, we found that aging plays a crucial role in regulating the density of CD31+ vessels. We noted significant differences between chronic alcoholics and both controls and age-matched alcoholics (groups A–C and groups B and C, respectively; Figure 2), thereby showing the possible effects of aging on vascular density. However, this decline in vasculature density was also mediated by the effects of alcohol, as evident from the significant differences obtained between controls and age-matched alcoholics (groups A and B; Figure 2). In previous studies, it has been shown that vascular density decreases with age in the human brain and is universally seen across all regions of the brain, although the rates of decrease may not be uniform [60,65–68]. Apart from the physiological aging-related decrease in the microvascular density, chronic ethanol intoxication has also been demonstrated to cause accelerated reductions in the terminal vascularization density due to disturbances in the angiogenesis [68].

CD31 is known to play a cytoprotective role in the endothelium [69]. Our findings on reduced expression of CD31 in alcoholics (Table 2; Figure 2) might indicate the increased vascular stress in the microvascular bed. Furthermore, in a study in human angiosarcoma samples, Venkataramani et al. demonstrated that the downregulation of CD31 expression led to loss of endothelial tube formation and an increased induction of antioxidative enzymes [70]. In addition, it has been shown that in PD patients, apart from a decline in the vascular density, there is a morphological transformation in the vessel structure. The vasculature becomes less branched along with the formation of endothelial cell "clusters", which may be formed due to capillary fragmentation [71]. The authors described a "ladder-like" effect with areas of absent staining followed by areas of clustered staining (as seen in Figure 2) [71].

### *4.3. Gray Matter Has Thinner and More Damaged Collagen-Iv-Containing Basement Membrane Than White Matter*

Our results indicate that there was a more global expression of collagen-IV coupled with smoother and more preserved integrity in the white matter vasculature than in the gray matter in both the SNpc and SNpr in controls (Tables 3 and 4). Animal studies have shown that the gray matter is more rigid, stiff, and fragile than the surrounding white matter [72,73] and accumulates with aging. Although this difference in rigidity comes from a multitude of factors, including physical cell–cell interactions, decreased collagen-IV thickness or expression per vessel in the microvasculature of the gray matter plays a contributing role in determining the overall tissue viscoelasticity [74].

Previous studies have quantified collagen-IV immuno-stained capillaries in various regions of the brain [30,32,75]. In a recent study, Hase et al. analyzed the capillary width using anti-collagen-IV antibody in the frontal cortex and the underlying white matter. The authors found that microvessels in the white matter in controls were significantly wider (or dilated) and were significantly more immunostained than the microvessels in the gray matter [51]. Our observations are consistent with the results obtained by these authors (Figure 3). Relative quantification of collagen-IV concentrations using ELISA showed higher protein concentrations in the deep gray matter, brainstem, and cerebellum as compared to white matter regions such as the *corona radiata* and *corpus callosum* [76]. Another study, however, reported no significant differences in collagen distribution between gray and white matter regions using Masson's trichrome staining [77].

We postulate that it is possible that the total content of collagen-IV may be higher in the gray matter due to a higher density of the microvasculature, although the distribution per vessel favors the white matter where the larger vessels have thicker BMs. Additionally, it is worth pointing out that in the previous studies the control samples studied represented older populations (55–92 years) as compared to the relatively younger controls (median age 31 years) we investigated in the present study. Studies in rat models have shown that the collagen-IV content decreases with aging and maturation, whilst the stiffness of the microvessels increases, showing the structural alterations the BM of the microvasculature undergoes [73,78,79].

Uspenskaia et al. showed that aging is associated with increased collagen type IV accumulation in the basal lamina of human cerebral microvessels [75]. Contrary to this, Rubio-Araiz et al. showed that in the post-mortem brain tissue samples obtained from the pre-frontal cortex of alcoholics, there were significant reductions in the immunoexpression of laminin and collagen-IV [80]. Using TEM, we observed at the ultrastructural level that the BM is multi-lamellar or split (Figure 6; Supplementary Table S4), which could potentially explain the differences noted by the previous authors, whereby a reduction in collagen-IV levels was noted. Furthermore, the differences in the vascular structure, spatial organization, and role and contribution of the string and coiled vessels towards the distribution and quantification of collagen-IV in the gray and white matter can explain the differences obtained in previous studies and ours.

### *4.4. The Dual Role of Upregulated Expression in Laminin-111*

As a major non-collagen protein component of the BM, laminins play a critical role in promoting endothelial differentiation and BBB stability, as demonstrated by rat knockout models showing complete BBB leakage to outright hemorrhage [22]. In fact, depletion of astrocytic laminin-111 leads to disturbances in the differentiation of pericytes, which negatively affects the BBB's integrity [81]. In agreement with our findings on increased laminin-111 expression, especially in chronic alcoholics (Tables 5 and 6), it has been demonstrated that in chronic mild hypoxia, upregulation of laminin-111 occurs, which bolsters the vascular structural and functional integrity against the insults [82]. Similar upregulated expression has also been reported in mice models who controlled blunt head trauma [83]. The authors found that the circumference of laminin-enriched vessels was surrounded by astrocytic processes, thereby continuously secreting laminin to seal the breaches in the BBB, restructure the basal lamina, and ultimately restore the BBB's integrity [83].

Apart from restoration of the BBB's integrity, laminins have also been shown to confer neuroprotection to neural cells. Increased expression of both laminins and collagen has been shown to reduce Aβ secretion, thereby playing a neuroprotective role in AD [84]. Emerging evidence also points to a possible interaction between laminins and microglia. It has been shown that laminins not only act as anchoring structures for microglia, but also act as "food" for microglial cells in order for these cells to maintain an amoeboid morphology [85]. Furthermore, laminins have been shown to induce a pro-inflammatory phenotype in the microglia (lower cell volume and ramification) and to promote neuroinflammation [86]. These facts correlate with our previous findings in the same cohort of individuals, whereby we showed a dystrophic microglial phenotype and migration of perivascular microglia to diffuse locations in alcoholics [46]. This migration of microglia can explain the increased accumulation of laminins in the BM, since there is an imbalance in the consumption–production ratio.

### *4.5. Increased Expression of Fibronectin Might Promote Endothelial Damage*

Fibronectin is largely absent in healthy adult brain tissue but is found abundantly during brain development [83]. During brain injury, the levels of fibronectin are known to increase, especially those of the soluble form, which possibly act as opsonins and aid macrophage clearance of the dead tissue and cellular debris [83]. An increased expression of fibronectin has been reported in post-mortem brain samples from both the frontal and temporal cortex in AD patients [87]. Evidence of an increase in the expression of fibronectin in stroke and a contrary decrease in expression in AD was reviewed by Thomsen et al. [20].

In our study, we found that alcoholics showed a sustained increase in fibronectin expression, indicating alcohol-mediated structural changes in the BBB. Furthermore, Nakakura et al. demonstrated that fibronectin is essential for the formation of fenestrae in the endothelial cells of the fenestrated capillaries [88]. These transcellular pores characterize the fenestrated endothelium found in the liver, kidneys, and different endocrine glands and aids in increasing the exchange of solutes. In contrast, the lack of fenestrae in the continuous endothelium of the BBB provides a strict endothelial control in a paracellular manner [89].

Our ultrastructural findings indicate alterations in the endothelial cell structure, thereby damaging the BBB (Figure 7). Such changes may be attributable to the increased amount of fibronectin in the vascular BM (Tables 7 and 8). However, the exact mechanisms of how fibronectin interacts with other ECM components in the context of alcoholism remain largely unknown, and future studies are needed to shed light on the neuroprotective role of fibronectin.

Finally, from the results and discussion presented above, it is evident that the imbalance in the ratio between synthesis–consumption rates of the BM proteins led to deposition and overexpression of ECM proteins in alcoholics. It has been suggested that this imbalance is astrocyte-mediated, whereby astrocytes presumably goes into "overdrive" mode (possibly in conjunction with pericytes) in response to BBB injury to quickly repair the BBB integrity and structure [20,90]. However, emerging evidence from other authors and from our previous study suggests a possible role for microglia in controlling the consumption part of the equation. It is intriguing that whilst on the one hand we saw an increased thickness or expression of ECM proteins, on the other hand we saw it to be associated with a more lamellar and discontinuous BM (Figure 6). This potentially could indicate two-pronged stimulation of astrocytes—one process driving the synthesis of ECM proteins, with the other driving the release of MMPs and other microglial ECM-degrading enzymes. This could explain the "ladder-like clustered" vessel appearance seen in the present study and by previous authors.

### *4.6. Potential Diagnostic Applications*

The results from the present study provide interesting insights into the vascular responses to alcohol exposure both without the effect of aging and with the aging. Changes in the vascular component of the blood–brain barrier can be quantified and assessed clinically using BOLD (blood oxygenation level-dependent) signals in fMRI (functional magnetic resonance imaging) [91]. Changes in endothelial function and increased thickness of the vessel wall due to collagen-IV, laminin-111, and fibronectin deposition would lead to decreased lumen in the vessel, along with increased viscoelasticity, which would affect the CVR (cerebrovascular reactivity) measurements in the fMRI [91,92]. Additionally, since we noted a global decrease in CD31+ vessels in the SN of alcoholics, such changes could reduce the rCBF (resting cerebral blood flow) when measured using doppler ultrasonography, radiotracer techniques, and phase contrast imaging [93–95]. Positron emission tomography can also be used for these purposes. A more detailed methodology for evaluating changes in vasculature clinically has been discussed elsewhere [91–95]. Since the present study was conducted on brain autopsy material, we cannot provide an exact clinical correlation of the observed changes in the present study with clinical parameters. Future studies to investigate this aspect are needed.

### *4.7. Limitations of the Present Study*

The results from the present study are nevertheless constrained by some limitations, which need to be discussed. Firstly, the number of investigated individuals per group was relatively low, and our study included only samples from male individuals in the control group (group A), whilst the alcoholics groups (groups B and C) had a mix of samples from both male and female individuals. This could have introduced some underlying gender bias in our results. However, we would like to point out that upon exclusion of the female individuals from groups B and C (i.e., only male individuals included), we found similar tendencies and trends as reported in the results section of the study. Future studies with larger and more diverse groups are needed to encapsulate the true effects of gender regarding changes in the BM composition and structure.

Secondly, more immunomarkers need to be explored, including junctional proteins such as claudins and occludins, as well as ECM component proteins such as nidogen and heparin sulphates, in order to understand the effects on the BBB as a whole. Additionally, the role of vascular growth or modeling enzymes, including matrix metalloproteinases

(MMPs) and vascular endothelial growth factor (VEGF), needs to be investigated. Thirdly, the functional morphologies of pericytes and astrocytes need to be examined in future studies to understand the effects of aging, alcohol, and oxidative stress on these cells. Finally, the results we obtained in the present study could not be quantified in absolute protein terms (due to the small tissue sample). Our observations could be affected by the differences in sectioning of the tissue material or 2D observational biases. However, it is important to highlight that we investigated a large enough number of vessels (approximately 8000–10,000 vessels per immunomarker) using two independent observers and presented the results in terms of weighted averages (thereby eliminating bias), which should bring the margin of error within acceptable limits.

## 5. Conclusions

In the present study, we demonstrate that in physiological conditions, the white matter shows significantly fewer CD31+ vessels in comparison to the gray matter, with the microvascular density being significantly decreased in both SN regions in response to chronic alcohol exposure. Furthermore, increased expression of BM proteins (collagen-IV, laminin-111, and fibronectin) was noted in both age-matched and chronic alcoholics, which leads to the thickening of the vascular BM. Alcohol exposure at the same time leads to increased splitting and disruption of the vascular membrane continuity, as evident from visualization of fenestrae and pores in ultrastructural and structural analyses.

All of these aforementioned changes lead to the formation of clustered, ladder-like vessels characteristic of the region, thereby contributing towards the dysregulation of the local tissue environment. Future studies with larger cohorts investigating other aspects of the BBB, including cellular responses, are needed to fully encapsulate the effects of aging and alcoholism on composition and structure of the vascular BM.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines10040830/s1: Table S1: Characteristics of the brain autopsy individuals. Table S2: Frequencies (%) showing the distribution of vessels based on the semi-quantitative grading scale for thickness in different regions of *substantia nigra* (SN). Table S3: Frequencies (%) showing the distribution of vessels based on the semi-quantitative grading scale for integrity in different regions of *substantia nigra* (SN). Table S4: Table of measurements for the thickness of the basement membrane of the microvessels (in nm). Figure S1: Corrected total cell fluorescence (CTCF) depicting the immunofluorescence signal intensity for the three-basement membrane proteins. Figure S2: Negative (phosphate-buffered saline—PBS) controls for immunohistochemistry (IHC) and immunofluorescence (IF) reactions in the SN region of the brain.

**Author Contributions:** S.S. and N.J. conceptualized the study, whilst S.S., M.S. and N.J. were responsible for the methodology. Data collection was performed by M.S., N.J. and S.S., whilst formal data analysis was performed by N.J. Graphic visualizations were performed by N.J., whilst structural and ultrastructural visualizations were performed by S.S. Validation of the study protocol, project supervision, and funding acquisition were performed by S.S. The original draft was prepared by S.S., N.J. and M.S., whilst final editing of the manuscript was performed by S.S., N.J., M.S. and M.M. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** In this study, informed patient consent was waived due to the use of post-mortem specimens taken during conventional autopsies. Protocols for obtaining post-mortem brain tissue complied with all institutional guidelines, especially in respect to identity confidentiality.

**Data Availability Statement:** All the data used in this study are available from the corresponding author upon request.

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# Neuroinflammation, Oxidative Stress, Apoptosis, Microgliosis and Astrogliosis in the Cerebellum of Mice Chronically Exposed to Waterpipe Smoke

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**Abstract:** Waterpipe smoking (WPS) is prevalent in Asian and Middle Eastern countries and has recently gained worldwide popularity, especially among youth. WPS has potentially harmful chemicals and is associated with a wide range of adverse effects on different organs. However, little is known regarding the impact of WPS inhalation on the brain and especially on the cerebellum. Presently, we aimed at investigating inflammation, oxidative stress and apoptosis as well as microgliosis and astrogliosis in the cerebellum of BALB/C mice chronically (6 months) exposed to WPS compared with air-exposed mice (control). WPS inhalation augmented the concentrations of proinflammatory cytokines tumor necrosis factor, interleukin (IL)-6 and IL-1β in cerebellar homogenates. Likewise, WPS increased oxidative stress markers including 8-isoprostane, thiobarbituric acid reactive substances and superoxide dismutase. In addition, compared with the air-exposed group, WPS caused an increase in the oxidative DNA damage marker, 8-hydroxy-2′-deoxyguanosine, in cerebellar homogenates. Similarly, in comparison with the air group, WPS inhalation elevated the cerebellar homogenate levels of cytochrome C, cleaved caspase-3 and nuclear factor-κB (NF-κB). Immunofluorescence analysis of the cerebellum showed that WPS exposure significantly augmented the number of ionized calcium-binding adaptor molecule 1 and glial fibrillary acidic protein-positive microglia and astroglia, respectively. Taken together, our data show that chronic exposure to WPS is associated with cerebellar inflammation, oxidative stress, apoptosis, microgliosis and astrogliosis. These actions were associated with a mechanism involving NF-κB activation.

**Keywords:** waterpipe smoking; cerebellum; neuroinflammation; oxidative stress; DNA damage

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## 1. Introduction

Tobacco smoking is considered one of the major causes of preventable diseases and premature death worldwide [1]. According to a previous report, in the last two decades there was a dramatic shift in the methods of tobacco consumption [2]. While cigarette consumption has declined [3], the use of waterpipe smoking (WPS) has substantially increased [4]. The prevalence of WPS is growing particularly among young people, and it became an emerging global epidemic that requires action [5]. This increase in WPS use is attributed to the misconception that WPS is less toxic and less addictive than cigarette smoking (CS) as well as to the use of sweetened and flavored tobacco, which makes it more appealing to consumers [6].

Experimental and clinical studies, including our own, have reported that WPS induces multiple adverse health effects on various organs such as the heart, lungs and kidneys [7–13]. However, its effect on the brain is underexplored.

It is well evidenced that CS is a risk factor in neurodegenerative diseases, and the associations between CS and neurological disorders such as stroke, Alzheimer's disease, and multiple sclerosis have been established by various studies [14–16]. High levels of biomarkers for Alzheimer's disease such as amyloid β42 levels and excessive oxidative stress and neuroinflammation have been reported in the brain of smokers [17]. Moreover, animal studies have revealed that CS exposure triggers multiple immune, inflammatory and oxidative responses in the brain that may play a crucial role in the pathogenesis of neurological diseases [18–21]. In the traumatic brain injury model, it was shown that there was CS-induced neuroinflammation and loss of blood–brain barrier integrity [22]. In addition, chronic exposure of rats to CS resulted in significant histological alterations, apoptosis, lipid peroxidation and mitochondrial dysfunctions in the brain [23]. Furthermore, the exposure of human premonocytic line U-937 and the rat insulinoma parental cell line RINm5F to tobacco smoke induced protein oxidation, DNA damage and cell death by apoptosis and necrosis [24].

In contrast, only a few experimental studies have explored the impact of WPS exposure on the brain. A clinical study has reported a reduced level of circulating brain-derived neutrophic factor in adolescent WPS smokers [25]. A prospective observational study has reported that exposure to WPS causes neuronal damage through the increase in cerebral blood flow rate, elevation in carboxyhemoglobin and S100 calcium-binding protein [26]. Furthermore, adverse effects on cognitive executive measures were recorded in humans following one session of WPS inhalation [27]. It has been reported that exposure to WPS induced inflammation in the mesocorticolimbic brain regions [28]. In addition, the occurrence of oxidative stress in the hippocampus and cognitive decline were shown in rats following WPS exposure [29,30].

The cerebellum represents about 10% of the mass of the brain [31]. It plays a crucial role in motor control, and recent studies have reported its involvement in cognition, emotions and social interaction [32–35]. A reduction in the cerebellar gray matter was shown in CS smokers compared to nonsmokers [36,37]. However, to the best of our knowledge, no study has investigated the effect of WPS exposure on the cerebellum. Therefore, in this study, we sought to examine the impact of chronic exposure to WPS in mice on cerebellar inflammation, oxidative stress, apoptosis, the expression of nuclear factor (NF)-κB and the occurrence of microgliosis and astrogliosis.

## 2. Materials and Methods

### *2.1. Animals and Treatments*

This study was reviewed and approved by the United Arab Emirates University animal ethics committee, College of Medicine and Health Sciences, and experiments were performed according to protocols approved by the Institutional Animal Care and Research Advisory Committee.

### *2.2. WPS Exposure*

BALB/C mice (animal house facility, College of Medicine and Health Sciences, United Arab Emirates University) were housed in a conventional animal house and maintained on a 12 h light–dark cycle (lights on at 6:00 a.m.), humidity of 60% and controlled temperature (22 ± 1 °C). Animals had free access to water and food ad libitum. Animals were indiscriminately separated into air- and WPS-exposed groups after one week of familiarization to their conditions.

Mice were placed in soft restraints and connected to the exposure tower as described previously [38–41]. Using a nose-only exposure system connected to a waterpipe (InExpose System, Scireq, Montreal, QC, Canada), the animals were exposed to either WPS or air through their noses. Animals were exposed to mainstream WPS generated by commercially available apple-flavored tobacco (Al Fakher Tobacco Trading, Ajman, UAE). Tobacco was lit with instant light charcoal. Similar to its use in humans, the smoke from the waterpipe passed through the water first before it was drawn into the exposure tower which was

controlled by a computerized system (In Expose System, Scireq, Montreal, QC, Canada). A computer-controlled puff was generated every minute, leading to a 2 s puff duration of WPS exposure followed by 58 s of fresh air. The duration of an exposure session was 30 min/day. Regarding the WPS group, mice were exposed to WPS 5 days/week for 6 months (mo), and control mice were exposed to air only [38–41].

*2.3. Cerebellum Collection and Homogenates Preparation*

At the end of the exposure period, WPS ($n$ = 8) and air ($n$ = 8) mice were sacrificed by decapitation. The brain was removed, and the cerebellum was immediately dissected out on ice, frozen in liquid nitrogen and stored at −80 °C until assayed. Cerebellar homogenates preparation for the measurement of markers of oxidative stress, inflammation and apoptosis were prepared as described before [42].

*2.4. Measurement of the Concentrations of Tumor Necrosis Factor α (TNFα), Interleukin (IL)-6 and IL-1β*

The concentrations of TNFα, IL-6 and IL-1β were measured using commercially available Elisa kits (Duo Set, R&D systems, Minneapolis, MN, USA).

*2.5. Quantification of Levels 8-Isoprostane, Thiobarbituric Acid Reactive Substances (TBARS) and Superoxide Dismutase (SOD)*

The concentrations of 8-isoprostane were assayed according to the protocols described by the manufacturer (Cayman Chemicals, Ann Arbor, MI, USA). NADPH-dependent membrane lipid peroxidation was measured as TBARS, using malondialdehyde as the standard (Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA). The activity of the antioxidant enzyme SOD was quantified as per the vendor's protocols (Cayman Chemicals, Ann Arbor, MI, USA).

*2.6. Assessment of 8-Hydroxy-2′-Deoxyguanosine (8-OHdG), Cytochrome C, Cleaved Caspase-3, Nuclear Factor-κB (NF-κB) and Phosphorylated (Phospho)-NF-κB*

Using commercially available ELISA kits, we measured in cerebellum homogenates the levels of 8-OHdG (Cusabio Biotech Co., Ltd., Wuhan, China), cytochrome C (R&D Systems, Minneapolis, MN, USA), cleaved caspase-3 (R&D Systems, Minneapolis, MN, USA), NF-κB and phospho-NF-κB (Cell Signaling Technology, Danvers, MA, USA) as described previously [43–45].

*2.7. Immunofluorescence Labeling*

For the immunofluorescence analysis we used a separate set of animals; at the end of 6 mo exposure to WPS, mice of each group WPS ($n$ = 5) and air ($n$ = 5) were deeply anesthetized with sodium pentobarbital (35 mg/kg, i.p) and perfused with 20 mL of phosphate-buffered saline followed by 100 mL of freshly prepared 4% formaldehyde as a fixative. The cerebellum was post-fixed with formalin and embedded with paraffin. Using microtome (Leica, RM2125RT, Wetzlar, Germany), 1 μm thick sagittal sections of the cerebellum were cut and mounted on gelatin-coated slides. Sections were deparaffinized with xylene and rehydrated with a graded series of ethanol, and heat-induced antigen retrieval was performed in citrate buffer (pH 6.0) using the microwave.

Double immunofluorescence labeling was achieved by incubation at 4 °C overnight with the primary antibodies. We used rabbit polyclonal anti-Iba-1 (cat#019-19741, Wako, Osaka, Japan, 1:1500) for microglia, guinea pig monoclonal anti-neuronal nuclear antigen antibody (Neun) (cat#ABN90P, MerckMillipore, Darmstadt, Germany, 1:1500) for neurons and rabbit polyclonal anti-GFAP (cat#Z0334, Dako, Glostrup, Denmark, 1:500) for astroglia. The next day, sections were washed three times in PBS, followed by incubation for 1 h at room temperature with secondary antibodies: donkey anti-rabbit conjugated to Alexa 488 (cat#A32790, Invitrogen, Rockford, USA, 1:200) and donkey anti-guinea pig conjugated to Rhodamine (cat#706-295-148, Jakson, PA, USA, 1:100). Next, sections were incubated with 4,6-diamidino-2-phenylindole (DAPI) for nuclei counterstaining for 5 min and then were washed in PBS three times. Slides were then cover-slipped with Fluoroshield mounting

medium (cat#ab104135, Abcam, Waltham, MA, USA, 1:100). Images were acquired using an EVOS M5000 microscope.

For quantification of Iba-1,GFAP and NEUN/DAPI positive microglia, astroglia and neurons, respectively, three sections of the cerebellum from each animal were used; five animals from each group. Cell counting was performed at 20X magnification in the whole sections of the cerebellum with ImageJ software (NIH, Bethesda, MY, USA).

### *2.8. Statistical Analysis*

The statistical analysis was performed using GraphPad Prism (version 7; GraphPad Software Inc., San Diego, CA, USA). Data were tested using the unpaired *t*-test for differences between the two groups. The results are expressed as the mean ± SEM, and *p*-values of <0.05 were considered to be significantly different.

## 3. Results

### *3.1. Effect of Chronic Exposure to WPS on the Concentrations of TNFα, IL-6 and IL-1β in the Cerebellum*

Figure 1 shows that, compared to the air-exposed group, WPS exposure for 6 mo caused a significant increase in the concentrations of TNFα (Figure 1A, $p < 0.001$), IL-6 (Figure 1B, $p < 0.0001$) and IL-1β (Figure 1C, $p < 0.01$) in the cerebellar homogenates.

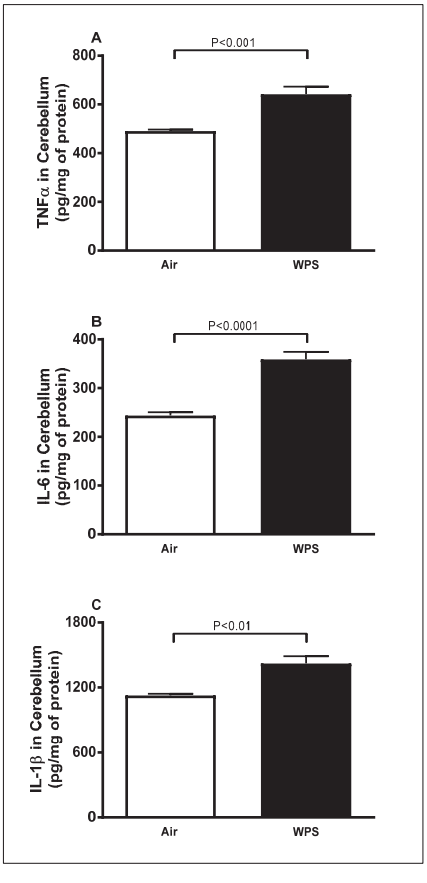


**Figure 1.** Tumor necrosis factor α (**A**), interleukin-6 (**B**) and interleukin-1β (**C**) concentrations in the cerebellar homogenates of mice exposed to either air or waterpipe smoke (WPS) for 6 months. Data are mean ± SEM ($n$ = 7–8).

*3.2. Effect of Chronic Exposure to WPS on the Concentrations of 8-Isoprostane and TBARS and Activity of SOD in the Cerebellum*

Compared to the air-exposed group, WPS inhalation for 6 mo induced a significant increase in the concentrations of 8-isoprostane (Figure 2A, $p < 0.0001$), TBARS (Figure 2B, $p < 0.05$) and activity of SOD (Figure 2C, $p < 0.01$) in the cerebellar homogenates.

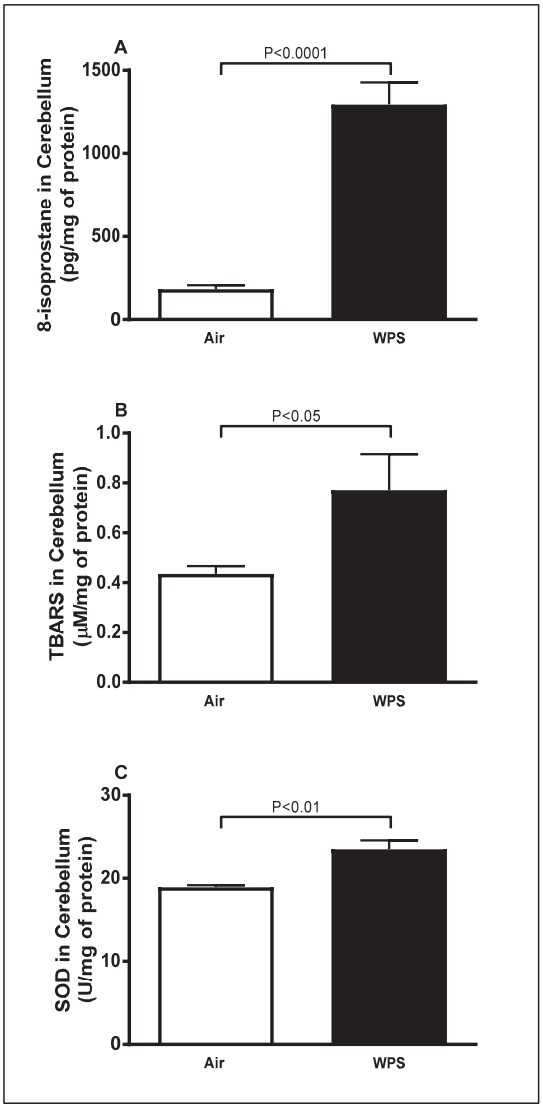


**Figure 2.** 8-isoprostane (**A**), thiobarbituric acid reactive substances (**B**) and superoxide dismutase (**C**) levels in the cerebellar homogenates of mice exposed to either air or waterpipe smoke (WPS) for 6 months. Data are mean ± SEM ($n$ = 7–8).

*3.3. Effect of Chronic Exposure to WPS on the Concentrations of 8-OHdG in the Cerebellum*

As illustrated in Figure 3, there was a significant ($p < 0.01$) increase in the concentrations of 8-OHdG, a marker of oxidative DNA damage, in the cerebellar homogenates following WPS exposure for 6 mo compared with the air-exposed group (Figure 3).

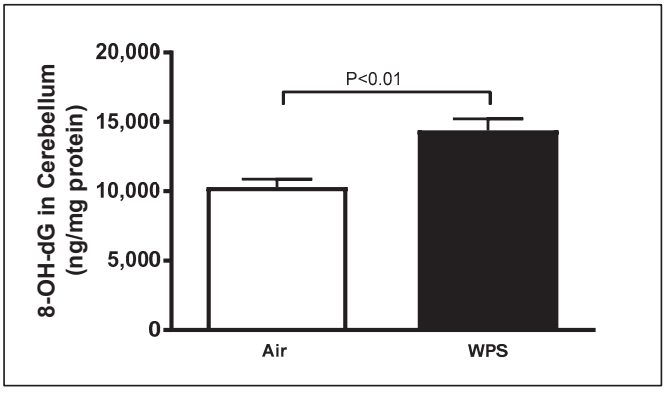


**Figure 3.** 8-hydroxy-2′-deoxyguanosine (8-OH-dG) concentrations in the cerebellar homogenates of mice exposed to either air or waterpipe smoke (WPS) for 6 months. Data are mean ± SEM ($n$ = 7–8).

*3.4. Effect of Chronic Exposure to WPS on the Levels of Cleaved Caspase-3 and Cytochrome C in the Cerebellum*

Figure 4 illustrates that WPS exposure for 6 mo induced a significant increase in the levels of cleaved caspase-3 ($p < 0.0001$) and cytochrome C ($p < 0.01$) in the cerebellar homogenates compared with the air-exposed group.

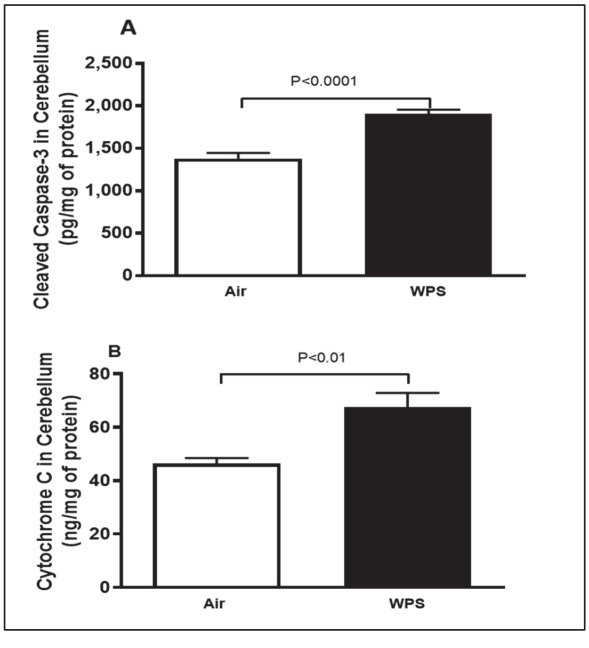


**Figure 4.** Cleaved caspase-3 (**A**) and cytochrome C (**B**) concentrations in the cerebellar homogenates of mice exposed to either air or waterpipe smoke (WPS) for 6 months. Data are mean ± SEM ($n$ = 7–8).

### *3.5. Effect of Chronic Exposure to WPS on the Expression of NF-κB and Phospho-NF-κB in the Cerebellum*

The total concentrations of NF-κB and phospho-NF-κB in the cerebellar homogenates are shown in Figure 5. WPS exposure for 6 mo resulted in a significant elevation ($p < 0.001$) in the total concentrations of NF-κB (Figure 5A) and phospho-NF-κB (Figure 5B) compared with the air-exposed group.

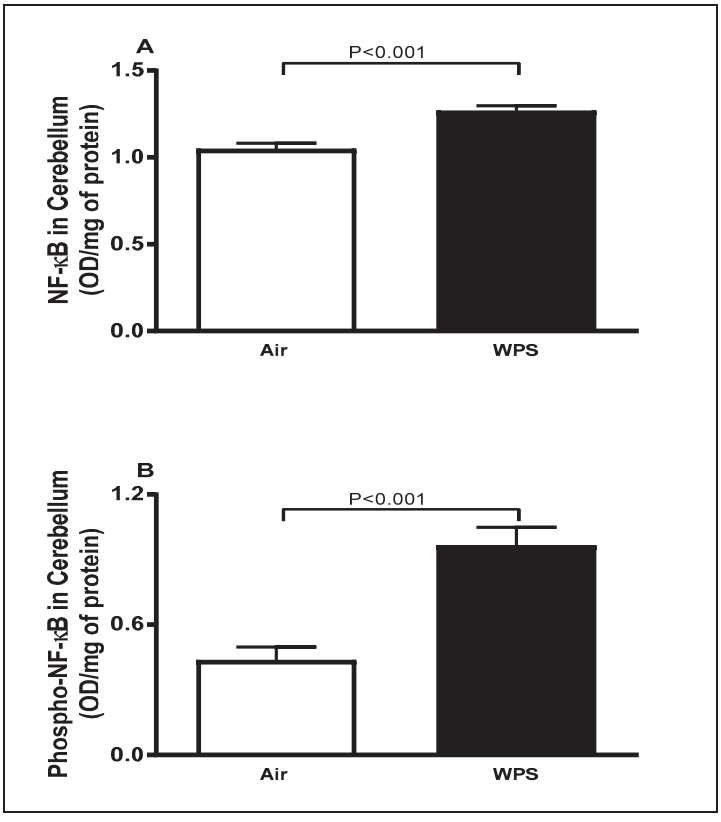


**Figure 5.** The total concentrations of nuclear factor kappa B (NF-κB) (**A**) and phosphorylated-NF-κB (**B**) in the cerebellar homogenates of mice exposed to either air or waterpipe smoke (WPS) for 6 months. Data are mean ± SEM ($n$ = 7–8).

### *3.6. Effect of Chronic Exposure to WPS on Iba-1 Labeling and Quantification of Iba-1-Positive Microglia in the Cerebellum*

The double immunofluorescence staining of microglia (Iba-1, green), neurons (NEUN, red) and the counterstain for nuclei (DAPI, blue) of sagittal sections of the cerebellum after 6 mo WPS exposure revealed an increase in the immunoreactivity of Iba-1 in different areas, indicating the occurrence of microgliosis (Figure 6E,H). In contrast, representative images of air-exposed mice showed a lower immunoreactivity of Iba-1 where the microglia appeared in their resting state with small cell bodies and thin processes in the white matter of the cerebellum (Figure 6A,D). The quantitative analysis showed that the number of Iba-1-positive microglia in the cerebellum of WPS-exposed mice was significantly higher ($p < 0.01$) compared with the air-exposed group (Figure 6I).

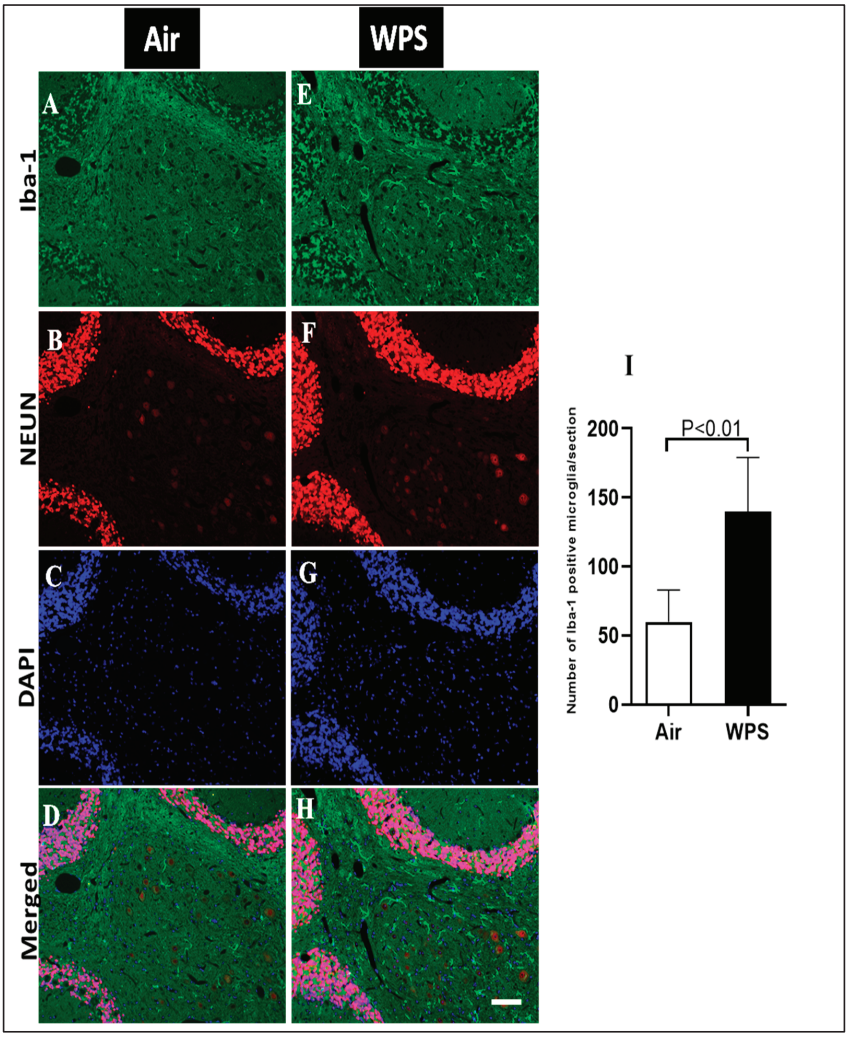


**Figure 6.** Representative images of double-immunofluorescence staining for anti-ionized calcium-binding adaptor molecule 1 (Iba-1, green), anti-neuronal nuclear antigen antibody (red) and 4, 6-diamidino-2-phenylindole (blue as a nuclei counterstain) of sagittal sections of the cerebellum of mice exposed to either air or waterpipe smoke (WPS) for 6 months. (**A**–**D**) Representative images of air-exposed mice showing ramified, resting microglia in the white matter of the cerebellum. (**E**–**H**) Representative images of WPS-exposed mice showing increased Iba-1 immunoreactivity in the white matter of the cerebellum. Scale bars = 50 μm, ($n$ = 5). (**I**) Bar graph showing cell counting of Iba-1 positive microglia that revealed a significant increase in the number of microglial cells in the cerebellum of mice exposed either to air or WPS for 6 months. Data are mean ± SEM ($n$ = 5).

The examination of the Iba-1 labeling of the cerebellum showed a slight increase in the number of Iba-1 positive microglia in the granular layer (Figure 7E,H) of the cerebellum after WPS exposure compared with the air-exposed group (Figure 7A,D).

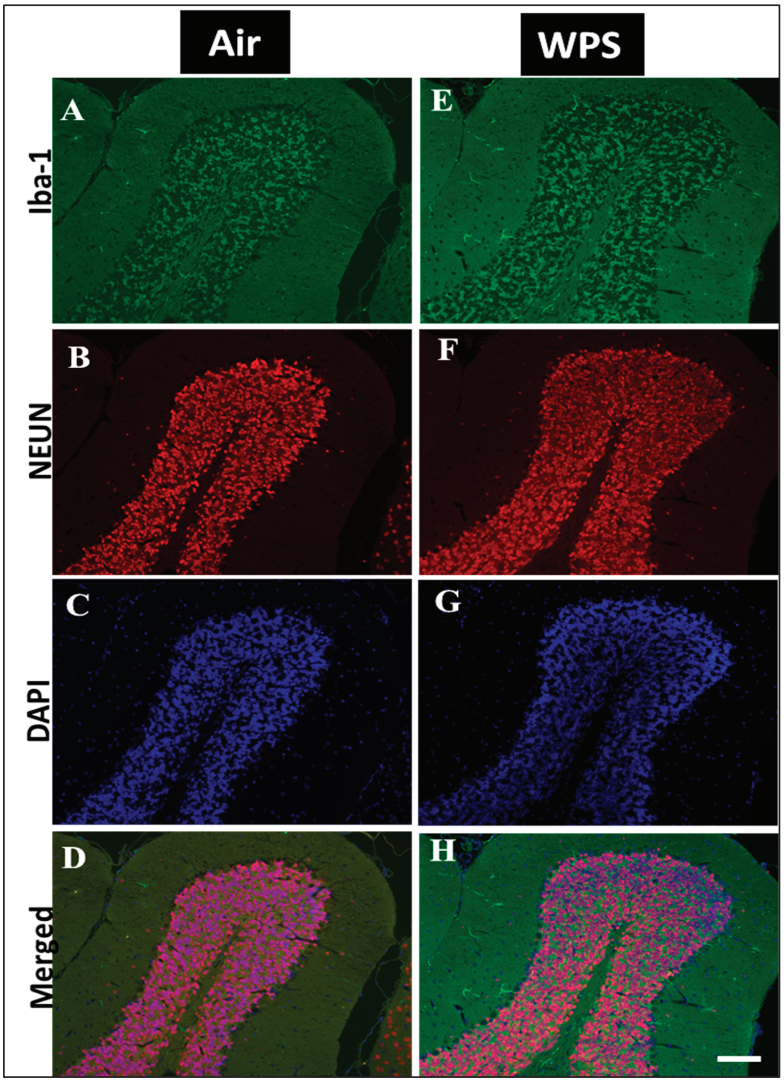


**Figure 7.** Representative images of double-immunofluorescence staining for Iba-1 (Iba-1, green), NEUN (red) and DAPI (blue as a nuclei counterstain) of sagittal sections of the cerebellum of mice exposed to either air or waterpipe smoke (WPS) for 6 months. (**A**–**D**) Representative images of air-exposed mice showing ramified, resting microglia in the granular layer of the cerebellum. (**E**–**H**) Representative images of WPS-exposed mice showing increased Iba-1 immunoreactivity in the granular layer of the cerebellum. Scale bars = 50 μm, ($n$ = 5).

### *3.7. Effect of Chronic Exposure to WPS on the GFAP Labeling and Quantification of GFAP-Positive Astroglia in the Cerebellum*

In order to examine the effect of WPS exposure for 6 mo on GFAP immunoreactivity in the cerebellum, a double immunofluorescent labeling of astroglia (GFAP, green), neurons (NEUN, red) and the counterstain for nuclei (DAPI, blue) was performed. Activated astrocytes underwent morphological changes and appeared hypertrophic and showed intense GFAP immunoreactivity in the white matter of the cerebellum of WPS-exposed mice (Figure 8E,H). In the air-exposed group, the astrocytes appeared with thin cell bodies

and short processes (Figure 8A,D). Figure 8I shows that the exposure to WPS for 6 mo resulted in a significant increase ($p < 0.01$) in the number of GFAP-positive astrocytes in the cerebellum compared with the air-exposed group. Moreover, Figure 9E,H shows more GFAP immunoreactivity in the granular layer after WPS exposure in comparison with the air-exposed group (Figure 9A,D).

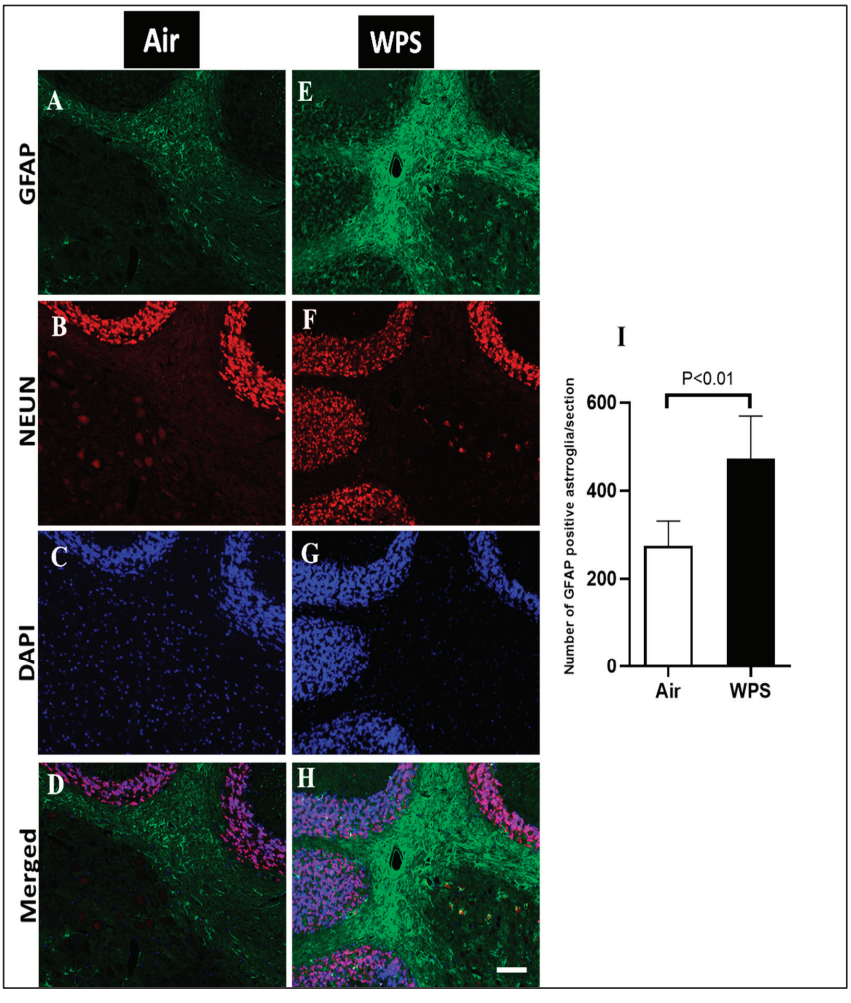


**Figure 8.** Representative images of double-immunofluorescence staining for GFAP (green), NEUN (red) and DAPI (blue as a nuclei counterstain) of sagittal sections of the cerebellum of mice exposed to either air or waterpipe smoke (WPS) for 6 months. (**A**–**D**) Representative images of air-exposed mice showing astrocytes with small cell bodies and thin processes in the white matter of the cerebellum. (**E**–**H**) Representative images of WPS-exposed mice showing increased GFAP immunoreactivity in the white matter of the cerebellum. Scale bars = 50 µm, ($n$ = 5). (**I**) Bar graph showing the cell counting of GFAP positive astroglia that revealed a significant increase in the number of astroglia cells in the cerebellum of mice exposed either to air or waterpipe smoking for 6 months. Data are mean ± SEM ($n$ = 5).

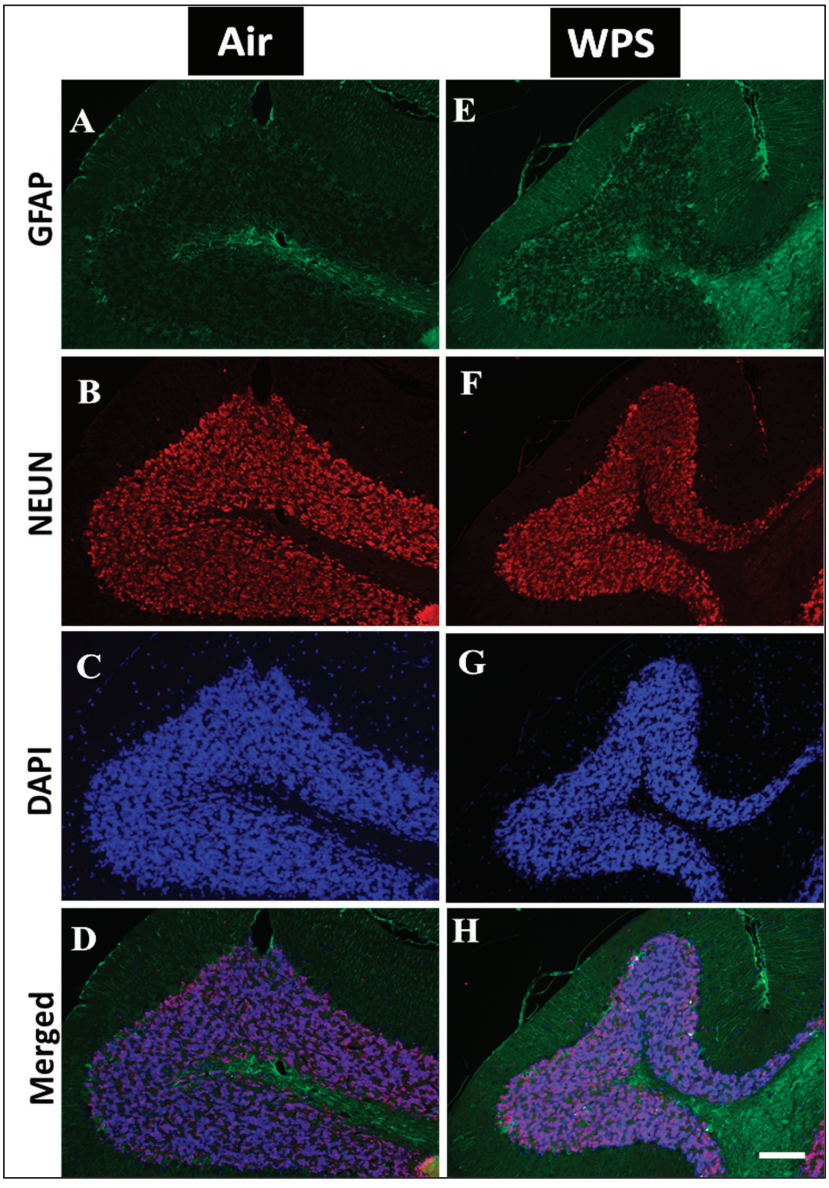


**Figure 9.** Representative images of double-immunofluorescence staining for GFAP (green), NEUN (red) and DAPI (blue as a nuclei counterstain) of sagittal sections of the cerebellum of mice exposed to either air or waterpipe smoke (WPS) for 6 months. (**A**–**D**) Representative images of air-exposed mice showing astrocytes with small cell bodies and thin processes in the granular layer of the cerebellum. (**E**–**H**) Representative images of WPS-exposed mice showing increased GFAP immunoreactivity in the granular layer of the cerebellum. Scale bars = 50 μm. Data are mean ± SEM ($n$ = 5).

### *3.8. Effect of Chronic Exposure to WPS on the NEUN/DAPI Labeling and Quantification of Neurons in the Cerebellum*

The analysis of NEUN/DAPI labeling of the cerebellum revealed no statistical difference in the number of neurons of mice exposed to WPS compared with the control group (Figure 10).

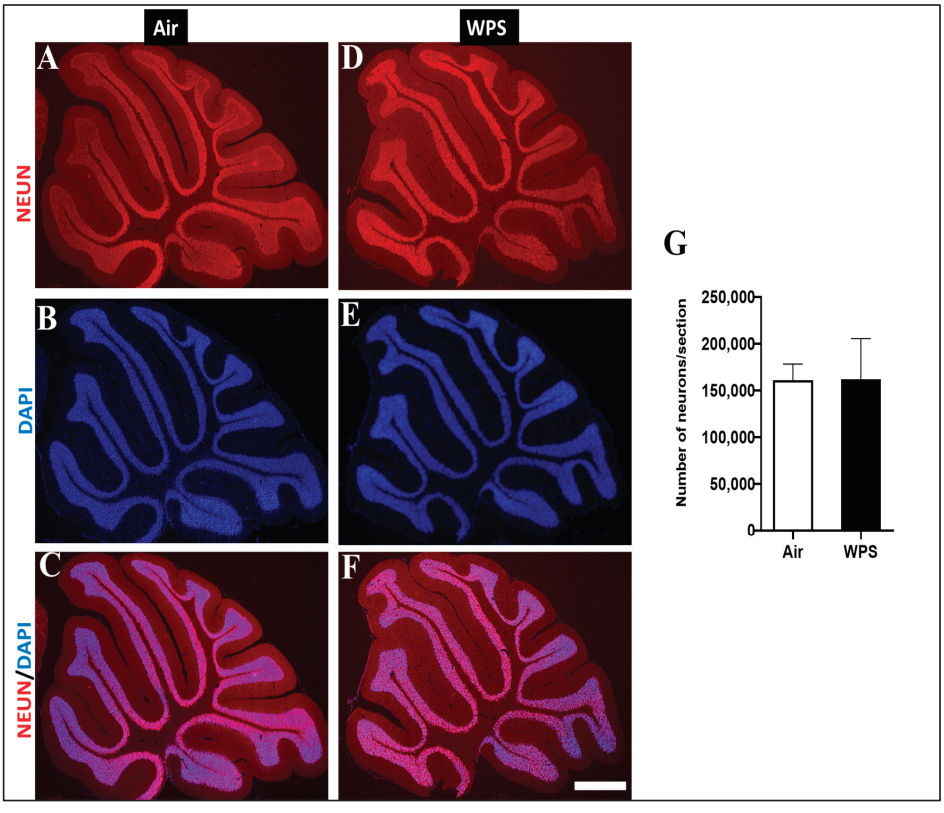


**Figure 10.** Representative images of double-immunofluorescence staining for anti-neuronal nuclear antigen antibody (red) and 4, 6-diamidino-2-phenylindole (blue as a nuclei counterstain) of sagittal sections of the cerebellum of mice exposed to either air or waterpipe smoke (WPS) for 6 months. (**A**–**C**) Representative images of the cerebellum of air-exposed mice. (**D**–**F**) Representative images of the cerebellum of WPS-exposed mice. Scale bars = 100 μm. (**G**) Bar graph showing neuron counting, demonstrating no statistical difference in the number of neurons in the cerebellum of mice exposed either to air or WPS for 6 months. Data are mean ± SEM ($n$ = 5).

## 4. Discussion

Waterpipe smoking is becoming a popular trend worldwide. Research in this area has mostly highlighted the cardiovascular and respiratory pathophysiologic effects of WPS. However, little is known regarding the role of WPS in causing biochemical and histopathological alterations in the brain and, more specifically, in the cerebellum. This study is the first of its kind that focused on the effects of WPS on the cerebellum in rodents. The data of the current study show that long-term exposure to WPS is associated with inflammation, oxidative stress, DNA damage, apoptosis, microgliosis and astrogliosis in the cerebellum.

Several studies have reported a consistent association between smoking and increased risk of dementia, including Alzheimer's disease (AD), vascular dementia and cognitive decline [46–48]. In addition, one of the hallmarks of neurodegenerative diseases is the increase in the concentration of proinflammatory markers in the brain [49]. The present study supports previous observations on the fact that neuroinflammatory responses are associated with smoking. Our data show that WPS exposure for 6 mo induced neuroinflammation, as indicated by the significant augmentation in the concentrations of proinflammatory cytokines TNFα, IL-6 and IL-1β in the cerebellar homogenate. In agreement with our results, WPS inhalation in rats was found to cause a significant increase in the expression of TNFα mRNA in mesocorticolimbic brain regions [28]. Moreover, high expression of proinflammatory cytokines in the brain was seen following 3–6 weeks of CS exposure in rats and mice [19,22]. It has been shown that the treatment with one of the most potent ingredients present in CS [48] and WPS [50], such as Benzo[a]pyrene diol epoxide (BPDE), led to an augmentation in mRNA and protein levels of the highly inducible inflammation factor cyclooxygenase-2 in the cortical cells of rats [51]. In support of the latter, treatment with 4-N-methyl-N-nitrosamino-1-(3-pyridyl)-1-butanone (NNK), a major nitrosamine formed in tobacco smoke, has been shown to induce a significant increase in the expression of TNFα, IL-6 and IL-12 both in vivo and in vitro [52].

Oxidative stress is a major trigger of cell damage, and it is well-established that it plays a role in the pathogenesis of various neurological disorders [53–55]. Our data show that long-term exposure to WPS caused a substantial increase in the concentrations of markers of lipid peroxidation TBARS and 8-isporstane. The increase of TBARS and 8-isporstane is an indication of the development of oxidative stress and the occurrence of lipid peroxidation in the cerebellum. Moreover, our results showed an increase in the activity of SOD. This increase might be considered to be a compensatory mechanism aiming at counterbalancing the ongoing oxidative injury induced by nose-only WPS inhalation in the cerebellum. Using whole body exposure system to WPS, Alzoubi et al. [29] reported the occurrence of oxidative stress in the hippocampus evidenced by a decrease in the activities of key antioxidant enzymes including glutathione peroxidase, catalase and SOD indicating a consumption of these antioxidants in the course of combatting WPS-induced oxidative damage. Inflammation and oxidative stress in cells often target the DNA. It is well known that repeated oxidative injury of the DNA is implicated in the pathogenesis of numerous diseases such as chronic pulmonary diseases, atherosclerosis and neurodegenerative disorders [56–58]. Our study showed that WPS-exposed mice had higher levels of the oxidative DNA damage marker 8-OHdG. The latter is the predominant form of free radical-induced oxidative lesions in nuclear and mitochondrial DNA [59]. This observation is in agreement with a previous study that showed high levels of 8-OHdG in the brain of rats exposed chronically to passive smoking [47].

Inflammation and oxidative stress in the brain are the main causes of DNA damage and cell death leading to neurodegeneration, which is the predominant phenomenon that underlies the symptoms of multiple human neurological disorders [60]. Cell death frequently implies the initiation of apoptosis via caspase activation [60]. Hence, caspase-3 is commonly used as a marker of apoptosis. In this work, we found that WPS-exposed mice displayed a significant augmentation in cleaved caspase-3. This observation is consistent with a study that showed that exposure to CS (6 cigarettes/twice a day) for 57 days significantly increased the immunoreactivity of cleaved caspase-3 in the white matter but not in the granular layer of the cerebellum of rats [61].

Cytochrome C is involved in both cellular energy and apoptosis [62]. Our data showed that 6 mo exposure to WPS induced a significant increase in the activity of cytochrome C. In line with our observation, Hosseini et al. [63] showed that the incubation of rat mitochondria with different concentrations of CS extract triggered the collapse of mitochondrial membrane potential, mitochondrial swelling and outer membrane rupture that resulted in the release of cytochrome C. We have previously shown that WPS inhalation in mice caused an elevation of cytochrome C in the kidney and testicular tissues [44,64].

In an attempt to investigate the underlying mechanism of the increase in oxidative stress and neuroinflammation observed in the present work, we measured the transcription factor NF-κB. The latter is commonly known as a critical regulator of both oxidative stress and inflammatory responses through the regulation of oxidative stress and numerous proinflammatory genes such as cytokines [65–67]. Our data show that WPS induced a significant increase in the levels of NF-κB. Consistent with our results, it has recently been demonstrated that WPS exposure for 4 weeks led to a significant increase in the expression of mRNA levels of NF-κB in different areas of the brain of rats including the prefrontal cortex, nucleus accumbens and ventral tegmental area [28]. We have previously reported that exposure to WPS in mice induced a marked increase in the expression of NF-κB in various organs including the heart, lungs and testicles [11,64,68]. Additional studies involving an inhibitory approach through repressing the NF-κB gene and/or the use of NF-κB knockout mice are needed to establish whether NF-κB plays a critical role in the observed effects.

Microglia are the smallest cells in the brain, and the resident macrophages in the brain [69], and continuously monitor the extracellular environment of neurons [70]. In response to neuronal insults, microglia cells change their morphology and their numbers increase noticeably, which is termed microgliosis [71]. Our study revealed a marked increase in the number of microglia in the granular layer and more abundantly in the white matter. It has been demonstrated that CS condensate accelerates the activation of microglia in the experimental autoimmune model [72]. In support of the latter, immunohistochemical staining revealed a massive microglial and astrocyte activation in the brain of mice following exposure to the tobacco carcinogen NNK [52].

Astrocytes are the most abundant cell type within the central nervous system [73]. They play a critical role in the health of the central nervous system by maintaining structural support, formation of the blood-brain barrier, neuronal metabolism and neurotransmitter synthesis [74]. In order to investigate the activation of astrocytes in response to various injuries, GFAP has been used as a primary marker for astrocytes [75]. Our results revealed an activation of the astrocytes all over the cerebellum and, more intensely, at the level of the white matter where we observed a hypertrophy of astrocytes. This observation is consistent with a prior study performed on rats demonstrating that gestational maternal exposure of rats to nicotine induced an elevation in the expression of GFAP in the cerebellum of the offspring at puberty [76]. Fuller et al. [77] reported an increase in the GFAP immunoreactivity in the cerebellum in adult rats exposed to CS for 3 weeks.

It has been shown that activated microglia promote stimulation of astrocytes through the secretion of IL-1β, which may work to increase the production of other cytokines such as IL-6, mainly from astrocytes [78]. In addition, it has been suggested that upon activation, astrocytes might play a potential role in the exacerbation of the neuronal and structural damage through the release of cytokines, chemokines, nitric oxide and reactive oxygen species, all of which can induce and potentiate inflammation [79]. The latter can cause DNA damage and apoptosis [80,81]. Therefore, we can speculate that the cerebellar inflammation and oxidative stress seen presently could plausibly be linked, at least partly, to the observed microgliosis and astrogliosis. Further experimental work is required to clarify this point.

In the current work, we did not correlate the observed biochemical and histological findings with cerebellar motor functions. Therefore, further studies are required to address this point using behavioral tests such as the rotarod test (to evaluate motor function and motor coordination) and treadmill gait analysis (to measure motor performance in mice by assessing quantitatively the gait).

In summary, we can conclude that our findings demonstrate for the first time, that chronic exposure to WPS triggers inflammatory responses, oxidative stress, DNA damage and apoptosis through mechanisms associated with NF-κB activation. These biochemical changes were accompanied with a marked astrogliosis and microgliosis in the cerebellum. Therefore, our data provide supporting evidence that WPS is a major risk factor for neuropathological alterations.

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**Institutional Review Board Statement:** This study was reviewed and approved by the United Arab Emirates University animal ethics committee, College of Medicine and Health Sciences, and experiments were performed according to protocols approved by the Institutional Animal Care and Research Advisory Committee (ERA_2021_8413).

**Informed Consent Statement:** Not applicable.

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

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**Conflicts of Interest:** The authors declare no conflict of interest.

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# Hypoxia Depresses Synaptic Transmission in the Primary Motor Cortex of the Infant Rat—Role of Adenosine $A_1$ Receptors and Nitric Oxide

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**Abstract:** The acute and long-term consequences of perinatal asphyxia have been extensively investigated, but only a few studies have focused on postnatal asphyxia. In particular, electrophysiological changes induced in the motor cortex by postnatal asphyxia have not been examined so far, despite the critical involvement of this cortical area in epilepsy. In this study, we exposed primary motor cortex slices obtained from infant rats in an age window (16–18 day-old) characterized by high incidence of hypoxia-induced seizures associated with epileptiform motor behavior to 10 min of hypoxia. Extracellular field potentials evoked by horizontal pathway stimulation were recorded in layers II/III of the primary motor cortex before, during, and after the hypoxic event. The results show that hypoxia reversibly depressed glutamatergic synaptic transmission and neuronal excitability. Data obtained in the presence of specific blockers suggest that synaptic depression was mediated by adenosine acting on pre-synaptic $A_1$ receptors to decrease glutamate release, and by a nitric oxide (NO)/cGMP postsynaptic pathway. These effects are neuroprotective because they limit energy failure. The present findings may be helpful in the preclinical search for therapeutic strategies aimed at preventing acute and long-term neurological consequences of postnatal asphyxia.

**Keywords:** hypoxia; postnatal asphyxia; motor cortex; synaptic transmission; adenosine; adenosine $A_1$ receptor; nitric oxide; cGMP; infant rat; neuroprotection

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## 1. Introduction

Perinatal asphyxia (or hypoxia–ischemia) is a lack of blood flow and/or gas exchange to the fetus/neonate immediately before, during, or after the birth process, due to compromised placental and/or pulmonary function. The incidence is between one and three per 1000 live births in developed countries, but the rate is up to about ten times higher in developing countries. Fifteen to twenty percent of the affected newborns die (accounting for an estimated 900,000 deaths each year) and up to 25% of survivors remain with permanent neurologic deficits. The significant human and social impact of perinatal asphyxia prompted a large number of experimental and clinical studies, which greatly improved our knowledge in this field. Short-term effects are hypoxic–ischemic encephalopathy and seizures, and long-term consequences include cerebral palsy, epilepsy, and behavioral/cognitive impairments [1–4]. The risk of cerebral palsy is increased threefold in the presence of neonatal seizures [5]; dyskinetic tetraplegic cerebral palsy is the most common subtype [6].

Notably, only a few studies have focused on asphyxia occurring later in the postnatal period, which can be due to various conditions, including pulmonary, neurological, or cardiovascular abnormalities, or positions of the body that prevent adequate breathing (positional asphyxia). The newborn's brain continues to develop after birth, and consequently the responsiveness to injuries such as asphyxia progressively changes [7]. The knowledge of the molecular processes involved in the response to asphyxia at defined ages is a necessary condition to offer appropriate targets for therapeutic intervention.

In both perinatal and postnatal asphyxia, the hypoxic insult can cause the immediate (necrosis) and delayed death (apoptosis) of neurons. No established treatment prevents necrosis, but apoptosis can be reduced by therapeutic interventions, which mainly consist of hypothermia to limit secondary energy failure. This treatment improves the outcomes, but its efficacy is limited, and its proper application requires expensive devices that can be prohibitive for some developing countries [8,9]. In an attempt to increase its effectiveness or replace it with new treatments, various promising agents have been used in pre-clinical studies, but the results are not yet satisfactory [10–12]. The severity of the long-term consequences is closely related to the amount of neurological damage that occurs within a few minutes of the onset of hypoxia, as a function of the balance between early disruptive and protective hypoxia-induced effects. Therefore, the understanding of the molecular mechanisms underlying the early changes occurring at the age of the hypoxic insult is important for developing appropriate therapeutic strategies.

Accordingly, experimental studies in animal models of hypoxia and hypoxia–ischemia have been performed at different ages [13–16]. The most used animals were rats and mice, with a minority of researchers studying larger animals such as pigs, sheep, or primates. Most of the studies in rat models have been performed in postnatal days (P) 0–10, and fewer studies after P11, based on comparative analyses suggesting that brain development of neonatal and infant rats up to about P10–13 days corresponds to the third trimester of human gestation. An analysis of experimental data from the literature estimated that the cerebral cortex of a P12–13 rat is developmentally comparable to that of a full-term newborn human [17]. A more recent study based on electrocortical brain activity recordings suggested that P1, P7, and P10 rats correspond to human 23, 30–32, and 40–42 weeks of gestation, respectively [18].

The most widely used animal model of hypoxia–ischemia is the unilateral carotid artery ligation of Rice–Vannucci [19]. Oxygen deprivation in neonatal and infant rats is the most used hypoxia-only model to focus on postnatal asphyxia and asphyxia-induced seizures, and to investigate electrophysiological and molecular mechanisms underlying their acute and long-term consequences [16]. Interestingly, Jensen et al. [20] found that oxygen deprivation induced epileptiform tonic/clonic behavior associated with epileptiform EEG activity significantly more frequently in P10–12 (64%) and P15–17 (62%) than in P5–7 (19%) rats. No epileptiform tonic/clonic behavior was observed in P25–27 and in P50–60 rats. In vitro hypoxia-only models are also extensively used; brain slice preparations proved to be particularly useful, because they allow for the combination of cellular and system level approaches.

Neurons mostly rely on oxidative metabolism for the maintenance of ion homeostasis and membrane potential; accordingly, electrophysiological recordings in brain slices have shown that neuronal membrane potential is highly sensitive to reduced oxygen availability. Depolarization and/or hyperpolarization may occur, depending on the brain region and the type of neuron, and even on the resting potential of the neuron [21]. Hypoxia-induced hyperpolarization is neuroprotective, as being associated with synaptic depression, it decreases energy consumption [22]. Notably, hypoxia-induced synaptic depression appeared later and was less pronounced in CA1 neurons in hippocampal slices from P1–P11 rats than in those from P14–21 and adult rats [23], and in II/III-layer neurons of somatosensory cortex slices from P5–8 and P14–18 rats than in those from adult rats [24,25].

Extracellular adenosine increases in the brain during hypoxia. Most of the studies performed in adult animal preparations show that the resulting increased activation of $A_1$ receptors may play a neuroprotective role, mainly by reducing glutamate release and NMDA receptor activation, and by inducing hyperpolarization through inwardly rectifying $K^+$ channel activation [26–32]. The protective effect is more pronounced in the early phase of the hypoxic event, and then it becomes progressively less efficient due to the internalization of $A_1$ receptors and the desensitization of $A_1$ receptor-mediated responses [33]. Notably, data obtained in infant animals suggest that increased adenosine $A_1$ receptor activation may exert adverse effects on the developing brain, rather than neuroprotection [34]. For

example, $A_1$ receptor stimulation could not prevent ischemic brain damage in 7-day-old rats, likely reflecting the lower number of receptors (23% of the adult levels) [35]. Moreover, a study performed in P3–14 mice exposed to hypoxia showed that these receptors play a prominent role in the development of hypoxia-induced ventriculomegaly [36].

Another interesting player in hypoxia is nitric oxide (NO), which has emerged as both a major neurotoxic agent and a potential therapeutic intervention. Despite the extensive therapeutic use of inhaled NO for pulmonary artery hypertension, its actions during brain hypoxia and ischemia (which coexist with pulmonary artery hypertension in 20–30% of affected infants) are still unclear, due to the high variability of the effects produced [37]. The effects may differ according to various factors, including age, temporal stage after hypoxia onset, brain region and cell type by which it is produced, NO synthase (NOS) isoform involved, amount of NO produced, cellular redox state, and underlying disease processes [37–40]. Simplifying a much more complex scenario, data obtained so far suggest that NO produced by endothelial NOS mainly plays a neuroprotective role, by maintaining cerebral blood flow (vasodilatory action) and by inhibiting platelet and leukocyte adhesion, whereas NO produced by inducible and neuronal NOS tends to be neurotoxic, leading to cascade reactions of excitotoxicity, inflammation, and apoptosis. Consequently, clinical trials with selective neuronal and inducible NOS inhibitors as potential agents for the treatment of hypoxic encephalopathy are now ongoing [39]. Notably, NO is a key signaling molecule that plays a critical role in a wide range of physiological functions in the brain, including synaptic plasticity and memory formation [41–43]. In hippocampal slices from adult rats, NO was found to contribute to synaptic depression induced by prolonged (8–45 min) but not by brief (2–3 min) hypoxic events in CA1 neurons [44]. In the same preparation, synaptic depression induced by a selective agonist of adenosine $A_1$ receptors resulted to be partly mediated by the NO/cGMP pathway [45].

Surprisingly, electrophysiological changes induced by hypoxia in the infant motor cortex have not been investigated so far, despite the critical involvement of this cortical area in epilepsy. In the present study, to shed light on the acute effects of the decrease in oxygen availability in this specific neuronal network, primary motor cortex slices obtained from the brains of infant rats were exposed to 10 min of hypoxia. We choose the age window P16–18 as a model of human postnatal asphyxia because it is characterized by a high incidence of hypoxia-induced seizures associated with epileptiform motor behavior [20]. Extracellular field potentials evoked by horizontal pathway stimulation were recorded in layers II/III of the primary motor cortex before, during, and after the hypoxic event to study the effect on glutamatergic synaptic transmission and neuronal excitability.

## 2. Materials and Methods

### *2.1. Animals*

Twenty-nine ($n$ = 29) P16–18 infant male Sprague Dawley CD IGS rats (Charles River Laboratoires Italia, Calco, Italy) were used in this study. Animals were treated in accordance with the European Community Directives 86/609/EEC and 2010/63/EU, and the 3R concept has been considered when planning the experiments. The animal study protocol was approved by the Ethical Committee of the University of Bologna. Rats were individually housed under controlled conditions (temperature: 24 ± 1 °C; humidity: 50 ± 5%), maintained on a 12:12 h light-dark cycle, and fed ad libitum.

### *2.2. Slice Preparation*

The experiments were carried out in coronal brain slices including the primary motor cortex (M1). The slices were prepared as previously described [46]. Briefly, rats were deeply anaesthetized using halothane and rapidly decapitated. Their brains were rapidly removed and immersed in ice-cold low-sodium, high-sucrose solution containing (in mM): 212.7 sucrose, 26.0 $NaHCO_3$, 2.6 KCl, 1.23 $NaH_2PO_4$, 2.0 $MgSO_4$, 10 dextrose, and 2.0 $CaCl_2$, bubbled with a mixture of 95% $O_2$ and 5% $CO_2$ at pH 7.4. Coronal slices, 400 μm thick, were cut using an oscillating tissue slicer (FHC, Bowdoin, ME, USA), beginning ~2 mm caudal to

the frontal pole. Slices were collected in a range from 3.7 to 1.7 mm anterior to Bregma [47] in artificial cerebro-spinal fluid (ACSF) of the following composition (in mM): 126.0 NaCl, 26.0 NaHCO3, 3.0 KCl, 1.25 $NaH_2PO_4$, 1.0 $MgSO_4$, 2.0 $CaCl_2$, and 10.0 dextrose, bubbled with a mixture of 95% $O_2$ and 5% $CO_2$ at pH 7.4 and maintained at room temperature. All chemicals were purchased from Sigma Adrich - Merck KGaA (Darmstadt, Germany). After a recovery time of at least 1 h, the slices were transferred into a submersion recording chamber perfused (3 mL/min) with warm (34 °C) ACSF and left undisturbed for at least another hour before starting recording.

*2.3. Extracellular Field Potential Recording*

Field excitatory postsynaptic potentials (fEPSPs) evoked by glutamatergic afferent pathway stimulation were recorded using glass micropipettes filled with 2.0 M NaCl (1–3 MΩ) and connected to a DC current amplifier by an Ag/AgCl electrode. Recording micropipettes were positioned in layers II/III of the primary motor cortex, 200–400 μm below the cortical surface and 2.5–4.5 mm lateral to the midline. Horizontal cortical pathways were stimulated with a concentric bipolar electrode (70–80 KΩ; FHC, Bowdoin, ME, USA) located in cortical layers II/III, at ~500 μm from the recording electrode [48]. Constant-current square pulses (0.2 ms) were applied at 0.1 Hz using the stimulus generator Master 8 (AMPI, Jerusalem, Israel); stimulus intensity was adjusted to the value needed to elicit the maximal synaptic response (we chose this intensity to be able to also induce a non-synaptic response large enough to be measured accurately). Only slices in which we could elicit a synaptic response of at least 0.8 mV amplitude were considered in this study. Before hypoxia induction, the perfusion was switched to an ACSF containing the $GABA_A$ receptor inhibitor (-)-bicuculline methobromide (BMI, 2 μM) and, when indicated, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 50 nM), 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ, 100 μM), and Nω-nitro-l arginine methyl ester (L-NAME, 2 mM) or DPCPX (50 nM) plus L-NAME (2 mM). In order to identify non-synaptic and synaptic components of the fEPSPs, at the end of all the experiments the perfusion was switched to an ACSF containing the AMPA and kainate glutamate receptors blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM).

*2.4. Hypoxia Induction*

Hypoxia was induced when the amplitude of the synaptic response was stable (none of the values exceeding the average by more than 10%): slices were perfused with ACSF equilibrated with 95% $N_2$ and 5% $CO_2$ (instead of 95% $O_2$ and 5% $CO_2$). Changes in $O_2$ pressure ($pO_2$) were monitored with an $O_2$ electrode (WPI, Sarasota, FL, USA) placed in the perfusing ACSF within the recording chamber. In order to avoid the onset of anoxic depolarization (AD) we set an anoxic perfusion time of 10 min (AD events were never observed in these conditions).

*2.5. Agents and Chemicals*

BMI, CNQX, DPCPX, ODQ, and TTX were purchased from Tocris - Biotechne (Minneapolis, MN, USA), and L-NAME from Sigma Adrich - Merck KGaA (Darmstadt, Germany). DPCPX was made in a 5 mM stock solution of 99% dimethyl sulfoxide (DMSO) and 1% 1 M NaOH; ODQ was dissolved in 99% DMSO. DPCPX and ODQ solutions were then diluted in ACSF. TTX was dissolved in distilled water and then diluted in ACSF. All the other drugs were directly dissolved in ACSF.

*2.6. Data Analysis and Statistics*

*Responses to single-pulse stimulation.* We measured the amplitude of the non-synaptic and the synaptic components of the fEPSPs by using the software Axon Clampfit 7.0 (Molecular Devices, San Jose, CA, USA). Six consecutive responses were pooled in each minute data and expressed as mean ± standard deviation. For each slice, data were normalized taking as 100% the averaged of the values obtained during the last ten minutes

before hypoxia onset. Statistical analysis within and among groups was performed by the one-way analysis of variance (ANOVA) followed by the post hoc Tukey's multiple comparison test.

*Responses to paired-pulse stimulation*. We measured the amplitude of the synaptic component of the fEPSPs evoked by two pulses delivered at a 50 ms interval. The average of six consecutive responses to the second pulse (R2) was divided by the average of six consecutive responses to the first one (R1) and expressed as paired-pulse ratio (mean ± standard deviation). Statistical analysis between pre-hypoxia and hypoxia values was performed using Student's *t*-test.

## 3. Results

Pyramidal cells in layer II/III make mono-synaptic connections via long horizontal collaterals with the proximal dendrites of layer II/III cells belonging to distant columns [48]. We found that the stimulation of these axon collaterals generates an fEPSP containing: (a) a first short-latency (1.4 ± 0.4 ms; $n = 18$) negative component, due to neuronal activation as it was sensitive to TTX (1 μM), but non-synaptic being resistant to CNQX (20 μM); (b) a second negative-going wave (latency: 2.8 ± 1.0 ms; $n = 23$), which is the glutamatergic synaptic component as it was sensitive to CNQX (20 μM).

### *3.1. Hypoxia-Induced Depression of Neuronal Excitability and Glutamatergic Synaptic Transmission*

The 10 min perfusion with anoxic ACSF caused a gradual fall in $O_2$ tension ($pO_2$) within the recording chamber, and both the depressed non-synaptic and synaptic components of the fEPSPs; $pO_2$ gradually decreased to about 20% of the control values, then rapidly recovered. The non-synaptic component of the fEPSPs was gradually depressed during and after hypoxia and reached the minimal mean value about 12 min after hypoxia onset. The synaptic component was more strongly depressed and reached the minimal mean value about 10 min after hypoxia onset. The effect on the two components of the fEPSPs is shown in Figure 1. The statistical analysis revealed that the means of the minimal values were significantly different with respect to the means of pre-hypoxia baseline values ($p < 0.001$), and that the mean of non-synaptic minimal values was significantly different with respect to the mean of the synaptic minimal values ($p < 0.001$) (Figure 1B). Both fEPSPs components recovered after 20 min of re-oxygenation (Figure 1B).

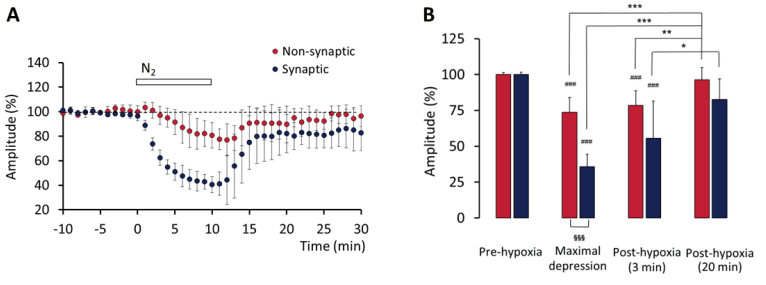


**Figure 1.** Hypoxia-induced changes on fEPSPs recorded in layers II/III of the primary motor cortex, in brain slices from infant rats. (**A**) Time course of the effect of hypoxia on the amplitude of non-synaptic (red symbols) and synaptic (blue symbols) components of the fEPSPs ($n = 5$). (**B**) Amplitude of non-synaptic (red histograms) and synaptic (blue histograms) components of the fEPSPs: comparison of the mean values (± SD) recorded during 10 min before hypoxia onset (pre-hypoxia), during the minute corresponding to the maximal hypoxia-induced depressive effect, and during min 3 and min 20 after re-oxygenation onset. ### $p < 0.001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; §§§ $p < 0.001$ (vs. the columns indicated).

*3.2. Involvement of Adenosine $A_1$ Receptors and the NO/cGMP Pathway in the Depressive Effects of Hypoxia*

To evaluate whether the adenosine $A_1$ receptor- and the NO/cGMP pathways were involved in the depressive effects of hypoxia, we compared the fEPSP components recorded in control conditions with those recorded in slices pre-treated with the adenosine $A_1$ receptor antagonist DPCPX (50 nM), the NO synthase inhibitor L-NAME (2 mM), or the NO-sensitive soluble guanylyl cyclase (GC) inhibitor ODQ (100 μM).

As shown in Figure 2, the effect of hypoxia on the amplitude of the non-synaptic component of the fEPSP (which is considered an index of neuronal excitability) was not significantly different in the control slices ($n$ = 5) with respect to those pre-treated with DPCPX ($n$ = 4; Figure 2A), L-NAME ($n$ = 5; Figure 2B), or ODQ ($n$ = 4; Figure 2C). These results suggest that the $A_1$ receptors and the NO/cGMP pathway do not play a major role in the depressive effect of hypoxia on neuronal excitability. Comparisons among the means of the values recorded during pre-hypoxia, maximal depression, and after 3 and 20 min of re-oxygenation, in control conditions and in the presence of DPCPX, L-NAME or ODQ, are shown in Figure 2D.

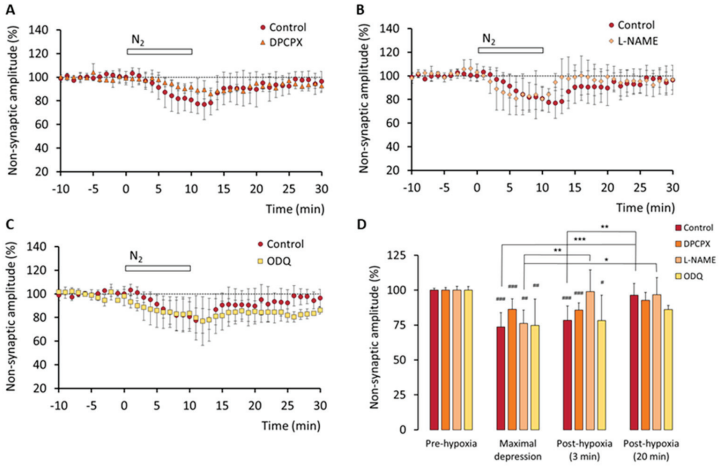


**Figure 2.** Adenosine $A_1$ receptors and NO/cGMP are not involved in hypoxia-induced depression of the non-synaptic component of the fEPSPs. Time course of hypoxia effect on the amplitude of the non-synaptic peak in control conditions ($n$ = 5) and after pre-treatment with: (**A**) the $A_1$ receptors antagonist DPCPX (50 nM; $n$ = 3); (**B**) the NO synthase inhibitor L-NAME (2 mM; $n$ = 5); (**C**) the NO-sensitive GC inhibitor ODQ (100 μM; $n$ = 4). (**D**) Comparison of the mean amplitude (± SD) of the non-synaptic peak recorded during the 10 min before hypoxia onset (pre-hypoxia), during the minute corresponding to the maximal hypoxia-induced depressive effect, and during min 3 and min 20 after re-oxygenation onset in the different conditions indicated. ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ (vs. pre-hypoxia). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (vs. the columns indicated).

By contrast, the peak amplitude of the synaptic component of the fEPSPs was much less depressed by hypoxia in slices pre-treated with DPCPX ($n$ = 4) than in control slices: the means of the values were significantly different at the maximal depression ($p < 0.001$) and after 3 min of re-oxygenation ($p < 0.05$) (Figure 3A,D). The effect of DPCPX suggests a major role of adenosine acting on $A_1$ receptors in the depressive effect of hypoxia on glutamatergic transmission. Comparisons among the means of the values recorded during pre-hypoxia, maximal depression, and after 3 and 20 min of re-oxygenation, in control conditions and in the presence of DPCPX (or the other drugs), are shown in Figure 3D.

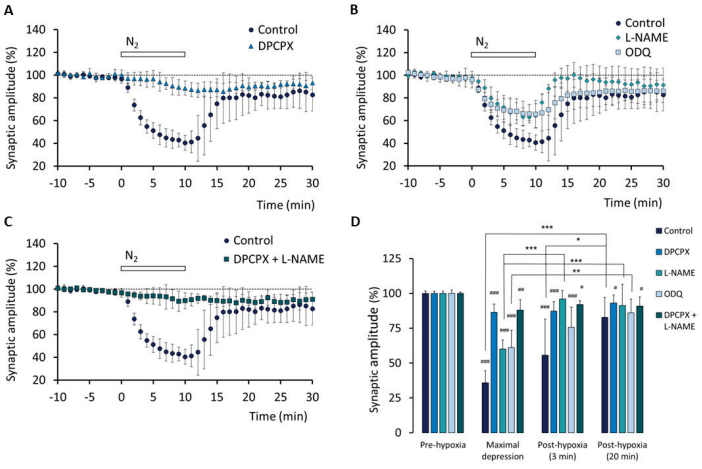


**Figure 3.** Involvement of adenosine $A_1$ receptors and NO/cGMP in hypoxia-induced depression of the synaptic component of the fEPSPs. Time course of hypoxia effect on the amplitude of the synaptic peak in control conditions ($n$ = 5) and after pre-treatment with: (**A**) the $A_1$ receptors antagonist DPCPX (50 nM; $n$ = 4); (**B**) the NO synthase inhibitor L-NAME (2 mM; $n$ = 5) or the NO-sensitive GC inhibitor ODQ (100 μM; $n$ = 5); (**C**) both DPCPX (50 nM) and L-NAME (2 mM) ($n$ = 4). (**D**) Comparison of the mean amplitude (± SD) of the synaptic peak recorded during the 10 min before hypoxia onset (pre-hypoxia), during the minute corresponding to the maximal hypoxia-induced depressive effect, and during min 3 and min 20 after re-oxygenation onset in the different conditions indicated. ### $p < 0.001$, ## $p < 0.01$; # $p < 0.05$ (vs. pre-hypoxia). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (vs. the columns indicated).

Figure 3B shows that also in the slices pre-treated with L-NAME ($n$ = 5), the synaptic peak amplitude was less depressed by hypoxia than in the control slices: the means of the values were significantly different at the maximal depression ($p < 0.01$) and after 3 min of re-oxygenation ($p < 0.01$) (Figure 3B,D). The effect of L-NAME suggests an involvement of NO in the depressive effect of hypoxia on glutamatergic transmission. The comparison between the effects of DPCPX and L-NAME (cf. Figure 3A,B and see Figure 3D) suggests that the NO contribution to hypoxia-induced synaptic depression is smaller than that of adenosine acting through $A_1$ receptors.

In addition, Figure 3B shows that also in ODQ-treated slices ($n$ = 5), the synaptic peak amplitude was less depressed by hypoxia than in the control slices; the means of the values were significantly different at the maximal depression ($p < 0.01$), but not after 3 min of re-oxygenation (Figure 3B,D). Nevertheless, the comparison between the effects of L-NAME and ODQ (Figure 3B) suggests a major involvement of the NO-sensitive GC in the effect of NO on synaptic transmission.

In a series of experiments, the slices ($n$ = 4) were treated with both DPCPX (50 nM) and L-NAME (2 mM). We found that the synaptic fEPSP component was depressed by hypoxia as in DPCPX-treated slices (cf. Figure 3A,C,D): the means of the values were significantly different vs. those recorded in control slices at maximal depression ($p < 0.001$) and after 3 min of re-oxygenation ($p < 0.05$).

### *3.3. Involvement of Changes at Pre-synaptic Level in Hypoxia-Induced Depression of Synaptic Transmission*

To assess whether hypoxia-induced depression of synaptic transmission involved changes at the pre-synaptic level, we used the paired-pulse paradigm, which provides a reliable indication of pre-synaptic events [49,50].

Under normoxic conditions ($n$ = 5), using a 50 ms inter-stimulus interval, the paired-pulse ratio (PPR) of synaptic amplitudes (responses to the second pulse divided by re-

sponses to the first one) was 0.831 ± 0.069 (i.e., paired-pulse depression). During hypoxia, PPR significantly ($p < 0.005$) increased (1.325 ± 0.304; i.e., paired-pulse facilitation), suggesting a decrease in glutamate release. PPR returned to pre-hypoxia values after re-oxygenation (0.871 ± 0.114) (Figure 4A).

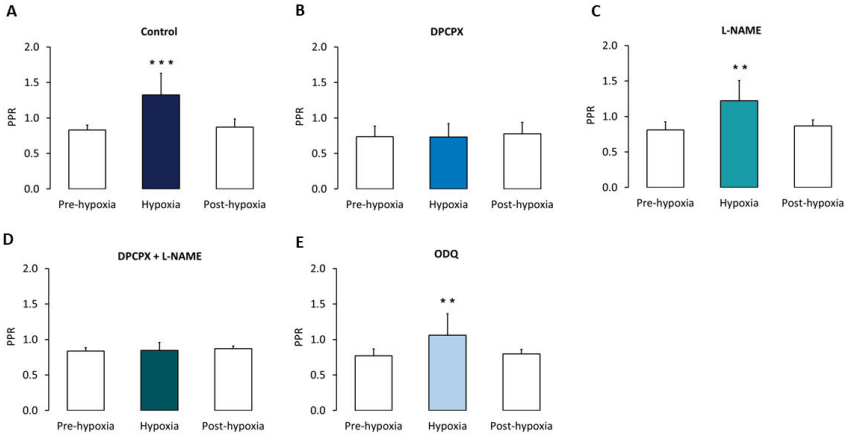


**Figure 4.** Paired-pulse ratio changes induced by hypoxia. (**A**–**E**) Comparison of the mean (± SD) paired-pulse ratio (PPR) of synaptic peak amplitudes (average of six consecutive responses to the second pulse (R2) divided by the average of six consecutive responses to the first one (R1)) recorded during the last minute before hypoxia onset (pre-hypoxia), during the minute corresponding to the maximal hypoxia-induced depressive effect of the response evoked by the first pulse (hypoxia), and during min 20 after re-oxygenation onset (post-hypoxia), in: (**A**) control conditions ($n = 5$); (**B**) after pre-treatment with the $A_1$ receptors antagonist DPCPX (50 nM; $n = 4$); (**C**) after pre-treatment with the NO synthase inhibitor L-NAME (2 mM; $n = 5$); (**D**) after pre-treatment with both DPCPX (50 nM) and L-NAME (2 mM) ($n = 4$); (**E**), after pre-treatment with the NO-sensitive GC inhibitor ODQ (100 μM; $n = 5$). *** $p < 0.005$, ** $p < 0.01$.

In DPCPX-treated slices ($n = 4$), PPR did not increase during hypoxia, suggesting a major involvement of adenosine, which through pre-synaptic $A_1$ receptors decreased glutamate release (Figure 4B).

By contrast, in L-NAME- ($n = 5$) and ODQ- ($n = 5$) treated slices, PPR significantly ($p < 0.01$ and $p < 0.05$, respectively) increased during hypoxia, suggesting that NO does not play a significant role in the hypoxia-induced decrease in glutamate release. PPR recovered after re-oxygenation (Figure 4C,E).

In DPCPX + L-NAME ($n = 4$) -treated slices, PPR did not increase during hypoxia (Figure 4D), as observed in slices only treated with DPCPX.

## 4. Discussion

In order to investigate the effects induced in the motor cortex by an asphyxia event occurring in the postnatal period, we exposed brain slices from infant rats in the age window P16–18, which is characterized by high incidence of hypoxia-induced seizures associated with epileptiform motor behavior, to 10 min of hypoxia [20].

The results obtained show that the hypoxic event caused a reversible depression of neuronal excitability and glutamatergic synaptic transmission (maximal depression: 26 and 64%, respectively) in the primary motor cortex. The effect of hypoxia on synaptic transmission is consistent with previous data obtained in CA1 neurons in rat hippocampal slices exposed to 2–4 min of hypoxia; compared to our results, the maximal synaptic depression was lesser in hippocampal slices from P1 and P4 rats (42 and 52%, respectively), and greater in those from P11, P14, P21, and adult rats (72, 100, 98, and 89%, respectively) [23]. The synaptic depression observed in the present study is also consistent with previous results obtained in

II/III-layer neurons in rat somatosensory cortex slices exposed to 2.5 [24] or 20 [25] min of hypoxia; compared to our results, the maximal synaptic depression was lesser in both studies, at all the ages considered: P5–8 (10 and 3%, respectively), P14–18 (15 and 42%, respectively), and adulthood (42% and 55%, respectively). Since about 33–50% of cerebral oxygen is used for synaptic transmission [22], its depression is strongly neuroprotective: by decreasing the mismatch between energy requirement and supplies, it contributes to the prevention of the early and long-term neurological consequences of hypoxia. Present results and the previous ones obtained in the hippocampus [23] and in the somatosensory cortex [24,25] suggest that this protective system develops and progressively becomes more and more effective, but at different speeds and reaching a different maximum level of efficacy in different brain regions in adulthood; the hippocampus appears to be the most protected brain region.

The strong effect of hypoxia on synaptic responses to paired-pulse stimulation (from depression to facilitation) observed in the present experiments suggests that synaptic depression is at least partly due to the inhibition of glutamate release. This view is further supported by the following evidence, which suggest the involvement of adenosine acting on pre-synaptic $A_1$ receptors: the specific $A_1$ antagonist DPCPX prevented the hypoxia-induced inhibition of synaptic responses, as well as the effect of hypoxia on synaptic responses to paired-pulse stimulation. Moreover, a previous observation in hippocampal slices from adult mice provided direct evidence that the neuroprotective role of adenosine during hypoxia depend on inhibition of synaptic transmission by the activation of presynaptic $A_1$ receptors: the focal deletion of these receptors on the Schaffer collateral input slowed the depression of CA3 fEPSPs in response to hypoxia and impaired the recovery of the fEPSPs after hypoxia [29]. In our experiments, we cannot exclude that post-synaptic adenosine $A_1$ receptors activation might contribute to synaptic inhibition, through the inhibition of NMDA receptors and/or activation of inwardly rectifying $K^+$ channels. However, the latter contribution appears negligible in present experimental conditions, as the specific $A_1$ antagonist DPCPX did not prevent a hypoxia-induced decrease in neuronal excitability. Regardless of the site of action, present results provide the first evidence of neuroprotection by adenosine, via $A_1$ receptors, against energy failure induced by hypoxia in the primary motor cortex of infant rats. This evidence appears particularly relevant, as previous studies had shown that the adenosine activation of $A_1$ receptors during hypoxia induced neurotoxic rather than neuroprotective effects in the developing brain [34–36].

The results obtained in the presence of the NO synthase inhibitor L-NAME suggest that NO was also involved in the hypoxia-induced depression of synaptic transmission, in accordance with previous observations in hippocampal slices from adult rats [44,51]. In our experiments, the NO contribution to synaptic depression was smaller than that of adenosine acting on $A_1$ receptors. Physiological NO signal transduction mainly occurs through the activation of intracellular GC-coupled receptors, leading to cGMP formation [52,53]. Present data suggest the involvement of the cGMP pathway in the contribution of NO to the depressive effect on synaptic transmission, since it is completely prevented by the GC inhibitor ODQ. This is consistent with the previous observation that an NO donor depresses synaptic transmission in hippocampal CA1 through an increase in cGMP [54], and with a recent study partly performed in our laboratory showing that the NO/cGMP pathway in the rat perirhinal cortex is involved in a form of long-term depression of glutamatergic synaptic transmission that is crucial for visual recognition memory [42]. Here, we also found that the blockade of either NO or cGMP synthesis does not prevent hypoxia-induced changes of synaptic responses to paired-pulse stimulation (from depression to facilitation), suggesting that NO depressed synaptic transmission by acting at a post-synaptic level rather than on glutamate release, in agreement with previous findings [54,55]. In hippocampal slices from adult rats, synaptic depression induced by a selective agonist of adenosine $A_1$ receptors resulted to be partly mediated by the NO/cGMP pathway [45]. The present results suggest that the two depressive mechanisms are instead independent in the motor cortex, because they act in different synaptic sites. Apart from the mechanism of action, the present data provide the first evidence in support of the hypothesis that in addition to adenosine, NO may also contribute to limiting the energy failure

induced by hypoxia in the primary motor cortex of infant rats. This potential neuroprotective action should be considered among the many contrasting effects induced by NO during and after hypoxic events.

The present results show that hypoxia also induced a minor and reversible $A_1$ receptor- and NO-independent depression of neuronal excitability; this effect contributes to neuroprotection by reducing action potential firing. In accordance with our data, previous studies in somatosensory cortex slices from adult rats have shown that hypoxia induces hyperpolarization and a decrease in input resistance, possibly through the activation of ATP-sensitive $K^+$ conductance [56,57]. Studies in hippocampal slices from adult rats have shown that the CA1 antidromic population spike amplitude is not (or is only slightly) depressed by hypoxia [58–60]; membrane hyperpolarization and decrease in input resistance were also observed [61,62].

## 5. Conclusions

The results of the present study provide the first evidence that hypoxia causes a reversible depression of glutamatergic synaptic transmission and neuronal excitability in primary motor cortex slices from infant rats. Data obtained in the presence of specific blockers suggest that synaptic depression was mediated by adenosine acting on presynaptic $A_1$ receptors to decrease glutamate release, and by an NO/cGMP postsynaptic pathway. Depression of synaptic transmission and neuronal excitability is neuroprotective because it limits energy failure: by decreasing the mismatch between energy needs and supplies, it contributes to the prevention of early and long-term neurological consequences of hypoxia. The present findings might be helpful in the search for therapeutic strategies aimed at preventing acute and long-term neurological consequences of postnatal asphyxia.

**Author Contributions:** Conceptualization and design of the work, I.Z. and G.A.; electrophysiological experiments, data acquisition and analysis, I.Z.; data interpretation, I.Z. and G.A.; original draft preparation and review, I.Z. and G.A.; approval of the submitted version, I.Z. and G.A. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The animal study protocol was approved by the Ethical Committee of the University of Bologna.

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

**Conflicts of Interest:** The authors declare no conflict of interest.

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# Temporary Occlusion of Common Carotid Arteries Does Not Evoke Total Inhibition in the Activity of Corticospinal Tract Neurons in Experimental Conditions

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**Abstract:** Temporary occlusion of the common cervical artery is the reason for ischemic stroke in 25% of patients. Little data is provided on its effects, especially regarding neurophysiological studies verifying the neural efferent transmission within fibers of the corticospinal tract in experimental conditions. Studies were performed on 42 male Wistar rats. In 10 rats, ischemic stroke was evoked by permanent occlusion of the right carotid artery (group A); in 11 rats, by its permanent bilateral occlusion (B); in 10 rats, by unilateral occlusion and releasing after 5 min (C); and in 11 rats, by bilateral occlusion and releasing after 5 min (D). Efferent transmission of the corticospinal tract was verified by motor evoked potential (MEP) recordings from the sciatic nerve after transcranial magnetic stimulation. MEPs amplitude and latency parameters, oral measurements of temperature, and verification of ischemic effects in brain slides stained with hematoxylin and eosin staining (H + E) were analyzed. In all groups of animals, the results showed that five minutes of uni- or bilateral occlusion of the common carotid artery led to alterations in brain blood circulation and evoked changes in MEP amplitude (by 23.2% on average) and latency parameters (by 0.7 ms on average), reflecting the partial inability of tract fibers to transmit neural impulses. These abnormalities were associated with a significant drop in the body temperature by 1.5 °C on average. Ten minutes occlusion in animals from groups A and B resulted in an MEP amplitude decrease by 41.6%, latency increase by 0.9 ms, and temperature decrease by 2.9 °C of the initial value. In animals from groups C and D, five minutes of recovery of arterial blood flow evoked stabilization of the MEP amplitude by 23.4%, latency by 0.5 ms, and temperature by 0.8 °C of the initial value. In histological studies, the results showed that ischemia was most prominent bilaterally in sensory and motor areas, mainly for the forelimb, rather than the hindlimb, innervation of the cortex, putamen and caudate nuclei, globulus pallidus, and areas adjacent to the fornix of the third ventricle. We found that the MEP amplitude parameter is more sensitive than the latency and temperature variability in monitoring the ischemia effects course following common carotid artery infarction, although all parameters are correlated with each other. Temporary five-minute lasting occlusion of common carotid arteries does not evoke total and permanent inhibition in the activity of corticospinal tract neurons in experimental conditions. The symptoms of rat brain infarction are much more optimistic than those described in patients after stroke, and require further comparison with the clinical observations.

**Keywords:** ischemic stroke; common carotid artery occlusion; corticospinal tract function; motor evoked potentials; temperature; rat

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## 1. Introduction

Ischemic stroke is a disease with a high mortality and an enormous socio-economic burden [1]. It is the second highest (87%) health problem in the world population, occurring more often than a hemorrhagic stroke (25%) [2]. The percentage of elderly patients with the incidence of ischemic stroke dramatically increases, but the average age of patients is decreasing [3]. Despite intensive experimental and clinical scientific efforts throughout recent decades, treatment options for acute ischemic stroke patients remain very limited [4]. Etiopathogenesis, the course and effects of ischemic stroke, seems to be the most important topic that impacts the best treatment strategies [5–8]. The cause of stroke is most often the aim of studies referring especially to people as well as the experimental studies on animals, and much is known on this topic [9]. However, effects of stroke before brain death seem to be unsatisfactorily described [10].

Among commonly used methods of clinical neurophysiology, such as electromyography and electroneurography, somatosensory (SEP) and motor evoked potentials (MEPs) were frequently chosen as precise methods in the evaluation of transmission in ascending and descending pathways in patients after stroke [11–13]. A number of studies in patients with ischemic stroke showed that MEP recordings are a sensitive prognostic indicator of the effects and orientation of treatment, aiming to restore the lost motor function [14–16]. MEPs recorded on the more paretic side were either absent or had low amplitude and prolonged duration, while the central efferent conduction time was only slightly increased [17–19]. Studies of MEPs in stroke patients provided descriptions of changes in their recording parameters up to 3 months after a middle carotid artery infarction [18].

Previous experimental studies with MEP recordings mainly from muscles, and less often from nerves, are aimed towards the description of ischemic stroke models following middle cerebral artery occlusion and include observations of changes in efferent transmission up to 6 h [20,21]. The reason this work has been undertaken is the phenomenon of variability in efferent transmission through the corticospinal tract as a result of occlusion in the common carotid artery, constituting the high incidence cause of ischemic stroke. Local carotid stenosis at 70% of its diameter occurs in 20% of stroke patients, but few papers clarified its effects [22].

Dougherty et al. [23] and Garcia [24] state that most rodents are the ideal models for observing the effects of stroke following selective carotid artery uni- or bilateral occlusion. The rat model commonly used for carotid artery occlusion is considered the most reliable in the evaluation of the effects of ischemic stroke on the function of the cortico-spinal system in experimental conditions [25], similarly, as applied in this study. Kety [26] believes that the blockage of the carotid artery does not cause significant changes in the cerebral circulation of most mammals, including humans, while Longa et al. [27] mention that the occlusion of intracranial carotid arterial branches in the rat does not cause stroke at all. In their opinion, this is due to numerous anastomoses forming connections between the branches of external and internal carotid arteries.

The available literature does not provide detailed descriptions of studies on the course of ischemic stroke in the rat after occlusion of the common carotid artery when transcranial magnetic field stimulation was used to assess its effects on cortical motor neuron excitability. Moreover, if such studies were undertaken, the recordings of evoked potentials from hindlimb muscles instead of nerves were performed. This last-mentioned site of recordings would increase the precision of measurements in the direct efferent transmission coming from the possible influence of anesthetics and changes in muscle motor end-plate function. Therefore, in this paper, an attempt was made to demonstrate the longest duration effect of cerebral ischemia in experimental conditions, which, despite its existence, is reversible in adverse effects on the activities of neurons in the motor cortex.

## 2. Materials and Methods

### *2.1. Animals and Study Design*

Studies were performed on 46 adult male Wistar rats weighing from 220 to 280 g, kept adaptively for a week in a laboratory animal house. Tests were conducted in the same room, at 21–23 °C, in a similar manner for all animals; firstly, supine, and later, the prone position with tetrapodal fixation. Animals (N = 42) were divided into four groups, depending on the different options of the induced brain ischemia by occlusion of the common carotid artery. In group A (N = 10), stroke was induced by permanent clamping of the right carotid artery; in group B (N = 11), by permanent bilateral clamping of the carotid arteries; in group C (N = 10), by temporary clamping of the right carotid artery with its releasing; and in group D (N = 11), by temporary clamping the carotid arteries on both sides with their later releasing (Figure 1).

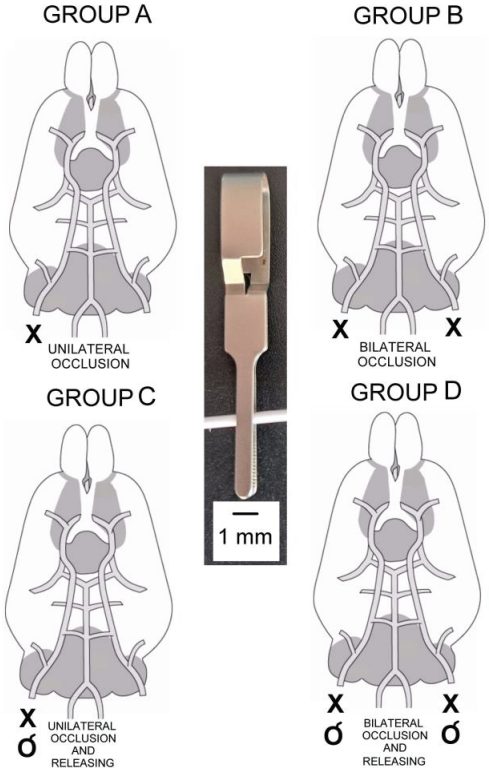


**Figure 1.** Diagrams of four types of induced ischemic stroke on plans of the rat brain vasculature. Group A—permanent unilateral occlusion of the common carotid artery (always right), group B—permanent bilateral occlusion of the common carotid arteries, group C—temporary unilateral occlusion of the common carotid artery (always right) with its releasing, group D—temporary bilateral occlusion of the common carotid arteries with their release. The "X" means occlusion, while "O" means the artery release. The center of the figure shows a surgical clamp used to occlude the common carotid artery.

The outcome data were parameters of the amplitude (in μV) and latency (in ms) of the motor evoked potentials and the oral temperature (in °C) measured before and every minute at the subsequent stages of experiments. In 4 out of 46 animals (control group), stroke had not been induced but MEP and temperature measurements were performed in order to obtain reference values for comparison to occlusion effects and to parameters of

other authors [21]. The total observation time in groups A and B was 10 min and 11 min in groups C and D. In animals from groups C and D, after five minutes of temporary occlusion of the common carotid artery on the right side or bilaterally, the "0 period" was applied for about one minute for the recovery of the arterial blood flow, including the elastic properties of the vessel after clamp release. The attempts to measure the blood pressure and $PCO_2$ with a small cannula from the vein were abandoned because they might influence the arterial flow, an essential factor of stroke induction. After experiments, the brains were collected for cutting onto slides for histopathological examination. Histological verification also confirmed the influence of transcranial magnetic stimulation on the brain tissue structure in animals from the control group. The MEP parameters and the results from histological brain slides were read out independently by three observers, then discussed with and verified to obtain the final conclusions.

The study was conducted in accordance with the Declaration of Helsinki. Ethical considerations were also in agreement with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010, on the protection of animals used for scientific purposes. Approval was also received from the Bioethical Committee of the University of Medical Sciences in Poznań (Poland), including studies on animals (decision no. 4/2008).

### *2.2. Anesthesia and Surgical Procedures*

The animals were anesthetized intraperitoneally with ketamine hydrochloride (Ketanest, Park-Davis, Germany) at a dose of 90 mg/kg body weight. The depth of anesthesia was verified by a pupil dilatation and no toe pinch reflex could be elicited after approximately 25 min. The effect of the drug started approximately 20 min after administration and lasted for around 2 h. Small doses of ketamine (10 mg/kg) were added, when necessary, to maintain the anesthesia. To prevent aspiration of saliva into the lungs, the animals were premedicated by intramuscular injections with atropine sulfate (Polpharma, Poland) at a dose of 0.05 mg/kg.

When animals were placed dorsally, the common carotid artery was dissected uni- or bilaterally, 2 mm before it splits to internal and external carotid arteries. Dissected common carotid arteries were occluded and/or released depending on the group of tested animals (Figure 1).

When animals were placed ventrally, the right sciatic nerve was carefully dissected from the surrounding tissues before it splits into common peroneal and tibial nerves and prepared for recordings of evoked potentials. Special attention was paid not to dry the dissected nerve branches; they were soaked with drops of warm paraffin oil. The head of the animal was fixed with ear bars in a frame of the stereotactic apparatus while the upper and lower limbs were fixed to its base. The head of the animal was maintained at a distance of approximately 0.5 cm from the base. The procedure of anesthesia can cause the rats to lose their body temperature control, thus, the mounting base temperature was kept constant with a feedback-controlled heating blanket. The temperature of the animals was measured orally with an electronic probe; the temperature measured rectally could have been influenced by the heated base. Moreover, we considered a more proper measurement of the animal's head area where the occlusion procedures were performed than the whole body heated from the base by the feedback system.

After completion of the experiments, a dose of ketamine two times greater than the lethal dose was administered intraperitoneally to animals. At the same time, the perfusion was performed (see Section 2.4). Next, a total craniotomy was performed, and brains were removed for histopathological procedures.

### *2.3. Electrophysiological Recordings*

In order to determine changes in the efferent transmission of the corticospinal tract following the artery occlusions, transcranial magnetic stimulation (TMS) was applied to the contralateral motor cortex and MEP recordings were performed from the right sciatic nerve. The methodology was similar to the description presented by Kamida et al. [28] and

our own experiences. After dissection of the sciatic nerve, it was placed on a bipolar, silver chloride recording electrode. The anode of the electrode pair was oriented closer to the spinal center while the cathode was distal. The distance between the poles of the recording electrodes was 3–4 mm. The ground electrode was placed on the muscle in close proximity to the recording electrodes.

TMS was performed with a 10 cm in diameter, circular magnetic coil (Medtronic A/S, Skøvlunde, Denmark) placed over the area of motor cortex for hindlimb innervation. The magnetic coil center ("hotspot") was positioned from 1 to 5 mm lateral to the bregma. The coil was moved craniocaudally ($\pm$5 mm relative to the bregma with 1 mm steps) to optimize recorded MEP amplitudes (the largest) and latencies (the shortest). The coil was not angulated but placed flat onto the calvarial bone. The left side of the motor cortex was always stimulated with standard single pulses, assuming that the lateral corticospinal tract transmits efferent impulses mainly (in approximately 80%) by crossed projections of axons to the spinal cord motor centers of the opposite side. Hence, the optimal recording of MEPs was always performed from the right sciatic nerve. Moreover, we expected a small influence of the magnetic field generated by the coil on the right common carotid artery, which was most frequently occluded on the right side in all groups of animals. According to descriptions of Hovey and Jalinous [29], the coil with a diameter of 10–14 cm induces the greatest magnetic field strength at 660 V/m in the range from 2.2 to 2.7 T. In this study, the magnetic stimulation strength was defined as the percentage of maximal output. In most experiments on the rodents, this strength ranges from 30 to 70% [30]. This study used minimally invasive single magnetic stimuli, which means that their strength was adjusted to elicit MEP potentials with supramaximal amplitudes while minimizing movement artifacts for the stimulated objects that could affect the recording conditions. The right motor cortex close to the midline for innervation of hindlimb muscles was always stimulated with the B side of the coil. The analyzed MEP parameters were the amplitude, measured in μV, from the negative to positive deflection of the potential, and their latency, measured in ms, which is the time from the moment of motor cortex activation with a magnetic pulse is seen in the recording as the stimulus artifact to the potential's onset. General rules for MEP recordings have been adopted for the standards described by Ferreira et al. [31], and the whole methodology on the TMS application in rats was very similar to the ones described by Nielsen et al. [32] and Linden et al. [33]. Motor evoked potentials were recorded using an integrated diagnostic system KeyPoint (Medtronic A/S, Skøvlunde, Denmark), configured with the R30 MagPro magnetic stimulator (Medtronic A/S, Skøvlunde, Denmark). All MEP recordings were made at 0.5 Hz with a low-pass filter setting while the upper-pass filter of KeyPoint was set at 2 kHz. The time base was set at 5 ms/D and the sensitivity of recording was from 0.5 to 10 mV/D.

### *2.4. Histological Verification of Ischemia*

After completion of electrophysiological recordings, the animals were placed in a supine position on the operating table, the hearts were exposed, and perfusion was performed with physiological saline. After the blood flow was clarified, the skulls were carefully opened, brains removed, and stored in a 30% formalin solution for a period of one month. Horizontal, 90 μm thickness sections of brains were performed using freezing microtome with $CO_2$ (Reichert, Austria). Cuts were performed sequentially at levels $-2$ mm, $-3.1$ mm, $-4.1$ mm, $-5.1$ mm from the bregma, placed on microscope slides, transferred through the histological reagents, stained with hematoxylin and eosin (H + E), and closed in DPX. Histological verification of the material obtained after staining procedures was performed in the bright field illumination microscopy with magnifications of 5$\times$ and 10$\times$ (Leitz, Germany). In most animals, the ischemic changes in brain tissue were due to changes either by direct necrotic ischemia or artifacts in the form of neural tissue defects resulting from cracks in small vessels. Locations of the changes in the microscopic image that were considered to be the result of ischemia were reconstructed on the transverse planes according to Paxinos and Watson's [34] rat brain atlas and in the frontal planes according to Pellegrino

et al. [35]. They were defined as the highest concentration in specific areas of the brain and their location was described according to the scheme of Garcia et al. [36].

*2.5. Statistical Analysis*

The results were analyzed using Statistica software STATISTICA v. 9.1 StatSoft (Kraków, Poland). Quantitative characteristics, such as amplitude, latency, and the temperature recorded in animals from groups A–D in every minute of the test, have been described by the mean value, standard deviation (SD), and the percentage of change with reference to the data obtained in a control group. Recordings of amplitudes of MEPs performed before occlusion of the common carotid artery were also treated as reference (control) values for each group of animals (100%). Wilcoxon test was used for comparisons of the amplitude and latency values from MEPs and the temperature at successive stages of observation (before and at subsequent minutes after arteries occlusion and/or releasing). The level of statistical significance was accepted at $p < 0.05$.

**3. Results**

The graphical presentation of the measured parameters variability is shown in the charts in Figure 2. A detailed summary of the results regarding the parameters of MEPs recorded in animals from groups A–D at various stages of observations are presented in Table 1.

There were no observed statistically significant changes between MEP parameters as well as temperatures recorded in animals from groups A–D after preparation of the surgical field at the neck prior to occlusion of the common carotid artery with the surgical clamp, and amplitudes ($p = 0.07$), latencies ($p = 0.09$), or temperatures ($p = 0.08$) measured in animals from the control group (Table 1). In four control animals, neither these results nor the histological verification confirmed the influence of transcranial magnetic stimulation on the brain tissue structure or the maneuvers during surgical preparation of the arteries to suspected MEP parameter fluctuations caused by the disturbed blood flow before occlusions.

In animals from groups A and B, results showed that five-minute uni- and bilateral occlusion of the common carotid arteries lead to alterations in brain blood circulation and evoked a significant decrease (at $p = 0.06$, see Table 2) in the MEP amplitudes by 19.1% and 23.1%, respectively (Table 1). Ten-minute occlusion in animals from groups A and B resulted in an MEP amplitude decrease (at $p = 0.01$ and $p = 0.009$) by 34.8% and 48.4%, respectively. In animals from groups C and D, after a similar course of the amplitude decrease during the first five minutes of occlusion by approximately 23.4% (at $p = 0.04$ and $p = 0.03$), the recovery in the arterial blood flow brought stabilization of the amplitude parameter at 11.9% and 23.0%, respectively, with reference to the initials values. The values of MEP amplitudes recorded in animals from groups C and D before occlusion did not differ significantly in the final observations after artery release (at $p = 0.06$ and $p = 0.05$, respectively).

The increase in MEP latency was by 0.9 ms on average (Table 1), reflecting the partial inability of corticospinal tract fibers to transmit neural impulses, which appeared to be significant only after the bilateral arteries occlusions in the fifth minute of recordings in animals from groups B and D (at $p = 0.04$, see Table 2). A significant change (at $p = 0.03$) in the average latency by 1.4 ms was observed in animals from group B in the final observation. However, the bilateral recovery of blood flow in animals from group D provided the subsequent shortening of this parameter in the last observation period, which did not differ significantly (at $p = 0.05$) from the initial value.

A similar course of the temperature change by 2.2 °C on average (Table 1) at $p = 0.04$ (Table 2) to the latency variability could be observed during the first five minutes of measurements in animals from groups B and D following bilateral occlusion. Permanent uni- or bilateral arterial ischemia in animals from groups A and B evoked a decrease in this parameter by up to 2.9 °C on average in the final observation (at $p = 0.03$ and $p = 0.02$, respectively). In both animals from groups C and D, a release of the common carotid

arteries resulted in the recovery of temperature parameters with no significant differences to the values recorded before occlusions.

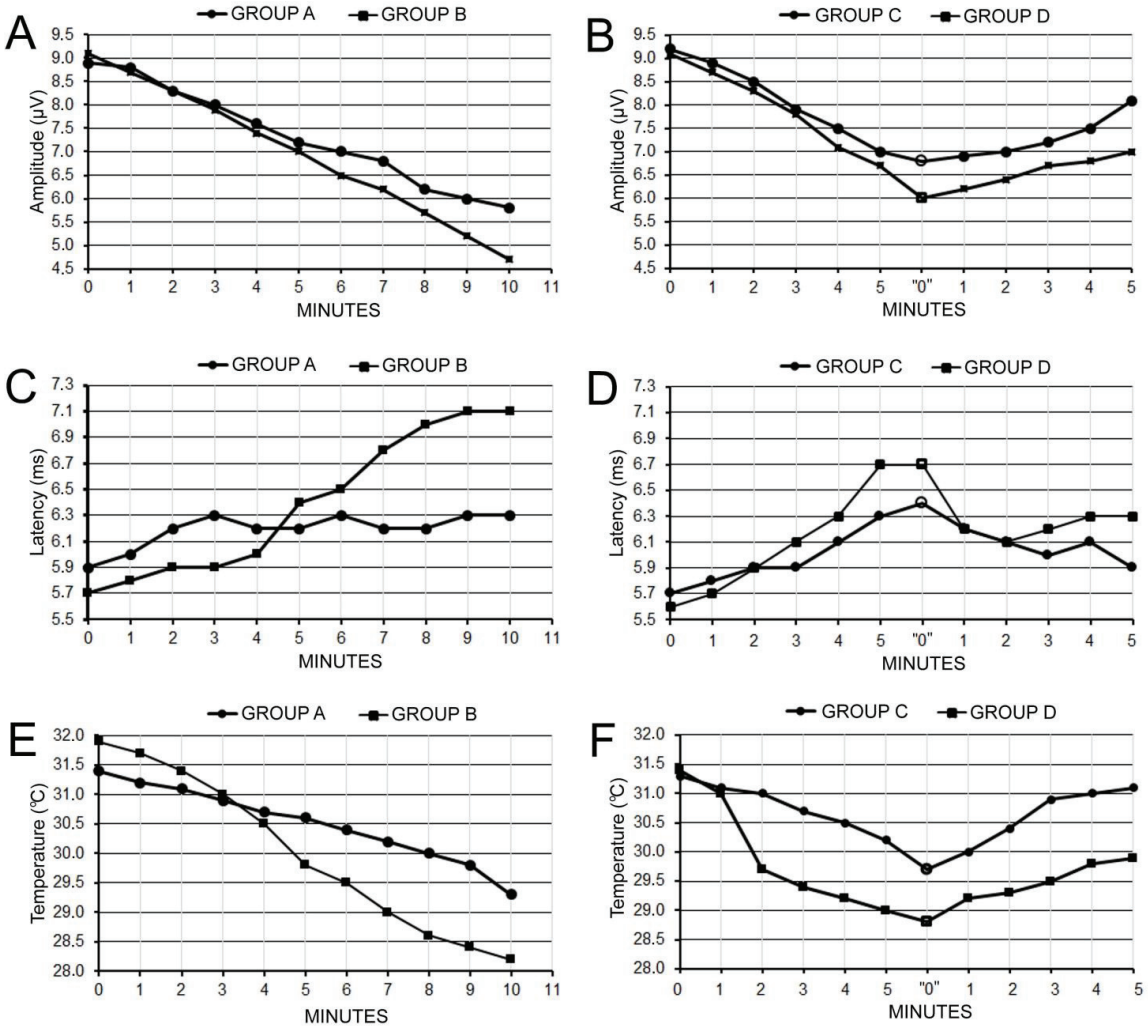


**Figure 2.** Variability of the amplitude (**A**,**B**) and latency (**C**,**D**) of the MEPs, and temperature (**E**,**F**) recorded in rats from groups A and B (**left side**) and C and D (**right side**) at various stages of observations. "0"—releasing the artery after five minutes of occlusion.

**Table 1.** Summary of mean values and standard deviations of motor evoked potential amplitudes (expressed in percentages) and latencies, as well as temperature values recorded in four groups of animals at different stages of observation. Data on the analogical parameters measured in the control group of animals with no surgeries are presented on the left side of the table.

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Amplitude (μV), % of Change** | | | | | | | | | | | | | |
| | | **Duration of Ischemia after Carotid Artery Occlusion (min)** | | | | | | | | | | | |
| Control | Animals Groups | Before | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| 9.2 ± 1.3 (N = 4) | A (N = 10) | 8.9 ± 1.1 −100% | 8.8 ± 1.0 −1.20% | 8.3 ± 0.9 −6.80% | 8.0 ± 1.1 −10.10% | 7.6 ± 0.9 −14.60% | 7.2 ± 1.2 −19.10% | 7.0 ± 1.1 −20.30% | 6.8 ± 0.9 −23.60% | 6.2 ± 1.0 −30.30% | 6.0 ± 1.2 −32.60% | 5.8 ± 1.1 −34.80% | |
| | B (N = 11) | 9.1 ± 1.3 −100% | 8.7 ± 1.0 −4.40% | 8.3 ± 0.7 −8.80% | 7.9 ± 0.8 −13.20% | 7.4 ± 0.9 −18.70% | 7.0 ± 1.0 −23.10% | 6.5 ± 1.1 −28.60% | 6.2 ± 0.9 −31.90% | 5.7 ± 1.1 −37.40% | 5.2 ± 1.0 −42.90% | 4.7 ± 0.9 −48.40% | |
| | | Occlusion duration (min) | | | | | | | Occlusion releasing duration (min) | | | | |
| | Animals Groups | Before | 1 | 2 | 3 | 4 | 5 | "0" | 1 | 2 | 3 | 4 | 5 |
| | C (N = 10) | 9.2 ± 1.2 −100% | 8.9 ± 1.0 −3.30% | 8.5 ± 1.0 −7.60% | 7.9 ± 1.0 −14.10% | 7.5 ± 0.8 −18.50% | 7.0 ± 1.0 −23.90% | 6.8 ± 0.9 −26.10% | 6.9 ± 1.0 −25.00% | 7.0 ± 0.9 −23.90% | 7.2 ± 1.2 −21.70% | 7.5 ± 1.1 −18.40% | 8.1 ± 1.1 −11.90% |
| | D (N = 11) | 9.1 ± 1.3 −100% | 8.7 ± 0.9 −4.40% | 8.3 ± 0.9 −8.80% | 7.8 ± 1.0 −14.30% | 7.1 ± 1.0 −21.90% | 6.7 ± 0.7 −26.30% | 6.0 ± 1.1 −34.10% | 6.2 ± 1.1 −31.80% | 6.4 ± 0.8 −10.90% | 6.7 ± 3.1 −11.00% | 6.8 ± 0.8 −25.20% | 7.0 ± 0.9 −23.00% |
| **Latency (ms)** | | | | | | | | | | | | | |
| | | **Duration of Ischemia After Carotid Artery Occlusion (min)** | | | | | | | | | | | |
| 5.8 ± 0.8 (N = 4) | Animals Groups | Before | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| | A (N = 10) | 5.9 ± 0.8 | 6.0 ± 0.8 | 6.2 ± 0.6 | 6.3 ± 0.9 | 6.2 ± 0.5 | 6.2 ± 0.9 | 6.3 ± 0.7 | 6.2 ± 0.7 | 6.2 ± 0.7 | 6.3 ± 0.7 | 6.3 ± 0.8 | |
| | B (N = 11) | 5.7 ± 0.7 | 5.8 ± 0.7 | 5.9 ± 0.8 | 5.9 ± 0.7 | 6.0 ± 0.7 | 6.4 ± 0.5 | 6.5 ± 0.8 | 6.8 ± 0.8 | 7.0 ± 0.9 | 7.1 ± 0.9 | 7.1 ± 0.8 | |
| | | Occlusion duration (min) | | | | | | | Occlusion releasing duration (min) | | | | |
| | Animals Groups | Before | 1 | 2 | 3 | 4 | 5 | "0" | 1 | 2 | 3 | 4 | 5 |
| | C (N = 10) | 5.7 ± 0.4 | 5.8 ± 0.8 | 5.9 ± 0.6 | 5.9 ± 0.4 | 6.1 ± 0.4 | 6.3 ± 0.4 | 6.4 ± 0.4 | 6.2 ± 0.4 | 6.1 ± 0.5 | 6.0 ± 0.5 | 6.1 ± 0.4 | 5.9 ± 0.6 |
| | D (N = 11) | 5.6 ± 0.7 | 5.7 ± 0.5 | 5.9 ± 0.6 | 6.1 ± 0.6 | 6.3 ± 0.6 | 6.7 ± 0.6 | 6.7 ± 0.5 | 6.2 ± 0.5 | 6.1 ± 0.5 | 6.2 ± 0.4 | 6.3 ± 0.4 | 6.3 ± 0.5 |

**Table 1.** *Cont.*

| | | Temperature (°C) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Duration of Ischemia after Carotid Artery Occlusion (min) | | | | | | | | | | |
| | Animals Groups | Before | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 31.6 ± 1.1 (N = 4) | A (N = 10) | 31.4 ± 1.0 | 31.2 ± 1.2 | 31.1 ± 1.2 | 30.9 ± 1.2 | 30.7 ± 1.1 | 30.6 ± 1.1 | 30.4 ± 0.9 | 30.2 ± 1.1 | 30.0 ± 1.2 | 29.8 ± 1.1 | 29.3 ± 1.1 |
| | B (N = 11) | 31.9 ± 1.2 | 31.7 ± 1.3 | 31.4 ± 1.2 | 31.0 ± 1.3 | 30.5 ± 0.9 | 29.8 ± 1.2 | 29.5 ± 0.9 | 29.0 ± 1.1 | 28.6 ± 1.0 | 28.4 ± 1.2 | 28.2 ± 1.2 |
| | | Occlusion duration (min) | | | | | | Occlusion releasing duration (min) | | | | |
| | | Before | 1 | 2 | 3 | 4 | 5 | "0" | 1 | 2 | 3 | 4 | 5 |
| | C (N = 10) | 31.3 ± 1.2 | 31.1 ± 1.2 | 31.0 ± 1.0 | 30.7 ± 0.9 | 30.5 ± 1.0 | 30.2 ± 1.0 | 29.7 ± 1.2 | 30.0 ± 1.1 | 30.4 ± 1.0 | 30.9 ± 0.9 | 31.0 ± 1.1 | 31.1 ± 1.0 |
| | D (N = 11) | 31.4 ± 0.9 | 31.0 ± 0.9 | 29.7 ± 1.0 | 29.4 ± 1.0 | 29.2 ± 1.0 | 29.0 ± 1.1 | 28.8 ± 0.9 | 29.2 ± 1.0 | 29.3 ± 0.9 | 29.5 ± 0.9 | 29.8 ± 0.9 | 29.9 ± 0.9 |

Abbreviations: Groups of animals A—permanent unilateral occlusion of the common carotid artery (always right), group B—permanent bilateral occlusion of the common carotid arteries, group C—temporary unilateral occlusion of the common carotid artery (always right) with its releasing, group D—temporary bilateral occlusion of the common carotid arteries with their releasing; "0"—releasing the artery after five minutes of occlusion.

**Table 2.** Differences ($p$) in parameters of MEPs and the temperature recorded at different periods of observations in the four groups of animals.

| Observation Periods (Minutes) | Group A | | | Group B | | | Group C | | | Group D | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amplitude | Latency | Temperature | Amplitude | Latency | Temperature | Amplitude | Latency | Temperature | Amplitude | Latency | Temperature |
| "0" vs. 5th | **0.06** | 0.05 | 0.05 | **0.06** | **0.04** | **0.04** | **0.04** | 0.05 | 0.05 | **0.03** | **0.04** | **0.04** |
| "0" vs. 10th | **0.01** | 0.05 | **0.03** | **0.009** | **0.03** | **0.02** | 0.05 | 0.07 | 0.07 | **0.04** | 0.05 | 0.05 |

Abbreviation: "0"—artery releasing; $p < 0.05$ significant differences are marked in bold.

Examples of recordings in Figure 3 are convincing regarding the effects of arterial blood changes on the fluctuations of the MEPs amplitude parameter. Note the subsequential decrease in the MEPs amplitude parameter in A recorded following the permanent unilateral artery occlusion, and a different sequence of changes (increase after a decrease) shown in B, when the recordings were performed in an animal with bilateral, temporary occlusions.

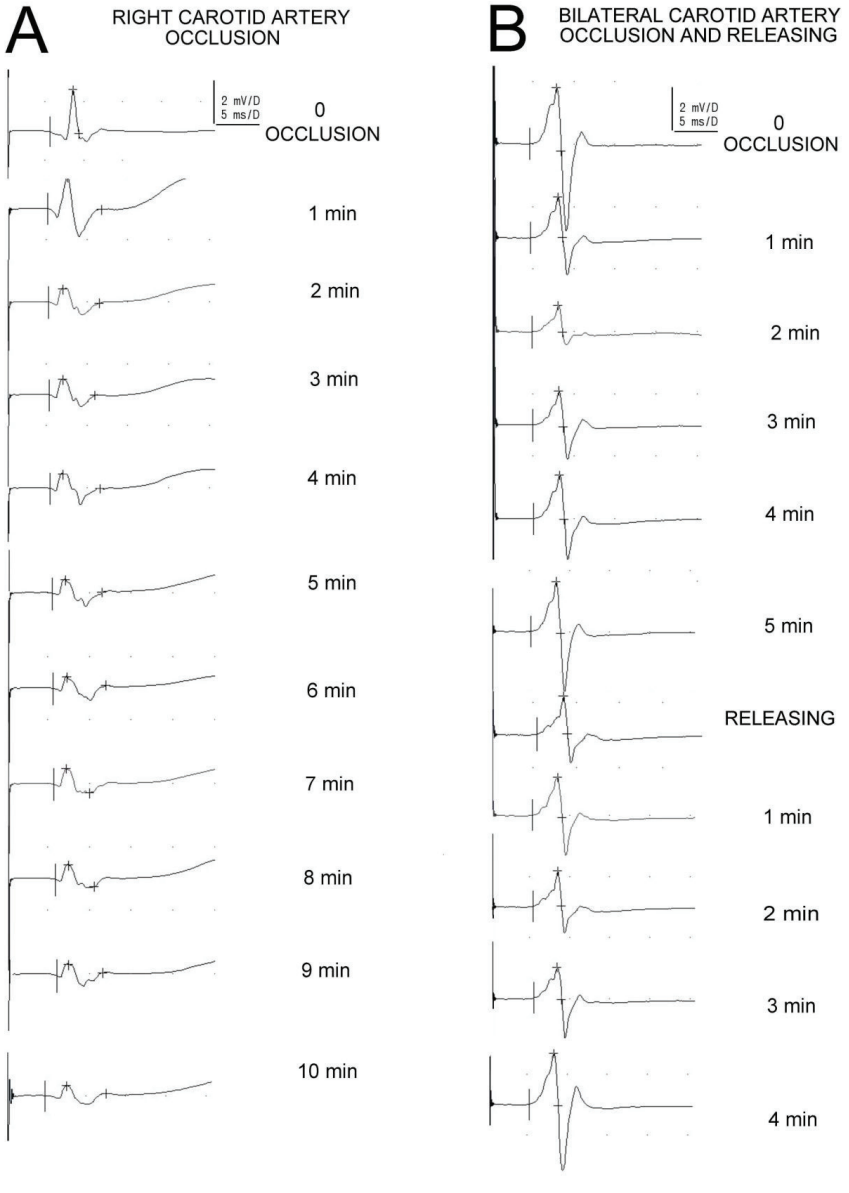


**Figure 3.** Examples of motor evoked potential recordings with the observation time of up to 10 min (**A**) in one of the animals from group A as a result of the right carotid artery occlusion, and (**B**) in one of the animals from group D with the bilateral artery occlusion and release.

In histological studies, results of ischemia were the most prominent bilaterally in sensory and motor areas mainly for the forelimb, rather than the hindlimb, innervation of the cortex, putamen and caudate nuclei, globulus pallidus, and areas adjacent to the fornix of the third ventricle (Figure 4).

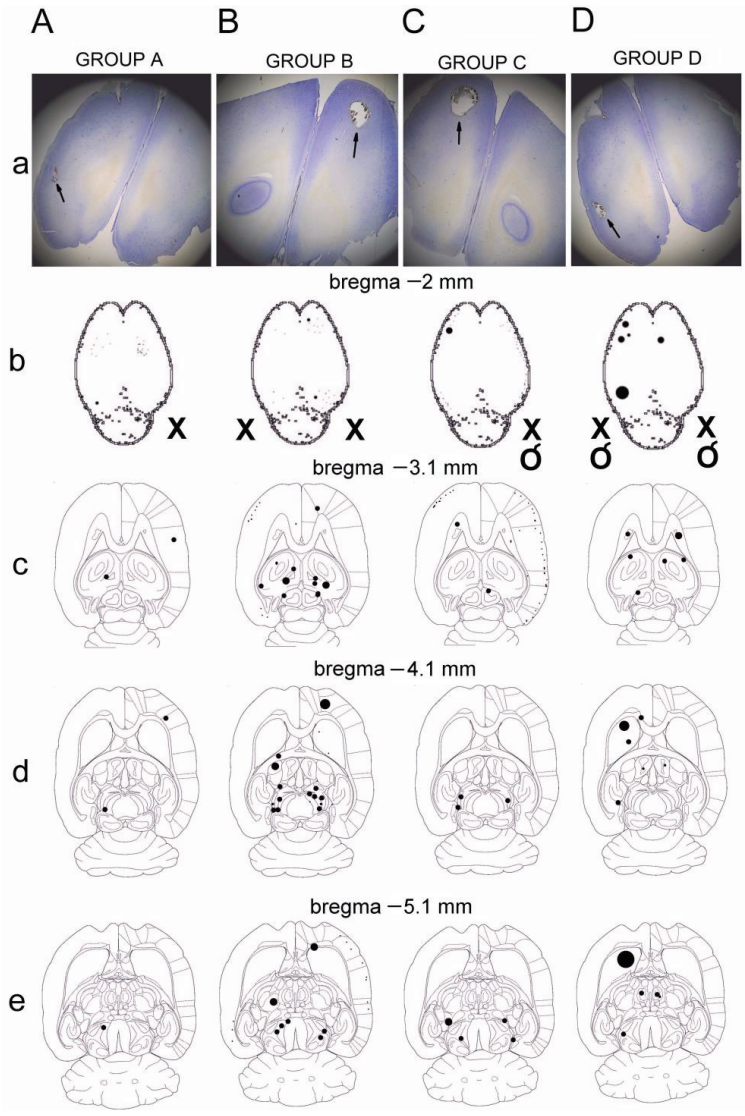


**Figure 4.** Locations of lesions resulting from the induction of ischemia with various variants found in animals from A–D groups ("X" means occlusion, while "O" is the artery releasing). Examples of microscopic images with the lesion sites marked with arrows in the four groups of animals (**A**–**D**) are shown in part "(**a**)" of the figure. The results of histological examinations (**b**–**d**) were re-drawn from the microscopic images and showed the location of lesions of varying severity, which are marked with circles of different diameters on the cross-sectional diagrams of the brain. In "(**b**)", cross-sections are shown at the levels −2 mm from the bregma point, in "(**c**)", −3.1 mm, in "(**d**)", −4.1 mm, and in "(**e**)", −5.1 mm. Black arrows indicate ischemic spots.

## 4. Discussion

In this study, we evaluated the parameters of transcranially evoked motor evoked potentials recorded from the hindlimb nerve branches in rats following 5 min of uni- or bilateral common carotid artery occlusion and its release, which did not reveal total inhibition but reversible changes in the activity of corticospinal tract. Data presented in the graphs in parts B, D, and F of Figure 2 are convincing enough that the MEP amplitude parameter and the animal's temperature, more than the MEP latency, are precise indicators of the reversible consequences of the ischemic stroke.

The neurophysiological method used in this study with the recording from nerves avoided the muscle stretch-related artifacts that could influence the precision of measurements of the MEP amplitudes and latencies, as well as the animals' temperature. Moreover, such an approach diminished the possible risk of the applied anesthetics on the release of acetylcholine at the level of the neuromuscular junction, which could have been a source of false negative or positive results [30,37,38]. Thus, we have verified the hypotheses included in the study aims. Our previous experiences confirmed that MEPs recorded from the hindlimb nerves of rats following transvertebral stimulation is precise enough for evaluation of the regeneration process in the motor fibers of rat's hindlimbs [30]. Moreover, MEPs evoked transcranially or transvertebrally are proven as a reliable and non-invasive diagnostic tool to assess the effects of stroke or peripheral nerve damage, and its subsequent spontaneous or therapeutically induced recovery [39–43]. A methodologically similar study by Kikonohana et al. [37] with transcranial motor evoked potentials revealed the high sensitivity of detection of the stroke effects after aortic occlusion in rats. In general, the morphology and parameters of the recorded MEPs before performing the ischemic procedures (see Table 1) is consistent with the description of the N1 and N2 components in the potentials recorded from the nerves by Shao et al. [21]. The MEP averaged amplitudes and latencies recorded in our study did not differ significantly from the parameters obtained in the works of Zhang et al. [38] and Fishback et al. [44], which were reported as 11.47 mV $\pm$ 5.25 mV and 5.1 ms $\pm$ 1.8 ms, respectively. The recorded MEPs main potential (see Figure 3), similar to the study of Linden et al. [33], reached the threshold amplitude at 15% of the stimulator output, while its maximal value was recorded at 60%.

A study by Konrad et al. [45] showed that MEPs morphology is altered more by pathological changes at the synaptic level than along the axons. Therefore, it should be assumed that in our studies, the expected changes after unilateral carotid artery clamping will show more changes at the level of the supraspinal centers of the brain than at the level of the spinal cord itself. According to Dougherty et al. [23] and Garcia et al. [24], unilateral or bilateral common carotid artery occlusion is an excellent experimental model of the effects of ischemic stroke. The results of our study seem to contradict the views of Longa et al. [27] regarding the lack of results of such a model due to numerous anastomoses compensating for the incorrect blood supply to the brain in critical conditions, and Kety et al. [26] that experimental blockade of the common carotid arteries does not cause significant changes in cerebral circulation in experimental conditions in animals and in clinical conditions in humans. In this study, we observed the effects of changes in cerebral circulation up to 10 min after the application of the effects of ischemia induced after temporary occlusion of the common carotid artery. Previous reports in related methodology included mainly long-term observations of the consequences of middle cerebral artery occlusion due to the frequency of stroke with this location, which is significant in the population of post-stroke patients, and therefore, worth a closer examination. In a study by Simpson and Baskin [20], MEPs that were recorded every hour for 6 h after occlusion of the middle cerebral artery showed significantly attenuated amplitudes. However, after about 5 h, the early latency components exceeded control values. These results indicate moderate reliability of the results of long-term ischemia in light of the long-term observation experimental studies.

The markings presented in Figure 4 show that the largest number of lesions resulting from ischemia at all examined levels of the brain from the bregma point were observed in animals from groups B and D, in which bilateral arterial occlusion was performed, also

with the variant of restoring their flow. In animals with an ischemia model of occlusion of the right common carotid artery (groups A and C), artifacts were less numerous. It can be concluded that in animals of groups B and D, the markings were most often present unilaterally in the area of sensory and motor innervation of the limbs, mainly the anterior rather than the posterior cerebral cortex, the area of the putamen and the caudate nucleus bilaterally, the area of the globus pallidus bilaterally, and the area adjacent to the fornix of the third ventricle bilaterally. It should be noted that such changes illustrate the effects of short-term ischemia in a short period of observation. Nevertheless, they are structurally closely related to functional changes in the transmission of the corticospinal tract, where changes in the MEPs amplitude parameter were also most often noted and most clearly marked in the tested animals from groups B and D.

The purpose of histological verification in this study was to confirm the consistency of the results with the reports of other authors of experiments on rats after occlusion of the common carotid artery, concerning the effects of ischemia in the brain. Our study was not intended to quantify the effects of ischemia in detail, but to specify their most common locations. In addition, as can be seen from the slices in Figure 4, a similar pattern of unilateral (A and C) or bilateral (B and D) occlusion effects can be observed, whether secondary releasing has occurred or not. Similar to the work of Mathew et al. [46], using H + E staining of brain sections, this study defined the aftermath of stroke in the brain tissue as clear structural defects or empty areas with stained edges, mainly due to vascular rupture. There were no signs of bleeding in the lesion areas. Most authors of other reports described ischemic changes as structural discolorations or lesions resulting from the rupture of small vessels, similar to the observations reported in this paper. Nagasawa and Kogure [47] quantified artifacts according to the number of ventricles, discolorations, and brain tissue defects after H + E staining, most often in areas of the anterior neocortex after carotid artery clamping. This location closely coincides with our observations after histological verification of the brains of group B and D animals. Similar results of the neuroimaging and histochemical studies following the stroke caused in rats by the common carotid artery infarctions have been presented by Carmichael in his review [48].

In light of the presented literature data, which are not fully consistent with the observations in this work, it can be concluded that the verification of the results of MEP tests on the described model of a stroke may provide interesting data regarding the critical time of carotid artery occlusion, significantly affecting the activity of the motor cortex and the efferent neural transmission of the corticospinal pathways. The change in cerebral vascularity is probably compensated by the mechanism of arterial pressure compensation through anastomoses. Seitz et al. [49] showed that compensation for the effects of ischemia or general changes in arterial blood flow in the brain is affected by anastomoses and microanastomoses occurring between the arteries, which expand, thus eliminating blood flow disorders. This phenomenon seems to be one of the safety mechanisms allowing reorganization of the human cerebral circulation following cerebral ischemia induced by a change in arterial pressure. Confirmation of this phenomenon is a completely different course of stroke accompanying bilateral carotid artery occlusion, in which a sharp decrease in the MEP amplitude was observed, without the possibility of secondary blood supply compensation after bilateral carotid artery release within 5 min. The MEP amplitudes recorded in these cases were always significantly reduced throughout the duration of the ischemic model used and their values accounted for approximately 40% of the initial value.

Considering the limitations of the presented study, one should remember that the MEP study method does not fully specify the pathological phenomena occurring within the neurons of the motor cortex as a result of temporary, unilateral, or bilateral inhibition of blood flow within the carotid arteries, such as in episodes often described in clinical practice [50], indicating only model similarities in the ischemic stroke consequences. Moreover, our study was conducted on young adult male Wistar rats whereas, clinically, stroke affects mostly aged and comorbid, including obesity, patients [51]. Taking into account the differences in the ability of the rodent brain and the human brain to withstand ischemic

consequences, such as stroke, it is important to note that this study was conducted using rats as subjects and further research is needed to determine whether these findings are applicable to humans.

A contemporary clinical observational study hypothesized that previous transient ischemic attack (TIA) had a favorable effect on early outcomes after acute nonlacunar ischemic stroke [52]. Prior TIA was associated with a favorable outcome in nonlacunar stroke, suggesting its neuroprotective effect possibly by inducing a phenomenon of ischemic tolerance, allowing better recovery from a subsequent ischemic stroke. The proposed model used in our study with the assessment of the corticospinal system function could also be used in the future for the evaluation of the transient ischemic attack effects under experimental conditions. Moreover, the future line of research on the discussed topic would be to study the inhibition of the corticospinal tract neuronal activity in experimental conditions in cases of acute small vessel disease versus other stroke subtypes. This recommendation seems to be reasonable because the pathophysiology, prognosis, and clinical features of small vessel acute ischemic stroke are different from other stroke subtypes [53].

## 5. Conclusions

The parameters of transcranially evoked motor evoked potentials recorded from the hindlimb nerve branches in rats following 5 min of uni- or bilateral common carotid artery occlusion and its release did not reveal total inhibition but instead reversible changes in the activity of corticospinal tract. A five-minute occlusion of the common carotid artery on one or both sides causes a decrease in body temperature by approximately 1.5 °C, while their releasing after 5 min of observation causes compensation of this parameter to a statistically comparable value before the ischemia was caused. The parameters of MEP amplitude and temperature variability are more sensitive in monitoring the efferent transmission of the corticospinal tract than the MEP latency parameter in observing the course of ischemic effects in experimental conditions. Results of ischemia in histological studies were the most prominent bilaterally in sensory and motor areas mainly for the forelimb, rather than hindlimb, innervation of the cortex, putamen and caudate nuclei, globulus pallidus, and areas adjacent to the fornix of the third ventricle. The symptoms of rat brain infarction are much more optimistic than those described in patients after stroke and require further comparison with the clinical observations.

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**Data Availability Statement:** All data described in this study are presented in the manuscript.

**Conflicts of Interest:** The authors declare no conflict of interest.

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Article

# Fatty Acid Levels and Their Inflammatory Metabolites Are Associated with the Nondipping Status and Risk of Obstructive Sleep Apnea Syndrome in Stroke Patients

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**Abstract:** Background: This paper discusses the role of inflammation in the pathogenesis of nondipping blood pressure and its role in the pathogenesis of obstructive sleep apnea syndrome. The aim of the study was to assess the impact of free fatty acids (FAs) and their inflammatory metabolites on the nondipping phenomenon and the risk of sleep apnea in stroke patients. Methods: Sixty-four ischemic stroke patients were included in the prospective study. Group I consisted of 33 patients with a preserved physiological dipping effect (DIP), while group II included 31 patients with the nondipping phenomenon (NDIP). All subjects had FA gas chromatography and inflammatory metabolite measurements performed with the use of liquid chromatography, their 24 h blood pressure was recorded, and they were assessed with the Epworth sleepiness scale (ESS). Results: In the nondipping group a higher level of C16:0 palmitic acid was observed, while lower levels were observed in regard to C20:0 arachidic acid, C22:0 behenic acid and C24:1 nervonic acid. A decreased leukotriene B4 level was recorded in the nondipping group. None of the FAs and derivatives correlated with the ESS scale in the group of patients after stroke. Correlations were observed after dividing into the DIP and NDIP groups. In the DIP group, a higher score of ESS was correlated with numerous FAs and derivatives. Inflammation of a lower degree and a higher level of anti-inflammatory mediators from EPA and DHA acids favored the occurrence of the DIP. A high level of C18: 3n6 gamma linoleic acid indicating advanced inflammation, intensified the NDIP effect. Conclusions: We demonstrated potential novel associations between the FA levels and eicosanoids in the pathogenesis of the nondipping phenomenon. There are common connections between fatty acids, their metabolites, inflammation, obstructive sleep apnea syndrome and nondipping in stroke patients.

**Keywords:** dipping; inflammation; eicosanoids; cardiovascular disease; leukotriene; saturated fatty acids

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## 1. Introduction

### *1.1. Fatty Acids and Stroke*

Arterial stiffness increases with age due to atherosclerosis, arteriosclerosis, and calcifications of the vessel. Vascular calcifications, resulting from nucleation of calcium and phosphate into crystals, is associated with a transformation of vascular smooth muscle cells into osteoblast-like cells [1]. Polyunsaturated fatty acids (PUFA) are recommended by the guidelines of the European Society of Cardiology to reduce blood pressure, low density lipoprotein (LDL) cholesterol, the synthesis of proinflammatory mediators, and to

increase the availability of nitric oxide in the vascular wall [1,2]. Prevalent vegetable oil users, especially extra-virgin olive oil and corn oil, had lower arterial stiffness compared to prevalent animal fat users in a study [3].

Serum fatty acid levels are associated with the risk of ischemic stroke (IS), however, the effect of saturated and unsaturated fatty acids on the risk of IS remains uncertain and the available results are inconsistent [4,5]. The role of n-3 and n-6 polyunsaturated fatty acids is also equivocal [6,7]. In the Framingham Study population, serum palmitic acid (C16:0) level, which is a saturated fatty acid (SFA), increased the risk of ischemic stroke (HR 1.76; 9% CI: 1.26–2.45) [6]. On the other hand, in the Atherosclerosis Risk in Communities (ARIC) Study, palmitic acid level was not found to be connected with the risk of IS [8]. The long chain n-3 polyunsaturated fatty acids (n-3 PUFA) which play an important role in cardiovascular risk include: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA). They decrease the risk of hypertension, dyslipidemia, endothelial dysfunction and thus they are supposed to have a protective impact on cardiovascular diseases [9]. The level of n-3 polyunsaturated fatty acids such as α-linolenic acid (C18:3n3; ALA) was not shown to affect the risk of ischemic stroke, while in another study such a relationship was confirmed [10,11]. In a meta-analysis from the year 2012, n-3 PUFA supplementation was not associated with a lower risk of stroke, but in a recent meta-analysis from 2018 the analyzed fish intake was reported to lower the risk of stroke [12,13]. Marine omega-3 PUFAs may protect against ventricular arrhythmias, and there is growing evidence for an effect of marine omega-3 PUFAs in the prevention and treatment of atrial fibrillation [14]. In a prospective study with a follow-up of 8.3 to 11.2 years, higher levels of circulating DPA and DHA were connected with a lower risk of IS [9]. Moreover, trans unsaturated fatty acids (from margarine, cakes, and pastries), are not recommended by the European and American guidelines. They impair postprandial vascular endothelial function but not arterial stiffness in humans [15].

How monounsaturated fatty acids (MUFA) influence the risk of stroke is yet to be clarified, as for the time being there is still limited data available in this area of research [16,17].

### *1.2. The Eicosanoids*

Ischemic stroke is associated with the presence of a strong inflammatory reaction, in which arachidonic acid (AA) plays a key role. Derivatives are synthesized using three lipoxygenase pathways—5LOX, 15LOX and cyclooxygenase COX1 and COX2. It seems that 12LOX is not involved in this process [18].

The leukotrienes (LT) are lipid mediators belonging to a large family of molecules named eicosanoids. They are generated from arachidonic acid (AA), a carbon-20 PUFA, through the 5-lipoxygenase (5-LOX) pathway. Cysteinyl leukotrienes (CysLTs) are potent lipid inflammatory mediators and play a crucial role in the pathogenesis of inflammation. Therefore, CysLT modifiers as synthesis inhibitors or receptor antagonists, may become a potential target for the treatment of other inflammatory diseases such as cardiovascular disorders. The cardioprotective effects observed by using CysLT modifiers are promising and contribute to elucidate the link between CysLTs and cardiovascular disease [19]. Chronic, low-grade inflammation has been involved in the pathogenesis of atherosclerosis. A specific group with an increased risk of cardiovascular diseases are women with PCOS who would also be included in clinical trials [20].

### *1.3. The Nondipping Blood Pressure Phenomenon and Inflammation*

Blood pressure fluctuates following the circadian rhythm, which originates in the suprachiasmatic nuclei of the anterior hypothalamus. Nocturnal dipping of blood pressure mainly results from this physiological rhythm, and a lack of dipping is associated with increased cardiovascular disorders and more severe end-organ damage [21,22]. The cause of the nondipping phenomenon lies in the inability to modulate autonomic tone [23]. The 24 h monitoring of blood pressure is performed with the use of so-called ambulatory blood pressure monitoring (ABPM). The physiological nocturnal reduction in both systolic and

diastolic blood pressure should range from 10% to 20%, defining individuals as dippers, while subjects that do not reach 10% are referred to as nondippers [24,25]. A nighttime blood pressure value in hypertensive patients is an independent risk factor of cardiovascular disease, defined as stroke or coronary artery disease [26]. Nondippers were found to have a significantly higher risk for cerebral infarction (RR 1.59 (95% CI, 1.03 to 2.46); $p = 0.04$), regardless of the use of antihypertensives [27]. The nondipping status plays an important role in the cardiovascular risk also in subjects without hypertension. The nondipping pattern in 24 h blood pressure values within the normal range was connected with an increased risk of cardiovascular mortality and greater organ damage [28].

The role of inflammation in the pathogenesis of the nondipping pattern is still being analyzed. The inflammatory markers were found to be elevated in hypertensive individuals and to correlate with the risk of stroke [29,30]. An increased level of high-sensitivity C-reactive protein (hsCRP) was detected in nondipping patients with obstructive sleep apnea syndrome (OSAS) and with newly diagnosed hypertension [31,32]. OSAS is a risk factor for cardiovascular episodes, including ischemic stroke. The frequency of the nondipping pattern in OSAS patients reaches up to 84% and increases with OSAS severity. The risk of the nondipping pattern is 1.5 times higher in OSAS patients, as reported by a recent meta-analysis [33]. The risk of OSAS may be assessed with the use of the Epworth sleepiness scale (ESS), whose sensitivity reaches up to 76% [34]. Fatty acids play a role in the inflammatory process. The main anti-inflammatory effect is ascribed to n3 fatty acids, especially DHA [35]. A role in proinflammatory reactions is associated with n-6 PUFA—arachidonic acid (AA) [36]. Several fatty acids, including n-3 and n-6, are associated with inflammatory markers in subjects with high cardiovascular risk [37].

There are limited available data regarding the association between dipping status, serum fatty acids and their inflammatory metabolites, either in the general population or in ischemic stroke patients. There is also limited information about the role of nondipping blood pressure in the pathogenesis of stroke. The inflammatory pathogenetical aspects of OSAS were taken into consideration by assessing its risk with the use of ESS.

## 2. Materials and Methods

### *2.1. Study Design and Population*

The study included 64 ischemic stroke patients who were hospitalized in the Neurology Department in the district hospital in Poland. All subjects were Caucasians, and all patients were treated with statins and acetylsalicylic acid, and previous hypotensive treatment was maintained. Group I consisted of 33 patients with preserved physiological dipping effect (DIP), while group II included 31 patients with the nondipping phenomenon (NDIP). The inclusion criterion was the detection of ischemic stroke on the basis of clinical symptoms and additional test results, with standard treatment [38,39]. Informed consent was obtained from all the patients.

The exclusion criteria included intracranial hemorrhage visible on brain imaging, speech or consciousness disturbances which would make obtaining the informed consent impossible, presence of active infection including body temperature of more than 37.4 °C, clinical or biochemical symptoms of infection, active autoimmune disorder or malignancy. The fasting blood samples were taken on the seventh day of hospitalization. The characteristics of both groups are presented in Table 1.

All patients had 24 h blood pressure measurement (ABPM). ABPM (Suntech Medical, USA, Morrisville, 2017) tests were performed between 4 and 6 days after the onset of a stroke. ABPM reading were recorded at 15 min intervals during the daytime and at 30 min intervals during the nighttime. Daytime and nighttime were defined as 6 a.m. to 10 p.m. and from 10 p.m. to 6 a.m., respectively. The recordings were analyzed using dedicated software and patients were excluded from the study if $\geq$20% of the measurements were not recorded successfully. Patients with mean nocturnal BP decline of $\geq$10% were defined as dippers, whereas those with a recorded decline < 10% were considered as nondippers [40]. The risk of OSAS has been assessed with the use of the Epworth sleepiness scale (ESS)

whose sensitivity reaches up to 76%. The scale is useful as a screening tool and to control patients with OSAS that undergo therapy with the use of positive airway pressure [34]. The ESS was used for each situational question on a 4-point scale (from 0 to 3). Patients rated their likelihood of dozing off or falling asleep while engaged in eight usual activities. The ESS score was the sum of all eight situational sub-scores, with the total ranging from 0 to 24. The risk of OSAS increases with general score obtained by a patient. Severe excessive daytime OSAS can be recognized when the total score is >15 points [40].

**Table 1.** Characteristics of the study groups.

| Parameter | Group I (DIP) Mean ± SD *n* = 33 | Group II (NDIP) Mean ± SD *n* = 31 | *p* Value |
|---|---|---|---|
| BMI (kg/m$^2$) | 29.13 ± 5.19 | 27.88 ± 4.17 | NS |
| Age (years) | 62.39 ± 11.95 | 58.45 ± 12.35 | NS |
| Diabetes (*n*) | 18/31 | 12/33 | <0.05 |
| Ischaemic heart disease (*n*) | 4/31 | 2/33 | NS |
| Hypertension (*n*) | 24/31 | 22/33 | NS |
| CRP (mg/L) | 2.91 ± 5.11 | 2.14 ± 2.08 | NS |
| TChol (mg/L) | 204.48 ± 48.03 | 195.94 ± 57.36 | NS |
| LDL (mg/L) | 120.7 ± 41.23 | 112.45 ± 47.62 | NS |
| HDL (mg/L) | 54.36 ± 17.45 | 50.97 ± 14.11 | NS |
| TG (mg/L) | 151.82 ± 72.9 | 162.58 ± 83.58 | NS |

NS—not statistically significant; CRP—C-reactive protein; TChol—total cholesterol; LDL—low density lipoprotein; HDL—high density lipoprotein; TG—triglycerides.

### *2.2. Free Fatty Acids and Eicosanoid Detection*

Fatty acid methyl esters were isolated from the serum with the use of the modified Folch and Szczuko methods [18,41]. The fatty acids profile was labeled by gas chromatography. The gas chromatography (GC) was performed with the use of the Agilent Technologies 7890A GC System (SUPELCOWAX™ 10 Capillary GC Column (15 mm × 0.10 mm, 0.10 μm); Supelco, Bellefonte, PA, USA). FAs were identified by comparing their retention times with those of Food Industry FAME Mix (Restek, Anchemplus, Poland).

The inflammatory metabolites were detected with the use of high-performance liquid chromatography (HPLC) (Agilent Technologies, UK). Among the analyzed mediators were: 9-HODE, 13-HODE, 5(S),6(R)-Lipoxin $A_4$, 5(S),6(R),15(R)-Lipoxin $A_4$, 5-HETE, 5-oxoETE, 12-HETE, 15-HETE, Leukotriene B4, Prostaglandin E2, Prostaglandin B2 16(R)/16(S)-HETE, 18-HEPE, 17-HDHA, 10(S)17(R)DiDHA (Protectin DX), Maresine1, and Rev D1, Rev E1. Detailed methodology was described elsewhere [18,42].

### *2.3. Statistical Analysis*

Statistica 13.0 (Statsoft, Kraków, Poland) was used to perform the calculation dates. The assumptions for the use of parametric or non-parametric tests were checked using the Shapiro–Wilks test. Significant differences in mean values between the groups (group I—DIP and group II—NDIP) were assessed using one-way ANOVA and Tukey's post hoc test. If the normality and homogeneity assumptions were violated, the Mann–Whitney non-parametric test was used. Statistical significance was set at $p < 0.05$. The FA's matrix and derivatives were then correlated with the Epworth scale (ESS) with consideration of both groups (DIP, NDIP and all patients). The marked correlation coordinates are significant with $p < 0.05$.

## 3. Results

There was no association between dipping status and blood lipid levels (Table 1).

An increased level of C16:0 palmitic acid (saturated fatty acid) was observed in the nondipping compared to the dipping group, while a decrease was observed in regard to the level of other saturated fatty acids (C20:0 arachidic acid, C22:0 behenic acid) and

a monounsaturated fatty acid (C24:1 nervonic acid) (Table 2). In comparisons between groups, the following tendency was observed with certain fatty acids: in the nondipping patients an elevated level of C20:3n3 cis-11-eicosatrienoic acid (eicosatrienoic acid, ETE, n3 PUFA), C22:4n6 docosatetraenoate acid (all-cis-4,7,10,13,16-docosapentaenoicacid, adrenic acid, n6 PUFA) and C22:2 cis-docosadienoic acid (docosadienoic acid, n6 PUFA) were recorded, while a decrease was observed in the level of C24:0 lignoceric acid (saturated fatty acid). The other fatty acids did not correlate with the dipping status (Table 2).

**Table 2.** Comparison of fatty acids between group I (DIP) and II (NDIP).

| FA [%] | Group I (DIP) *n* = 33 | Group II (NDIP) *n* = 31 | *p* Value |
|---|---|---|---|
| C13:0 tridecanoic acid | 0.307 ± 0.1 | 0.31 ± 0.09 | 0.904 |
| C14:0 myristic acid | 1.265 ± 0.331 | 1.16 ± 0.435 | 0.277 |
| C14:1 myristolenic acid | 0.075 ± 0.036 | 0.066 ± 0.040 | 0.338 |
| C15:0 pentadecanoic acid | 0.221 ± 0.106 | 0.224 ± 0.124 | 0.925 |
| C15:1 cis-10-pentadecanoic acid | 0.078 ± 0.035 | 0.088 ± 0.04 | 0.295 |
| C16:0 palmitic acid | 26.294 ± 1.598 | 27.242 ± 1.649 | 0.023 * |
| C16:1 palmitoleic acid | 2.261 ± 0.715 | 2.119 ± 0.791 | 0.453 |
| C17:0 heptadecanoic acid | 0.309 ± 0.045 | 0.294 ± 0.06 | 0.262 |
| C17:1 cis-10-heptadecanoic acid | 0.089 ± 0.038 | 0.098 ± 0.031 | 0.308 |
| C18:0 stearic acid | 13.216 ± 2.041 | 13.602 ± 1.948 | 0.443 |
| C18:1n9 ct oleic acid | 22.363 ± 4.108 | 22.117 ± 2.610 | 0.778 |
| C18:1 vaccinic acid | 1.996 ± 0.403 | 1965 ± 0.313 | 0.729 |
| C18:2n6c linoleic acid | 11.552 ± 2.328 | 11.757 ± 2.427 | 0.732 |
| C18:2n6t linoleic acid | 6.47 ± 1.784 | 5.865 ± 2.199 | 0.229 |
| C18:3n6 gamma linoleic acid | 0.417 ± 0.191 | 0.359 ± 0.165 | 0.202 |
| C18:3n3 linolenic acid | 0.524 ± 0.148 | 0.467 ± 0.139 | 0.119 |
| C18:4 stearidonate | 0.061 ± 0.026 | 0.052 ± 0.025 | 0.175 |
| C20:0 arachidic acid | 0.215 ± 0.054 | 0.187 ± 0.041 | 0.024 * |
| C22:1/C20:1 cis11-eicosanic acid | 0.178 ± 0.064 | 0.171 ± 0.035 | 0.592 |
| C20:2 cis-11-eicodienoic acid | 0.152 ± 0.032 | 0.156 ± 0.037 | 0.581 |
| C20:3n6 eicosatrienoic acid | 1.347 ± 0.307 | 1.277 ± 0.321 | 0.378 |
| C20:4n6 arachidonic acid | 6.316 ± 1.355 | 6.364 ± 1.36 | 0.887 |
| C20:3n3 cis-11-eicosatrienoic acid | 0.029 ± 0.013 | 0.035 ± 0.016 | 0.062 |
| C20:5n3 eicosapentaenoic acid | 0.668 ± 0.301 | 0.574 ± 0.21 | 0.157 |
| C22:0 behenic acid | 0.245 ± 0.084 | 0.199 ± 0.08 | 0.028 * |
| C22:1n9 13 erucic acid | 0.06 ± 0.018 | 0.061 ± 0.026 | 0.355 |
| C22:2 cis-docodienoic acid | 0.015 ± 0.011 | 0.02 ± 0.011 | 0.08 |
| C23:0 tricosanoic acid | 0.212 ± 0.092 | 0.23 ± 0.169 | 0.59 |
| C22:4n6 docosatetraenoate | 0.202 ± 0.099 | 0.26 ± 0.137 | 0.054 |
| C22:5w3 docosapentaenate | 0.442 ± 0.108 | 0.502 ± 0.339 | 0.348 |
| C24:0 lignoceric acid | 0.166 ± 0.068 | 0.135 ± 0.068 | 0.068 |
| C22:6n3 docosahexaenoic acid | 1.799 ± 0.591 | 1.713 ± 0.518 | 0.538 |
| C24:1 nervonic acid | 0.451 ± 0.24 | 0.328 ± 0.195 | 0.028 * |

* statistically significant; FA—fatty acids; DIP—dipping effect; NDIP—non-dipping effect.

The HPLC analysis of the level of inflammatory mediators between the study groups showed that only the leukotriene B4 level was lower in the nondipping group (Table 3). There were no differences found in relation to the other tested inflammatory mediators.

The obtained results indicated that FA, pro and anti-inflammatory mediators in all analyzed patients showed no correlation with the ESS scale (Table S1). Only after introducing the division into the DIP and NDIP groups, the number of correlations with the ESS scale was found. The correlation between ESS and FAs in group I (DIP) showed a direct association with lowered C20: 0 arachidic acid, C22: 0 behenic acid, C22: 1n9 13 erucic acid, C22: 5w3 docosapentaenate, C24: 0 lignoceric acid and C24: 1 nervonic acid. Moreover, it was also shown that elevated levels of C15: 0 pentadecanoid acid, C17: 0 heptadecanoic acid, C22: 4n6 (docosatetraenoate) correlated with ESS. In the DIP group there was also a

positive correlation of the ESS scale with eicosanoids such as prostaglandin E2, protectin D1, 17RS HDHA and negative correlation with 16RS HETE.

**Table 3.** Comparison of eicosanoids level between group I (DIP) and II (NDIP).

| Eicosanoids [μg/mL] | Group I (DIP) $n = 33$ | Group II (NDIP) $n = 31$ | $p$ Value |
|---|---|---|---|
| resolvin E1 | 0.043 ± 0.041 | 0.080 ± 0.131 | 0.120 |
| prostaglandin E2 | 3.57 ± 5.248 | 2.701 ± 2.590 | 0.407 |
| resolvin D1 | 0.180 ± 0.2 | 0.181 ± 0.328 | 0.971 |
| ** LTX A4 5S, 6R | 0.00 | 0.041 ± 0.23 | 0.306 |
| LTX A4 5S, 6R, 15R | 0.027 ± 0.05 | 0.021 ± 0.037 | 0.53 |
| protectin D1 | 0.047 ± 0.08 | 0.049 ± 0.049 | 0.938 |
| maresin 1 | 0.032 ± 0.012 | 0.031 ± 0.019 | 0.61 |
| leukotriene B4 | 0.031 ± 0.011 | 0.0225 ± 0.014 | 0.033 * |
| 18RS HEPE | 0.113 ± 0.039 | 0.104 ± 0.038 | 0.311 |
| ** 16 RS HETE | 0.006 ± 0.039 | 0.00 | 0.337 |
| 13S HODE | 0.037 ± 0.037 | 0.030 ± 0.022 | 0.317 |
| 9S HODE | 0.039 ± 0.035 | 0.028 ± 0.017 | 0.122 |
| 15S HETE | 0.309 ± 0.194 | 0.286 ± 0.242 | 0.678 |
| 17RS HDHA | 0.115 ± 0.077 | 0.130 ± 0.08 | 0.435 |
| 12S HETE | 1.918 ± 1.19 | 1.680 ± 1.138 | 0.418 |
| 5 oxo ETE | 0.187 ± 0.084 | 0.184 ± 0.125 | 0.91 |
| 5 HETE | 0.026 ± 0.009 | 0.025 ± 0.017 | 0.806 |

* statistically significant; LTX—lipoxin; HETE—hydroxyeicosatetraenoic acids; HODE—hydroxyoctadecadienoic acid; ** in only a few patients samples the concentration was at the limit of quantification.

In group II, correlation between ESS and both FAs and eicosanoids showed a direct association with C14:1 myristolenic acid, C17:1 cis-10- heptadecanoid acid, C18:3n6 gamma linoleic acid, maresina 1, 18RS HEPE, 17RS HDHA, 5 oxo ETE. In the NDIP group, only the correlation between ESS and C18: 3n6 gamma linoleic acid was positive (Table S1).

## 4. Discussion

This is the first study to have shown possible pathogenetical connections between all the above-mentioned factors. We observed associations between the dipping status and the fatty acids in stroke patients. In the nondipping stroke subjects we detected lower levels of C24:1 nervonic acid (n9 MUFA), and diverse associations between SFAs and dipping status (Table 2). Palmitic acid is the most common SFA that can be provided by the diet and synthesized endogenously. Maintaining the proper ratio of n3 and n6 PUFA is crucial for keeping the membrane phospholipids in balance. The excessive accumulation of tissue palmitic acid results in hyperglycemia, fat accumulation, dyslipidemia and increased inflammation via Toll-like receptor 4 [43]. The palmitic acid level was found to be higher in patients with pathological, nondipping effect. This acid may play a role in cardiovascular disorders, inflammatory reactions and can increase the risk of stroke [6,8]. SFAs, including palmitic acid, were reported to be elevated in epicardial adipose tissue in patients with coronary artery disease [44]. The information regarding the role of arachidic acid is lacking, but we suggest that its decreased level in the nondipping group may result from its potential oxidation into a palmitic acid [45]. Another possible explanation involves the potential elongation of C14:0 myristic acid into the palmitic acid C16:0, because we detected lower levels of these acids in the NDIP group. Moreover, in our study myristolenic acids negatively correlated with the ESS.

We suggest that palmitic acid may act as an activator of inflammation in NDIP patients. A potential mechanism of proinflammatory activity can involve the Toll-like receptor 4 (TLR4) which is the main signaling pathway that triggers the obesity-induced inflammatory response. It is induced by the SFA and can be attenuated by the n3 PUFA by either lipopolysaccharides (LPS) or saturated fatty acids [46]. Further signaling processes lead to activation of transcription factor NF-κB that activates inflammatory cytokines such as Il-1,

Il-6, Il-8 and TNF-α [35,47]. The anti-inflammatory effect of n-3 PUFA in the reduction in experimental brain damage due to hypoxia may be achieved by the effect in microglia by inhibiting NF-κB activation [48].

From the outcomes reported by other authors we conclude that the levels of SFAs including palmitic acid were associated with higher levels of low-density lipoprotein cholesterol (LDL-C), TC/high-density lipoprotein cholesterol (HDL-C) ratio, triglycerides, ApoB, ApoB/A1 ratio, hsCRP, and lower levels of HDL-C and ApoA1 [49]. No association was found between palmitic acid and coronary heart disease [50]. It seems reasonable that palmitic acid may be a significant proinflammatory factor that leads to the development of cardiovascular disorders, such as atrial fibrillation and heart failure [51]. Moreover, palmitic acid was positively associated with incident type 2 diabetes, but available data do not indicate a relationship between the very long chain saturated fatty acids such as arachidic acid and the risk of type 2 diabetes [52,53]. Contrary to palmitic acid, other SFAs such as arachidic and behenic acids were detected at lower levels in nondipping patients. There can be diverse mechanisms for their inflammatory effects or SFAs, such as arachidic acid and behenic acid, can be the substrates in the inflammatory process and thus their lower levels were observed in patients with the detrimental nondipping phenomenon. On the other hand, palmitic acid can have a harmful impact, while the other aforementioned SFAs can play a protective a role in the pathogenesis of stroke. The role of SFAs in the pathogenesis of stroke and in the inflammatory status is not yet unequivocal and needs further investigation, especially when taking into consideration the results of a recent meta-analysis that showed a protective role of SFAs in the risk of ischemic stroke [54].

Other fatty acids analyzed in our study including n3 and n6 as well as lipid levels did correlate with the dipping status. In particular, C18: 3n6 gamma linoleic acid in NDIP and the lower levels of C22: 0 behenic acid, C24: 0 lignoceric acid and C24: 1 nervonic acid in the DIP group are noteworthy, which correlated with the ESS scale (Table S1). Furthermore, the higher level of C22: 4n6 (docosatetraenoate) favored the occurrence of the dipping phenomenon with the ESS scale (Table S1). It was observed that, behenic acid and nervonic acid were decreased in metabolic syndrome and polycystic ovary syndrome (PCOS) patients, while a positive association was found in relation to HDL-C level and an inverse association with triglyceride levels [55,56].

A lower level of nervonic acid was found in the group of nondipping patients compared to dippers. Nervonic acid, which is a very long chain monounsaturated fatty acids (VLCFA), is an intermediate in the nerve cell myelin synthesis and is a component of membrane sphingolipids. The dietary interventions with this fatty acid are beneficial in the treatment of adrenoleukodystrophy [57,58]. Other authors showed that the level of nervonic acid is inversely correlated with LDL-C, HDL-C and directly associated with heart failure, cardiovascular risk, cardiovascular and all-cause mortality, markers of inflammation and endothelial activation (hsCRP, IL-6, ICAM-1) [59]. Nervonic acid is negatively connected with Il-1b level and IFN gamma, with no association with Il-6, Il-8, Il-10 [29]. Nervonic acid was also reduced along with a reduction in body weight in obese women and may have protective effects in obesity-related metabolic risk factors such as lipid levels, fasting blood glucose, CRP and leptin [60,61]. Lower levels of nervonic acid in patients with the nondipping phenomenon may indicate that it is potentially an inflammatory substrate and is used in the inflammatory cascade, as is the case with the SFAs. The VLCFAs may modulate the inflammation via the beta-oxidation in peroxisomes, which may lead to the synthesis of plasmalogens that are associated with oxidative stress and chronic inflammation [62]. The VLCFAs are elongated under the control of peroxisome proliferator-activated receptor (PPARα) leading to a decrease in nervonic acid level. Moreover, nervonic acid could be lower in nondipping stroke patients, not because of being a cause but a result of inflammation, because atherosclerosis may lower the peroxisomal activity and lead to degradation of VLCFAs [63].

In our study the leukotriene B4 level was lower in nondipping patients compared to the dipping group. On the other hand, low levels of oxo ETE and EPA and DHA derivatives (17RS HDHA, 18RS HEPE) were associated with the absence of the nondipping phenomenon. While the level of Protectin D1 and 17RS HDHA favored its occurrence in correlation with the ESS scale (Table S1). The n3 and n6 fatty acids affect the inflammation by cell membrane activation throughout the conversion by cyclooxygenases (COXs) and lipooxygenases (LOXs). The conversion products include prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) [64]. Leukotrienes are synthesized in the inflammatory state especially in neutrophils and alveolar macrophages in the arachidonic cascade. Arachidonic acid is metabolized by 5-lipoxygenase to produce leukotrienes ($LTB_4$, LTC4, LTD4 and LTE4). Leukotrienes, in turn, play a role in the pathogenesis of chronic inflammation, oedema, leukocyte infiltration, while the level of 5-lipoxygenase correlates with the severity of atherosclerosis and atherosclerotic plaque instability. LTB4 activates the higher-affinity leukotriene receptor 1 (BLT1) which induces inflammation, enhances cytokine production and phagocytosis. Leukotriene B4 also activates the lower-affinity leukotriene receptor 2 (BLT2), but it is less known. Both receptors provoke NFκB activation and potentiate Toll-like receptor sterile inflammation. Therefore, LTB4 can play a central role in the development of metabolic diseases [65,66]. Taking into consideration the proinflammatory effects of LTB4, the explanation of its decreased level in nondipping stroke patients poses a real challenge. On the other hand, the direct association between LTB4 and ESS in the NDIP group may add to the pathogenetic importance of LTB4, because of the pathogenetic link between OSAS, inflammation and the nondipping phenomenon. As ESS is a tool for assessing the risk of OSAS, it is noteworthy that patients with OSAS have higher levels of LTB4. Additionally, the level of LTB4 was shown to be correlated with carotid atherosclerosis [67]. The role of leukotrienes in atherosclerosis development was documented at several levels, i.e., lipid retention and modification, intimal hyperplasia, endothelial dysfunction, atherosclerotic plaque formation, plaque rupture, myocardial and cerebral ischemia [68]. Nondipping patients have higher levels of arterial stiffness and cardiovascular risk [69]. The nondipping pattern has an additional negative effect on endothelial functions in hypertensive patients [63]. The inflammatory markers were shown to be increased in nondipping patients with OSAS (interleukin-2, CRP), while others were not different in such patients (IL-6, IL-8, IL-10, IL-12, and TNF-α) [31,32,70]. The nondipping status is not only related to proinflammatory but also to the procoagulant activity by increasing D-dimer, plasminogen activator inhibitor-1, von Willebrand factor, soluble intercellular adhesion molecule-1 and platelet-to-lymphocyte ratio [71,72]. The obtained results are shown in Figure 1.

We also demonstrated a direct association between MUFA acids (Table S1), anti-inflammatory protectin D1, 17RS HDHA and ESS score. Protectin D1 (10S,17S-dihydroxydocosahexaenoic acid;), 17RS HDHA and maresin D1 (7S,8R,17S-trihydroxy-4Z,9E,11E,13Z, 15E,19Z-docosahexaenoic acid) belong to a family of specialized anti-inflammatory lipid mediators. These anti-inflammatory molecules are synthesized in the later stage of inflammation from EPA and DHA. The anti-inflammatory mediators play a role in the cardioprotective effects of n3 PUFAs beside the suppressive effect on arachidonic acid [73–75]. We detected a negative correlation between 16RS HETE and the ESS in stroke patients with the physiological dipping pattern. It means that EPA and DHA derivatives may have a beneficial effect in the pathogenesis of ESS and presumably, it could subsequently be involved in the protection against nondipping BP status. There are no such studies available to confront, but protectin D1 has anti-inflammatory properties, which allows us to justify our presumptions [76]. The role of ESS in cardiovascular disorders was elucidated in a recent study [77]. Such findings need further investigation because a potential link between OSAS and anti-inflammatory molecules in the pathogenesis of stroke and the nondipping phenomenon cannot be excluded. As with the anti-inflammatory properties of protectin D1 and resolving D1, MUFAs are supposed to function mainly as anti-inflammatory factors. They constitute an important ingredient of the Mediterranean diet. They inhibit the activation of NF-κB, NLR family pyrin domain containing 3 (NLRP3)

and macrophages [78]. Higher intake of MUFAs is associated with lower pro-inflammatory (CRP, Il-6, I-18, IFN-γ, MCP-1, TNF-α) and elevated anti-inflammatory molecules (PPARγ, Il-4, Il-10) [79]. The role of anti-inflammatory lipids mediators and MUFAs in the risk of OSAS in the context of pathogenesis and the risk of stroke needs further studies. In our previous article, we demonstrated the effect of cyclooxygenase on the abolition of the thromboxane mediated dipping effect [80,81]. In the current study, we supplement the knowledge in terms of the participation of cyclooxygenase and the 5-LOX pathway with the participation of leukotriene.

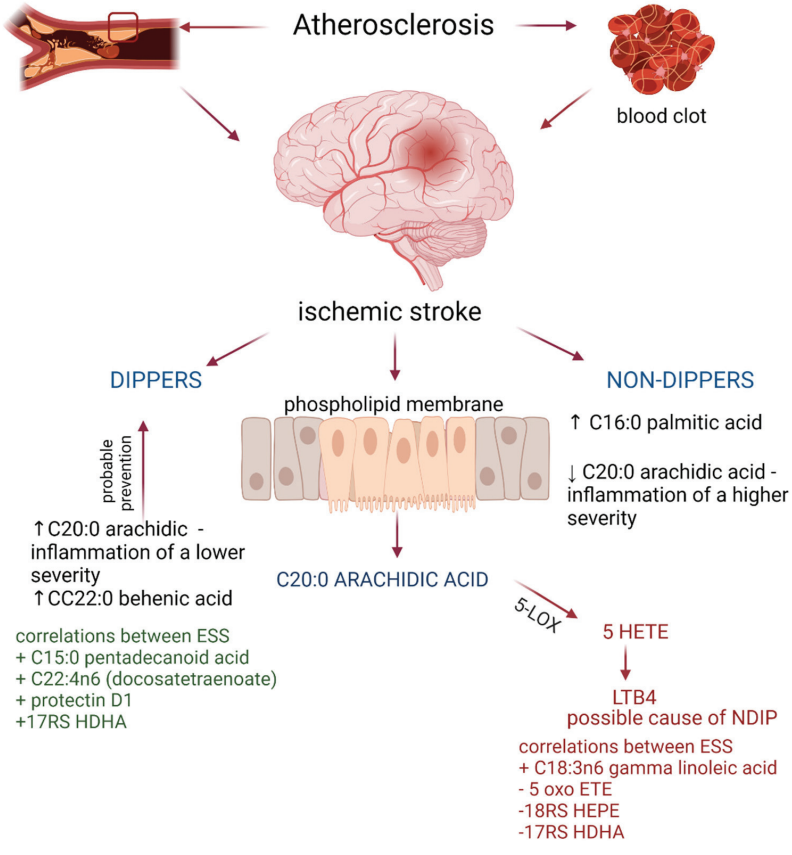


**Figure 1.** The participation of fatty acids and their mediators in the course of the DIP mechanism. (Created with BioRender.com https://app.biorender.com/, accessed on 8 August 2022). 5-LOX—lipooxygenase; 5HETE—5-hydroxyeicosatetraenoic acid; $LTB_4$—leukotriene B; ESS—Epworth sleepiness scale.

The limitation of our study is the use of ESS as a screening test for detection of OSAS, while the results can also be influenced by other factors that change the quality of sleep. The classic definition and set hours of daytime and nighttime could be affected by the individual sleep-wake cycles connected with personal physiological differences and individual habits. When comparing the groups in the context of their characteristics, there was a higher prevalence of diagnosed diabetes in the dipping study patients. The observation is inconsistent with other studies which reported associations between diabetes and nondipping status [82]. We are also aware of the limited number of patients included in our study, but we suggest interpreting the presented results as a novel, pilot study requiring further analysis.

## 5. Conclusions

In our study we demonstrated the potential novel associations between the blood fatty acid levels and their inflammatory metabolites in the pathogenesis of the nondipping phenomenon in stroke patients. We suggest there are common connections between certain fatty acids, leukotriene B4, inflammation, hypertension, obstructive sleep apnea syndrome and nondipping profile. All these factors may play a role in the pathogenesis of stroke and need further investigation.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines10092200/s1, Table S1: Significant correlations between ESS, fatty acids and their inflammatory metabolites (separately for group I, group II).

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**Institutional Review Board Statement:** All procedures involving human participants were performed in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration as amended or in compliance with comparable ethical standards. The study was approved by the Bioethics Commission at the District Medical Council in Zielona Góra (decision number 08/73/2017, 27 February 2017).

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

**Data Availability Statement:** The data will be made available on request.

**Conflicts of Interest:** The authors declare no conflict of interest.

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# NLRP3 Inhibition Reduces rt-PA Induced Endothelial Dysfunction under Ischemic Conditions

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**Abstract:** Thrombolysis with recombinant tissue plasminogen activator (rt-PA) is a mainstay of acute ischemic stroke treatment but is associated with bleeding complications, especially after prolonged large vessel occlusion. Recently, inhibition of the NLRP3 inflammasome led to preserved blood–brain barrier (BBB) integrity in experimental stroke in vivo. To further address the potential of NLRP3 inflammasome inhibition as adjunct stroke treatment we used immortalized brain derived endothelial cells (bEnd5) as an in vitro model of the BBB. We treated bEnd5 with rt-PA in combination with the NLRP3 specific inhibitor MCC950 or vehicle under normoxic as well as ischemic (OGD) conditions. We found that rt-PA exerted a cytotoxic effect on bEnd5 cells under OGD confirming that rt-PA is harmful to the BBB. This detrimental effect could be significantly reduced by MCC950 treatment. Moreover, under ischemic conditions, the Cell Index—a sensible indicator for a patent BBB—and the protein expression of Zonula occludens 1 stabilized after MCC950 treatment. At the same time, the extent of endothelial cell death and NLRP3 expression decreased. In conclusion, NLRP3 inhibition can protect the BBB from rt-PA-induced damage and thereby potentially increase the narrow time window for safe thrombolysis in stroke.

**Keywords:** NLRP3; inflammasome; MCC950; rt-PA; blood–brain barrier; Cell Index; ASC; ischemic stroke; i.v. thrombolysis

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## 1. Introduction

Ischemic stroke (IS) is one of the leading causes of death and disability worldwide [1]. Thrombolysis and mechanical thrombectomy are applied early after stroke onset to achieve rapid recanalization as a prerequisite for a good functional outcome. However, rapid infarct growth from stroke onset until the initiation of these interventions increases the probability of futile or even harmful recanalization, mainly due to bleeding complications [2]. In particular, the application of recombinant tissue plasminogen activator (rt-PA) increases the risk of hemorrhagic stroke transformation beyond 4.5 h of stroke onset. Furthermore, there is experimental evidence that rt-PA can impair the blood–brain barrier (BBB) [3–6].

Inflammasomes are molecular protein complexes which sense cellular deviation from homeostasis and subsequently initiate inflammatory responses [7]. Inflammasomes consist of three components: firstly, a cytosolic pattern recognition receptor (PRR)—most notably the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3)—causing the oligomerization of the sensor by recognizing inflammatory mediators and DAMPs in sterile inflammatory processes such as IS [8,9]. Secondly, the recruitment of ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) is induced. Thirdly, ASC filaments provide a docking site for pro-caspase 1 as effector molecule [10,11]. This triggers the autoactivation of caspase 1 and the downstream activation of the pro-inflammatory cytokines IL-1β and IL-18 as well as the pyroptosis-inducing protein Gasdermin D [12,13], which are significantly expressed during IS [14–16].

We could recently show that NLRP3 inflammasome is upregulated early after stroke onset in neurons and glial cells, but also in endothelial cells (EC) [16]. This upregulation is of particular interest, as EC die under hypoxic conditions. This leads to a BBB breakdown, which responds to NLRP3 inhibition [15]. Our goal, now, was the quantification of the endothelial barrier function in the bEnd5 cell model dependent upon the period of oxygen and glucose deprivation (OGD) and additional rt-PA administration. Furthermore, we tested whether NLRP3 inhibition can mitigate rt-PA induced damage of the BBB.

## 2. Materials and Methods

### *2.1. Materials*

A total of 10 mg of the inflammasome-inhibitor MCC950 (NLRP3 Inhibitor, MCC950, #538120, Merck, Darmstadt, Germany) were dissolved in 1 mL 1× phosphate-buffered saline (PBS) (Dulbecco's Phosphate Buffered Saline, #D8537, Merck). MCC950 was further diluted with cell culture medium to 100 µmol/L. rt-PA (Actilyse, PZN-03300636, Boehringer Ingelheim, Ingelheim am Rhein, Germany) was diluted with cell culture medium to a final concentration of 50 ng/mL in line with the average concentration of rt-PA detected in human pial blood samples [17]. Dulbecco's Modified Eagle's Medium (DMEM) high glucose (4.5 g/L) (Dulbecco's Modified Eagle's Medium—high glucose, #D5671), DMEM low glucose (1 g/L) (Dulbecco's Modified Eagle's Medium—low glucose, #D5921), sterile water (Water, #W3500), L-Glutamine (L-Glutamine Solution 200 mM, #59202C), Trypsin (Trypsin-EDTA solution 0.25%, #T4049), and PI (Propidium iodide solution—solution 1.0 mg/mL in water, #P4864) were all purchased by Merck. Calf serum (FCS) (Sterile Plasma Derived Bovine Serum, #60-00-850) was provided by First Link (UK) Ltd. (Birmingham, UK). The T75-cell culture flasks (Cellstar Cell Culture Flasks, 75 $cm^2$, #658170) were provided by Greiner Bio-One GmbH (Frickenhausen, Germany), the 96-well plates (Nunc™ Cell-Culture, 96-Well, #165306), DAPI (ProLong™ Gold Antifade Mountant with DAPI, #P36931) and as secondary antibodies Alexa Fluor™ 488 goat anti-mouse IgG (Goat anti-Mouse IgG (H+L), Alexa Fluor 488, #A11001) and Alexa Fluor™ 546 goat anti-rabbit IgG (Goat anti-Rabbit IgG (H+L), Alexa Fluor 546, #A11035) were provided by Thermo Fisher Scientific (Waltham, MA, USA). For the xCELLigence experiments, xCELLigence E-Plates (xCELLignece E-Plate VIEW 16, #300601140) were purchased by Agilent (Santa Clara, CA, USA).

### *2.2. Cell Culture*

We purchased bEnd5 cells from Merck (bEnd5, #96091930). They were grown in DMEM (high glucose, 4.5 g/L), supplemented with 10% FCS and 1% L-Glutamine (200 mM), in a humidified (95%) 37 °C incubator with 5% $CO_2$ and 21% $O_2$. We plated bEnd5 in 75 $cm^2$ culture flasks and subcultured them using 0.25% (*w*/*v*) trypsin in 0.02% (*w*/*v*) EDTA at 80% confluence. We changed media every 2 d. We passaged the cells chosen for experimentation between 18 and 26 times. For resistance measurements, we transferred bEnd5 cells on gold electrode plates (ACEA, San Diego, CA, USA) and for immunofluorescent microscopy on 96-well plates 24 h prior to commencement of the respective trials.

### *2.3. Oxygen and Glucose Deprivation (OGD)*

We exposed confluent monolayers of bEnd5 cells to hypoxic (1% $O_2$, 95% humidity, 5% $CO_2$, 37 °C) and aglycemic conditions by replacing the culture medium with low glucose-medium (hypoxic DMEM low glucose with 1% L-Glutamine without FCS). We preincubated the OGD medium for 24 h under hypoxic conditions before administration.

### *2.4. Treatment Regimes*

We used a rt-PA concentration of 50 ng/mL corresponding to the average value of recently analyzed human post occlusive blood samples [17].

A total of 100 µmol/L of the specific NLRP3 inflammasome-inhibitor MCC950 were used to treat bEnd5 cells. MCC950 was dissolved as described above. As vehicle treatment,

the same amount of pure culture or OGD medium was added. The investigators were blinded to the group allocation.

### *2.5. xCELLigence Assay*

For label-free real-time assessment of transendothelial resistance, we used an Agilent xCELLigence RTCA DP system. It records the impedance changes compared to the background of cell-free electrodes at three different alternating current frequencies expressed as the dimensionless Cell Index [18]. It correlates to the transendothelial electrical resistance but does additionally reflect the capacitance of the cell layer. When confluent after 24 h of growth, evidenced by a plateau of the Cell Index, we treated the cells with MCC950, rt-PA, MCC950 and rt-PA at the same time, or rather vehicle, under either normoxic or OGD conditions as indicated. Data was processed by the xCELLigence RTCA DP software 1.2.1 (Agilent, Santa Clara, CA, USA).

### *2.6. Immunofluorescence Microscopy*

For histology we fixated bEnd5 cultures with a glyoxal solution containing 40% glyoxal, acetic acid, water, and 100% ethanol. Cell cultures were dyed with DAPI, PI, and antibodies against NLRP3 (anti-NLRP3/NALP3, mAb (Cryo-2), #AG-20B-0014, 1:100, Adipogen Life Sciences, San Diego, CA, USA), Zonula occludens 1 (ZO-1 Polyclonal Antibody, #61-7300, 1:1000, Thermo Fisher Scientific), and ASC (Anti-ASC (AL177), #AG-25B-0006, 1:100, Adipogen). We used secondary antibodies in a dilution of 1:100. For recording, we used a Leica microscope (Leica DMi8, DMC 2900/DFC 3000G camera control, LAS X software (Leica, Wetzlar, Germany)). To measure cell death bEnd5 cells cultivated on 96-well plates were visualized with transmitted light microscopy and apoptotic cells with fluorescence measurements after PI (1:200) staining with the above-mentioned microscope. The red fluorescent cells were counted. For measurement of NLRP3, ASC, and ZO-1 intensity, images of the cell cultures were recorded with the same microscope. Subsequently, after converting the images into 16-bit black and white files, the intensities of the respective stainings were determined with ImageJ Analysis Software 1.52a (National Institutes of Health, Bethesda, MD, USA).

### *2.7. Statistics*

Results are presented as grouped summary data indicating median and standard deviation for each time point. For statistical analysis the GraphPad Prism 8 software (GraphPad Company, San Diego, CA, USA) was used. Data was tested for Gaussian distribution with the D'Agostino-Pearson omnibus normality test and then analyzed by 1-way analysis of variance (ANOVA) with post hoc Tukey adjustment for $p$ values. Probability values $< 0.05$ were considered to indicate statistically significant results.

## 3. Results

### *NLRP3 Inhibition Improves Endothelial Barrier Function and EC Survival after rt-PA Administration under OGD*

The Cell Index is a sensitive read-out for endothelial monolayer integrity. Using the xCELLigence real-time cell analysis (RTCA) system an accelerating decrease in the Cell Index could be detected under OGD, which amplified significantly after rt-PA administration. Coincubation with MCC950, though, stabilizes the Cell Index of the rt-PA + MCC950 group until 15 h of OGD in comparison to rt-PA administration only (Figure 1A). Under normoxic conditions no significant differences between the single treatment regimens stood out (Figure 1B). In a next step, the Cell Index under OGD was correlated with the quantified cell death: while the number of dead EC was the highest in the rt-PA treatment group with a significant reduction in the rt-PA + MCC950 group, the cell death was lowest in the MCC950 only group, as shown by propidium iodide (PI) staining (Figure 1C,D). To verify the role of NLRP3 within the loss of endothelial barrier function during OGD and rt-PA treatment, an immunofluorescent microscopic analysis of the cell culture was performed. It

showed an increasing NLRP3 signal during OGD incubation in the vehicle group with a considerable gain of NLRP3 intensity in the rt-PA group and an unambiguous reduction in the respective MCC950 groups. After 1 h of OGD only a faint NLRP3 signal was detected in the MCC950 only group. The staining of the apoptosis-associated speck-like protein containing a CARD domain (ASC), the essential adaptor molecule for inflammasome activation, which is described as a readout parameter for inflammasome activation, showed similar results (Figure 2) [19].

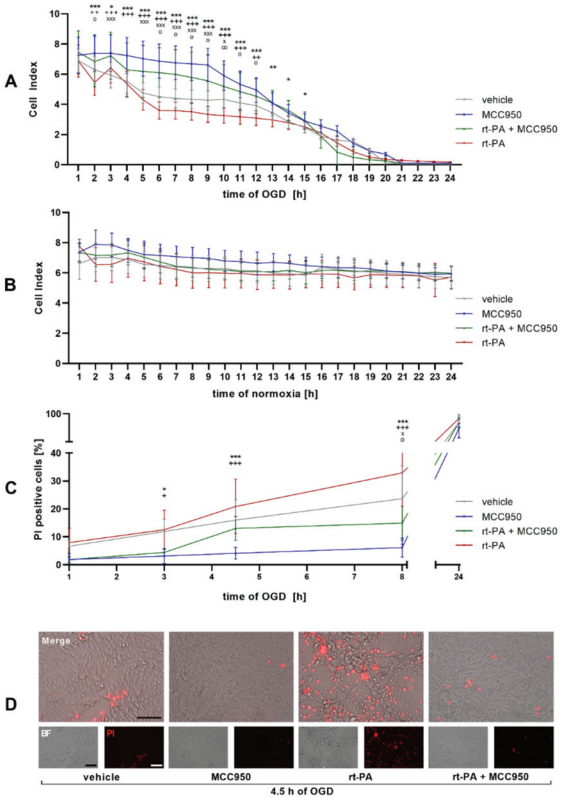


**Figure 1.** NLRP3 inhibition stabilizes the Cell Index of endothelial cells and reduces endothelial cell death under OGD. (**A**) The Cell Index over a period of 24 h of OGD (1% $O_2$, 5% $CO_2$, 95% humidity, 37 °C, 1 g/L glucose) as measured with the ACEA xCELLigence DP system. bEnd5 were either treated with vehicle, MCC950, rt-PA, or rt-PA and MCC950 ($n$ = 24 out of 3 independent experiments). (**B**) The Cell Index over a period of 24 h of normoxia. bEnd5 were either treated with vehicle, MCC950, rt-PA, or rt-PA and MCC950 ($n$ = 24 out of 3 independent experiments). (**C**) Percentage of apoptotic, propidium iodide (PI) positive, bEnd5 cells per well after 1 h, 3 h, 4.5 h, 8 h, and 24 h of OGD depending on the treatment regime ($n$ = 15 out of 3 independent experiments). (**D**) Representative microscopic brightfield images of bEnd5 after 4.5 h of OGD and visualization of treatment-dependent PI (red) uptake; 10× objective; scale bar = 100 µm. Data was analyzed by 1-way ANOVA with post hoc Tukey adjustment * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. Significance as indicated by * refers to the comparison between the rt-PA treated group and the rt-PA + MCC950 treated group, by $^{+}$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ to the comparison between the vehicle treated group and the MCC950 treated group, by $^{o}$, $^{oo}$ $p < 0.01$ to the comparison between the vehicle treated group and the rt-PA treated group and by $^{x}$, $^{xxx}$ $p < 0.001$ to the comparison between vehicle and the rt-PA + MCC950 treated group. Merged images are enlarged by a factor of 2.

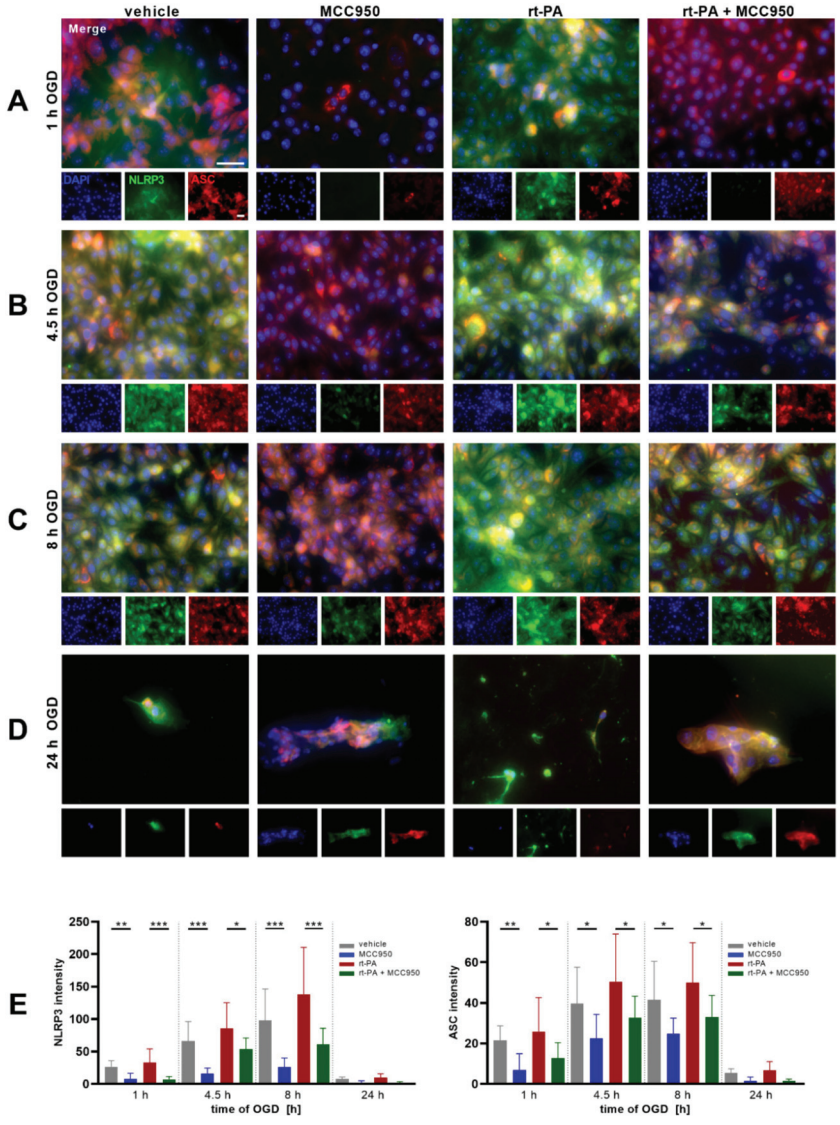


**Figure 2.** MCC950 treatment reduces NLRP3 and ASC expression under OGD. Representative NLRP3 (green), ASC (red), and DAPI (blue) immunofluorescence stainings of bEnd5 after (**A**) 1 h, (**B**) 4.5 h, (**C**) 8 h, or (**D**) 24 h of OGD (1% $O_2$, 5% $CO_2$, 95% humidity, 37 °C, 1 g/L glucose) either with vehicle, MCC950, rt-PA, or dual rt-PA and MCC950 treatment. (**E**) Quantification of NLRP3 (left) and ASC (right) expression by intensity measurement of the immunofluorescent stainings ($n$ = 15 out of 3 independent experiments); 20 × objective; scale bar = 20 μm; scale bars account for all images. Merged images are enlarged by a factor of 3. Data was analyzed by 1-way ANOVA with post hoc Tukey adjustment. * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$.

The characteristic high transendothelial resistance of brain endothelial monolayers is achieved by the formation of tight junctions (TJ) that seal the intercellular clefts. These transmembraneous multi-protein complexes are linked to the cytoskeleton by intracellular adapter proteins such as zonula occludens-1 (ZO-1). Thus, ZO-1 reduction after OGD exposure and rt-PA stimulation, as analyzed by immunofluorescent microscopic analysis,

points to a loss of TJ integrity. After 8 h of OGD, the vehicle group showed a distinctly reduced ZO-1 signal while it vanished nearly completely in the rt-PA group. In the MCC950 treated groups, though, a distinct intensity remained even after 8 h of OGD (Figure 3).

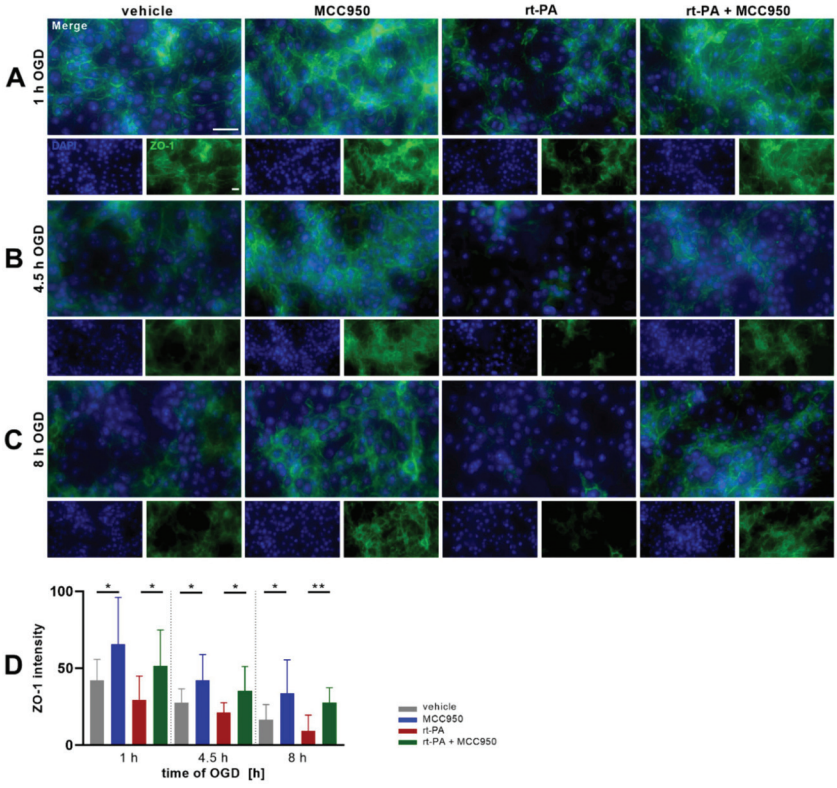


**Figure 3.** MCC950 treatment improves ZO-1 expression under OGD. Representative ZO-1 (green) and DAPI (blue) immunofluorescence stainings of bEnd5 after (**A**) 1 h, (**B**) 4.5 h, or (**C**) 8 h of OGD (1% $O_2$, 5% $CO_2$, 95% humidity, 37 °C, 1 g/L glucose) either with vehicle, MCC950, rt-PA or dual rt-PA and MCC950 treatment. (**D**) Quantification of ZO-1 expression by intensity measurement of the immunofluorescent stainings ($n$ = 15 out of 3 independent experiments); 20× objective; scale bar = 20 µm; scale bars account for all images. Merged images are enlarged by a factor of 2. Data was analyzed by 1-way ANOVA with post hoc Tukey adjustment. * $p < 0.05$; ** $p < 0.01$.

## 4. Discussion

Intravenous rt-PA treatment in acute ischemic stroke is restricted to 4.5 h after stroke onset, partly due to an increasing risk of side effects, such as bleeding complications, with progressing occlusion time. As our principal finding, we show that targeting the NLRP3 inflammasome can protect brain endothelial cells from unwanted toxic side effects of the thrombolytic agent rt-PA under ischemic conditions.

Prolonged OGD of bEnd5 cells, as a model system of the brain microvasculature during large vascular occlusion in IS, triggers cell death over time [15]. This is accompanied by a rise in NLRP3, as well as ASC protein expression and the disruption of endothelial barrier function. Of note, further deterioration of hypoxic endothelial damage by rt-PA treatment was significantly attenuated when MCC950 was co-administered. Importantly, to achieve the greatest possible transferability of the in vitro model, the very same concentration of rt-PA was applied to the cells that was averagely measured in IS patients behind the occluding thrombus before mechanical thrombectomy [17]. The observed disruption of

barrier integrity in the rt-PA treated group within 6 h of OGD resembles data of human rt-PA observational studies. Here, the number needed to treat (NNT) rises from 10 (3 h) to 19 (4.5 h) and further to 50 (6 h), while the number needed to harm equals the NNT after 6 h [20].

In the given in vitro setting, clinically important variables such as symptom severity, possible side effects of the treatment, or the overall outcome cannot be examined, which we consider as a study limitation [21]. Furthermore, to evaluate detailed molecular mechanisms of NLRP3 activation and corresponding downstream inflammatory processes when endothelial cells are exposed to rt-PA under ischemic conditions, additional studies are needed.

Taken together, our study provides evidence that early application of a NLRP3 inhibitor does not only preserve the patency of the BBB in middle cerebral artery occlusion, but furthermore protects hypoxic endothelial cells from side effects of concomitant rt-PA treatment. Therefore, NLRP3 inhibition presents as a promising therapeutic target to extend the narrow window of opportunity for acute stroke treatment.

**Author Contributions:** Conceptualization, M.B. and M.K.S.; methodology, M.B., L.Z., A.H. and M.K.S.; validation, M.B., A.T.R., M.K.S. and G.S.; formal analysis, M.B.; investigation, M.B. and A.T.R.; writing—original draft preparation, M.B.; writing—review and editing, A.T.R., L.Z., A.H., M.K.S. and G.S.; visualization, M.B.; supervision, M.K.S. and G.S.; project administration, M.K.S.; funding acquisition, M.B., M.K.S. and G.S. All authors have read and agreed to the published version of the manuscript.

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# Expression of Amyloid Precursor Protein, Caveolin-1, Alpha-, Beta-, and Gamma-Secretases in Penumbra Cells after Photothrombotic Stroke and Evaluation of Neuroprotective Effect of Secretase and Caveolin-1 Inhibitors

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**Abstract:** Our studies reveal changes in the expression of the main participants in the processing of amyloid precursor protein (APP) in neurons and astrocytes after photothrombotic stroke (PTS). Here we show the increase in the level of N- and C-terminal fragments of APP in the cytoplasm of ischemic penumbra cells at 24 h after PTS and their co-immunoprecipitation with caveolin-1. The ADAM10 α-secretase level decreased in the rat brain cortex on the first day after PTS. Levels of γ-secretase complex proteins presenilin-1 and nicastrin were increased in astrocytes, but not in neurons, in the penumbra after PTS. Inhibitory analysis showed that these changes lead to neuronal death and activation of astrocytes in the early recovery period after PTS. The caveolin-1 inhibitor daidzein shifted APP processing towards Aβ synthesis, which caused astroglial activation. γ-secretase inhibitor DAPT down-regulated glial fibrillary acidic protein (GFAP) in astrocytes, prevented mouse cerebral cortex cells from PTS-induced apoptosis, and reduced the infarction volume. Thus, new generation γ-secretase inhibitors may be considered as potential agents for the treatment of stroke.

**Keywords:** amyloid precursor protein; photothrombotic stroke; ischemia; alpha-secretase; beta-secretase; gamma-secretase; caveolin-1; apoptosis; astrocyte activation

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## 1. Introduction

Stroke is the second leading cause of mortality and the major cause of physical disability in adults worldwide [1]. Preclinical testing of potential drugs has not yet found agents without serious side effects, limiting the propagation of pathological processes from the primary site of damage to surrounding healthy tissues, and protecting nerve cells [2,3]. Thus, deep and comprehensive studies on molecular mechanisms of the pathological processes after stroke are required.

Our previous proteomic studies have shown that the expression of amyloid precursor protein (APP), as well as nicastrin and apolipoprotein E (APOER or LRP1), involved in the processing and functioning of APP, increased in the penumbra at 1–4 h after acute photothrombotic exposure; hence, we decided to study APP and its proteolytic system understroke [4].

The APP protein has been intensively studied since the 1980s due to its central role in the development of Alzheimer's disease (AD). Its fragment β-amyloid peptide (Aβ) accumulates in amyloid plaques in the brain of AD patients. APP is an evolutionarily conserved protein [5,6]. A high level of APP expression in the brain indicates its important role in the nervous system. It is involved in the development, differentiation, and function

of neurons, neurite growth, synapse and long-term memory formation, brain integrity maintenance, and neuronal response to damage [7,8]. However, the specific biochemical and physiological functions of APP and its proteolytic products are still unknown. The accumulation of APP in damaged neurons after ischemic stroke indicates its important role in stroke-induced pathological processes in the nerve tissue [9,10].

APP is a large transmembrane glycoprotein that crosses the plasma membrane (PM) once. Its large N-terminal domain faces the extracellular environment, while its small C-terminal domain faces the cytoplasm. APP undergoes proteolytic cleavage by α-, β-, and γ-secretases to form several peptides: sAPPα (soluble amyloid precursor protein alpha), sAPPβ (soluble amyloid precursor protein beta), Aβ (β-amyloid peptide), AICD (amyloid precursor protein intracellular domain), and some less studied peptides. APP proteolytic products have independent activity and are involved in various cellular processes. There are amyloidogenic and non-amyloidogenic pathways of APP proteolysis.

In the non-amyloidogenic pathway, α-secretase cleaves a large N-terminal fragment of sAPPα from APP. At the same time, Aβ (the APP fragment that is immersed in the membrane) is cleaved and inactivated. Then γ-secretase cleaves off the intracellular AICD peptide that degrades in the cytoplasm. Cleavage of APP by α-secretase occurs mainly on the cell surface, although some part of APP is cleaved along the pathways of processing and traffic in cisterns and vesicles of the endoplasmic reticulum and Golgi apparatus [11,12]. Proteinases of the ADAM (a disintegrin and metalloproteinase) family act as α-secretase in mammalian cells and ADAM10 protein plays the main role in APP processing in the non-amyloidogenic pathway [13,14]. Amyloidogenic processing of APP occurs in specialized regions of the cell membranes, the lipid rafts. However, ADAM10-mediated non-amyloidogenic processing is believed to occur in the non-raft region of the membrane [15–17].

β-secretase is an aspartyl protease represented by two main isoforms: BACE1 (beta-site APP cleaving enzyme 1) and BACE2. BACE1 is abundant in the nervous system (in neurons, astrocytes, and oligodendrocytes) while BACE2 is abundant in peripheral tissues (melanocytes or pancreatic β-cells).

In the amyloidogenic pathway, BACE1 and γ-secretase cleave APP in the plasma membrane so that the Aβ peptide is released into the environment and AICD is released into the cytoplasm. During cerebral ischemia, BACE1 is activated as a result of oxidative stress and stimulation of the oxygen sensor HIF1α [18,19]. γ-secretase is a large multi-subunit enzyme consisting of presenilin-1 (PS1) that performs a proteolytic function, presenilin-2 (presenilin enhancer 2, PEN-2) that associates and causes the endoproteolysis of PS1 into the N-terminal fragment (NTF) and C-terminal fragment (CTF), involved in the substrate recognition of nicastrin (NCT), and the anterior pharynx-defective 1 (APH-1) protein that forms a platform for subunit binding [20]. The results of γ-secretase activity include the release of the amyloid peptide Aβ into the extracellular environment promoting the development of AD and the release of the rest into the cytoplasm as the transcription factor AICD is regulating the expression of proapoptotic genes [11]. The subcellular localization of APP influences its proteolytic processing and Aβ formation, not only the localization of β- and γ-secretases in lipid rafts and their co-localization with caveolin-1 but also the exclusion of ADAM10 from lipid rafts lead to Aβ formation [16]. On the other hand, non-amyloidogenic APP processing occurs on the cell surface, where α-secretase is localized while amyloidogenic APP processing occurs after the internalization of APP from the cell surface via endocytosis and only partially on the cell surface [21,22]. Caveolin-1 positively regulates APP cleavage by α-secretase [23] and, vice versa, down-regulates BACE1 activity [24], suggesting that caveolae and caveolins may play a key role in APP proteolysis. Caveolin-1 level significantly increases in neurons with aging [25,26] and under oxidative stress [27].

In this study, we investigated the expression and localization of APP in rat brain cells after photothrombotic stroke (PTS). We studied the expression and localization of α-secretase ADAM10, β-secretase BACE1, and components of the γ-secretase complex pre-

senilin 1 and nicastrin involved in APP proteolysis in rat brain neurons and glial cells after PTS. The effect of ischemia on caveolin-1 levels and co-immunoprecipitation of caveolin-1 with N- or C-terminal fragments of the APP (N-APP and C-APP) and ADAM10 was also investigated. In addition, we studied the effect of secretase inhibitors and caveolin-1 inhibitors on apoptosis, expression of the glial fibrillary acidic protein (GFAP) in astrocytes, and the volume of mouse brain infarction after PTS.

## 2. Methods

### *2.1. Antibodies*

For Western blot analysis and immunofluorescence microscopy, rabbit antibodies were used: anti-ADAM-10, C-terminus (A2726), anti-BACE1 (SAB2100200), anti-nicastrin (ab1) (PRS3983), anti-presenilin-1 (PRS4203), anti-caveolin-1 (marker of lipid raft) (A19006); mouse antibodies NeuN (marker of neurons) (MAB377), GFAP (marker of astrocytes) (SAB5201104), and anti-caveolin-1 (marker of lipid raft) (SAB4200216). To determine the expression and localization of the APP protein by Western blot analysis, immunofluorescence microscopy and immunoelectron microscopy in nerve cells, we used antibodies that specifically recognize the N- or C-terminal fragments of the APP protein (N-APP and C-APP). According to the manufacturer (Merck, Darmstadt, Germany), the anti-rabbit N-APP antibody (SAB4200536) recognizes the N-terminal extracellular domain of human, rat, or mouse APP, and its proteolytic products sAPPα. The anti-rabbit C-APP antibody (A8717) is specific for the sequence of amino acids 676–695 at the C-terminal of APP and anti-β-Amyloid antibody (A8354).

All antibodies and reagents used were purchased from the Moscow branch of Merck (Merck Life Science LLC, Moscow, Russian Federation), except for rabbit antibodies anti-caveolin-1 (A19006) from ABclonal (Woburn, MA, USA).

### *2.2. Animals*

The experiments were carried out on adult male rats (3–4 months, 200–250 g). The experiments with inhibitors were performed on male mice of the outbred CD-1 stock (14–15 weeks old, 20–25 g). Outbred Wistar rats and CD-1 mice were purchased from a farm in Pushchino, Moscow Region (http://www.spf-animals.ru/animals/rats/, accessed on 13 May 2020). Outbred white mice and rats were obtained from the vivarium of the Rostov Scientific Research Institute of Microbiology and Parasitology. Animals were kept in standard cages in groups of 4–5 animals with free access to food and water under standard conditions: 12 h light/12 h dark cycle, 22–25 °C, air exchange rate 18 changes per hour. International, national, and institutional guidelines for the care and use of animals were followed. All experimental procedures were carried out in accordance with European Union directives 86/609/EEC on the use of experimental animals and local legislation on the ethics of animal experimentation. Animal protocols were evaluated and approved by the Animal Care and Use Committee of the Southern Federal University (Permit No. 08/2016). For the entire period of detention and before the experiment the animals were properly cared for with daily veterinary examination (body position in space, activity), thermometry, and weighing of each one. The adequate depth of anesthesia was achieved in about 30 min. The depth of anesthesia was assessed by the absence of a plantar reflex and a reaction to pinching the membrane between the fingers), a decrease in or absence of muscle tone in the limbs, and a slow regular heart rate and respiratory rhythm. The following measures of physiological support of an animal during anesthesia and experimental procedures were obtained: prevention of dry eyes and damage to the cornea by placing an ophthalmic ointment in the conjunctival sac and temperature maintenance via an electrically heated mat. After the surgical intervention, each animal was placed into a separate warm clean cage until the complete recovery from anesthesia. Further postsurgical care included the administration of analgesics, antimicrobials, daily monitoring of the animals' state for signs of pain and distress; special attention was paid to the condition of sutures and the irradiation area.

*2.3. Photothrombotic Stroke Model*

For a model of ischemic stroke, we used unilateral photothrombotic stroke (PTS) in the somatosensory cerebral cortex of rats or mice. In PTS, local laser irradiation induces photoexcitation of the introduced photosensitizing dye Bengal rose. Due to its physical properties, it does not penetrate cells and remains in blood vessels. After laser irradiation, highly reactive singlet oxygen is generated and damages the vascular endothelium, causing platelet aggregation and vascular thrombosis [28].

Experiments were performed as described before [29]. Briefly, rats were anesthetized with intraperitoneal injections of telazol (50 mg/kg) and xylazine (10 mg/kg). The animals were fixed, the periosteum was removed, and a longitudinal incision was made in the skull skin. Rose Bengal (20 mg/kg) (R4507, Merck, Moscow, Russia) was injected into the subclavian vein. Then the somatosensory cortex (3 mm lateral to the bregma) was irradiated through the relatively transparent cranial bone with a diode laser (532 nm, 60 mW/cm$^2$, Ø3 mm, 30 min). This mode of exposure induces the formation of an infarction core with a diameter of about 3 mm surrounded by a penumbra about 1.5 mm wide [30]. After anesthesia, the rats were decapitated in 4 h or 24 h or 7 days after PTS. The brain was removed and a section of the cortex corresponding to the infarction core was removed on ice with a cylindrical knife (Ø 3 mm) and then a 2-mm ring was cut around the irradiation zone with another knife (Ø 7 mm), approximately, corresponding to the penumbra tissue (experimental sample, respectively, PTS4, PTS24, and PTS7d). The control groups included sham-operated (SO) animals subjected to the same procedures but without the photosensitizer administration. The obtained tissue samples were further used for Western blot analysis.

Experiments with inhibitors of α-, β-, and γ-secretases were carried out on mice. Mice were anesthetized at 25 mg/kg telazol and 5 mg/kg xylazine. Rose Bengal at a concentration of 15 mg/mL was administered intraperitoneally at a dose of 10 μL/g of body weight. At 5 min after the photosensitizer administration, the area of the mouse skull free from the periosteum was irradiated with a diode laser in the sensorimotor cortex area (2 mm lateral to the bregma). Irradiation parameters: wavelength 532 nm, intensity 0.2 W/cm$^2$, beam diameter 1 mm, duration 15 min. The control groups included SO animals subjected to the same operations but without the photosensitizer administration. At 3, 7, and 14 days after laser irradiation, the mice were decapitated and the brain was removed to study the extent of damage, the level of apoptosis of cells in the perifocal region and the expression of the GFAP protein in astrocytes. The surgery is non-invasive with 100% survival of animals before the decapitation.

*2.4. Cytoplasmic and Nuclear Fractions of Brain Tissue Extraction*

Cytoplasmic and nuclear fractions were obtained using the CelLytic™ NuCLEAR™ Extraction Kit (NXTRACT, Sigma-Aldrich, Darmstadt, Germany). To do this, the samples were homogenized on ice for 3 min using a Vibra-Cell VCX 130 ultrasonic homogenizer (Sonics, Newtown, CT, USA) in Lysis Buffer, which is included in the CelLytic™ NuCLEAR™ Extraction Kit, supplemented with a mixture of inhibitors, proteases, and phosphatases (PPC1010, Sigma-Aldrich, Darmstadt, Germany), necessary for the preservation of proteins and their phosphorylated forms, as well as nuclease benzonase (E1014, Sigma-Aldrich, Darmstadt, Germany), which destroys nucleic acids. After the homogenization, the samples were centrifuged for 20 min at 10,000–11,000× *g* at 4 °C in a Mikro 220 R centrifuge (Hettich, Tuttlingen, Germany). Then, the supernatant containing cytoplasmic proteins was collected and nuclear proteins were extracted from the sediment containing cell fragments and cell nuclei using the Nuclear Extraction Buffer included in the NXTRACT Reagent Kit. To carry out this process, the pellet was resuspended and incubated for 40 min with this buffer. The lysate was centrifuged for 5 min at 20,000–21,000× g at 4 °C.

In the resulting supernatant, containing nuclear proteins and the previously obtained cytoplasmic fraction, the protein content was determined using the Bradford reagent (B6916,

Sigma-Aldrich). The lysates were then aliquoted, frozen in liquid nitrogen, and stored at −80 °C for further Western blot analysis.

The purity of the fractions was checked as follows: negative control of the cytoplasmic marker in the nuclear fraction was used and, vice versa, negative control of the nuclear marker in the cytoplasmic fraction was used as well. The acetylated histone protein H4 (ac-H4) was used as a nuclear fraction marker. We used an H4 anti-acetyl-Histone antibody obtained from rabbits (No. 06-866, Merck), diluted at 1:500. Proteinglyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a marker of a cytoplasmic fraction. We used an anti-GAPDH antibody obtained from rabbits (G9545, Sigma-Aldrich) at a 1:1000 dilution. The cytoplasmic fraction was confirmed by the absence of ac-H4, and the nuclear fraction was confirmed by the absence of the cytoplasmic fraction marker GAPDH (Figure S1).

### *2.5. Immunoblotting*

Expression of C-APP, N-APP, Aβ, ADAM10, BACE1, presenilin 1, nicastrin, and caveolin-1 in the cytoplasmic fraction of rat cerebral cortex cells after PTS was studied using the Western blot method as described previously [20]. Briefly, the rat cortical tissue samples were homogenized on ice using a Vibra-Cell VCX 130 ultrasonic homogenizer. Nuclear and cytoplasmic fractions were isolated using the CelLytic NuCLEAR Nuclear Fraction Extraction Kit.

Samples containing 10–20 μg of protein per 15 μL were subjected to electrophoretic separation in a polyacrylamide gel (7–10%) in the presence of sodium dodecyl sulfate in a mini-PROTEAN Tetra cell (Bio-Rad, Hercules, CA, USA). ColorBurst Electrophoresis Marker (C1992, Sigma-Aldrich) was used as a standard protein marker. After the separation, the proteins were subjected to electrophoresis onto a PVDF membrane (polyvinyl difluoride membrane 162-0177, Bio-Rad) using the Trans-Blot® Turbo Transfer System (Bio-Rad, USA). After washing with PBS, the membrane was successively incubated for one hour in blocking buffer (TBS 1% Casein Blocker, Bio-Rad) and overnight at 4 °C with primary rabbit anti-C-APP (A8717, Merck, 1:500) or anti-N-APP (SAB4200536, Merck, 1:500) antibodies; anti-ADAM-10, C-terminus (A2726); anti-BACE1 (SAB2100200); anti-nicastrin (ab1) (PRS3983); anti-presenilin-1 (PRS4203); anti-caveolin-1 (A19006, ABclonal, 1:500); mouse anti-β-actin antibodies (A5441, 1:5000), mouse monoclonal anti-β-Amyloid antibody (A8354, 1:500) (Table S1 Supplementary).

After the incubation, the membranes were washed in Tris buffer with the addition of 0.1% Tween-20 (TTVS, 10 mM; pH 8) and incubated for one hour at room temperature with a secondary anti-rabbit antibody IgG peroxidase (A6154, Merck, 1:1000). Protein detection was performed on Clarity Western ECL Substrate (Bio-Rad). Chemiluminescence was analyzed using the Fusion SL gel documentation system (Vilber Lourmat, Collégien, France). The obtained images were processed using the VisionCapt software package (https://visioncapt.software.informer.com/, accessed on 20 August, 2020).

### *2.6. Co-Immunoprecipitation*

Co-immunoprecipitation (Co-IP) was performed to confirm the fact of protein–protein interaction between C-APP, N-APP, ADAM-10, and caveolin-1. Co-immunoprecipitation was performed according to the Sileks commercial kit (Sileks, Moscow, Russia) using magnetic particles with protein G (SileksMag-Protein G, cat. no. K0182) in accordance with the manufacturer's recommendations. For this purpose, in a cytoplasmic protein extract of penumbra tissue, obtained 24 h after photothrombotic exposure (PTS24), endogenous caveolin-1 proteins were immunoprecipitated with anti-caveolin-1 antibody and co-precipitated C-APP, N-APP, or ADAM-10 were subsequently detected by antibodies against respective proteins. To visualize the protein–protein interaction, the resulting immunoprecipitate was subjected to immunoblotting.

The primary antibodies: rabbit anti-C-APP (A8717, 1:500), anti-N-APP (SAB4200536, 1:500), anti-ADAM-10 (A2726, 1:500) caveolin-1 (SAB4200216, 1:500) were used. The expression level of the caveolin-1 protein served as a Co-IP control. HRP-conjugated

antibodies (goat anti-rabbit IgG-HRP, Merk A6154, 1:1000; goat anti-mouse IgG-HRP, Amersham NIF825, 1:1000) were used as secondary antibodies. Proteins were identified in immunoblotting.

*2.7. Immunofluorescence Microscopy*

The double immunofluorescence method was used to evaluate the expression and distribution of α-, β- and γ-secretases, and caveolin-1 in penumbra neurons and astrocytes in rats at 4 and 24 h, and 7 days after PTS. The isolated rat brain was fixed in 4% paraformaldehyde overnight and placed in a 30% sucrose solution. Frontal 20 μm thick brain slices (+4.5 mm to −2.5 mm from bregma), obtained using a Leica VT1000 S vibratome (Leica Biosystems, Deer Park, IL, USA), were washed in PBS and incubated in 5% bovine serum albumin c 0.3% TritonX-100 in PBS for one hour at room temperature and then incubated overnight at 4 °C in the same BSA solution with antibodies added: anti-C-APP (1:500), anti-N-APP (1:500), anti-ADAM10 (1:500), anti-BACE1 (1:500), anti-nicastrin (ab1) (1:500), anti-presenilin-1 (1:500), and anti-caveolin-1 (A19006, ABclonal, 1:500), anti-Caspase 3, active (C8487, 1:500), as well as antibodies to NeuN (neuron marker) (1:1000) and GFAP (astrocyte marker) (1:1000), and then with fluorescent secondary anti-rabbit CF488A (SAB4600045, 1:1000) or anti-mouse CF555 (SAB4600302, 1:1000) antibodies. Hoechst 33342 was used as a marker of cell nuclei. After washing in PBS, slices were incubated for one hour with fluorescent secondary anti-rabbit CF488A (SAB4600045, 1:1000) or anti-mouse antibodies CF555 (SAB4600302, 1:1000). The slices were then mounted on glass slides in 60% glycerol/PBS. The results were analyzed using Nikon Eclipse FN1 fluorescent microscope equipped with a Nikon Digital Sight DS-5Mc digital camera (Nikon, Tokyo, Japan) with NIS Elements and Olympus BX51 microscope equipped with an OrcaFlash 4.0 V3 digital camera with HCImage Live software (Hamamatsu, Hamamatsu City, Japan).

Quantitative evaluation of the fluorescence of the experimental and control preparations was carried out on 10–15 images obtained with the same digital camera settings. To isolate and calculate the fluorescence intensity, we used the "Threshold" method of the Adjust menu in the ImageJ application (http://rsb.info.nih.gov/ij/, NIH, USA, accessed on 20 October 2021). For better isolation, cells were cut off background pixels using the Subtract background feature in the Process menu. Next, using the capabilities of the Analyze Particles and ROI Manager menus, cells were isolated and their total fluorescence intensity was measured. The data were normalized after background subtraction:

$$I = \frac{I_m - I_b}{I_b},$$

where $I_m$ is the average cell fluorescence intensity and $I_b$ is the average background fluorescence outside the cells. Protein co-localization was assessed using the ImageJ application with the JACoP plugin. The co-localization coefficient M1 represents the proportion of pixels in the green channels relative to the total signal recorded in the red channel (marker).

*2.8. Inhibitor Assay*

Batimastat (batimastat (BB-94); SML0041) was used as an α-secretase inhibitor; LY2886721 (SML3013), as a β-secretase inhibitor); DAPT (D5942), as an inhibitor of γ-secretases.

Batimastat was dissolved in DMSO and administered intraperitoneally to CD-1 mice at a dose of 50 mg/kg (or 3 mg/mL) one hour after irradiation for five days. Batimastat was previously shown to efficiently penetrate the brain when administered intraperitoneally [31].

LY2886721 is a potent and selective active site inhibitor of β-secretase (BACE1,2) without inhibition of other proteases such as cathepsin D, pepsin, and renin [32]. The LY2886721 preparation was dissolved in 6.7% DMSO and 5% Tween 20 in PBS and administered to animals intraperitoneally at a dose of 10 mg/kg/day for five days.

DAPT, a γ-secretase inhibitor, was dissolved in 5% DMSO and administered to animals intraperitoneally at a dose of 10 mg/kg/day for five days [33].

Daidzein (Sigma-Aldrich, 486-66-8), a caveolin-1 inhibitor was dissolved 1:10 in a solution of dimethyl sulfoxide:phosphate-buffered saline (pH 7.2) and administered at 0.4 mg/kg/day, subcutaneously from 1st to 14th day after PTS [27].

*2.9. Assessment of the Cerebral Cortex Infarction Volume in Mice after PTS*

To assess the infarction volume, brain slices of mice were stained with 2,3,5-triphenyltetrazolium chloride (TTX; T8877, Sigma) at 3, 7, and 14 days after PTS. After decapitation, the brain was quickly removed and placed in a pre-chilled brain matrix of adult mice (J&K Seiko Electronic Co., Ltd.). The matrix with brain tissue was transferred to a freezer (−80 °C) for 3–5 min and cut into 2 mm thick sections. These sections were stained with 1% TTX for 30 min in the dark at 37 °C. Using the ImageJ image analysis application (http://rsb.info.nih.gov/ij/), the areas of infarction zones on each slice were measured, summed, and multiplied by the slice thickness (2 mm).

*2.10. Estimation of the Number of Apoptotic Cells*

Apoptotic cells were visualized using the TUNEL (TdT-mediated dUTP-X nick-end labeling) method which marks DNA strand breaks using the In Situ Cell Death Detection Kit, TMR red (no. 12156792910, Roche). At 3, 7, and 14 days after PTS and administration of inhibitors, mice were decapitated and frontal sections of the brain 20 μm thick were made on Leica VT 1000 S vibratome (Germany). The sections were treated with the reagents of the kit according to the manufacturer's recommendations with the addition of Hoechst 33342 at a final concentration of 10 μg/mL and incubated for one hour at 37 °C.

The apoptotic index (AI) was calculated for TUNEL-positive cells (red fluorescence) in the perifocal region and the cortex of sham-operated animals within the whole area of the mount at a magnification of 20× using the formula: AI = (TUNEL-positive cell number)/(Total cell number (stained with Hoechst 33342)) × 100.

*2.11. Electron Immunohistochemistry*

Animal brain fixation was performed via transcranial perfusion under anesthesia (Nembutal at a dose of 60 mg/kg) using the Perfusion Two perfusion system(Leica Biosystems, Deer Park, IL, USA) equipped with an automatic pump. Perfusion was first carried out with a phosphate buffer solution, pH 7.4, brought to 37 °C (Merck, Darmstadt, Germany), and then with a cooled fixative solution, 4% paraformaldehyde (Merck, EMS, Kenilworth, NJ, USA) in phosphate buffer (pH 7.4). Then the brain was removed and placed in a fixative solution for additional fixation overnight at a temperature of 4 °C. After the post-fixation, a section was isolated from the brain along the coordinates: the first incision was 0.2 mm rostral from the bregma, the second incision was 6.04 mm caudal from the bregma while the brain was not dissected laterally. The excised brain fragment was glued at the caudal side of the cut down to the vibratome table VT 1000E (Leica Biosystems, Deer Park, IL, USA). Next frontal sections 60 μm thick were made.

Electronic immunohistochemistry was performed on rat brain slices according to the pre-embedding protocol. The pre-embedding method (before embedding) is based on the fact that the incubation of slices with primary and secondary antibodies as well as the detection of immune complexes takes place before wiring and embedding the sections in epoxy resin for electron microscopy. Vibratome slices of 60 μm were placed alternately in solutions of 6%, 15%, and 30% sucrose for cryoprotection. Unmasking of antigenic activity was carried out by instantaneous freezing of sections over vapors of liquid nitrogen and subsequent thawing in phosphate buffer. The slices were then incubated in primary anti-APP antibodies supplemented with 0.1% sodium azide to prevent bacterial growth. Incubation was carried out for four days at 20 °C. After washing in a phosphate buffer, the slices were incubated in secondary antibodies RTU Envision Flex/HRP anti-mouse, anti-rabbit (Dako, Glostrup, Denmark) for 24 h at 20 °C. Immune complexes were detected using the EnVision HRP + Peroxidase imaging system (Dako, Glostrup, Denmark). Then, tissue processing was carried out by standard methods for electron microscopic examination.

After washing in a phosphate buffer for at least 15 min, the slices were additionally post-fixed in 1% OsO4 solution in the phosphate buffer for 1.5 h. Then all tissue samples were dehydrated in ascending alcohols and absolute ethanol, processed in three shifts of propylene oxide and embedded in an epoxy resin based on Epon-812. The slices were placed in a drop of resin between two glass slides coated with a water-soluble anti-adhesive Liquid Release Agent (EMS, USA). Polymerization of brain tissue was carried out at 70 °C overnight. Fragments of the studied zones were excised from the sections obtained after polymerization with a blade under a stereotaxic magnifying glass and polymerized to a prefabricated block of epoxy resin. Single and serial (up to 20 sections in one tape) 70 nm thick ultrathin slices were made using an EM UC 7 ultramicrotome (Leica, Germany) and an ultra 45° diamond knife (Diatome, Nidau, Switzerland), counterstained with uranyl acetate and lead citrate and viewed under an electron microscope Jeol Jem 1011 (Jem, Akishima, Tokyo, Japan) with an accelerating voltage of 80 kV.

### *2.12. Randomization and Blinding*

Randomization was applied by randomly choosing the animals from their cages. Blinding was performed at different stages of the experiments: PTS or sham PTS procedure, sacrifice after a certain period post-(sham)-PTI, obtaining brain samples, microscopy, measurement, and statistical processing. Blinding was performed by different researchers.

### *2.13. Statistical Analysis*

In studies with a single effective factor (time post-PTS), differences between sample groups were statistically estimated using one-way ANOVA (Figures 1–8). In studies where different-target inhibitors were used (secretase types in Figure 8), we applied the Student's *t*-test.

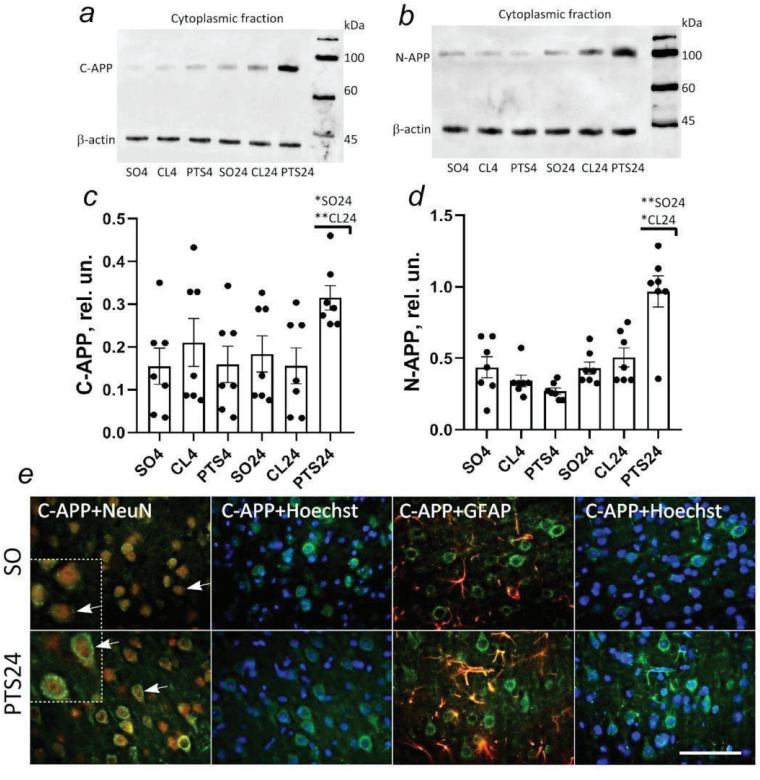


**Figure 1.** *Cont.*

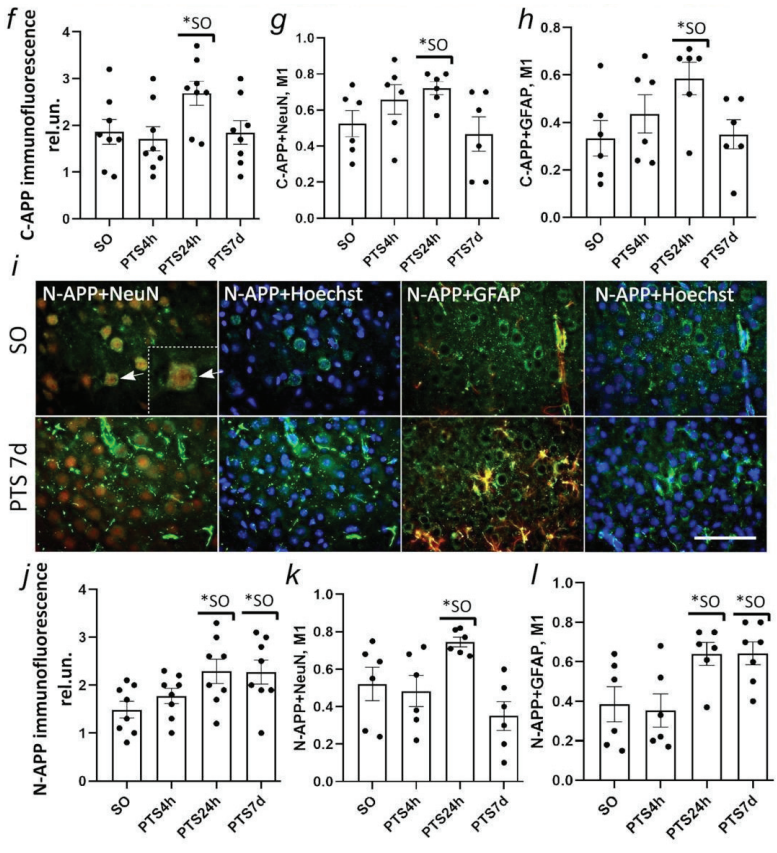


**Figure 1.** (**a**,**b**) Immunoblotting of C- and N-APP in the cytoplasmic fraction of the cortex of sham-operated animals (SO4 and SO24), at 4 (PTS 4 h) and 24 (PTS 24 h) hours after photothrombotic stroke in the cerebral cortex of rats and the contralateral cortex of the same rats (CL4 and CL24, respectively); (**c**,**d**) C- and N-APP level in relative units at different times after PTS; (**e**,**i**) Immunofluorescence of C- and N-APP (green); image overlay of C- and N-APP with NeuN (neuron marker, red), GFAP (astrocytes marker, red), and 33342 Hoechst (nuclei marker, blue). Scale bar 100 μm. Image overlay of ADAM10 with NeuN and with Hoechst. Scale bar 100 μm. Insert: enlarged image. Scale bar 50 μm. Arrows show granularity in the cytoplasm. (**f**,**j**) C- and N-APP fluorescence in penumbra cells after PTS and the cortex of sham-operated rats. (**g**,**k**) Localization of C- and N-APP in penumbra neurons. (**h**,**l**) Localization of C- and N-APP in penumbra astrocytes. The indices of the groups of sham-operated animals at 4 h, 24 h, and 7 days after irradiation had no statistically significant differences, therefore the indices of the three groups were combined (SO). One-way ANOVA. M ± SEM. $n = 6–8$. * $p < 0.05$; ** $p < 0.01$.

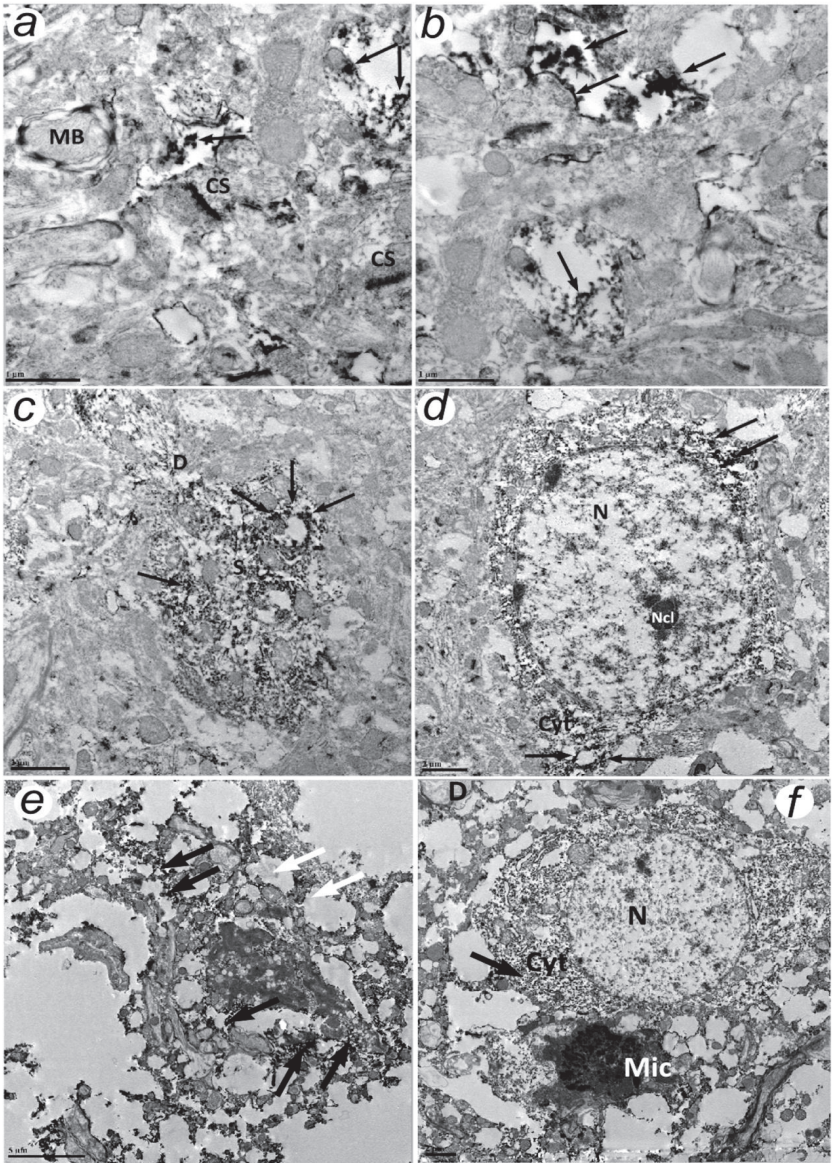


**Figure 2.** Electron immunohistochemistry with antibodies to C- and N-terminal fragments of APP protein in rat brain neocortex of the control group (**a**,**b**) and 4 h (**c**,**d**) or 24 h (**e**,**f**) after photothrombotic stroke. (**a**) Localization of APP (C-terminal domain): the medium amount of protein associated with cellular elements. (**b**) Localization of APP (N-terminal domain): filling of individual cell processes with APP. (**c**) APP (N-terminal domain) localization: numerous processes containing APP fragments. (**d**) APP (N-terminal domain) localization in neuronal cytoplasm. (**e**) The destruction of the neuropil, lysis of the cytoplasm of the cell and processes. (**f**) A neuron containing the APP and an APP-negative microglial cell. Legend: CS—chemical synapse, MB—myelin branch, S—neuronal soma, D—dendrite, N—nucleus, Ncl—nucleolus, Cyt—cytoplasm, Mic—microglia, the products of the reaction are indicated by arrows. Magnification: a and b ×10,000 (Scale bar 5 μm); c,d,f ×20,000 (Scale bar 2 μm); e ×50,000 (Scale bar 1 μm).

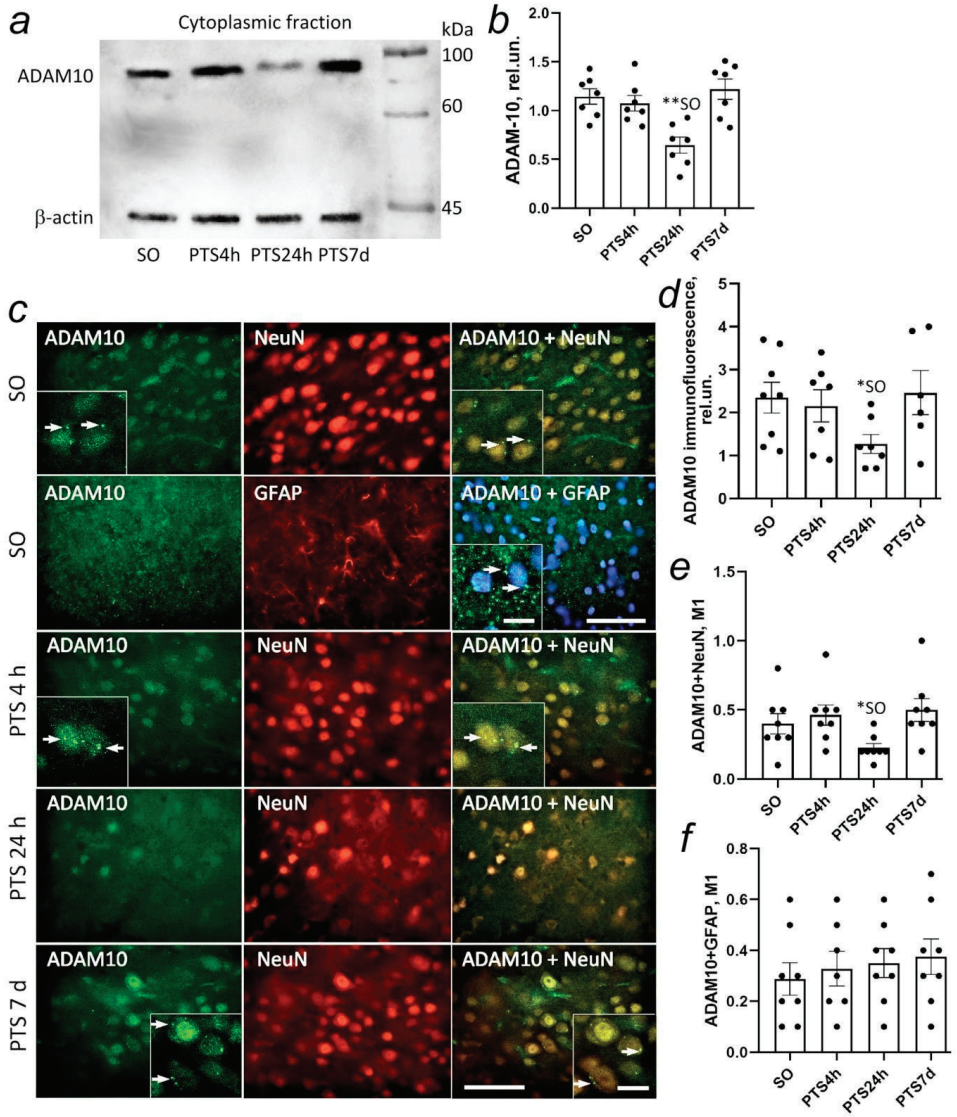


**Figure 3.** Changes in ADAM10 expression in the penumbra at 4 h, 24 h, and 7 days after PTS in the rat cerebral cortex (PTS 4 h, PTS 24 h, and PTS 7d, respectively) relative to the cortex of sham-operated rats (SO). (**a**) ADAM10 in the cytoplasmic fraction of the cortex of sham-operated animals (SO) and at 4 (PTS 4 h), 24 (PTS 24 h), and 7 days (PTS 7d) after PTS; (**b**) ADAM10 level in relative units at different times after PTS. The indices of the groups of sham-operated animals at 4 h, 24 h, and 7 days after irradiation had no statistically significant differences, therefore the indices of the three groups were combined. (**c**) Immunofluorescence and image overlay of ADAM10 (green), NeuN (red), and GFAP (red). Image overlay of ADAM10 with NeuN and with Hoechst. Scale bar 100 μm. Insert: enlarged image. Scale bar 50 μm. Arrows show granularity (clusters) in the area of the cytoplasmic membrane. (**d**) ADAM10 fluorescence in penumbra cells after PTS and in the cortex of sham-operated rats. (**e**) Localization of ADAM10 in penumbra neurons. (**f**) Localization of ADAM10 in penumbra astrocytes. One-way ANOVA. M ± SEM. $n = 6–8$. * $p < 0.05$ compared to the SO. ** $p < 0.01$ compared to the S.O.

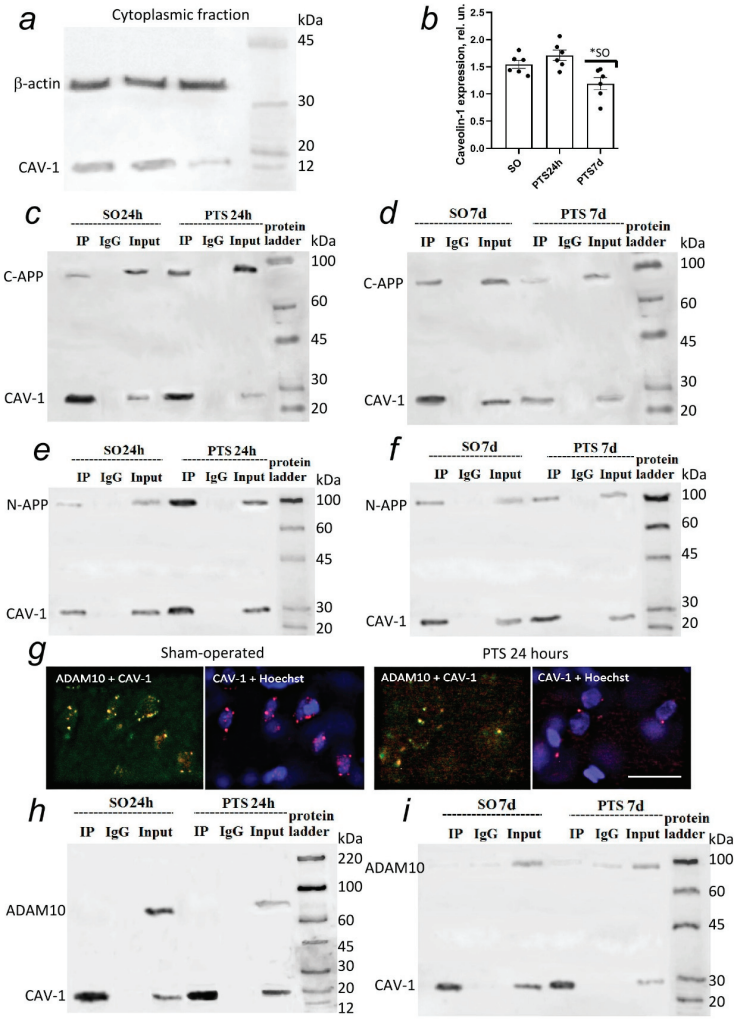


**Figure 4.** (**a**) Caveolin-1 (CAV-1) in the cytoplasmic fraction of the cortex of sham-operated animals (SO) and at 24 (PTS 24 h) and 7 days (PTS 7d) after PTS; (**b**) Caveolin-1 level in relative units at different times after PTS. The indices of the groups of sham-operated animals at 24 h, and 7 days after irradiation had no statistically significant differences. The result of Western blot analysis of co-immunoprecipitation of caveolin-1 protein and C-APP (**c**,**d**), or N-APP (**e**,**f**), or ADAM10 (**h**,**i**) in the cytoplasmic fraction of penumbra tissue at 24 h (PTS 24 h) and 7 days (PTS 7d) after PTS. Control group: cerebral cortex of sham-operated rats (SO24h or SO7d, respectively). Endogenous caveolin-1 proteins were immunoprecipitated (IP) with anti-caveolin-1 antibody, and co-precipitated C-APP or N-APP, or ADAM10 proteins were subsequently detected with anti-C-APP or N-APP, or ADAM10 antibodies, respectively. The expression level of caveolin-1 served as a Co-IP control. Negative control: normal IgG: 3 μg normal mouse IgG was added instead of anti-precipitating protein antibodies to exclude non-specific binding. Positive control: the original sample, not subjected to immunoprecipitation (input). To identify proteins on the blot, a molecular weight marker (protein ladder) was used. (**g**) Immunofluorescence and image overlay ADAM10 (green) and caveolin-1 (red) or caveolin-1 (red) and Hoechst (blue) in the cortex of sham-operated animals (SO) and at 24 h (PTS 24 h) after PTS. Scale bar 50 μm. One-way ANOVA. M ± SEM. $n = 6–8$. * $p < 0.05$ compared to the SO.

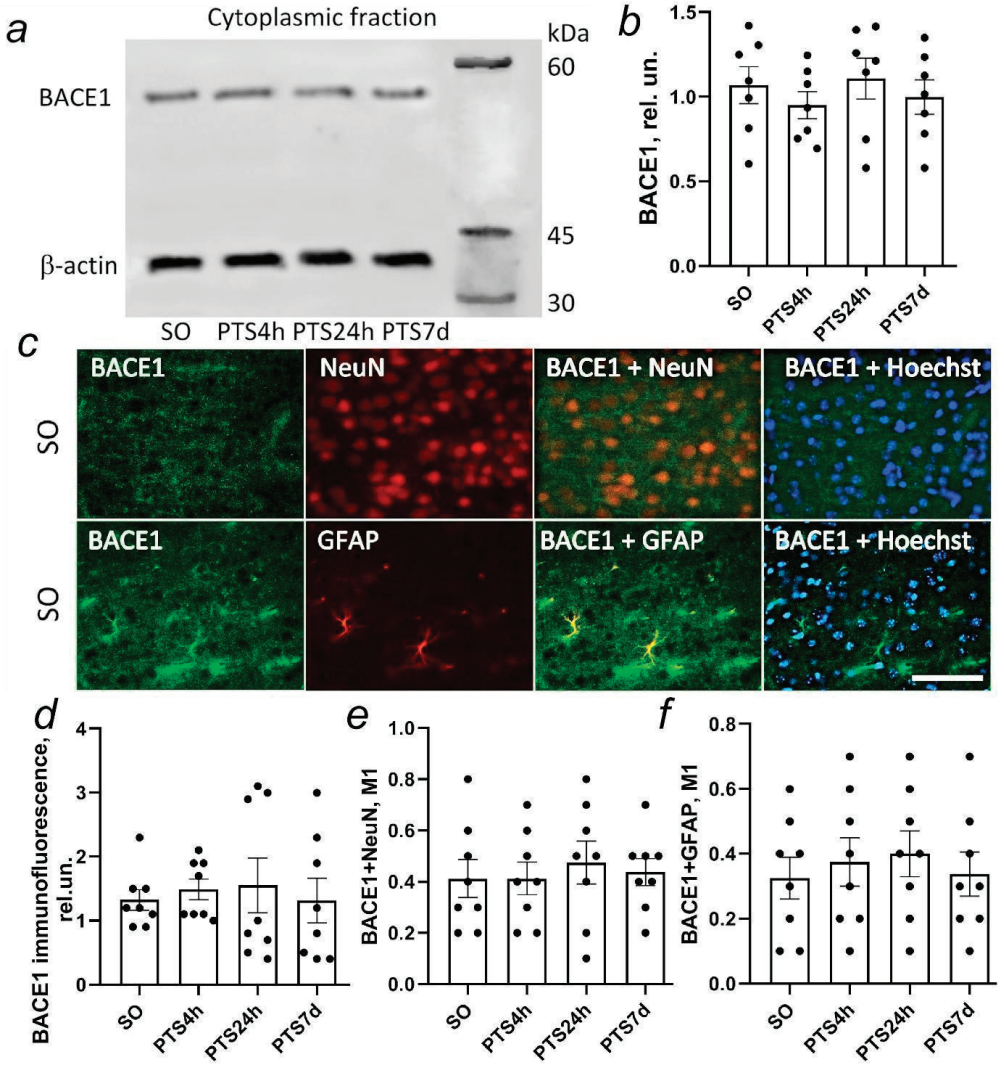


**Figure 5.** (**a**) Immunoblotting of BACE1 in the cytoplasmic fractions of the cortex of sham-operated animals (SO) and at four (PTS 4 h), 24 (PTS 24 h), and 7 days (PTS 7d) after PTS; (**b**) BACE1 level in relative units at different times after PTS. The indices of the groups of sham-operated animals at 4 h, 24 h, and 7 days after PTS had no statistically significant differences, therefore the indices of the three groups were combined. (**c**) Immunofluorescence of BACE1 (green), NeuN (neuron marker, red), and GFAP (astrocytes marker, red); image overlay of BACE1 with Neun, GFAP, and 33342 Hoechst (nuclei marker, blue). Scale bar 100 μm. (**d**) Changes (in relative units) in BACE1 fluorescence in penumbra cells after PTS and in the cortex of sham-operated rats. (**e**) Localization of BACE1 in penumbra neurons. (**f**) Localization of BACE1 in penumbra astrocytes. One-way ANOVA. M ± SEM. $n$ = 7–8.

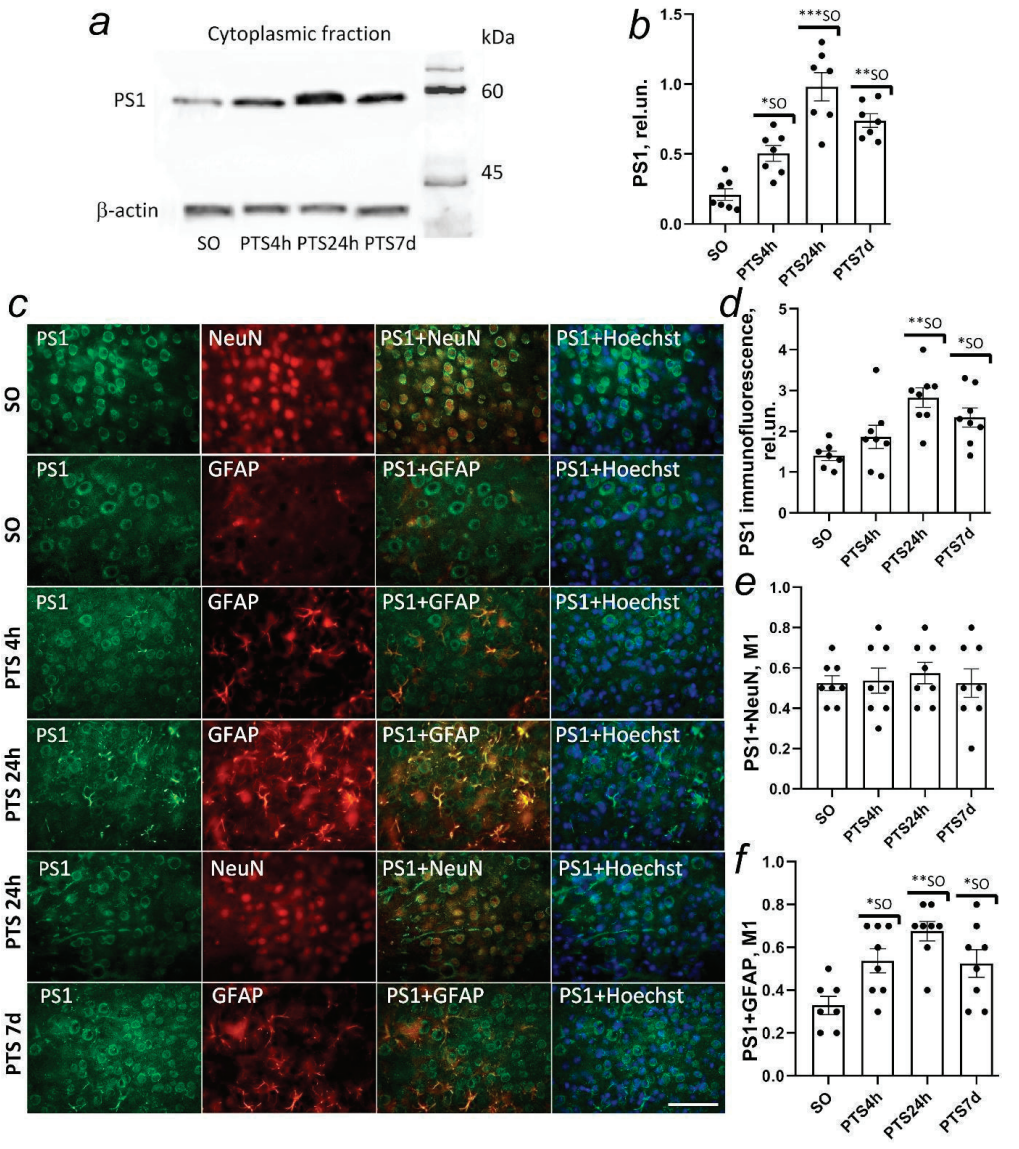


**Figure 6.** (**a**) Immunoblotting of presenilin-1 (PS1) in the cytoplasmic fraction of the cortex of sham-operated animals (SO) and at 4 h (PTS 4 h), 24 h (PTS 24 h), and 7 days (PTS 7d) after PTS; (**b**) PS1 level in relative units at different timepoints after PTS. The indices of the groups of sham-operated animals at 4 h, 24 h, and 7 days after PTS had no significant differences, therefore the indices of the three groups were combined. (**c**) Changes in PS1 expression in the penumbra at 4 h, 24 h, and 7 days after PTS in the rat cerebral cortex (PTS 4 h, PTS 24 h, and PTS 7 d, respectively) relative to the cortex of sham-operated animals (SO). Immunofluorescence of PS1 (green), GFAP (astrocytes marker, red), and NeuN (neuron marker, red); image overlay of PS1 with GFAP, Neun, and Hoechst 33342 (nuclei marker, blue). Scale bar 100 μm. (**d**) Changes (in relative units) in PS1 fluorescence in penumbra cells after PTS and in the cortex of sham-operated rats. (**e**) Localization of PS1 in penumbra astrocytes. (**f**) Localization of PS1 in penumbra neurons. One-way ANOVA. M ± SEM. $n$ = 7–8. * $p < 0.05$ compared to the SO; ** $p < 0.01$ compared to the SO; *** $p < 0.001$ compared to the SO.

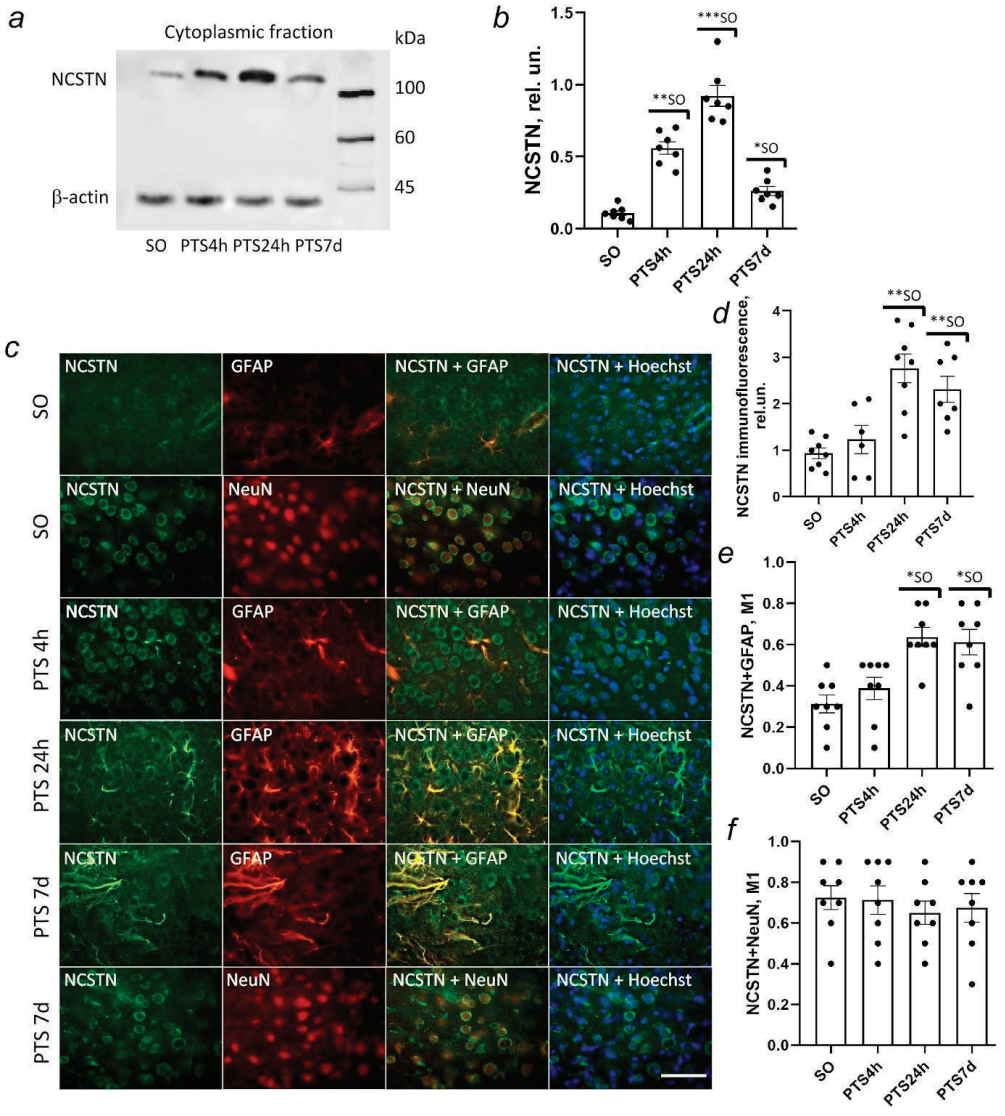


**Figure 7.** (**a**) Immunoblotting of nicastrin (NCSTN) in the cytoplasmic fraction of the cortex of sham-operated animals (SO) and at 4 h (PTS 4 h), 24 h (PTS 24 h), and 7 days (PTS 7d) after PTS; (**b**) Nicastrin level in relative units at different timepoints after PTS. The indices of the groups of sham-operated animals at 4 h, 24 h, and 7 days after PTS had no statistically significant differences, therefore the indices of the three groups were combined. (**c**) Immunofluorescence of nicastrin (NCSTN) (green), GFAP (astrocyte marker, red), and Neun (neuron marker, red); image overlay of NCSTN with Neun, GFAP, and Hoechst (nuclei marker, blue). Scale bar 100 μm. (**d**) Changes (in relative units) of nicastrin fluorescence in penumbra cells after PTS and in the cortex of sham-operated rats. (**e**) Localization of nicastrin in penumbra astrocytes. (**f**) Localization of NCSTN in penumbra neurons. One-way ANOVA. M ± SEM. n = 7–8. * $p < 0.05$ compared to the SO; ** $p < 0.01$ compared to the SO; *** $p < 0.001$ compared to the SO.

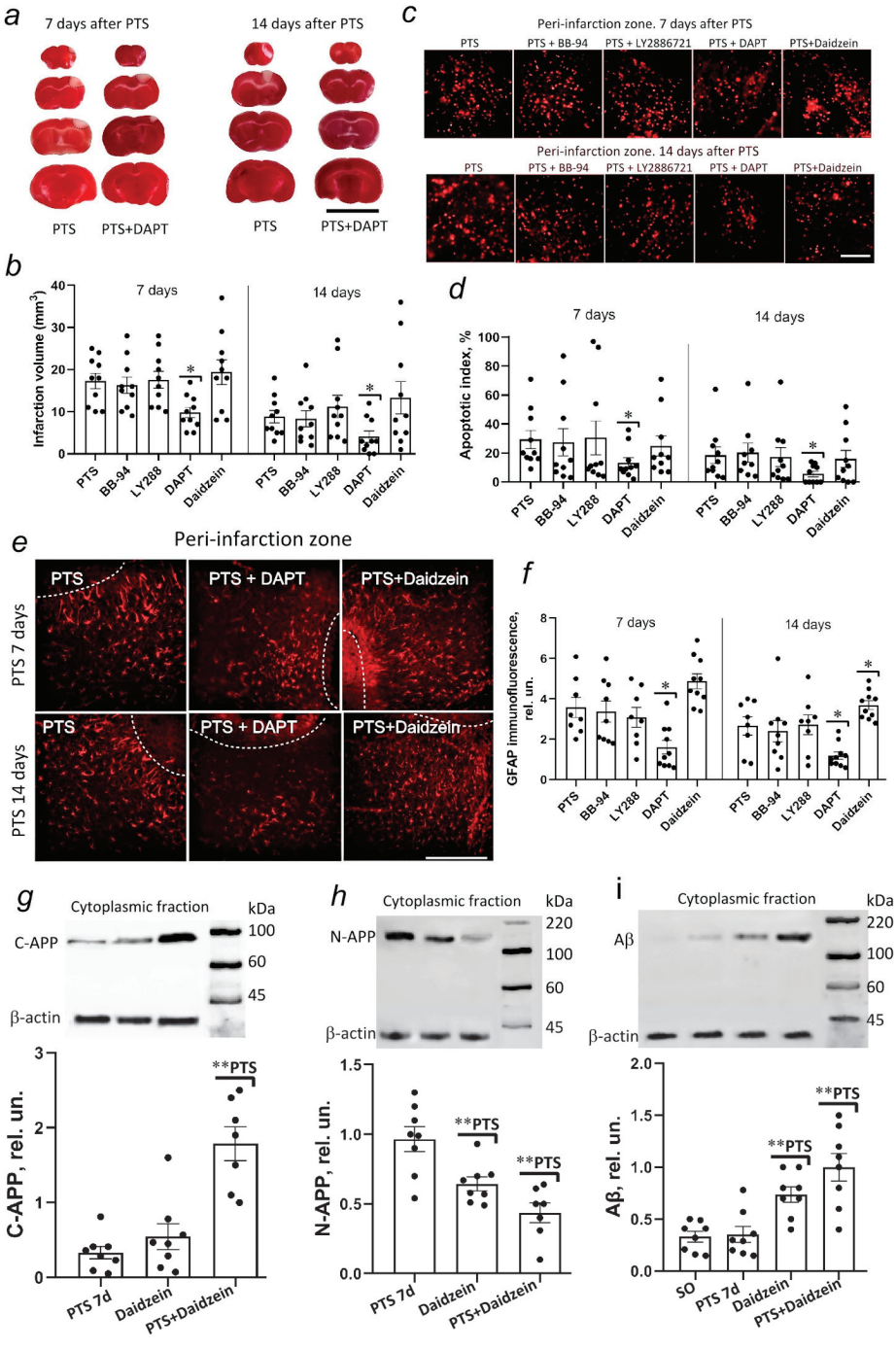


**Figure 8.** Effects of secretase inhibitors (LY2886721, an inhibitor of β-secretases BACE1 and BACE2; DAPT, an inhibitor of γ-secretase) and daidzein, an inhibitor of caveolin-1, on the volume of the infarction nucleus, apoptosis, and expression of GFAP in the peri-infarction region in the mouse cerebral cortex on days seven and 14 after PTS. (**a**) View of the mouse brain slices seven and 14 days

after PTS and against the background of the DAPT administration, stained with TTS. The dotted line is the zone of infarction. (**b**) Average values of the infarct core volume ($mm^3$) in the control groups (PTS without inhibitors) and the experimental groups (administration of inhibitors) seven and 14 days after PTS. Scale bar 1 cm. (**c**) Representative images of cortical areas stained with TUNEL (red fluorescence of apoptotic cells) seven and 14 days after PTS. Experimental groups: BB-94, an inhibitor of α-secretase ADAM10; LY2886721,an inhibitor of BACE1 and BACE2 β-secretases; DAPT is a γ-secretase inhibitor and daidzein, an inhibitor of caveolin-1. Scale bar 100 μm. (**d**) Changes in the apoptotic index (AI, %) in mice 7 and 14 days after PTS and after administration of inhibitors. (**e**) Immunofluorescence of GFAP (red) in the peri-infarction region of PTS in the mouse cerebral cortex on days 7 and 14 after PTS and against the background of the introduction of DAPT and daidzein. The dotted line is the zone of infarction. Scale bar 200 μm. (**f**) Changes (in relative units) of GFAP fluorescence in the peri-infarction region of PTS and against the background of the introduction of inhibitors. (**g**) Immunoblotting of C-APP in the cytoplasmic fraction of the cortex at 7 days (PTS 7d) after photothrombotic stroke in the cerebral cortex of mice and after administration of daidzein. (**h**) Immunoblotting of N-APP in the cytoplasmic fraction of the cortex at 7 days (PTS 7d) after photothrombotic stroke in the cerebral cortex of mice and after administration of daidzein. (**i**) Immunoblotting of amyloid beta (Aβ) in the cytoplasmic fraction of the cortex at 7 days (PTS 7d) after photothrombotic stroke in the cerebral cortex of mice and after administration of daidzein. T-test. M ± SEM. $n$ = 7–10. * $p < 0.05$ compared to the PTS; ** $p < 0.05$ compared to the PTS.

For a posteriori (post-hoc) test in ANOVA, Holm–Sidak's test was applied all-pairwise (for three 4 h groups and three 24 h independently in Figure 1c,d) or with the control comparison type (Figures 1–8). Differences were considered significant at $p < 0.05$. Data were presented as mean ± SEM.

## 3. Results

### *3.1. APP Expression in the Rat Cerebral Cortex after PTS*

According to the immunoblotting data, PTS stimulates the accumulation of the C-terminal APP fragment in the ischemic penumbra. In the cortex of control animals, the level of C-APP in the cytoplasmic fraction is not high (Figure 1a). At four hours after PTS, the level of C-APP in the penumbra tissue did not differ from the control. However, after 24 h, it significantly increased in the cytoplasmic fraction (Figure 1a,c). The level of N-APP was also low in the cytoplasmic fraction at 24 h but not at 4 h after PTS (Figure 1b,d).

The intensity of C-APP fluorescence increased by 45% ($p < 0.05$) at 24 h after PTS but it did not differ from the control level at 7 days after PTS (Figure 1f). An increase in the level of the C-terminal fragment of APP was observed both in neurons (Figure 1e,g) and in astrocytes at 24 h after PTS (Figure 1e,h). This was indicated by an increase in the co-localization coefficient of C-APP with the neuronal marker NeuN by 36% (Figure 1f) and with the astrocyte marker GFAP by 79% (Figure 1g).

According to immunofluorescent analysis, the increase in the level of N-APP at 24 h after PTS persisted for up to 7 days (Figure 1i,j). The level of the N-terminal fragment of APP was increased in neurons (by 44%) and in astrocytes (64%) at 24 h after PTS, and N-APP accumulated mainly in astrocytes on the 7th day after PTS (Figure 1i,l).

A more detailed picture of the intracellular distribution of the APP is provided by the electron immunohistochemistry data.

### *3.2. Subcellular Distribution of N- and C-Terminal Fragments of APP in Rat Brain Cells in Normal Conditions and on the First Day after Photothrombotic Stroke*

In the brain samples of sham-operated rats after the immunohistochemical reaction with antibodies to APP fragments, it was shown that the reaction products in thin unmyelinated processes were visualized in the cerebral cortex. The N- and C-terminal fragments of APP were also found in large processes containing mitochondria. In addition to transected axons and dendrites along and across, there were active zones of chemical synapses in

the field of view, while the reaction products seemed to accumulate in the postsynaptic part (Figure 2a).

In some cases, processes absorbed a larger amount of electron-dense flocculent material as a result of immunohistochemical reactions. The reaction products were also coupled to the plasma membranes of the processes (Figure 2c).

Electron microscopic examination of the brain neuropil 4 h after PTS and the study of the expression of the N- and C-ends of APP showed that both of them accumulate in the processes and the cytoplasm of individual neurons (Figure 2c).

The ubiquitous localization of APP fragments in the neuropil was observed at lower magnifications: in the longitudinally and transversely cut dendrites of neurons, as well as in processes with the absence of organelles and single mitochondria (presumably glial processes); in the zone of chemical synapses (Figure 2d).

Individual neurons containing APP fragments were observed. Such neurons contain nuclei without APP with several nucleoli and the cytoplasm with evenly distributed N- and C-terminal fragments of the APP (Figure 2c,d).

Significant tissue damage occurred 24 h after PTS: lysis of nuclei, destruction of cells and processes. At the same time, some elements of the destroyed tissue accumulated a significant amount of APP (Figure 2). Even in the destroyed tissue, the reaction products were localized mainly in the cytoplasm; neuropil elements, negative for APP, were also visualized (Figure 2f). For example, a neuron containing APP in the cytoplasm and a microglial cell with a dark nucleus negative for APP are localized among the destroyed tissue (Figure 2h). Thus, the reaction products of both C-terminal and N-terminal fragments of APP accumulate in thin processes, axons, and dendrites, and in chemical synapses. There were no differences in the localization or accumulation dynamics of the C- or N-terminal APP fragments at the ultrastructural level. At the level of individual neurons, APP fragments were associated with the cell cytoskeleton, rough endoplasmic reticulum, and ribosomes were absent in nuclei. The density of the reaction products depended on the time after exposure. Nerve cells contained the greatest amount of protein at 24 h after PTS. However, some glial cells and neurons did not accumulate APP. It is necessary to understand in further research what kind of cells do not contain APP and why.

### *3.3. ADAM10 Expression in the Rat Cerebral Cortex after Photothrombotic Stroke*

According to the immunoblotting data on α-secretase, ADAM10 is mainly present in the cytoplasmic fraction of the penumbra tissue (Figure 3). A sufficiently high level of protein is noticed in sham-operated animals and at 4 h after PTS. The level of ADAM10 decreased 2-fold ($p < 0.01$) in the cytoplasmic fraction relative to sham-operated animals 24 h after PTS. PTS caused a significant increase in ADAM10 in the cytoplasmic fraction by 97% ($p < 0.01$) 7 day after PTS compared to the protein level at 1 day after PTS approaching the values of the group of sham-operated animals (Figure 3a,b).

A more detailed picture of the intracellular distribution of the protein is provided by immunofluorescence microscopy data. The immunofluorescence signal of ADAM10 in brain tissue is low. ADAM10 expression in neurons, but not in the penumbra astrocytes of sham-operated animals, is quite high (Figure 3c,d). The protein is localized mainly in the cytoplasm of neurons [34]. At 24 h after PTS, an almost 2-fold decrease in the level of ADAM10 compared with the control level is observed due to its decrease in neurons (Figure 5e) but not in penumbra astrocytes (Figure 5f). Noteworthy is the clustering of ADAM10 on the surface of the cytoplasmic membrane. It is known that APP, Aβ, BACE1, and presenilins were found in caveolae and the main structural component of caveolae, caveolin-1 (CAV-1), is able to interact directly with APP [25] and BACE1 [16,24]. At the same time, ADAM10 is largely excluded from lipid rafts [15,16] but APP processing by α-secretase was found in the caveolae of nerve cells [23]. Despite the contradictory data, there is no doubt that caveolae and caveolin-1 in nerve cells are directly involved in APP processing affecting the balance between amyloidogenic and non-amyloidogenic pathways. Thus, at the next stage of the study, we investigated the change in the level of caveolin-1

in penumbra cells at different time points after PTS and its co-localization with C- and N-terminal fragments of APP and ADAM10.

### *3.4. Caveolin-1 Expression and Its Interaction with C- and N-APP and ADAM10 in the Rat Cerebral Cortex after Photothrombotic Stroke*

The level of caveolin-1 in the cytoplasmic fraction of penumbra cells decreased by 23% on the 7th day after PTS compared to sham-operated animals (Figure 4a,b).

Only a small part of the C- and N-terminal APP forms a bond with caveolin-1 in the cytoplasmic fraction of the brain cortex cells of sham-operated animals (Figure 4c–f). Co-immunoprecipitation data have shown the increase in APP-caveolin-1 interaction at 24 h after PTS and its decrease on the 7th day after PTS that correlates with caveolin-1 decrease in penumbra cells at this time point (Figure 4c–f). Interestingly, co-immunoprecipitation occurred both in the case of using an antibody to C-APP that allows detection of the C-terminal residue of APP from amino acids 676 to 695 and in the case of using antibodies to the N-terminal residue of APP that allows the detection of fragments of sAPP$\alpha$.

We also studied ADAM10 co-localization with caveolin-1 to describe the nature of ADAM10-containing clusters on the surface of the cerebral cortex cell membranes more accurately (Figure 3c). Co-localization of caveolin-1 with ADAM10 was observed by immunofluorescence microscopy (Figure 4g). However, the results of co-immunoprecipitation showed no direct interaction between caveolin-1 and ADAM10 after PTS (Figure 4h,j).

### *3.5. BACE1 Expression in the Rat Cerebral Cortex after Photothrombotic Stroke*

β-secretase BACE1 is practically absent in the nuclear fraction of rat cerebral cortex cells. Our study of BACE1 expression by Western blot at different times after PTS did not reveal any change in BACE1 level in the cytoplasmic fractions of the penumbra tissue compared to the group of sham-operated animals (Figure 5a,b).

BACE1 expression in neurons and astrocytes of the rat cerebral cortex was not high (Figure 5c,d), as indicated by Western blot data.

The results of the immunofluorescent analysis also did not reveal changes in BACE1 expression, either in neurons (Figure 5e) and astrocytes (Figure 5f) of the penumbra on the first day or in the early recovery period after a PTS (Figure 5d).

### *3.6. Presenilin-1 Expression in the Rat Cerebral Cortex after Photothrombotic Stroke*

According to Western blot analysis, the level of presenilin-1 in both fractions of the cortex of sham-operated animals was low. There was an increase in the protein level in the cytoplasmic fraction of the penumbra tissue at 4 h after PTS (by 49%, $p < 0.5$) (Figure 6a,b). Protein overexpression in the cytoplasmic fraction increased 2.5-fold compared to the control ($p < 0.01$) at 24 h after PTS and 1.5-fold compared to the protein level at 4 h after PTS ($p < 0.01$) (Figure 6a,b). On day 7 after PTS, the protein level in the cytoplasm of penumbra cells significantly decreased compared to 24 h after PTS, returning to control values (Figure 6a,b).

Immunofluorescent analysis showed that the increase in presenilin-1 expression is associated with its increase in astrocytes but not in penumbra neurons (Figure 6c,e,f). The dynamics of changes in the co-localization coefficient of presenilin-1 with the astrocyte marker GFAP was almost the same as in the total cytoplasmic fraction (Figure 6b).

Presenilin-1 was localized exclusively in the cytoplasm in neurons both in normal conditions and after PTS (Figure 6c). An increase in the protein level on the first day after PTS persisted during the recovery period and was not associated with its growth in neurons since the co-localization of presenilin-1 with the neuronal marker NeuN did not differ significantly from that in sham-operated animals (Figure 6f).

*3.7. Nicastrin Expression in the Rat Cerebral Cortex after Photothrombotic Stroke*

Nicastrin belongs to the γ-secretase protein complex. Little is known about the function of nicastrin in neuronal injury after stroke. In the proteomic studies, we have shown that the expression of nicastrin in the penumbra increased on the first day after PTS [4].

The initial level of the nicastrin protein in the cytoplasmic fraction of the cortex of sham-operated animals was not high (Figure 7a).

The protein level was practically not identified in the nuclear fraction. It increased 6-fold relative to sham-operated animals in the cytoplasmic fractions of the penumbra tissue at 4 h after PTS (Figure 7b). The protein level in the cytoplasmic fraction continued to rise and was 10-fold higher as compared to the control value ($p < 0.01$) 24 h after PTS and 6-fold higher as compared to the protein level 4 h after PTS ($p < 0.01$) (Figure 7a). The level of nicastrin significantly decreased at the 7th day after PTS compared to 4 and 24 h after PTS, but remained higher than the control group ($p < 0.05$) (Figure 7b).

The data from fluorescence microscopic studies confirm the conclusions of the Western blot analysis. Nicastrin is present in the cytoplasm of neurons and nuclei, cytoplasm, and astrocytes processes. Although there is a lot of protein in the brain cells of sham-operated animals, its level increased after PTS (Figure 7c). An increase in the level of nicastrin was observed at 24 h and persisted up to 7 days after PTS (Figure 7d). The increase in protein expression is associated with its high content in astrocytes (Figure 7e) but not in penumbra neurons (Figure 7f).

Thus, the expression of the main proteins of the γ-secretase complex, presenilin-1, and nicastrin, increased in penumbra astrocytes on the first day after PTS and in the early recovery period probably due to their involvement in the reaction of astrocytes to damage and during repair after PTS.

*3.8. Effect of α-, β-, γ-Secretase and Caveolin-1 Inhibitors on Infarction Volume, Apoptosis Level, and Expression of GFAP in the Brain of Mice after Photothrombotic Stroke*

Batimastat (BB-94) was used as an α-secretase inhibitor. The inhibitor molecule mimics the metalloproteinase substrate and therefore acts by competitive reversible inhibition [35]. BB-94 is used to study APP processing [36] and the role of its proteolytic products in memory and AD pathogenesis [37]. Metalloproteinases are currently considered effective antitumor agents in experiments with the administration of batimastat in rodent tumor models [35,38]. Batimastat is almost completely insoluble and therefore has very low oral bioavailability. Thus, the only way to administer batimastat is by direct injection into various body cavities (abdominal and pleural) [28]. In our work on PTS model in mice, batimastat (SML0041, Sigma-Aldrich) was dissolved in DMSO and administered to animals intraperitoneally at a dose of 50 mg/kg (or 3 mg/mL) one hour after irradiation once a day for five days [35,39–41]. It has been previously demonstrated that BB-94 efficiently enters the brain when administered intraperitoneally [41,42]. The selected dose of BB-94 has been shown to work in a mouse model of focal cerebral ischemia [31,33]. In addition, similar doses are effective in rodent tumor models [38]. However, we have not detected the effect of the drug on changes in infarct volume or the level of apoptosis of mouse cerebral cortex cells (Figure 8).

LY2886721 is a potent and selective active site inhibitor of β-secretase (BACE1,2) without inhibition of other proteases such as cathepsin D, pepsin, and renin [32,42]. Here, LY2886721 was dissolved in 6.7% DMSO and 5% Tween 20 in PBS and administered to animals intraperitoneally at a dose of 10 mg/kg/day for 5 days. As in the case of batimastat, we did not detect the effect of LY2886721 on changes in infarct volume or the level of apoptosis of mouse cerebral cortex cells after photothrombotic stroke (Figure 8).

DAPT (N-[N-(3,5-difluorophenacetyl)-1-alanyl]-Sphenylglycine t-butylester) is a γ-secretase inhibitor. The dose of DAPT was selected based on the literature data [43] and verified in preliminary experiments. The agent was dissolved in 5% DMSO and administered to animals intraperitoneally at a dose of 10 mg/kg/day for 5 days. Among the three secretase inhibitors studied, only DAPT reduced the infarction volume on the 7th

and 14th days after PTS, preventing the growth of mouse cerebral cortex cell apoptosis in the area adjacent to the infarction zone (Figure 8).

In this work, we showed that photothrombotic stroke caused an increase in the level of γ-secretase protein subunits of presenilin-1 and nicastrin in astrocytes, but not in penumbra neurons. We evaluated reactive astrocyte marker GFAP expression in astrocytes of the perifocal region adjacent to the infarction zone after PTS and after the administration of the γ-secretase inhibitor DAPT. The inhibitor was shown to reduce the level of GFAP in cortical astrocytes (Figure 8g,h). At the same time, the effect was already seen on the 7th day after the administration of the agent and persisted up to 14 days after the PTS (Figure 8e,f).

Daidzein is known as an inhibitor of caveolin-1 [27,44]. Subcutaneous administration of daidzein from the 1st to 14th day after PTS had no significant effect on infarct volume or apoptosis of penumbra cells (Figure 8), but a decrease in caveolin-1 expression caused an increase in the level of GFAP in astrocytes of the perifocal region of infarction by 7 and 14 days after PTS (Figure 8e,f). The size of the cell body and the thickness of the astrocytic processes increased, the processes of astrocytes strongly overlapped, polarization towards the site of damage occurred, the position of each astrocyte was blurred, and a glial scar of considerable thickness was formed compared to the group that did not receive daidzein (Figure 8e).

We also studied the effect of daidzein on C- and N-terminal APP and Aβ levels after PTS. Inhibitor administration slightly increased C-APP levels (+67%, $p \geq 0.05$) in intact mice (Figure 8g). However, administration of daidzein at 7 days after PTS caused a 6-fold increase in the C-terminal fragment of APP that is known to be derived from the β-secretase processing of APP. A decrease in the expression of caveolin-1 together with the administration of daidzein caused an increase in the level of Aβ, especially after PTS by 233% (Figure 8j). According to immunofluorescent analysis, the level of the N-terminal fragment of APP that is mainly a product of α-secretase processing of APP increased significantly at 24 h after PTS and remained high for up to 7 days (Figure 1i,j). The administration of daidzein reduced the level of N-APP not only in intact mice (by 40%, $p \leq 0.05$) but also by 60% after PTS (Figure 8h).

## 4. Discussion

APP processing and traffic and secretases cleaving it are described in detail in some reviews [11,45,46]. After a ribosomal synthesis of the rough endoplasmic reticulum (RER), APP undergoes processing along the RER—Golgi apparatus (AG)—TGN (trans-Golgi network) vesicles—plasma membrane (PM) pathway (Figure 9). Only about 10% of APP reaches the neuronal membrane as part of TGN vesicles, while the rest is localized in AG and TGN. After incorporation into the PM, APP is internalized within a few minutes and enters endosomes. Part of its amount is recycled and again enters PM, while the other part is degraded in lysosomes. After passing through the TGN in neurons, transport vesicles are transported along the microtubules of axons and dendrites to the periphery of these neurites. It is important to know where APP proteolysis occurs and where its products are generated. A significant fraction of the sAPPα domain is generated by α-secretase inside TGN vesicles and it is exported to the external environment after their incorporation into the PM. BACE1 is first expressed in TGN vesicles and also is concentrated in lipid rafts of the plasma membrane. After reaching the PM, most of BACE1 is internalized and enters endosomes where the acidic environment promotes APP proteolysis (pH 4.5 is optimal for BACE1). Most of the vesicles carrying APP and BACE1 are spatially segregated, both in cultured neurons and in mouse or human brains [47]. Although both APP and BACE1 are synthesized via ER→Golgi, BACE1 is subsequently present in recycling endosomes (Figure 9). Thus, APP and BACE1 are transported in different vesicles and this simple spatial separation limits APP cleavage by BACE1 under normal conditions.

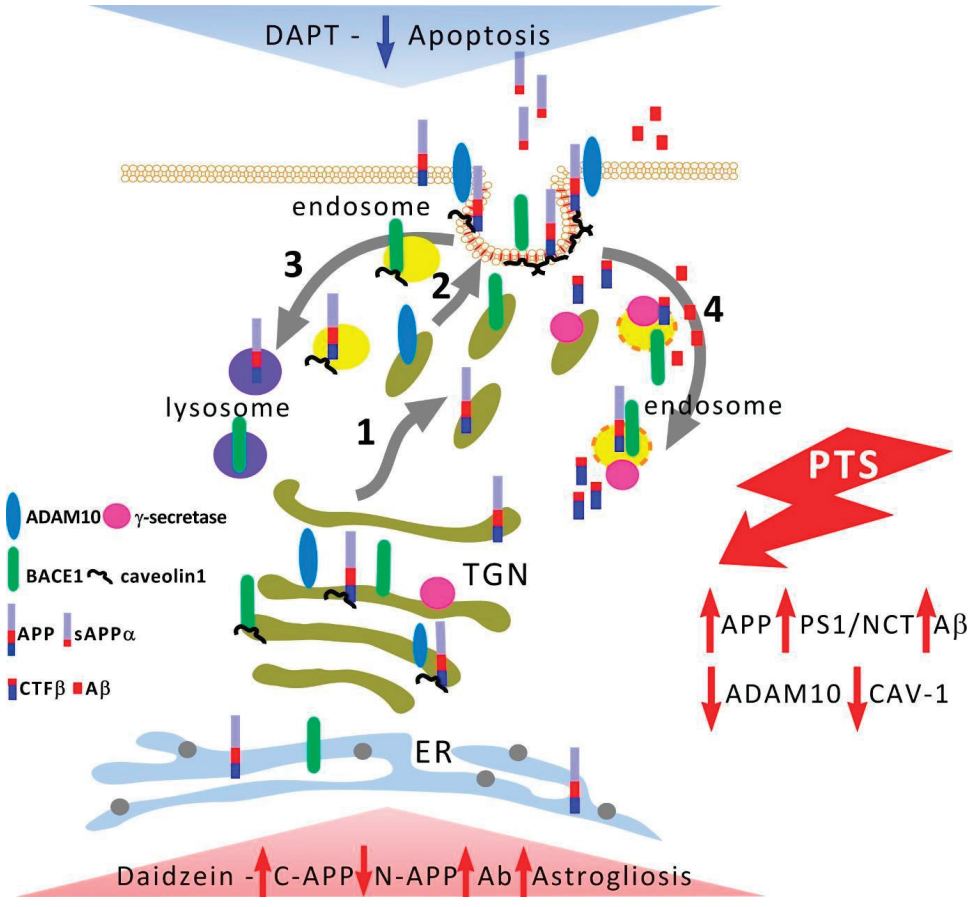


**Figure 9.** APP processing and effect of secretase and caveolin-1 inhibitors after PTS. Newly synthesized APP, BACE1, α-, and γ-secretases (1) are transported to the plasma membrane (2). Caveolae provide a platform for the regulation of APP processing. APP physically interacts with caveolin-1, providing its high concentration on the cytoplasmic membrane. APP is cleaved at the plasma membrane by α-secretase releasing sAPPα. After APP is cleaved by α-secretase, sAPPα is released into the extracellular space in a soluble form, while CTFα remains bound to the membrane where it is cleaved in lysosomes or further cleaved by γ-secretase. Most of APP, BACE1, and γ-secretase are internalized into early endosomes from the cell surface back into the cell and degraded in lysosomes (3). Delivery of APP or reduction of its internalization from the cell surface by binding to caveolin-1 promotes non-amyloidogenic APP processing. Amyloidogenic processing begins with the internalization of APP to endosomes. β-secretase, located on the endosomal membrane, cleaves APP at low pH into two forms in the lipid bilayer: the soluble fragment of APP (sAPPβ) and the membrane-bound β-carboxyl terminal fragment (CTFβ) (4). Further cleavage of CTFβ by γ-secretase produces Aβ monomers and the APP intracellular domain (AICD). The fate of APP along the amyloidogenic or non-amyloidogenic pathway largely depends on the co-localization of APP and secretases that can be influenced by caveolin-1, PTS causes an increase in the level of C- and N-terminal fragments of APP, proteins of the γ-secretase complex of presenilin-1 (PS1) and nicastrin (NCT) and Aβ with a decrease in the expression of ADAM10 and caveolin-1 (CAV-1). Decreased expression of caveolin1 by administering its inhibitor daidzein shifts APP processing towards Aβ synthesis that leads to hyperactivation of astrocytes. The administration of the γ-secretase inhibitor DAPT reduces the amount of PTS damage by reducing the level of apoptotic cell death in the peri-infarction area.

Here, we showed that C- and N-terminal fragments of APP are found not only in neurons but also in astrocytes after PTS. PTS caused the growth of both C- and N-terminal regions of APP already after 24 h. The level of the C-terminal fragment of APP decreased to control values by the 7th day after PTS and was localized in neurons, not astrocytes. A high level of N-APP (that could be predominantly a product of α-secretase (sAPPα) activity) persisted in astrocytes, but not in neurons at the 7th day after PTS. Interestingly, according to electron microscopy data, some neurons, astrocytes, and microglial cells did not contain APP. The reason for this selectivity remains to be seen. Little is known about the role of APP in astrocytes during cerebral ischemia. Although astrocytes express low levels of APP at rest, its level is strongly increased in models of brain injury where extensive gliosis occurs [48]. Astrocyte activation is an early sign of Alzheimer's disease and may be a source of beta-amyloid that forms neuropathological plaques in Alzheimer's disease [49]. Aβ is actively produced by reactive astrocytes as early as 3 days after MCAO, and peptide production decreases only 60 days after ischemia [50]. A 10 min cardiac arrest causes the growth of full-length amyloid precursor protein in reactive astrocytes up to 7 days after ischemia, and Aβ and C-terminus of APP only after 6 months when extensive loss of neurons and the onset of brain atrophy have been observed [51].

APP and its secretases' distribution in different membrane domains and cell compartments (Figure 9) can affect the balance between amyloidogenic and non-amyloidogenic processing pathways. Cell line studies have shown that APP and BACE1 convergence occurs at the plasma membrane in detergent-resistant regions of the membrane [15,16] or possibly near it, but more recent data suggest that these two proteins converge within early endosomes [45,46,52]. Thus, the degree of co-localization of secretases remains unclear.

The proteinases of the "disintegrin and metalloproteinases" or ADAM family, such as ADAM9, ADAM10, TACE/ADAM17, and ADAM19, act as α-secretase in mammalian cells [14]. However, in neurons, α-secretase activity is associated with ADAM10 [53]. α-secretases cleave Notch receptors and ligands, tumor necrosis factor α, cadherins, the IL-6 receptor, EGF receptor ligands, and several other transmembrane proteins to release their extracellular domain [54]. Protein kinase C is known to promote the processing of α-secretases and the secretion of the APP ectodomain [11]. Cleavage of CTFα by γ-secretase releases the 3 kD p3 peptide and AICD. Studies have shown that p3 can have neurotoxic effects such as neuronal apoptosis [55]. Chronic cerebral hypoperfusion caused an increase in the levels of sAPPα, ADAM10, and ADAM17 in the hippocampus of rats against the background of an even more significant increase in the levels of sAPPβ, BACE, and BACE1 contributed to the promotion of the amyloidogenic pathway of APP processing and caused cognitive impairment [34]. It is possible that the decrease in ADAM10 expression that we observed after PTS is associated with a general decrease in protein biosynthesis needed during the most acute period of stroke. A comparison of the behavior of adult mice showed that the loss of ADAM10 in A10cKO mice leads to a decrease in neuromotor abilities and a decrease in learning ability that was associated with a change in the activity of neurons in the CA1 region of the hippocampus and impaired synaptic function [56]. Histological and ultrastructural analysis of the brain of A10cKO mice revealed astrogliosis, microglial activation, and disturbances in the number and morphology of postsynaptic structures [56]. In a rat PTS model, a decrease in the enzyme in neurons occurred on the first day after PTS but by the seventh day, the protein level was restored to control values. At the same time, ADAM10 expression decreased in neurons, but not in astrocytes, where N-APP also remained high (Figure 1i,l). Probably, the increase in immunofluorescence of N-APP both in neurons on the first day after PTS and in astrocytes on the 7th day after PTS was not associated with the accumulation of ADAM10 activity products sAPPα, but was associated either with the accumulation of a full-length protein APP or accumulation of products of further processing of the amino-terminal region of APP that could be nonspecifically detected by the antibody we used.

Inhibition of BACE1 cleavage of neuregulin-1 (NRG1) and possibly neuregulin-3 (NRG3) causes a decrease in the thickness of the myelin sheath of axons of both periph-

eral nerves (sciatic nerve) and central optic nerves and impairs remyelination of injured nerves [57]. Proteolytic processing of the neuregulin-1 (NRG1) BACE1 protein is associated with the activation of ErbB receptor tyrosine kinases. This signaling pathway is involved in synapse formation, plasticity, neuronal migration, myelination of central and peripheral axons, and in the regulation of neurotransmitter expression and function. It is possible that seven days after the neurotrauma time point was an insufficient time to activate the expression of the enzyme since the available data indicate that high BACE1 activity in neurons may be associated with repair processes after brain cell damage.

γ-secretase catalyzes the final cleavage of APP with AICD and either Aβ in the case of the amyloidogenic pathway of APP proteolysis or p3 in the non-amyloidogenic variant of APP proteolytic formation. γ-secretase is composed of four integral membrane proteins: presenilin (PS) 1 or 2, nicastrin (NCSTN), PEN-2, and APH-1. Assembly of the complex begins with the stabilization of PS with nicastrin and APH-1, after which, the last component of the PEN-2 protein complex is added [58]. Biochemical studies show that PS1 and PS2 (or APH-1a and APH-1b and their alternatively spliced forms) never coexist in the same complex, suggesting that there are at least six different γ-secretase complexes in humans. PS1 is located mainly in the endoplasmic reticulum [59]. However, significant amounts of PS1 bound to NCSTN were found in the plasma membrane and endosomes/lysosomes, indicating that completely assembled complexes leave the endoplasmic reticulum and translocate to the plasma membrane. All four components of the γ-secretase complex are localized in the active form on the plasma membrane and lysosomes [59]. Our studies have shown that the expression of proteins of the γ-secretase complex, PS1 and nicastrin, increases in astrocytes, but not in penumbra neurons on the first day after PTS and remains high up to seven days after PTS. However, in the model of global ischemia, PS1 expression decreased from two up to seven days but the trend was reversed on day 30 [60].

Electron microscopy shows that the C- and N-terminal fragments of APP were associated with the plasma membranes of the processes of nerve cells. Our attention was drawn to the pronounced clustering of APP and ADAM10 but not BACE1. This is unexpected since BACE1 and presenilin1 (PS1), the catalytic unit of γ-secretase, are localized mainly in detergent resistant membranes (DRM) or lipid rafts, while ADAM10 is localized mainly in non-lipid raft domains.

Caveolae are a subset of lipid rafts that are characterized by small membrane invaginations and the presence of caveolin-1 [23,25–27]. Caveol-like membrane domains have been characterized in nerve cells [23,61,62]. In Alzheimer's disease, caveolar dysfunction can cause a decrease in α-secretase activity and accumulation of toxic amyloid Aβ peptide [15,25]. Caveolin-1 is known to physically interact with APP [23,25] and BACE1 [24], and overexpression of caveolin-1 attenuated γ-secretase-mediated proteolysis of APP and Notch [63]. Caveolin-1 was weakly expressed in rat brain cells, and PTS caused a further decrease in its level. Immunofluorescent analysis indicates a high co-localization of caveolin-1 with ADAM10 after PTS. However, the results of immunoprecipitation indicate an increase in the interaction between caveolin-1 and C-APP and, to a much lesser extent, between N-APP, as well as the absence of direct physical interaction between caveolin-1 and ADAM10 (Figure 4). Our results confirm the data that the caveolin-1 binding motif is located on the C-terminal cytoplasmic tail of APP [23]. The observed co-localization in fragments of APP and ADAM10 with caveolin-1 indicates the localization of APP and ADAM10 in areas of brain cells rich in caveolin both on the membrane and outside it. Our data indicate the accumulation of APP fragments in axons and dendrites and the zones of chemical synapses. It is assumed that axonal APP is concentrated in the caveolar structures of neurons [64]. Caveolin-1 can act independently of caveolae in ischemia [65]. Caveolin can be found in the trans-Golgi network (TGN) in the cytosol or separate structures, such as caveosomes (early endosomes) and TGN, which can be the site of APP processing to form Aβ [66]. Thus, the balance of caveolin-1 during ischemia may affect APP processing and the degree of damage to brain cells after ischemia. Caveolin-1 may play an important role in protecting the brain from stroke. Mice with caveolin-1 knockout had less lesions,

lower neurological deficits, and less cerebral edema after intracerebral hemorrhage [67] but caveolin-1 knockout mice showed a high level of apoptotic death of penumbra cells after ischemic stroke [68].

We carried out an inhibitory analysis to understand the significance of the detected changes in the expression of α-, β-, γ-secretase proteins, and caveolin-1.

Batimastat, or BB-94, was used as an α-secretase inhibitor. Some studies have demonstrated the neuroprotective effect of the inhibitor with a decrease in infarct volume [31], an improvement in neurological functions, and a decrease in mortality in various models of ischemic stroke in rats and mice [69,70], as well as excitotoxic damage to neurons in cell culture [71]. However, we have not detected the effect of the drug on changes in infarction volume or the level of apoptosis in cells of the mouse cerebral cortex.

LY2886721 demonstrates an effective dose-dependent decrease in the level of Aβ and sAPPβ in different experimental models: in HEK293 cells with the APP751 mutation; in primary cortical neurons of PDAPP-mutated mice [72]; in vivo animal models (3–30 mg/kg PDAPP mice, 1.5 mg/kg beagle dogs; orally) [22,52–54]. In transgenic mice, doses of 3–30 mg/kg reduced Aβ levels by 20–65%. The effect lasted up to nine hours after the application of the drug. A decrease in amyloid production has been observed in plasma and cerebrospinal fluid after the administration of LY2886721 [32,42]. In a beagle dog model, oral administration (1.5 mg/kg) showed a significant and persistent reduction in Aβ levels in the cerebrospinal fluid [73]. However, we could not detect the effect of LY2886721 on changes in infarct volume or the level of apoptosis of mouse cerebral cortex cells after ischemic stroke.

DAPT (N-[N-(3,5-difluorophenacetyl)-1-alanyl]-Sphenylglycine t-butylester) is a γ-secretase inhibitor. Among the three secretase inhibitors studied, only DAPT reduced the infarct volume on days 7 and 14 after PTS, preventing the increase in apoptosis of mouse cerebral cortex cells in the area adjacent to the infarction zone. This inhibitor was used to treat neurodegenerative diseases and modulated the differentiation of progenitor neurons and apoptotic cascades in neurons during cerebral ischemia [33]. DAPT protects the brain from cerebral ischemia [74] by influencing inflammatory processes, suppressing the expression of NF-κB, a family of transcription factors involved in ischemic injury, promoting inflammatory processes and inducing neuronal apoptosis [75–77]. DAPT has been shown to have an expressed neuroprotective effect in a mouse model of ischemia/reperfusion (I/R) caused by occlusion of the middle cerebral artery. DAPT significantly improved neurobehavioral performance and reduced neuronal morphological damage. It reduced the level of GFAP as well as the number of apoptotic cells by reducing the content of interleukin-6 and tumor necrosis factor-α [77]. Here, we also showed that photothrombotic stroke causes an increase in the level of γ-secretase proteins PS1 and nicastrin mainly in astrocytes and the administration of the inhibitor reduces the level of GFAP. It is most likely that inhibition of γ-secretase enhances the anti-inflammatory response and reduces the activation of astrocytes, contributing to the decrease in the level of apoptosis and, as a consequence, the amount of damage after ischemia. A possible mechanism for this drug effect could be a decrease in the synthesis of Aβ that can activate apoptosis both externally and internally [78]. A decrease in the level of Aβ in astrocytes that increases after ischemia [50,51] can also contribute to the reduction of astrogliosis after PTS against the background of DAPT administration.

Caveolin-1-deficient cells are known to exhibit significantly increased co-localization of γ-secretase with clathrin-coated non-caveolar endocytic vesicles [63] and a redistribution of γ-secretase between caveolar and non-caveolar membranes may stimulate Aβ formation against the background of a decrease in caveolin-1 levels that is observed at the 7th day after PTS. The administration of daidzein caused an increase in the level of C-APP and Aβ and a decrease in the N-terminal fragment after PTS (Figure 8g,h,j). Thus, the present study shows that APP is concentrated in caveolae-rich membrane regions not only in the cytoplasm but also may be in endosome membranes (caveosomes) and in TGN where caveolin-1 ensures the concentration of APP in these membrane microdomains increasing

the activity of α-secretase. It remains to be determined whether caveolin-1 is part of the retromer complex or interacts with it to sort out APP from β- and γ-secretase [79] in late endosomal compartments, resulting in decreased Aβ production. Moreover, the inactivation of γ-secretase will help to reduce the formation of Aβ peptides. However, the transport of caveolin-1 is also regulated by presenilins [80]. PS1 deficiency can lead to a serious loss of caveolae slowing repair processes after ischemia and activating astrocytes that make blocking of the expression of presenilin-1 (rather than a decrease in γ- secretase activity) unacceptable. We showed that the decrease in the level of caveolin-1 that is caused by the administration of daidzein contributed to the activation of astrocytes and the development of astrogliosis in the long term. A study of Cav-1 knockout mice showed reduced neovascularization and modified astrogliosis without proper glial scar formation around the infarct core 3 days after stroke [81]. In addition, knockout or knockdown of caveolin-1 increased blood–brain barrier (BBB) permeability and cell damage after cerebral ischemia-reperfusion by activating the NO/Cav-1/MMP signaling cascade [82].

The shift of APP processing towards the amyloidogenic pathway and the formation of Aβ, as well as full-length APP, can be a link between cardiovascular and neurodegenerative diseases [83,84]. The accumulation of full-sized APP in the mitochondria of brain cells, as well as cells of peripheral tissues, causes mitochondrial dysfunction and impairs energy metabolism [83]. APP is involved in the activation of endothelial cells and increases the expression of pro-inflammatory proteins, cyclooxygenase-2, and vascular cell adhesion molecule-1, as well as the cytokine IL-1β [85]. The accumulation of Aβ in the blood, vascular walls, and heart causes endothelial activation, inflammation and tissue damage, and impaired glucose metabolism, which contributes to the development of atherosclerosis and the formation of blood clots, chronic inflammation, and diabetes mellitus [83,84]. APP and its processing products are the link between aging and cardiovascular disease, and possibly vice versa. On the one hand, a violation of the blood–brain barrier during a stroke can cause an increase in APP and Aβ in the blood and their accumulation in the tissues of peripheral organs, disrupting metabolism. On the other hand, an increase in the level of Aβ in the brain after a stroke and its toxicity to endotheliocytes causes the development of inflammation, disruption of endothelial repair processes, cytokine-induced damage to BBB component cells, alteration of leukocyte–endothelial interactions, and the development of neurodegeneration. In this regard, in the future it will be interesting to study the balance of markers of BBB permeability impairment (neuron-specific enolase (NSE), GFAP, α-glycoprotein, etc.), inflammation markers in the peripheral blood after a stroke and their correlation with the level of APP and Aβ in the brain and blood in the recovery period.

Thus, inhibitory analysis showed that the decrease in ADAM10 expression in neurons and the increase in the expression of PS1 γ-secretase complex nicastrin against the background of the decrease in caveolin-1 in astrocytes promote a shift in APP processing towards the amyloidogenic pathway, which would lead to neuronal death and the development of astrogliosis and inflammation in the early recovery period after PTS. DAPT may be considered as a potential drug for stroke treatment. However, DAPT and LY2886721 inhibited the γ-secretase complex containing PS1 rather than the γ-secretase complex with PS2 in humans (in contrast to the results obtained in mice) [86]. The potential side effects caused by blocking Notch signaling must be considered [87].

## 5. Conclusions

The processing of APP is involved in the pathogenesis of many neurodegenerative disorders. We investigated the expression of APP and its processing proteases such as α-secretase of ADAM10, β-secretase of BACE1, γ-secretase subunits of PS1 and NCT, and caveolin-1 in a PTS model. The results showed an increase in the level of N- and C-terminal fragments of APP in the cytoplasm of ischemic penumbra cells 24 h after PTS and their co-immunoprecipitation with caveolin-1. α-secretase of ADAM10 was proven to be mainly present in the cytoplasmic fraction of the penumbral tissue and was increased significantly

in neurons at 24 h after PTS. However, no significant difference was found at different times after PTS for the expression of β-secretase of BACE1, although the expression of BACE1 in neurons and astrocytes of the rat cerebral cortex seemed relatively low. Interestingly, both γ-secretase subunits of PS1 and NCT were shown to increase in astrocytes at 24 h after PTS. The caveolin-1 inhibitor daidzein shifted APP processing towards Aβ synthesis, which caused astroglial activation. To further discover the roles of these secretases, Batimastat (BB-94), an α-secretase inhibitor, LY2886721, a β-secretase inhibitor, and DAPT, a γ-secretase inhibitor, were used in a PTS mice model. Among these, only DAPT can reduce the infarct volume on days 7 and 14 after PTS, preventing the growth of mouse cerebral cortex cell apoptosis in the area adjacent to the infarction zone. Regarding the positive correlation of the expression of APP, α-secretase of ADAM10, and γ-secretase subunits of PS1 and NCT after PTS, further research to reveal the exact roles of these proteins in stroke would be interesting and valuable to explore.

In conclusion, we demonstrated that a photothrombotic stroke led to reduced expression of ADAM10 α-secretase in neurons and increased the levels of the γ-secretase subunits of PS1 and NCT in astrocytes. Furthermore, the inhibitory assay showed that only the γ-secretase inhibitor of DAPT reduces GFAP levels and decreases brain infarct volume, suggesting γ-secretase appears to be a therapeutic target and its inhibitor of DAPT may have the therapeutic potential for the treatment of stroke. An important direction will be the development of very selective γ-secretase modulators targeting one subunit of the enzyme.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biomedicines10102655/s1, Figure S1. An example of Western blot evaluation of the purity of the obtained nuclear and cytoplasmic fractions of the cerebral cortex of sham-operated rats, penumbra tissue after 4 (PTS 4 h), 24 (PTS 24 h) hours, and 7 days (PTS 7d) after photothrombotic stroke (PTS) in rats. The acetylated histone H4 protein (ac-H4) was used as a nuclear fraction marker. Anti-acetyl-Histone H4 produced in rabbit (#06-866, Merck) diluted 1:500 was used. The protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a marker of the cytoplasmic fraction. Anti-GAPDH antibody produced in rabbit (G9545, Sigma-Aldrich) diluted 1:1000 was used. Table S2. List of primary antibodies used in this study. Primary antibodies used for WB assay are listed.

**Author Contributions:** Conceptualization, S.S. and S.D.; data curation, S.S. and S.D.; formal analysis, S.S., V.G., V.D., A.L., Y.K., N.D., Y.L. and L.C.; funding acquisition, B.H. and S.D.; investigation, S.S., V.G., A.L., A.K., Y.K., N.D., Y.L., L.C., B.H. and S.D.; methodology, S.S., V.G., V.D., A.L., Y.K., Y.L., L.C. and S.D.; project administration, S.S. and S.D.; resources, S.S.; software, S.S., A.K., Y.K., Y.L. and S.D.; supervision, S.S. and S.D.; validation, S.S., V.G., V.D., A.L., A.K., Y.K., N.D., Y.L. and B.H.; visualization, S.S., Y.K. and N.D.; writing—original draft, S.S. and S.D.; writing—review and editing, S.S., B.H., S.D. and A.K. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Animal Care and Use Committee of the Southern Federal University (Approval No. 08/2016). Our study did not involve any human subjects.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data are available on demand to corresponding author S.V. Demyanenko; B. He.

**Conflicts of Interest:** The authors declare no conflict of interest.

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