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Special Issue Reprint

Neurotrauma

Mechanisms, Pathways, and Emerging
Therapeutic Interventions

Edited by
Kevin Pierre and Brandon Lucke-Wold

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Neurotrauma: Mechanisms, Pathways, and Emerging Therapeutic Interventions

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Guest Editors

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This is a reprint of the Special Issue, published open access by the journal *Biomedicines* (ISSN 2227-9059), freely accessible at: https://www.mdpi.com/journal/biomedicines/special_issues/5A53K62TEM.

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

Lastname, A.A.; Lastname, B.B. Article Title. <i>Journal Name</i> Year , Volume Number, Page Range.
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ISBN 978-3-7258-4625-2 (Hbk)

ISBN 978-3-7258-4626-9 (PDF)

<https://doi.org/10.3390/books978-3-7258-4626-9>

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About the Editors

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Kevin Pierre is a PGY3/R2 diagnostic radiology resident at the University of Florida Department of Radiology. His academic qualifications include an MD from the University of Florida College of Medicine, where he graduated with Honors in Research and received the Society of Teaching Scholars Student Excellence in Medical Education award. Dr. Pierre's research interests encompass neurotrauma, emergency radiology, and medical education innovation. He has produced extensive work on traumatic brain injury mechanisms and diagnostic imaging applications, with over 60 peer-reviewed publications. As co-creator of the award-winning WIDI Case-Based Introduction to Radiology platform, he developed over 140 educational modules that have transformed radiology education at UF. His work earned him the 2023 UF GME Innovation of the Year award, and subsequently the RSNA Roentgen Resident/Fellow Research Awards in 2024 and 2025. Dr. Pierre serves as Secretary of the Society of Haitian Neuroscientists and maintains active roles in medical education, mentoring students while pursuing a subspecialization in neuroradiology.

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Preface

Neurotrauma remains a leading cause of death and disability worldwide, and affects individuals across all age groups and socioeconomic backgrounds. Despite decades of research, no pharmacological interventions have yet been successfully translated from bench to bedside for traumatic brain or spinal cord injury. This Special Issue Reprint aims to address this translational gap by bringing together diverse perspectives on neurotrauma mechanisms and emerging therapeutic strategies.

The eight contributions in this Reprint represent the work of multiple research teams with focuses ranging from post-injury cascades to the implementation of artificial intelligence in clinical settings. Several papers directly address the secondary injury processes—inflammation, oxidative stress, and excitotoxicity—that propagate neural damage and represent therapeutic targets. Others examine novel delivery systems, diagnostic tools, and the complex syndrome of chronic traumatic encephalopathy.

This Reprint is intended to serve as a useful resource for neuroscientists, neurologists, neurosurgeons, rehabilitation specialists, and translational researchers who are invested in improving outcomes for neurotrauma patients. We acknowledge the contributing authors for their efforts and thoughtful analyses. We also thank the editorial staff at *Biomedicines* for their support throughout the Special Issue process, and the peer reviewers whose critical evaluations strengthened each manuscript. We hope that this collection will stimulate further investigation and collaboration with the aim of developing effective neurotrauma therapies.

Kevin Pierre and Brandon Lucke-Wold

Guest Editors



Review

Navigating the Complexities of Traumatic Encephalopathy Syndrome (TES): Current State and Future Challenges

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Abstract: Chronic traumatic encephalopathy (CTE) is a unique neurodegenerative disease that is associated with repetitive head impacts (RHI) in both civilian and military settings. In 2014, the research criteria for the clinical manifestation of CTE, traumatic encephalopathy syndrome (TES), were proposed to improve the clinical identification and understanding of the complex neuropathological phenomena underlying CTE. This review provides a comprehensive overview of the current understanding of the neuropathological and clinical features of CTE, proposed biomarkers of traumatic brain injury (TBI) in both research and clinical settings, and a range of treatments based on previous preclinical and clinical research studies. Due to the heterogeneity of TBI, there is no universally agreed-upon serum, CSF, or neuroimaging marker for its diagnosis. However, as our understanding of this complex disease continues to evolve, it is likely that there will be more robust, early diagnostic methods and effective clinical treatments. This is especially important given the increasing evidence of a correlation between TBI and neurodegenerative conditions, such as Alzheimer's disease and CTE. As public awareness of these conditions grows, it is imperative to prioritize both basic and clinical research, as well as the implementation of necessary safe and preventative measures.

Keywords: chronic traumatic encephalopathy (CTE); Alzheimer's disease (AD); traumatic encephalopathy syndrome (TES); traumatic brain injury (TBI); repetitive head impacts (RHI)

1. Background

Chronic traumatic encephalopathy (CTE) is a distinct neurodegenerative disease and is often associated with a history of repetitive head impacts (RHI) in the context of sports or combat settings. The defining neuropathological characteristic of CTE includes hyperphosphorylated tau at the depths of cortical sulci and peri-vascular regions [1]. CTE was first reported in a group of boxers, who were described as “punch drunk” by Martland in 1928 [2]. The report described a group of boxers who had suffered repetitive head blows throughout their sporting careers, with clinical presentations of behavioral symptoms as well as severe memory and neurocognitive deficits. The newly defined neurodegenerative condition was labeled “dementia pugilistica” [3], and eventually CTE in 1949 [4]. More recently, Omalu et al. reported finding evidence of CTE in three retired football players [5–7]. McKee reported similar findings in three new individuals when reviewing the world literature on CTE, including one football player, as well as a multitude of reports and case studies of evidence of CTE in athletes and veterans who were exposed to repetitive head trauma.

In 2014, research diagnostic criteria for traumatic encephalopathy syndrome (TES) were proposed for use in clinical research settings to diagnose CTE in patients while alive by Montenegro et al. [8]. They proposed five general criteria for diagnosis of TES, which included (1) a history of multiple head impacts, (2) no other neurological disorders accounting for all the clinical features, (3) clinical features present for a minimum of 12 months, (4) at least one core clinical feature must be present, and (5) the presence of at least two supportive features.

The core features included cognitive deficits, behavioral symptoms, and mood symptoms. The cognitive symptoms, supported by detailed neuropsychological assessments, included changes in episodic memory, executive function, and attention, as defined by 1.5 standard deviations below normal. Behavioral symptoms included verbal or physical aggression, while mood symptoms included feeling depressed or hopeless. The supportive features included impulsivity, anxiety, apathy, paranoia, suicidality, headache, motor signs including dysarthria and dysgraphia, documented decline, as well as a delayed onset.

Mez et al. examined the validity of 336 brain donors with a prior history of repetitive head impacts due to etiologies, including contact sports, military settings, and/or due to violent trauma [9]. The TES criterion had a reported sensitivity and specificity of 0.97 and 0.21, respectively. Cognitive symptoms were significantly associated with CTE pathology. Modifying the TES criterion using cognitive deficits resulted in improved specificity (0.48) and a mild reduction in sensitivity (0.90), respectively. The authors found that having cognitive symptoms was significantly associated with CTE pathology, increasing the odds by 3.6-fold.

This review endeavors to offer a comprehensive exploration of the underlying pathophysiological mechanisms of traumatic encephalopathy syndrome (TES) and chronic traumatic encephalopathy (CTE). Additionally, it provides an in-depth survey of the present neuroimaging and plasma biomarkers employed in the diagnosis of traumatic brain injury (TBI). Furthermore, it scrutinizes the currently utilized clinical regimens in TBI treatment (refer to Figure 1). Given the rapid evolution of the research field concerning TES, CTE, and TBI at large, this review aims to encapsulate some of the most promising research trajectories in the field [10].

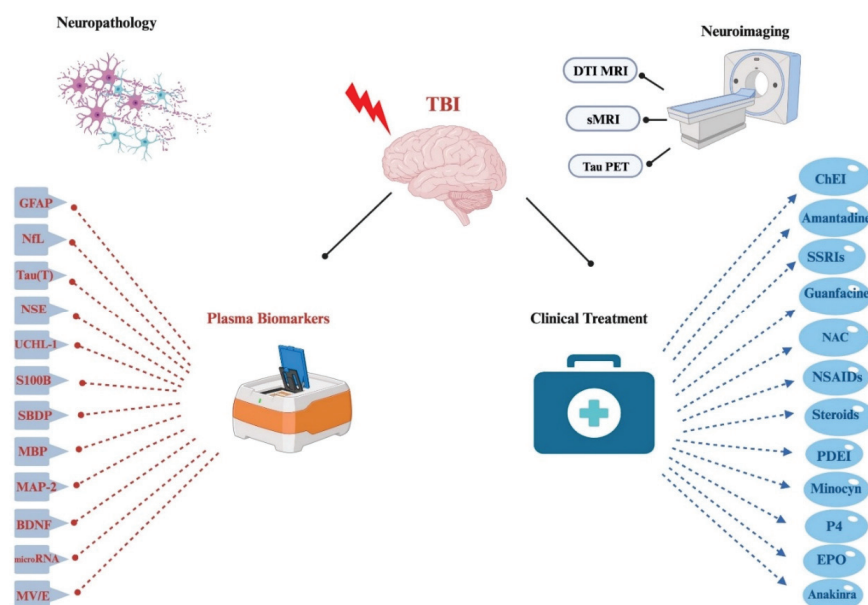


Figure 1. Schematic illustration of major plasma markers of TBI including GFAP (Glial Fibrillary Acidic Protein), NFL (Neurofibrillary Light Chain), total tau, NSE (Neuron Specific Enolase), UCHL-1 (Ubiquitin C-terminal hydrolase-1), S100B, SBDP (Spectrin Breakdown Products), MBP (Myelin Basic Protein), MAP-2 (Microtubule-Associated Protein-2), BDNF (Brain-Derived Neurotrophic Factor), microRNA, and MV/E (Microvesicles and Exosomes). Also included are the major clinical treatment options used in treatment of TBI patients, including cholinesterase inhibitors (ChEI), NMDA receptor antagonist (Amantadine), SSRIs, guanfacine, NSAIDs, Nutraceuticals such as NAC, phosphodiesterase inhibitor (PDEI), Minocycline (Minocyn), glucocorticoids and progesterone (P4), Erythropoietin (EPO) and Anakinra. Some of the current promising neuroimaging tools for clinical TBI patients include diffusion tensor imaging (DTI) imaging, structural MRI with corresponding volumetric analysis, as well as Tau PET imaging. Images created with BioRender.com (accessed on 22 October 2023).

2. Neuropathology

Coresellis et al., 1973 assessed 15 boxers who were diagnosed with dementia pugilistica. The reported neuropathological findings included neurofibrillary tangles (NFTs) without accompanying amyloid plaques, more prominently found in the medial temporal lobes and brainstem, substantial nigral atrophy with NFTs, cerebellar tonsils gliosis, and cavum septum pellucidum [11]. The neuropathological characterization of CTE has undergone further iterations [12]. As per the National Institute of Neurological Disorders and Stroke (NINDS)-funded study entitled “Understanding Neurologic Injury in Traumatic Encephalopathy” (UNITE), CTE’s characteristic neuropathological changes include NFTs in astrocytes, accumulated around blood vessels, and at the depth of sulci in an irregular fashion. As per NINDS panel consensus, the pattern of p-tau is distinct from other neurodegenerative conditions. Furthermore, the tau filament in CTE has been shown to have a unique conformation of the β -helix region with a hydrophobic cavity [13,14].

McKee et al. reported β -amyloid deposition, an essential feature of AD, in 43% of CTE cases [15]. Gardner et al. examined the autopsies of 85 athletes and found that only 20% had pure CTE, 52% with CTE and another co-morbid neuropathology, 5% with no CTE, and 24% with no observed neuropathology. These studies, in turn, highlight the inherent heterogeneity of CTE [16].

As defined by McKee et al., CTE pathological progression could be conceptualized in 4 distinct stages. In stage 1, the brain has typically normal weight, with NFTs and perivascular p-tau and astrocytic tangles predominantly in the superior and dorsolateral frontal cortices. In stage 2, there are more NFTs throughout the superficial cortical layers, as well as locus coeruleus and substantia innominata. In stage 3, there is a reduction in brain weight with accompanying cortical atrophy and ventricular dilation, with frequent cavum septum pellucidum. In addition, there is notable depigmentation of locus coeruleus and substantia nigra, as well as atrophic changes of mamillary bodies, thalamus, and hypothalamus, as well as white matter tracts, including corpus callosum. There are also widespread NFTs in various subcortical areas, including olfactory bulbs, amygdala, hippocampi, hypothalamus, mamillary bodies, nucleus basalis of Meynert, substantia nigra, dorsal and median raphe nuclei, locus coeruleus, and entorhinal cortex. In stage 4, there is substantial global cortical atrophy, including medial temporal lobes, and thalamus, hypothalamus, and mamillary bodies, with complete depigmentation of the locus coeruleus and substantia nigra [1] (Figure 2).

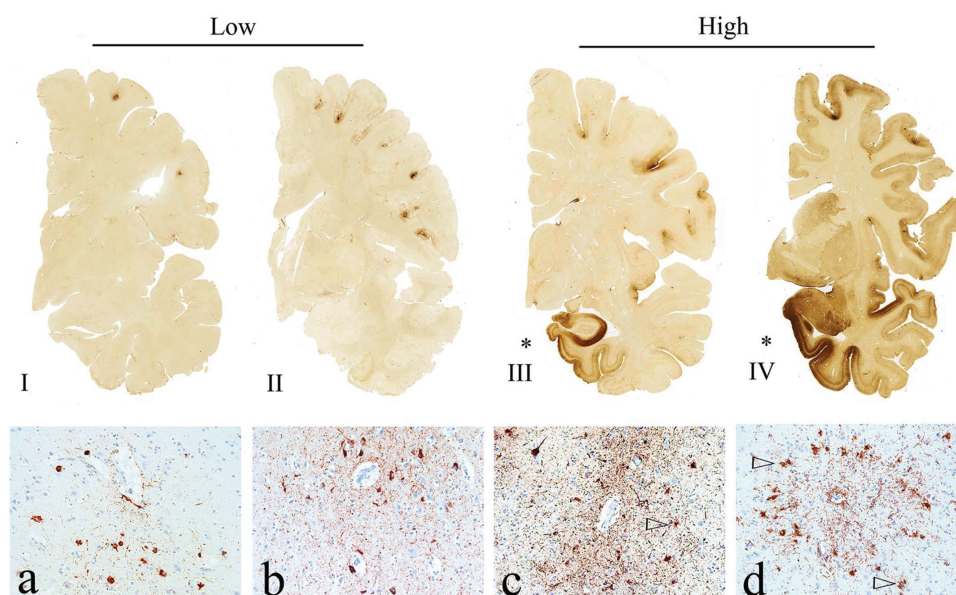


Figure 2. Top panel: Depiction of McKee staging system (I–IV). McKee stage I CTE is defined by one or two isolated CTE lesions at the depths of the cortical sulci. In stage II, there are typically three or more cortical CTE lesions. In stage III CTE, there are multiple loci of CTE lesions and diffuse NFTs in the medial temporal lobe. In stage IV CTE, CTE lesions and NFTs are ubiquitously distributed throughout

the cerebral cortex, diencephalon, and brainstem. Bottom panel: Characteristic stages: (a) CTE stage I perivascular AT8 positive NFTs and neurites. (b) CTE stage II lesions comprised of several AT8 positive NFTs and neurites; (c) Characteristic stage III CTE lesions, comprised of several perivascular AT8 positive NFTs and neurites; (d) A large accumulation of several AT8 positive NFTs and neurites (Images adopted from McKee et al. *Acta Neuropathologica* 2023 [17]) The triangular shapes refer to neurofibrillary tangles and neuritis which are AT8 stain positive. * refers to extensive degeneration of amygdala and entorhinal cortex.

CTE staging criteria are based on a series of multiple case studies and are largely cross-sectional and not longitudinal [1]. The study of Bieniek et al. reported CTE in 32% of the 66 studied athletes [18]. Another study by Stern et al. focused on the clinical presentation of 36 neuropathologically confirmed CTE patients [19]. The study reported two distinct clinical phenotypes: A younger (n = 22) group initially presented with behavioral and mood changes, and an older (n = 11) group presented predominantly with cognitive changes. Another notable study involving retired NFL players by Hampshire et al. reported abnormal connectivity changes on functional MRI (fMRI) involving the dorsal frontoparietal network [20].

3. Potential TES Biomarkers

As CTE is largely a postmortem diagnosis, the precise diagnosis of traumatic encephalopathy syndrome remains elusive. The clinical diagnosis of TES largely depends on neuroimaging, along with CSF and plasma biomarkers (see Table 1), that are measurable in a clinical setting. Although there is no consensus on a set of TES biomarkers, there is agreement on a set of converging neuroimaging; therefore, CSF/plasma markers warrant further discussions.

Table 1. TBI Plasma Biomarkers. Summary of reported TBI serum markers including GFAP (Glial Fibrillary Acidic Protein), NfL (Neurofibrillary Light Chain), total tau, NSE (Neuron Specific Enolase), UCHL-1 (Ubiquitin C-terminal hydrolase-1), S100B, SBDP (Spectrin Breakdown Products), MBP (Myelin Basic Protein), MAP-2 (Microtubule-Associated Protein-2), BDNF (Brain-Derived Neurotrophic Factor), microRNA, and MV/E (Microvesicles and Exosomes).

Serum Biomarker	TBI Outcomes
GFAP	Clinical TBI studies have reported longitudinal elevation in GFAP levels [21]. GFAP was also recently approved by the FDA as a TBI outcome clinical measure [22].
NfL	Clinical TBI studies have reported elevated NfL serum levels both acutely and longitudinally [21].
Tau (total)	Total tau elevation has been reported both acutely and chronically in TBI populations [21].
NSE	NSE elevated levels have been reported in both mild and more severe TBI populations [23,24].
UCHL-1	UCHL-1 has been shown to be robustly elevated in both mTBI and more severe TBI patients [25]. UCHL-1 was recently FDA-approved as a TBI outcome clinical measure [22].
S100B	S100B has been reported to be more acutely elevated in various TBI severity cases [26,27].
SBDP	SBDPs are products of calpain and caspase-3 post-TBI and have been reported to be elevated in both preclinical and clinical studies [28,29].
MBP	MBP is an oligodendrocyte protein and a product of proteases, including calpain, and is reported to be elevated in severe TBI patients [30,31].
MAP-2	An emerging biomarker for TBI patients [32].
BDNF	Mainly reported in the preclinical TBI studies, with potential application to the clinical TBI population [33].
microRNA	A class of small endogenous RNA molecules that have been reported to be elevated in biofluid (CSF, serum, or plasma) in several rodent models of TBI of various severities [34].
MV/E	Lipid-bilayered, encapsulated particles (10–100 nm in diameter) that are released from cells into the CSF and blood during TBI [35]. Reported elevated MV/E released into CSF in TBI patients [36].
Pro-inflammatory cytokines (IL-6, IL-1, IL-8, IL-10, TNF α , CRP)	Pro-inflammatory markers, especially IL-6 and CRP, have been shown to have robust diagnostic and prognostic value [20].

3.1. Neuroimaging

Asken et al. studied the structural MRI scans of nine patients with TES [37]. The regions of interest (ROIs) included the dorsal frontal, ventral frontal, temporal, parietal, occipital, thalamus, and medial temporal lobes. All nine patients had reported cavum

septum pellucidum (CSP), with no clear differences between patients with high CTE vs. those without CTE. There was prominent medial temporal atrophy amongst all nine patients. Other ROIs reported to have undergone notable atrophy included the thalamus, ventral frontal cortex (8/9), dorsal frontal cortex (8/9), and orbitofrontal cortex, as well as the right posterolateral frontal cortex [38].

The use of diffusion tensor imaging (DTI) has not been standardized for the diagnosis and prognosis of TES patients. There are a number of promising studies, including Strain et al., based on the DTI imaging analysis of 26 retired NFL players, which reported a significant association between depression and integrity of white matter [38]. These patients also had an increase in deep white matter lesions on T2-weighted fluid inversion recovery (FLAIR), compared with matched unimpaired NFL player control subjects [37]. In the study by Asken et al. [38], six patients underwent antemortem DTI ($n = 3$ with High CTE; $n = 1$ with Low CTE; $n = 2$ with no CTE). All six patients had significantly diminished fractional anisotropy (FA) along the fornix, irrespective of neuropathological diagnoses. Five of the six patients had significantly decreased FA in the genu of the corpus callosum (genu CC ROI median W score = -1.24) and medial temporal white matter in the areas of the uncinate fasciculus and cingulum–hippocampal bundle.

In another study by Asken et al. [37], five patients ($n = 2$ High CTE, $n = 1$ Low CTE, $n = 2$ no CTE) underwent FDG-PET imaging. Major hypometabolic ROIs included the thalamus (4/5 patients), medial temporal lobes (4/5 patients), and left dorsal frontal cortex (3/5 patients). Another promising PET imaging modality includes the use of tau-based ligands, which have already been utilized in AD patients [39]. The exploration of the use of tau PET imaging for the identification of p-tau aggregates that would be potentially specific and sensitive for CTE patients is currently in progress.

In a study by Stern et al. [40] involving 26 former NFL players and 31 control subjects, flortaucipir positron-emission tomography (PET) and florbetapir PET were employed to measure the deposition of tau and amyloid-beta, respectively, in the brains of former NFL players with cognitive and neuropsychiatric symptoms, and in asymptomatic men with no history of traumatic brain injury. The regional tau standardized uptake value ratio (SUVR), which is the ratio of radioactivity in a cerebral region to that in the reference cerebellum, was used to explore the associations of SUVR with symptom severity and with years of football play in the former-player group versus the control group. The inclusion criteria for the former players were male sex, age 40 to 69 years, a minimum of 2 years playing football in the NFL, a minimum of 12 years of total tackle football experience, and cognitive, behavioral, and mood symptoms reported by the participant through telephone screening. Each participant underwent flortaucipir PET, florbetapir, and T1-weighted volumetric MRI of the head. The mean flortaucipir SUVR was higher among former players than among controls in three regions of the brain: bilateral superior frontal (1.09 vs. 0.98; adjusted mean difference, 0.13; 95% confidence interval [41], 0.06 to 0.20; $p < 0.001$); bilateral medial temporal (1.23 vs. 1.12; adjusted mean difference, 0.13; 95% CI, 0.05 to 0.21; $p < 0.001$); and left parietal (1.12 vs. 1.01; adjusted mean difference, 0.12; 95% CI, 0.05 to 0.20; $p = 0.002$). All of the former NFL players in the study reported cognitive symptoms, and more than 35% had impaired delayed recall scores on an objective memory test.

3.2. CSF

CSF biomarkers, including neurofilament light (NfL) and tau protein, are potential CTE biomarkers, as they have been shown to be elevated in boxers within days to weeks following injury [37]. A prior study reported total tau elevation (>3.56 pg/mL) in former NFL players, including cases of remote injuries. However, the total tau levels did not correlate with neurocognitive deficits [42]. The t-tau elevation has also been reported in other neurodegenerative conditions, such as AD and FTD, as well as cerebrovascular conditions, limiting its utility [42], but it has less specificity for CTE.

3.3. Plasma

Asken et al. examined antemortem plasma GFAP, NfL, and total tau for eight of the nine patients with TES [38]. Five patients had longitudinal GFAP and NfL data, and two had longitudinal total tau data. Most patients had elevated plasma GFAP, NfL, and total tau at their initial visit compared to age-matched healthy controls. Three of five patients with longitudinal GFAP and NfL data demonstrated increasing concentrations over time, and four had increasing NfL over time.

In aging cohorts, plasma GFAP was tightly linked to AD-related amyloid β 42 plaque [21,43,44]. The mechanisms underlying plasma GFAP changes in patients without AD remain to be determined but could reflect astrocytic dysfunction and inflammation in non-AD disease pathogenesis. AD-based biomarkers such as A β -PET, CSF amyloid β 42 and phosphorylated tau, or plasma phosphorylated tau in studies of patients with previous repetitive head impacts and TES may add to more specific biomarker signatures that are specific to CTE and minimize the risk of misattributing biomarker changes to AD (co)pathology.

Neuron-specific enolase (NSE), also known as gamma-enolase or enolase 2, exists in mature neurons and neuroendocrine cells [20]. NSE has been reported to be elevated in the blood of both mTBI, as well as more severe TBI patients [23,24,45–48]. NSE is also abundantly expressed in red blood cells, making it less specific and requiring hemolysis correction for accurate blood measurement [49]. Ubiquitin C-terminal hydrolase-L1 (UCH-L1) is a protein that mainly resides in the neuronal cell body cytoplasm [32,46,50–53]. UCH-L1 was first reported to be released into CSF and serum among severe TBI patients. The use of CSF UCH-L1 is a potentially robust clinical outcome predicting marker of mortality following non-penetrating TBI [54]. UCHL-1 was also reported to be elevated in serum/plasma in mTBI, including athletes after concussion [30,55]. Based on the results of a multi-center TBI study (ALERT-TBI), GFAP and UCHL-1 have shown high sensitivity (0.976) and negative predictive value (0.996) for the detection of traumatic intracranial injury in the acute setting [56]. These robust TBI biomarker findings ultimately led to FDA approval of GFAP and UCHL-1 to aid in TBI evaluation in a clinical setting [57].

S100B, an astroglial calcium-binding protein, has been extensively studied as a TBI marker [22,26] and of various degrees of injury severity [27,58]. An important confound is the multiple potential sources of S100B, which includes adipose tissues, as well as cardiac/skeletal muscles. S100B has been reported to be elevated in orthopedic trauma without accompanying head injuries [59]. However, S100B remains a sensitive predictive marker for CT abnormality and the development of post-concussive syndrome (PCS) among mTBI patients [60–62].

C-terminal breakdown products (BDPs), which include SBDP120, SBDP 145, and SBDP150, are byproducts of calpain and caspase-3. These markers have been shown to be elevated in preclinical models of TBI, as well as human CSF samples [28,59,63–69]. Another spectrin product, the N-terminal spectrin fragment (SNTF), was also reported to be elevated after concussion. One potential confound is the fact that the α II-spectrin protein is expressed in other organs and peripheral blood mononuclear cells (PBMC), limiting its clinical interpretation [28,59,63,69].

Myeline basic protein (MBP) is an oligodendrocyte-based protein and an essential component of the myelin sheath. TBI leads to the activation of calpain and an increase in MBP degradation. MPB has been reported to be elevated in severe pediatric and adult TBI patients [54,70].

A novel set of TBI biomarkers include dendritic protein microtubule-associated protein-2 (MAP-2) [31,51], brain-derived nerve growth factor (BDNF) [29], and postsynaptic protein neurogranin [71]. Moreover, microRNA (miRNA) has been reported to be elevated in biofluid (CSF, serum, or plasma) in a number of preclinical TBI models [33]. There are also a number of candidate miRNA biomarkers reported in clinical TBI in both mild and severe cases [20,34,72,73].

There are reported CSF levels of MV/E containing SBDPs, synaptophysin, UCHL-1, and GFAP [74,75]. Elevated levels of circulating tau-containing exosomes has been proposed to be a promising predictive risk marker for CTE in chronic TBI patients [35].

TBI induces a cascade of secondary biological phenomena, which includes the production of a series of pro- and anti-inflammatory cytokines [36]. In individuals with severe TBI, increased levels of (IL)-6, IL-1, IL-8, IL-10, and tumor necrosis factor-alpha (TNF α) were associated with worse clinical outcomes [76]. Based on a recent meta-analysis, IL-6 was shown to have robust potential as a pro-inflammatory marker in acute mild TBI patients [77]. IL-6 has also been reported to have potential prognostic biomarker value for clinical outcomes post-TBI [78]. Moreover, as one of the outcomes of the Transforming Research and Clinical Knowledge in Traumatic Brain Injury (TRACK-TBI) study, high-sensitivity C-reactive protein (hsCRP) measured within 2 weeks of TBI was found to be a prognostic biomarker of disability 6 months later [79]. Intriguingly, post-traumatic stress disorder (PTSD), a highly co-morbid psychiatric illness in brain injury patients [80], has been shown to be a pro-inflammatory condition associated with the elevation of pro-inflammatory markers, including CRP, IL-6, and TNF α [81]. Depression, another highly co-morbid psychiatric condition with TBI [82], has also been shown to be associated with low-grade inflammation, as manifested by mildly elevated CRP levels [83].

4. Treatment

Although there are no established treatments for TES, there are pharmacological and non-pharmacological treatment options for the treatment of TBI/TES symptoms. The following section provides a wide scope of potential pharmacological and non-pharmacological treatment options for TBI/TES patients.

4.1. Non-Pharmacological Management

Potential non-pharmacological TBI regimens include outpatient regular cognitive rehabilitation therapy, mood and psychotherapy-focused treatment, including regimented cognitive behavioral therapy and mindfulness/stress reduction techniques, as well as a Mediterranean diet and aerobic exercise. Other therapeutic modalities include vestibular rehabilitative therapy, occupational/ocular therapy, and physical/motor therapy sessions when indicated [84].

4.2. Clinical Pharmacological Management

Currently, there are no FDA-approved disease-modifying regimens available for chronic traumatic encephalopathy (CTE). The existing treatments are considered “off-label” and primarily focus on alleviating symptoms. To address memory impairment, which is a common issue in CTE, medications originally developed for Alzheimer’s disease, such as galantamine, donepezil, and rivastigmine, have been repurposed for CTE patients [84]. Additionally, to tackle apathy symptoms, stimulants like methylphenidate and dopamine agonists such as carbidopa/levodopa, pramipexole, amantadine, and memantine may be employed. Amantadine has been the subject of extensive examination in moderate to severe TBI patients. A recent meta-analysis, encompassing 14 clinical trials and 6 observational studies, demonstrated the cognitive benefits of amantadine for this patient population. Notably, the improvements in cognition were more prominent in younger patients with less severe TBIs [85]. Stimulants can also prove beneficial in managing attention and concentration deficits. When dealing with depression and anxiety symptoms, selective serotonin reuptake inhibitors (SSRIs) like sertraline and escitalopram can be used, but caution is advised due to the potential risk of suicidality, as suicide cases have been documented in CTE [82]. Another promising approach for addressing working memory (WM) deficits resulting from traumatic brain injury involves the use of an alpha-2-adrenergic receptor agonist known as guanfacine. Through functional MRI imaging, a study by McAllister et al. demonstrated improvements in verbal WM in 13 mild TBI

patients one month after their injury. Moreover, the group treated with guanfacine exhibited increased activation in WM circuitry, particularly in the prefrontal cortex (PFC) region [86].

4.3. Clinical Use of Nutraceutical Regimen

There is an increased level of reactive oxygen species (ROS) and reactive nitrogen species (RNS) production post-TBI due to the excitotoxic nature of injury [87]. Anti-oxidants have been widely studied as a potent treatment modality to diminish the level of ROS and RNS post-injury [88]. There are several anti-oxidant therapies used in the treatment of TBI, including ascorbic acid (vitamin C), N-acetylcysteine (NAC), flavonoids, resveratrol, alpha-tocopherol (vitamin E), coenzyme Q10, carotenoids, omega-3 fatty acids, and Pycnogenol® [87,88].

4.4. Preclinical Investigational Pharmacological Intervention

A wide array of preclinical models of TBI have been utilized, including fluid percussion injury (FPI), the blast wave injury model, the weight drop injury model (WDI), and controlled cortical impact models. More novel preclinical injury models include the closed-head impact model of engineered rotational acceleration (CHIMERA) and closed-head projectile concussive impact (PCI). The TBI preclinical models have induced a range of neuropathological changes, including microgliosis, tauopathy, endoplasmic reticulum stress (ER), excitotoxicity, and white matter injury [89,90]. These models also capture the long-lasting, chronic cognitive deficits and mood fluctuations associated with TBI [90–97]. Moreover, there are several prospective treatment targets, encompassing tau acetylation, tau phosphorylation, and the realms of neuroinflammation and immunotherapy.

4.5. Targeting Tau Acetylation

Tau phosphorylation follows tau acetylation, and it is often induced by neuroinflammation and oxidative stress [84,96,98]. A tau acetylation modulating agent is salsalate, which has been shown to reduce inflammation, provide neuroprotection, and enhance neurogenesis via gene regulation. Salsalate has been largely studied in preclinical models [99,100]. Methylene blue, which modulates K280/K281 acetylation activity, was reported to increase neuroprotection, diminish behavioral deficits and mood changes, and minimize neuronal degeneration, neuroinflammation, lesion volume, microgliosis, and mitochondrial dysfunction in TBI rodent models [101–103]. Histone deacetylase 6 (HDAC) and sirtuins (SIRT1 and SIRT2) increased tau deacetylation, presenting another potential treatment methodology that targets the same pathway mechanism but in a different manner [104].

4.6. Targeting Tau Phosphorylation

Inhibition of kinases has been studied in preclinical TBI models. One such target kinase is glycogen synthase 3 beta (GSK-3 β), induced by p-tau, which leads to further downstream tau phosphorylation and amyloidopathy, diminishing anti-oxidant defenses such as nuclear factor E2-related factor 2 (Nrf2) [84,105–107]. Agents such as dimethyl fumarate (DMF) and lithium modulate GSK-3 β activity and have been reported to reduce neurodegenerative processes, diminish lesion size in preclinical TBI models, and improve neurocognitive outcomes. Lithium may also modulate behavioral symptoms such as mood, impulsivity, and suicidal behavior [108]. Another kinase inhibitor, roscovitine, inhibits cyclin-dependent kinase (CDK) and has been reported to modulate neuroinflammation, diminish neurodegeneration, and improve cognitive outcomes in rat preclinical TBI models [108–112]. Another preclinical study reported a potential synergistic role in diminishing p-tau levels in repetitive mild TBI models [97]. Using a preclinical CTE model based on combined repetitive mild TBI and chronic stress, Tang and Fesharaki-Zadeh et al. examined the long-term pharmacological use of Fyn kinase inhibition, AZD0530. Post-injury Fyn inhibition led to a reduction of focal phospho-tau accumulation, as well as neurobehavioral rescue as measured by rescuing object recognition and improving spatial memory function (Figure 3) [90].

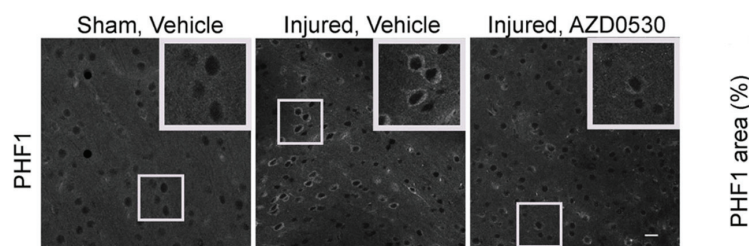


Figure 3. Representative images using immunofluorescent staining for PHF1 of coronal cerebral cortex sections within 0.5–1 mm medial to the site of injury in 7.5-month-old control mice from SV (Sham Vehicle treated), IV (Injured Vehicle treated), and IA (Injured AZD0530 treated) groups. (Images adopted from Tang et al. 2020 [90]).

4.7. Targeting Inflammation

Damage and cellular demise lead to the extracellular release of various ions, molecules, and proteins collectively known as damage-associated molecular patterns (DAMPs) [113,114]. These DAMPs encompass ATP and K^+ , double-stranded DNA, and the high mobility group 1 (NMG1) chromatin protein. ATP binds and activates $P2 \times 7$ receptors, while elevated K^+ stimulates pannexin receptors [115]. DAMPs bind extracellular receptors that activate intracellular inflammasomes [116]. Activated inflammasomes in neurons and astrocytes convert pro-IL-1 β and pro-IL-18 into their biologically active forms [117]. Extracellular IL-1 β and IL-18 levels increase acutely post-injury and are the main inducers of microglia and other early inflammatory processes [115]. TNF α , IL-6, IL-12, and interferon γ are additionally released in the acute phase of injury [115]. Neurovascular changes, infiltration of peripheral inflammatory cells, and activation of resident microglia and astrocytes lead to a more global release of cytokines, chemokines, and bioactive lipids [118,119]. The alteration of microglia activation is a key event in switching from inflammation with early and largely deleterious effects to a later phase of tissue repair and remodeling [118]. Microglia can differentiate into either pro-inflammatory M1 or anti-inflammatory M2 phenotypes [120]. M1 microglia intensify inflammation, bolster the presence of pro-inflammatory cells, and facilitate the clearance of apoptotic cells. They secrete pro-inflammatory cytokines, including IL-1 β , TNF α , and IL-6, along with chemokines that attract more inflammatory cells to the site of injury. Moreover, M1 microglia amplify oxidative stress through elevated expression of NADPH oxidase and iNOS [121]. The M2 microglial has been reported to have an anti-inflammatory role [122]. The precise identification of the inflammatory mediators essential for achieving optimal therapeutic effects remains an ongoing challenge [123].

Previous investigations have aimed at addressing the intricate inflammatory cascade and metabolic changes in preclinical models of CTE. A recent study specifically centered on the potential application of the pyrimidine derivative OCH, which is believed to safeguard mitochondrial function and maintain adequate ATP synthesis following traumatic brain injury (TBI) [124]. OCH demonstrated enhancements in ATP production, respiratory efficiency, and cerebral blood flow, coupled with reductions in glycolysis activity, CTE biomarker levels, and β -amyloid concentrations. Furthermore, OCH treatment effectively preserved sensorimotor function [124]. The use of salubrinal (SAL), a stress modulator, significantly diminished ER stress, oxidative stress, pro-inflammatory cytokines, and inducible nitric oxide synthase. SAL treatment also reduced impulsive-like behavior in rodent models of repetitive TBI [125]. Calpain-2 has been implicated in the progression of neurodegeneration after TBI. The application of a selective calpain-2 inhibitor, known as C2I, resulted in a significant reduction in calpain-2 activation. This intervention effectively halted the elevation of tau phosphorylation and TDP-43 alterations, curbed astrogliosis and microgliosis, and successfully mitigated cognitive impairment in a preclinical model of repeated mild traumatic brain injury [126]. Inhibiting monoacylglycerol lipase (MAGL), responsible for the metabolism of 2-arachidonoylglycerol (2-AG), yielded significant reductions in neurodegeneration, tau phosphorylation, TDP-43 aggregation, astrogliosis, and pro-inflammatory cytokines. This intervention also resulted in improved cognitive

outcomes in a rodent model of repetitive mild TBI. Additionally, the application of 2-AG enhanced blood–brain barrier integrity and reduced the expression of inflammatory cytokines when utilized in a preclinical CHI rodent model [116].

Glucocorticoids exert a broad anti-inflammatory effect by inhibiting the synthesis of interleukins and bioactive lipids. They also suppress cell-mediated immunity and reduce leukocyte count and activity [117]. Despite several preclinical studies, none have investigated whether the anti-inflammatory properties of dexamethasone translate into improved brain function [126]. Clinical trials have yielded limited success, likely due to a narrow therapeutic window [127]. A significant phase III trial, known as CRASH (corticosteroid randomization after significant head injury), included 10,008 adults with TBI and a Glasgow Coma Score (GCS) ≤ 14 [127]. Within 8 h of the injury, these patients received a 48-h infusion of methylprednisolone or a placebo. Intriguingly, the methylprednisolone group exhibited a higher risk of mortality compared to the placebo group, irrespective of the injury severity, thus diminishing the potential clinical efficacy of this regimen.

Non-steroidal anti-inflammatory drugs (NSAIDs) represent a class of medications known for their potent analgesic, antipyretic, and anti-inflammatory properties achieved through the inhibition of COX-1 and COX-2 [128]. COX-2 selective drugs such as carprofen, celecoxib, meloxicam, nimesulide, and rofecoxib have undergone testing in various pre-clinical TBI models [129]. Despite their anti-inflammatory potential, these agents have not proven sufficiently effective in targeting COX-1 or COX-2, making them less promising as therapeutic options for TBI treatment [114]. TNF α , a pro-inflammatory cytokine induced post-TBI, was targeted using HU-211, a synthetic cannabinoid, leading to sustained improvements in various cognitive and motor functions when administered within two hours post-injury [118]. Another TNF α antagonist showed effectiveness in reducing IL-1 β and IL-6 at 3 days post-injury and TNF α at both 3 and 7 days post-injury [129]. Similarly, IL-1 β , an acute pro-inflammatory cytokine post-injury, was modulated in mice overexpressing IL-1ra, resulting in reduced edema and improved neurological scores [130]. Anakinra, a human IL-1 receptor antagonist, when administered two hours after traumatic brain injury, had a limited impact in various assessments [131]. In a clinical trial, administering anakinra within 24 h of injury modified the neuroinflammatory response, but the study's size prevented a clear determination of its therapeutic effect [132].

Rolipram, a phosphodiesterase IV inhibitor, altered both histology and function when administered 30 min before injury [114]. When given 30 min after injury, rolipram similarly lowered IL-1 β and TNF α levels three hours after injury. However, the lesion size was increased compared to vehicle controls [133].

Minocycline is a lipophilic tetracycline-based antibiotic that can cross the BBB with anti-inflammatory action at higher concentrations [134]. Multiple prior studies have demonstrated the anti-inflammatory effects of minocycline [25,135]. Administering minocycline between 5 min and 1 h after injury enhanced performance in various behavioral tasks, such as novel object recognition, the elevated plus maze, the Morris water maze, and active place avoidance in preclinical TBI studies [25,136]. The reduction in IL-1 β production is believed to be the mechanism behind minocycline's inhibitory effect on microglia [137]. Combining minocycline with N-acetylcysteine (NAC) synergistically improved memory in the active place avoidance (APA) task, a complex spatial memory test [136].

Progesterone, a gonadal hormone, has various anti-inflammatory effects. When given 30 min after injury, progesterone initially raised IL-1 β levels at 6 h, followed by a decrease at 24 h. It inhibited IL-6 at both 6 and 24 h post-injury, reduced TNF α at 6 h, and increased TGF β levels at 24 h [138]. In the PROtect phase II trial, patients receiving progesterone within 11 h of injury had a lower 30-day mortality rate than those receiving a placebo. Patients with moderate traumatic brain injury showed better outcomes on clinical scales. However, a large Phase III PROtect III trial involving 882 patients was terminated as it showed no significant effect of progesterone on functional recovery compared to placebo based on the extended Glasgow Outcome Score 6 months post-injury [128]. Another trial

(SYNAPSE) with 569 severe TBI patients did not find differences in outcomes between progesterone and control groups based on various assessments at different time points.

Erythropoietin, responsible for regulating the growth of red blood cell precursors in the bone marrow, has demonstrated anti-apoptotic, anti-oxidative, angiogenic, and neurotrophic effects in various preclinical models of traumatic brain injury (TBI) and stroke [139]. When administered five minutes after injury, erythropoietin effectively reduced IL-1 β , IL-6, and CXCL2 [140]. A one-hour dosing of erythropoietin prevented increased IL-1 β and microglia later after injury in a model combining weight drop and hypoxia [141]. In a study involving 200 closed head injury patients with a Glasgow Coma Score > 3 [142], erythropoietin was compared with high or low hemoglobin transfusion. Transfusion, initiated within 6 h post-injury, aimed to maintain a hemoglobin threshold of 7 or 10 g/dL and included either erythropoietin or a placebo. In a separate observational study, erythropoietin therapy administered within the first 2 weeks post-injury resulted in patients on erythropoietin having significantly shorter stays in the intensive care unit, which is potentially suggestive of a longer survival [143]. The evidence for the use of erythropoietin has not reached the threshold for its use in a phase III trial [114].

Anakinra, a recombinant human IL-1 receptor antagonist (IL-1ra), was studied in a phase II randomized control clinical trial assessing neuroinflammatory modulation using anakinra following TBI [144]. This trial involved the study of 20 TBI patients with a Glasgow Coma Score of ≤ 8 , who were recruited within the first 24 h after the injury. Using microdialysis probes within the brain parenchyma, various cytokines, including IL-1ra, were examined. CCL22 levels were reported to be significantly lowered in the anakinra group. The study was too small to establish anakinra as an effective clinical regimen but provided an intriguing approach for the use of extracellular fluid as a probe as opposed to baseline serum or CSF markers.

A limited number of previous studies have explored the use of statins for TBI, with two notable large observational trials. In one of these trials, conducted by Schneider et al., 523 patients with moderate to severe TBI (Abbreviated Injury Score of ≥ 3) were observed. Among the patients, 22% were regular users of statins [145]. The statin users were found to have a lower risk of in-hospital death. At a one-year assessment of the Extended Glasgow Outcome Scale of the 264 remaining patients, statin users had a small but significantly higher likelihood of more optimal recovery, but the net therapeutic effect of statins was not measurable once controlled for cardiovascular comorbidities in statin users.

4.8. Immunotherapy

Immunotherapy employing monoclonal antibodies has also been a subject of investigation in preclinical studies focused on tauopathies [84]. A recent study demonstrated that the delivery of an adeno-associated virus (AAV) vector coding for an anti-p tau antibody reduced CNS p tau levels in rodent models of repeated traumatic brain injury [146]. Furthermore, in an in vitro study, several tau antibodies demonstrated their efficacy in preventing neuronal tau uptake. Specifically, the antibody 6C5 successfully thwarted interneuronal propagation and the progression of tau aggregation after cellular uptake [147].

Specific antibodies targeting the pathogenic cis-P-tau post-TBI have been reported to lead to improved structural and functional outcomes [148–150]. Removing microglia with PLX5622, a colony-stimulating factor 1 receptor (CSF-1R), was found to have minimal impact on traumatic brain injury (TBI) outcomes. However, encouraging the turnover of these cells through pharmacological or genetic methods leads to a neuroprotective microglial phenotype and significant recovery after TBI. The positive effects of these replenished microglia rely heavily on interleukin-6 (IL-6) trans-signaling through the soluble IL-6 receptor (IL-6R) and strongly support adult neurogenesis [151].

4.9. Potential Dietary Targets

The consumption of a Western diet (WD) and the associated obesity have been consistently linked to systemic inflammatory responses, cognitive decline, and worsened outcomes following brain injuries [152–154]. WD-induced secretion of interleukins such as

IL-1 b and IL-6 can disrupt neural circuits involved in cognition and memory [155]. In a preclinical study focused on the secondary injury outcomes resulting from a closed head injury (mTBI), obese C57 BL/6 mice fed a WD were compared to lean mice. At a chronic time point (30 days), the obese mice displayed significantly increased microglial activation and a chronic state of inflammation [156].

The Ketogenic diet (KD) is a fat-rich diet low in proteins and carbohydrates, with low obesogenic, and has been demonstrated to be neuroprotective [152,157,158]. Unlike the WD, the KD can reduce neuronal inflammation [159], rescue behavioral patterns of depression in animal models [160], diminish cognitive defects [161], and modulate neuronal injury [162]. In addition, Mediterranean diet (MD) consumption has also been associated with reduced risk of dementia and better memory and language performance [163]. Preclinical studies have reported that diets rich in anti-oxidants and flavonoids derived from fruits and vegetables effectively mitigate neuro-inflammation by modifying oxidative stress and apoptosis. This is achieved through the inhibition of NF-KB-dependent inflammatory signaling pathways [164].

5. Future Directions

Chronic traumatic encephalopathy (CTE) and traumatic encephalopathy syndrome (TES) have gained special attention in the public discourse. The diagnosis of CTE remains predominantly pathological, in turn, making the diagnosis of TES and emerging CTE pathological diagnosis challenging. As TES is a relatively novel clinical diagnostic classification, the exact prevalence of TES amongst athletes, combat veterans, and civilians remains largely unknown. A recent study encompassed 176 participants, consisting of 110 boxers and 66 mixed martial artists (MMA), who were all included in the analysis. Among them, 72 individuals (41% of the total) were categorized as having traumatic encephalopathy syndrome (TES), with the likelihood of TES increasing as age advanced. TES-positive (TES+) participants were more likely to be boxers, initiated their fighting careers at a younger age, engaged in more professional fights, and experienced more frequent knockouts [165].

There are a number of diagnostic challenges, which include limited research pertaining to diagnostic validity [164]. There are also limitations pertaining to the absence of universally agreed-upon biomarkers [20]. Despite proposed neuroimaging correlates for TBI and CTE, their diagnostic and prognostic utility remain elusive. Diagnostic challenges include small sample size, inherent heterogeneity of TBI/CTE among injured individuals, as well as the widely varying interval between injury and clinical assessment [84]. The majority of the completed studies lack female study participants, a significant limitation that hinders clinical applicability. Given the tauopathy nature of CTE/TES, wider use of tau markers, including tau PET ligands such as flortaucapir is needed [166]. Identification of sensitive and specific CTE/TES biomarkers would facilitate early diagnosis, monitoring of disease progression, and assessment of disease prognosis. Access to validated biomarkers would also provide the necessary basis to study the natural progression of the disease in a more systematic way.

Currently, there are no FDA-approved drugs for CTE/TES that would offer a disease-modifying effect. Although a number of preclinical studies have proposed potential therapeutic effects for CTE [95], there is an urgent need for large-scale clinical trials. Moreover, there are a number of proposed preclinical models of CTE [90,95,167–169], but there is no agreement on a specific animal model of CTE. The lissencephalic nature of the rodent cortex also adds a layer of complexity to its application to clinical studies. Moreover, the central role of neuroinflammation is increasingly recognized in TBI [84,170]. The development of disease-specific immunomodulating agents, including humanized monoclonal antibodies, is, quite possibly, on the CTE/TES treatment horizon [171].

Given the lack of current treatment options, the most viable CTE/TES treatment option is prevention and safe practices. There is a great need to continue the optimization of protective sports gear, vigilant enforcement of sports contact rules and protocols, and raising public awareness [172,173].

One area poised for significant future advancements is the development of highly sensitive and specific assays for traumatic brain injury (TBI) in serum and cerebrospinal

fluid (CSF). Despite numerous studies, there is currently a lack of consensus regarding proposed biomarkers for TBI. The recent FDA approval of GFAP and UCHL-1 for acute assessment of TBI in the ED setting and examining the necessity of CT neuroimaging, is a major step forward [56,57]. Research on other TBI biomarkers has produced mixed results. For example, two prior studies found no correlation between serum S100B concentration and clinical outcomes, as measured by tools such as the Glasgow Outcome Scale (GOS), Glasgow Outcome Scale-Extended (GOS-E), or imaging studies [174,175]. Similarly, another study found that GFAP was not an effective clinical predictive marker based on GOS-E and functional timeline [176], while an earlier study demonstrated that serum-cleaved tau (c-tau) was also not a reliable predictor after mild TBI [177].

The research and clinical management of chronic traumatic encephalopathy (CTE) and traumatic encephalopathy syndrome (TES) are rapidly evolving areas as our current understanding of their neuropathological mechanisms continues to expand [10]. Advancements in ultra-sensitive biofluid assays are crucial for earlier and more accurate clinical detection of CTE/TES, as well as the potential for more effective treatments. In addition, the development and refinement of disease-specific markers such as tau PET ligands would further expand the much-needed arsenal for timely and effective management of this complex disease, both in research and clinical settings (Table 2).

Table 2. TBI pharmacological treatments reported in preclinical and clinical studies. An overview of the reported TBI pharmacological regimen examined in preclinical and clinical studies. A number of these regimens, including cholinesterase inhibitors, NMDA receptor antagonists, SSRIs, guanfacine, NSAIDs, as well as glucocorticoids and progesterone, are cross-purposed medications that have been utilized in the treatment of TBI clinical studies. Tau phosphorylation, tau acetylation, and immunotherapy regimens have largely been examined in the preclinical TBI setting.

TBI Pharmacological Regimen	Proposed Mechanism
Cholinesterase Inhibitors	Cholinesterase inhibitors, including galantamine, donepezil, and rivastigmine, have been repurposed for TBI patients [84].
NMDA receptor antagonists	NMDA receptor antagonist, amantadine, has been shown to improve cognition in moderate to severe TBI patients [85].
SSRIs	Selective serotonin reuptake inhibitors (SSRIs) like sertraline and escitalopram have been utilized to manage behavioral symptoms in TBI patients [82].
Guanfacine	Guanfacine has been reported to improve working memory deficits in mild TBI patients [86].
Nutraceuticals	A number of nutraceuticals have been utilized in the treatment of TBI in preclinical and clinical studies, including N-acetylcysteine (NAC), flavonoids, resveratrol, alpha-tocopherol (vitamin E), coenzyme Q10 [87].
NSAIDs	COX-2 selective drugs like carprofen, celecoxib, meloxicam, nimesulide, and rofecoxib have undergone testing in various preclinical TBI models with no significant degree of established efficacy [114].
Glucocorticoids	Despite several promising preclinical studies, clinical trials have resulted in limited success, likely due to a narrow therapeutic window [127].
Phosphodiesterase Inhibitors	Phosphodiesterase inhibitors have been utilized mostly in preclinical studies and have not been systematically studied in a clinical trial setting [133].
Minocycline	In prior preclinical studies, minocycline given between 5 min and 1 h after injury improved performance on a variety of neurobehavioral tests [136].
Progesterone	A large, multi-center Phase III PROtect III trial, as well as a second larger-scale trial (SYNAPSE), examined progesterone and did not establish clinical efficacy [178].
Erythropoietin	Despite preclinical studies' success, the evidence for the use of erythropoietin has not reached the threshold for its use in a phase III trial [126].
Anakinra	A small phase II randomized controlled clinical trial reported anti-inflammatory benefits in an Anakinra-treated group; the study size was too small to establish efficacy but provided an intriguing potential future approach [144].
Tau phosphorylation targets	The studies focusing on tau-phosphorylation targets have been mostly preclinical, with possible future clinical applications [90].
Tau acetylation targets	Tau acetylation inhibitors, including salsalate and methylene blue, as well as histone deacetylase 6 and sirtuins, have largely been examined in the preclinical setting [104].
Immunotherapy	Specific antibodies targeting the pathogenic cis-P-tau post-TBI have been reported to lead to improved structural and functional outcomes [148] but have yet to be examined in larger clinical trial settings.

Funding: This research received no external funding.

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

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Review

Neurotrauma—From Injury to Repair: Clinical Perspectives, Cellular Mechanisms and Promoting Regeneration of the Injured Brain and Spinal Cord

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Abstract: Traumatic injury to the brain and spinal cord (neurotrauma) is a common event across populations and often causes profound and irreversible disability. Pathophysiological responses to trauma exacerbate the damage of an index injury, propagating the loss of function that the central nervous system (CNS) cannot repair after the initial event is resolved. The way in which function is lost after injury is the consequence of a complex array of mechanisms that continue in the chronic phase post-injury to prevent effective neural repair. This review summarises the events after traumatic brain injury (TBI) and spinal cord injury (SCI), comprising a description of current clinical management strategies, a summary of known cellular and molecular mechanisms of secondary damage and their role in the prevention of repair. A discussion of current and emerging approaches to promote neuroregeneration after CNS injury is presented. The barriers to promoting repair after neurotrauma are across pathways and cell types and occur on a molecular and system level. This presents a challenge to traditional molecular pharmacological approaches to targeting single molecular pathways. It is suggested that novel approaches targeting multiple mechanisms or using combinatorial therapies may yield the sought-after recovery for future patients.

Keywords: CNS; traumatic brain injury; spinal cord injury; neuroregeneration; neurotrauma; neuroprotection

1. Introduction

Trauma, a physical injury resulting from an external force, is ubiquitous across geographical and societal groups. Whilst many body tissues are capable of significant biological and functional repair, the human central nervous system (CNS) is not. In contrast to the peripheral nervous system (PNS), CNS neurons do not replicate to replace cells lost after injury, and surviving neurons are not capable of regenerating their axons [1]. Due to the unique and profound functions of the CNS, the ramifications of neurotrauma without recovery are of enormous significance to individuals, their families and wider society. Amongst several possible and valid definitions of neurotrauma, the present review will consider neurotrauma as: “traumatic injury to the brain or spinal cord”.

Neurotrauma is an enormously heterogeneous disease state, with a variety of possible clinical and biological phenomena that occur after the initial insult. Even the simple anatomical dichotomy between injury to the brain and spinal cord can be undermined by the increasing incidence of comorbid injury to both, termed “tandem” neurotrauma. Beyond this, there are infinite permutations of injury biomechanics and comorbidities, as well as a broad spectrum of clinical severities and differing relative burdens of discrete deleterious post-injury biological mechanisms. As such, an appreciation of the varying clinical contexts and management strategies is important in understanding the complexities involved in developing therapeutics for promoting functional recovery. As such, the following review will first describe the overall

importance, classification, pathophysiology and clinical management of traumatic brain injury (TBI) and spinal cord injury (SCI). The molecular and cellular basis of neurotrauma in general will then be described, given the considerable commonalities between TBI and SCI, and this will form the basis of a discussion on contemporary approaches to promoting neural repair and regeneration after injury.

2. Traumatic Brain Injury

2.1. Importance

TBI is a significant global health challenge, with no disease-modifying treatment shown to improve outcomes. A principle cause of morbidity and mortality in young adults, the incidence of TBI in Europe is estimated at 1012 cases per 100,000 people per year and 939 per 100,000 globally [1,2]. TBI disproportionately affects low-to-middle-income countries and is a significant financial burden to economies worldwide; the total annual cost globally is estimated to be in the region of £47 billion [3,4]. The risk of suffering TBI is present across society, and injuries are often sustained through road traffic collisions, falls or assault. A wide range of life-changing sequelae may result from injury, including: motor and sensory deficits, cognitive dysfunction, impaired consciousness, depression, behavioural changes, and increased mortality, including an increased risk of suicide [5–9]. These consequences are as yet untreatable beyond supportive therapy in the acute setting, and rehabilitative therapy thereafter [10].

2.2. Classification

TBI is typically stratified by either symptomatic severity or anatomical measures. The clinical presentation of a patient can be used to stratify severity based on the Glasgow Coma Scale (GCS) [11], where a GCS score of 13–15 is mild, 9–12 is moderate and ≤ 8 is severe [12]. The duration of loss of consciousness or post-traumatic amnesia can also be used to classify injury severity [13]. Anatomical measures can be used, for example, to classify TBI by location of haemorrhage, presence of diffuse axonal injury or the presence/absence of multiple variables, as in the computerised tomography (CT)-derived Marshall or Rotterdam grading systems [14,15]. Emerging alternative stratification tools, for example, using immunohistochemical markers, are in their early research phases and are not yet widely accepted. The differences in outcomes, management and pathophysiology vary enormously across this spectrum of injury sub-types. Though termed “mild” TBI (also termed concussion), the long-term consequences can have a severe impact on quality of life and ability to function [5–9]. Mild TBI accounts for up to 90% of TBI, and persistent symptoms occur in up to one-third of people. Severe TBI is invariably a life-changing injury, with mortality rates as high as 40%, and often results in long-term significant disability [5].

2.3. Pathophysiology

The damage resulting from trauma to the brain is typically considered in two parts: ‘primary’ and ‘secondary’. ‘Primary brain injury’ is sustained by the immediate event of trauma itself, whilst ‘secondary brain injury’ occurs after injury due to a variety of adverse sequelae resulting in further cell death and damage [16]. Though primary injuries are modifiable (through personal and public health measures to reduce the incidence and severity of injuries), acute medical care interventions typically focus on the mitigation of secondary injury mechanisms as modifiable targets to improve patient outcomes. Secondary injury to neural tissue can occur through a broad variety of mechanisms, spanning cellular, systemic and anatomical processes. On a cellular level, mitochondrial dysfunction after injury can result in metabolic failure and oxidative stress, which have a role in the propagation of injury and trigger apoptotic cell death after TBI, with associated effects on long-term function [17,18]. “Metabolic crisis” is a phenomenon in TBI that results in severe metabolic dysfunction despite adequate provision of metabolic substrates [19]. Neuroinflammatory processes, whilst essential for wound healing and restoration of the blood–brain barrier (BBB), result in the harmful propagation of injury into the penumbra (areas of the brain

with lesser injury, surrounding an injury focus). This principally involves microglial activation: a predominance of M1 (proinflammatory) over M2 (pro-repair) phenotypes within populations of these resident tissue macrophages of the CNS [20].

The initial injury and ensuing necrosis result in the dysregulated release of neurotransmitters (such as glutamate) [21]. Their activation of local synapses leads to uncontrolled regional depolarisation, known as excitotoxicity. Locally, this can compound metabolic dysfunction and result in regional dysregulated cellular activation (cortical spreading depolarisation) or global seizures [22,23]. The development of cerebral oedema and the expansion of surgical mass lesions, result in increasing intracranial pressure (ICP). Due to the fixed volume of the intracranial space, expansion of intracranial contents results in increasing ICP, as first described in the Monro–Kellie hypothesis [24]. This can result in a variety of harmful sequelae and is the prevailing cause of mortality in the early phase after TBI. Anatomically, this can lead to compression (and obfuscation in extremis) of arteries, cranial nerves and ultimately brain parenchyma. Where this includes compromise of the delicate structures of the brainstem, including regions of respiratory control, mortality rates are high. Increasing ICP also results in global compromise of cerebral blood flow, with unfavourable effects on brain oxygenation and the provision of essential metabolites (predominantly glucose), which further propagate cellular dysfunction after injury.

Whilst the pathophysiology of TBI has been the subject of many years of scientific research, the processes that propagate neural damage after injury are not fully elucidated. More recent observations have been attributed to supporting emerging hypotheses of further injury mechanisms. For example, damage to the BBB through injury may result in dysregulated entry of systemic molecules into the CNS, such as pro-inflammatory cytokines. Similarly, the dysregulated release of adenosine triphosphate (ATP) from damaged neurons has been hypothesised to contribute to increased neuroinflammation and cellular apoptosis [25].

2.4. Clinical Management

Whilst there is a growing understanding of the pathophysiological mechanisms of TBI, the opportunities to measure and correct these processes are limited. Contemporary therapeutic paradigms in severe TBI are predominantly based on the monitoring of ICP, informing clinical decision-making to offer intervention for correction and normalisation of these indices as supportive measures [26]. ICP monitoring is typically performed using a temporarily implantable fibre optic probe, which is placed within the brain parenchyma to a depth of 1.5–2 cm. This probe connects externally to a transducer and a user interface, allowing real-time pressure readings to be presented to the clinician. The procedure to implant an ICP probe requires a hole to be drilled through the skull, and an opening in the dura and cortex is made via a sharp puncture. The entry point is typically at Kocher’s point (an anatomical point 11 cm posterior to the nasion in the mid-pupillary line). A plastic self-tapping, hollow bore “bolt” is screwed into the skull hole to house and secure the ICP wire. Similarly, placement of an intraventricular catheter (external ventricular drain (EVD)) can be used to monitor intracranial pressure via the transduction of pressure within the ventricle.

Acute management of TBI is predominantly targeted at control of ICP alongside general supportive intensive care and management of other traumatic injuries. Whilst this paradigm has been the subject of some historical debate, ICP remains at the centre of clinical guidelines [26]. Therapeutic interventions to reduce ICP use are as follows: (1) patient head positioning, (2) therapeutic hypocapnia, (3) sedation and paralysis, (4) osmotic therapy, (5) diversion of cerebrospinal fluid (CSF), (6) barbiturate-induced coma and (7) decompressive craniectomy [26–31]

Since the establishment of ICP monitoring as a standard of care in TBI, additional monitoring capabilities have been integrated with an ICP probe into cranial access to increase the scope of the “bolt” paradigm (Figure 1). This has seen the greatest success with the introduction of partial pressure of brain tissue oxygen (PbtO₂) monitoring and, to a

lesser extent, with microdialysis probes [32–34]. The extent to which the inclusion of these technologies provides information that is of value for clinical decision-making is yet to be conclusively determined, and as such, they are variably implemented. Despite this, early investigations of PbtO₂ monitoring have shown a trend towards lower mortality with its use, and it has been included in TBI guidelines for ensuring adequate cerebral oxygenation during hyperventilation therapy [26,35]. Beyond these invasive monitoring techniques, there are a few established methods to identify and monitor the pathological processes that occur after injury [36–38]. As such, there are limited opportunities to direct targeted therapies at specific secondary mechanisms.

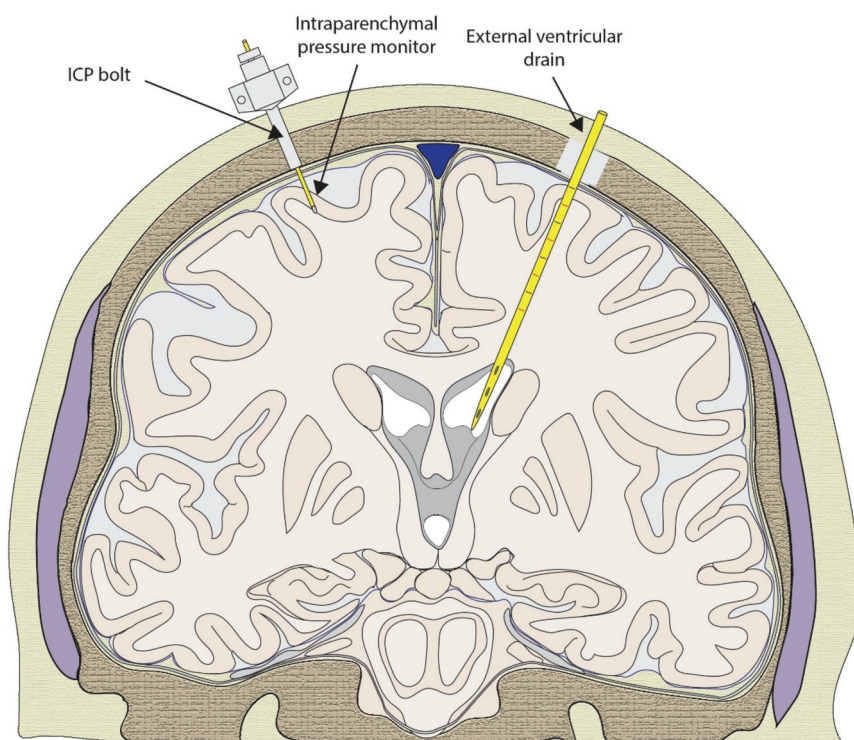


Figure 1. Schematic diagram of a coronal section of the head with an intraparenchymal pressure monitor (with bolt) and external ventricular drain in situ. Both devices may be used to measure pressure from their respective compartments via a transducer.

3. Spinal Cord Injury

3.1. Importance

Traumatic SCI is damage to the spinal cord sustained by mechanical trauma, resulting in a deficit in neurological function [39]. Injuries affect people across society and the globe, with a bimodal age distribution. Injury may occur from falls, road traffic collisions, or sporting accidents, and less commonly from assaults and penetrating or blast injuries [40]. SCI is of increasing prevalence, with 2500 new cases occurring each year in the UK alone [41], resulting in additional lifetime costs of around £2.8 billion each year [41,42]. The lifelong disabilities caused by SCI are typically profound: loss of motor and sensory function, loss of bladder, bowel and sexual function, as well as neuropathic pain and, in some cases, tetraplegia and loss of respiratory function [39]. Whilst some recovery can be anticipated in incomplete injuries through rehabilitation, the loss of function sustained in SCI is typically permanent, as the spinal cord, like the rest of the CNS, has no innate capacity for repair [43].

3.2. Classification

SCI is most commonly classified using the American Spinal Injury Association (ASIA) impairment scale [44], which classifies injury based on neurological impairment, measured by a thorough and standardised International Standards for Neurological Classification of

Spinal Cord Injury (ISNCSCI) clinical examination [44]. This identifies whether an injury is “complete” (i.e., no preservation of any neurological function below the level of injury) or “incomplete” (i.e., partial preservation of motor, sensory or sacral function below the level of injury), as well as identifying the neurological level of injury (the lowest (most caudal) spinal cord segment with intact neurological function). Both the neurological level and severity of the injury indicate the prognosis for functional outcome [45]. The severity of injury has been correlated with the likelihood of recovering the ability to walk independently, with complete injury (ASIA A) associated with the lowest probability of independent ambulation (Table 1) [45,46].

Table 1. Likelihood of independent ambulation after one year (positive predictive value (PPV)) with 95% confidence interval (CI) based on ASIA impairment scale classification (based on van Middendorp et al., 2011 [46]).

ASIA Grade	PPV	95% CI
A	8.3%	5.2–12.6
B	39.4%	27.6–52.2
C	61.8%	50.0–72.8
D	97.3%	92.2–99.4

The spinal cord is a complex structure, composed of grey matter (unmyelinated) and white matter (myelinated). Three columns can be recognised as structures of the cord, which run bilaterally in a rostro-caudal plane: the dorsal, ventral and lateral columns (Figure 2). Within these columns are more focal tracts, categorised as ascending (afferent/sensory), descending (efferent/motor) and mixed (Figure 2). SCI can be classified by spinal cord regions damaged/affected by the injury and may be complete (with all tracts affected) or incomplete (with some tracts preserved) [46–48]. The regions affected and corresponding clinical presentations of incomplete SCI syndromes are shown in Figure 3. Similarly, the variety of pre-clinical models for the study of traumatic SCI result in differing neurological deficits dependent on the anatomical location of the injury.

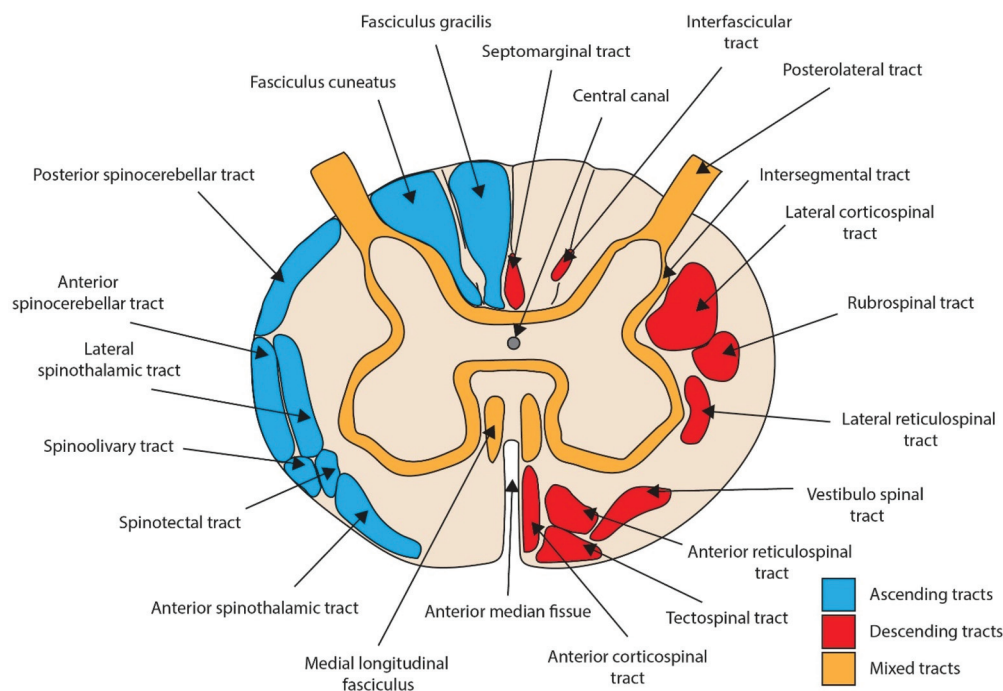


Figure 2. Schematic diagram of an axial cross-section of the spinal cord with labelled ascending, descending and mixed tracts (structures exist bilaterally).

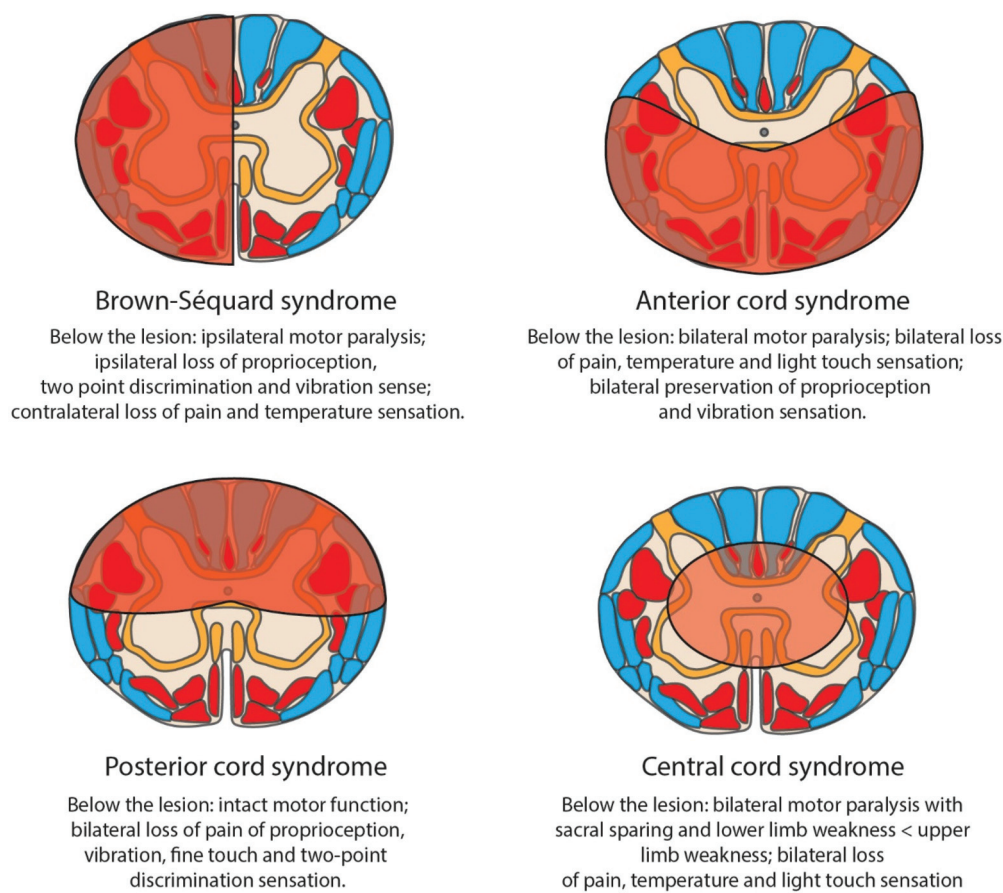


Figure 3. Schematic diagram of four classically described incomplete spinal cord injury syndromes with brief descriptions of their typically associated clinical features. Damaged regions are denoted in translucent red. Motor tracts = opaque red; sensory tracts = blue. Brown-Séquard syndrome (hemisection of the cord) can occur following trauma, particularly penetrating injuries, or from the expansion of tumours. Anterior cord syndrome can occur during trauma or ischaemia. Posterior cord syndrome typically follows posterior spinal artery occlusion. Central cord syndrome is a cervical SCI that can occur after a hyperextension injury with pre-existing cervical stenosis.

3.3. Pathophysiology

The pathophysiological phenomena of SCI, as an acute traumatic insult to the tissues of the CNS resulting in swelling within an enclosed and fixed bony space, hold many similarities to those described above for TBI. Similarly, the injury mechanisms can be considered “primary” (occurring directly from trauma) and “secondary” (as subsequent events and consequences following the primary injury) [39,45,49]. Primary injury can result from direct spinal cord trauma (from penetrating objects or primary blast trauma) or, more commonly in civilian settings, from mechanical force and pressure from fracture and/or dislocation of the surrounding spinal column. Bony displacement, fragments, or the resultant haemorrhage can all mediate primary injury [39,45,49]. Though primary injury typically occurs at the time of the trauma, the primary neuronal injury can be delayed from the index traumatic event: trauma that compromises the mechanical stability of the spinal column can result in delayed mechanical injury to the spinal cord only after weight-bearing [39,49].

The initial trauma to the spinal column and spinal cord commences a complex cascade of secondary injury mechanisms, as seen in TBI [39,45]. In the acute post-injury phase, vascular or bony injury can compromise arterial supply to the spinal cord, resulting in prolonged ischaemia and ongoing neuronal injury, whilst resultant haemorrhage can cause direct pressure effects with compressive effects on local tissue [39,45]. Dysregulated necrotic

release of neurotransmitters such as glutamate from neurons and astrocytes can result in excitotoxicity, intracellular calcium influx and ultimately cell death via apoptosis or necrosis, accompanied by sodium influx resulting in oedema [39,45,50,51]. The release of reactive oxygen species (ROS) and free radicals from necrotic or dysfunctional cells can result in oxidative stress and lipid peroxidation [49,52]. Metabolic failure further contributes to this ionic and oxidative disturbance [52,53]. Damage to the blood-spinal cord barrier (BSCB) disturbs its protective function and allows unregulated migration of inflammatory cells and cytokines into the area to perpetuate the local inflammatory response, contributing to local spinal cord oedema, which can in turn result in further damage [39,54,55].

In the sub-acute phase, there is ongoing apoptotic activation within and surrounding the umbra of the injury site [56]. Growth cone collapse and aborted axonal regeneration, along with demyelination and continuation of the inflammatory response that initiates glial scar formation, ensue [43,57,58]. In the chronic phase, cavitation and maturation of the glial scar, along with degeneration and regression of the remaining axons, occur [45].

3.4. Clinical Management

In further similarity to TBI, current therapeutic paradigms for SCI focus primarily on the mitigation and prevention of secondary damage, particularly via mechanical and hypoxic damage [39,49]. Initial assessment and management follow Advanced Trauma Life Support guidelines and involve resuscitation for maintenance of spinal perfusion pressure [39,59,60]. Immobilisation of the spinal column for resuscitation is recommended where possible to avoid additional damage through the mechanical effects of instability [61]. More definitive management of bony instability is typically achieved via fixation, accompanied (where indicated) by bony decompression of the spinal cord [39,62–65]. Along with the supportive management of blood pressure dysregulation (through neurogenic shock, orthostatic hypotension and autonomic dysreflexia), targeted blood pressure regulation and augmentation to optimise spinal cord perfusion is also a common feature of contemporary management [66,67]. Management beyond these targeted therapies is supportive, managing the complications of injury and promoting functional recovery through physical therapies and rehabilitation [39].

4. Molecular and Cellular Responses to Neurotrauma

The general principles of promoting survival and repair of the CNS neuron after traumatic injury can be considered across both TBI and SCI. All CNS neurons do not regenerate once injured, and typically enter apoptosis or a senescent state. Lost or dysfunctional neurons cannot be replaced through proliferation since neurons are post-mitotic. The resulting immediate cell loss and damage (primary injury) from the direct effects of the trauma are not modifiable once sustained. As such, current interventions in neurotrauma care aim to mitigate secondary injuries. In TBI, optimisation of ICP decreases early mortality and mitigates pressure-induced brain injury. In SCI, spinal decompression and fixation may mitigate the propagation of secondary injury through pressure effects or prevent subsequent mechanical injury through bony instability. Despite much research into neuroprotection and neuroregeneration, however, no therapeutic intervention is presently available that improves functional outcomes through promoting survival or repair of neurons after injury. Long-term rehabilitation may improve functionality, facilitated through neural plasticity and the use of physical aids. However, the capacity for functional recovery by these means is extremely limited at present, particularly in severe injuries, owing largely to the innate failure of the CNS to repair or regenerate neurons.

CNS responses to injury can be categorised into three phases, which are not entirely distinct but represent the principal processes occurring over general time periods after injury [68]:

1. Acute phase (I) (0–3 days post-injury);
2. Subacute phase (II) (3–14 days post-injury);
3. Chronic/consolidation phase (III) (14 days onwards post-injury).

4.1. The Acute Phase

4.1.1. Haemorrhage

Along with direct traumatic injury to neural tissue and necrosis of directly damaged cells, the index traumatic event results in damage to local blood vessels, leading to haemorrhage into the injury site [39]. This, if of large volume, can result in not only direct compressive effects but also the delivery of cytokines, blood-derived immune cells (lymphocytes, neutrophils and macrophages), clotting factors and growth factors into the injured neural tissue, usually excluded by the blood–CNS barrier. Activation of the coagulation cascade and platelet degranulation results in the release of transforming growth factor beta (TGF β) and platelet derived-growth factor (PDGF) [69,70].

4.1.2. Inflammatory Cascade

The presence of TGF β and proinflammatory mediators activates the inflammatory cascade, directly and indirectly via chemokine release (activating migrated blood-derived immune cells (macrophages, neutrophils and lymphocytes) and resident glia (microglia and astrocytes)) (Figure 4). This activation potentiates the inflammatory response, releasing further TGF β , as well as interleukins (ILs) (IL-1 α/β , IL-2, IL-6 and IL-8 [71]) and epidermal growth factor (EGF) [70]. Local chemokines also result in tissue remodelling via upregulation of matrix metalloproteinases (MMPs) and plasminogen activator 1 (PA-1). Whilst the initial neuroinflammatory response is triggered during this acute phase, the immune response persists throughout these three phases [68]. To an extent, leucocyte activation is favourable in traumatic injury for the restoration of blood–CNS barrier integrity and wound sterilisation and debridement. Neutrophils sterilise the wound of foreign pathogens by phagocytosis, with further debridement and the release of inflammatory mediators, MMPs and ROS. Monocytes deposit extracellular matrix (ECM) and initiate angiogenesis via the release of vascular endothelial growth factor (VEGF). Macrophages and resident microglia have a multi-faceted role, with favourable effects (mitigating local excess neurotransmitters from necrotic release, tissue remodelling and growth factor release) and unfavourable effects (myelin phagocytosis, demyelination and astrogliosis).

Populations of microglia, the resident macrophages of the CNS, demonstrate a biphasic response after trauma, with peaks within acute/sub-acute as well as chronic consolidation phases [68,69,72]. However, of greater importance than microglial presence in a favourable or unfavourable environment post-injury is their phenotype. Microglia may become polarised to an M1 phenotype (pro-inflammatory) or an M2 phenotype (anti-inflammatory, pro-repair) [20,69,73,74]. Microglia in the M1 state are understood to release pro-inflammatory cytokines/chemokines, including TNF α , IL-6 and IL-1 β and increase surface expression of cluster differentiation (CD)16, CD32, CD40 and CD86 (Figure 4). Conversely, M2 phenotype microglia increase expression of CD163 and CD206, producing anti-inflammatory mediators (IL-10), growth factors (insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF)) and neurotrophic factors (e.g., nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF)) [74,75]. In addition to well-characterised inhibitors/mediators of inflammation, there are emerging microglial-neuronal crosstalk mechanisms such as direct synaptic interfaces, extracellular vesicles and communication via gap junctions. Together, these suggest a complex and pivotal role for microglia in neurotrauma pathophysiology [76].

Macrophages (monocyte-derived) exhibit similar M1/M2 phenotypes as microglia, with corresponding pro- and anti-inflammatory roles within the CNS after injury. M1 phenotypic switching in SCI has been related to the presence of extracellular myelin [77], present in abundance in the context of axonal disruption within white matter tracts. Macrophages, alongside microglia, clear myelin and other cellular debris from necrosis and axonal shearing by phagocytosis, with greater macrophage residence in the lesion core and greater microglia accumulation within the penumbra. Foam macrophages, derived from macrophage phagocytosis of myelin, can result in paradoxical damage once formed [69]. Influx of peripherally circulating T lymphocytes similarly can promote microglial (M1)

activation through the release of interferon-gamma ($\text{IFN}\gamma$) [78] and perpetuate increased permeability of the blood–CNS barrier through the release of perforin [79]. Conversely, T cell responses to myelin basic protein (MBP) have been associated with chaperoned microglial phenotype shifting to M2 [80].

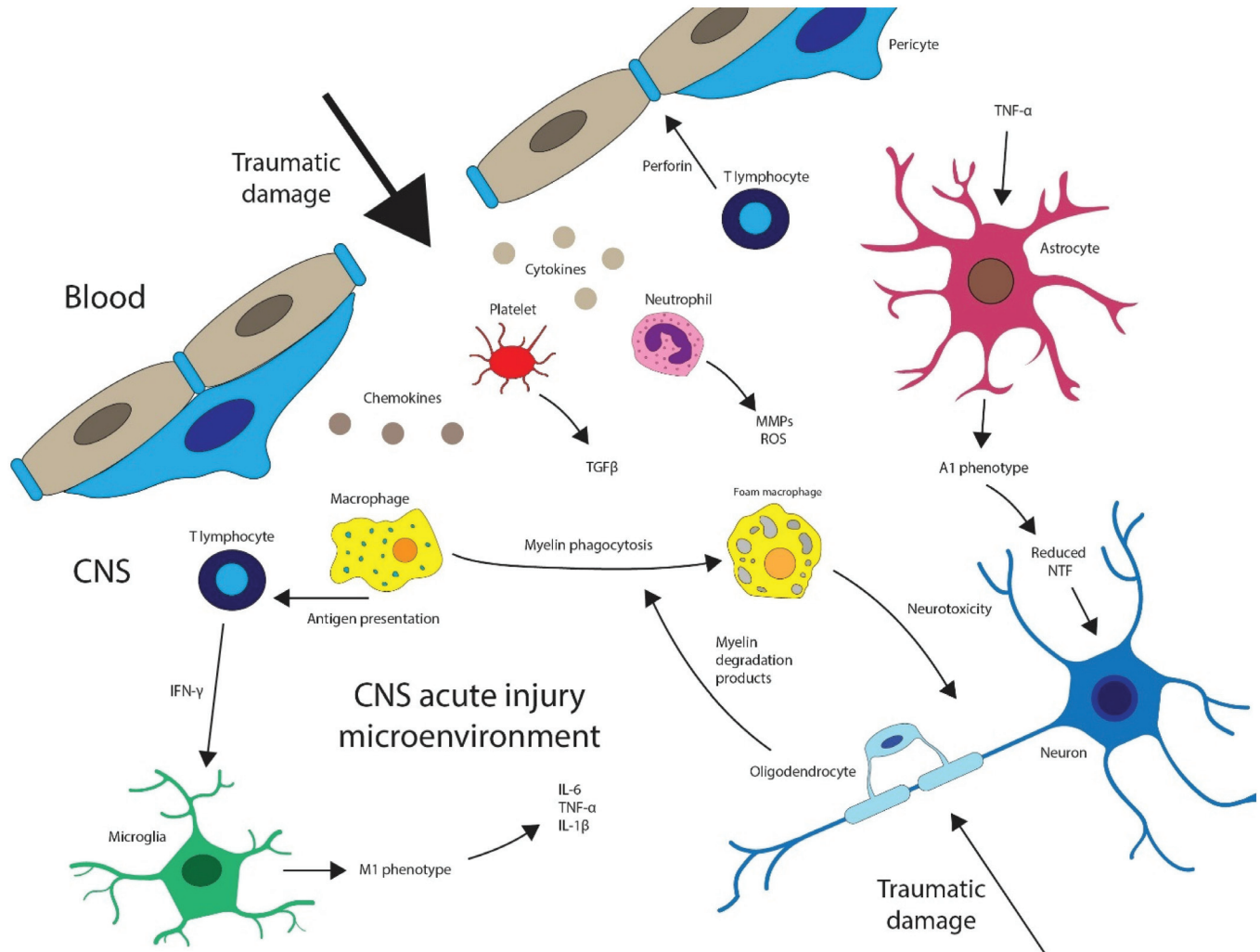


Figure 4. Schematic diagram of the acute phase of injury, following compromise of the blood–CNS barrier and entry of blood-derived cells and signalling proteins, detailing pro-inflammatory signalling leading to microglial/astrocytic polarisation.

4.1.3. Compromise of the Blood–CNS Barrier

Loss of integrity of the blood–CNS barrier from the immediate trauma, sustained by the molecular activity described above by T-lymphocytes, amongst other mechanisms, permits continued compromise of the exclusion of the CNS from the blood-derived immune cells and circulating inflammatory mediators, which in turn sustains the neuroinflammatory response and contributes to developing oedema. This process is illustrated in Figure 4.

4.1.4. Excitotoxicity

Excitotoxicity results from the increased and uncontrolled release of excitatory neurotransmitters after trauma, principally glutamate [16,68]. Glutamate release from damaged axons in the spinal cord and pre-synaptic terminals in the brain results in accumulation within the injury microenvironment [21], compounded later by impaired reuptake due to decreased astrocytic expression of glutamate transporters glutamate aspartate transporter (GLAST) and glutamate transporter (GLT)-1 [81]. Glutamate activates α -amino-3-

hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors, which permit influx of cations (K^+ , Na^+ , and Ca^{2+}) and depolarisation, with excessive activation resulting in intracellular Ca^{2+} accumulation, compromising mitochondrial function [16], contributing to ROS production and activating apoptotic pathways. This process is illustrated in Figure 5.

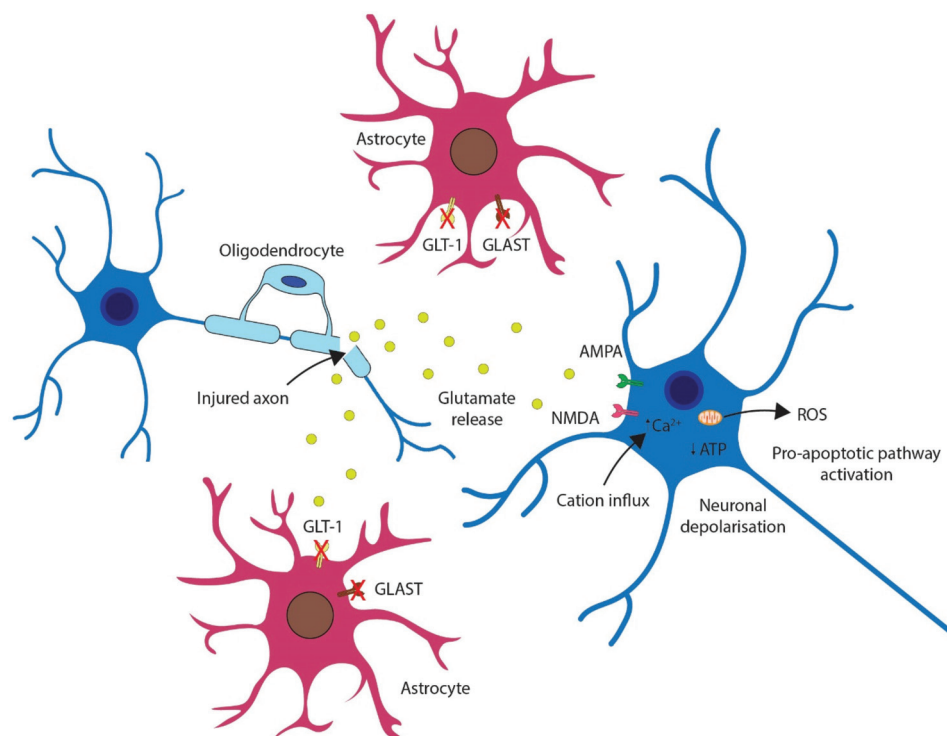


Figure 5. Schematic diagram of the excitotoxicity resulting from uncontrolled glutamate release from severed pre-synaptic axons, perpetuated by downregulation of astrocytic capacity for scavenging free glutamate.

4.1.5. Oedema

Oedema in neurotrauma is a significant mechanism of secondary injury propagation, which occurs through three mechanisms. Cytotoxic oedema is a result of the failure of ATP-dependent Na^+ - K^+ pumps (particularly in astrocytes), resulting in the accumulation of Na^+ (and consequently, water via aquaporin water channels and the G protein-coupled receptor, GPRC5B [82–85]) within the cell. Ionic oedema follows, with the diffusion of Na^+ ions across the intact blood–CNS barrier into the extracellular space to replenish those sequestered intracellularly by cytotoxic oedema. Vasogenic oedema occurs through the influx of water and solutes across a compromised blood–CNS barrier (particularly large proteins such as albumin) into the interstitium of the CNS.

4.2. Sub-Acute Phase

Axonal sprouting: initial early axonal sprouting of damaged neurons after injury can be observed in the early sub-acute phase after injury. This is later aborted, as the initial modest release of neurotrophic factors after injury is not sustained. The generation of a non-permissive injury microenvironment via other mechanisms inhibits any remaining drive for growth from residual neurotrophic factors. This is in contrast to peripheral nervous system injury, where Schwann cells produce a consistent and graded concentration of neurotrophic factors to support axonal regeneration [86,87].

4.2.1. Astrocyte Activation

Astrocytes, the multifunctional support cell of the CNS, become activated after traumatic injury, resulting in their proliferation within the lesional area, transformation to “reactive” astrocytes (astrogliosis) and upregulation of the expression of glial fibrillary acidic protein (GFAP) [88]. Reactive astrocytes have two characterised phenotypes: A1 proinflammatory/neurotoxic astrocytes and A2 anti-inflammatory/pro-survival astrocytes (akin to the phenotypic polarisation of reactive microglia) [16,69,89,90]. A1 astrocytes are formed via the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells pathway (induced by microglial secretion of IL-1 α and TNF α) and secrete an uncharacterised neurotoxin that triggers neuronal and oligodendrocyte cell death [90,91] (Figure 4). Expression of component C3 is used to identify A1 astrocytes [69]. A2 astrocytes were initially identified as being polarised by ischaemic injury and are specifically induced via TNF α /IL-1 β /IL-6-mediated activation of the signal transducer and activator of transcription 3 (STAT3) pathway [89,92,93]. Scar formation via A2 astrocytes can create a more permissive environment for regeneration through an astroglial scar [94], with A2 astrocytes playing a role in increasing the availability of neurotrophins [91]. A2 astrocytes may be identified by their specific expression of S100A10, pentraxin-3 (PTX3), S1Pr3 and Tweak [69,95].

4.2.2. Initiation of the Glial Scar

Immediately after injury, a lesion core is formed through haemorrhage as a collection of non-neuronal cells, blood products, CSF and serous fluid that accumulate through the damaged blood–CNS barrier. Astrocytes migrate to the periphery of this core lesion site and begin to form a network of tightly connected peripheral processes to surround and corral the lesion core to effect a physical barrier between the lesion core and the penumbral neural tissue [69]. Fibroblasts (cells that form connective tissue) from dura/blood and pericytes (endothelial cells of capillary networks and blood–CNS barrier interfaces) also migrate to this zone and proliferate [69,96]. Upon forming a network, astrocytes, pericytes and fibroblasts begin to form an ECM with the secretion of laminin, collagen (type IV), fibronectin and chondroitin sulphate proteoglycan (CSPG), which form the molecular meshwork of the glial scar [69,88]. Whilst the presence of the glial scar, particularly the presence of CSPG, forms both a physical barrier and a non-permissive chemical microenvironment, the presence of an ECM appears necessary for axonal regeneration, with matrix proteins such as laminin acting as an intercellular skeleton, as total scar suppression impairs stimulated axonal regeneration [94].

4.2.3. Demyelination

Acute damage to myelin can occur either by direct damage to the myelin sheath itself (alongside axonal injury) or due to damage to the supporting oligodendrocyte from which the myelin is derived [16,68,97]. The damaged myelin also contributes to this non-permissive microenvironment via the release of inhibitory proteins such as myelin-associated glycoprotein (MAG), neurite outgrowth inhibitor-A (Nogo-A) and oligodendrocyte-derived myelin glycoprotein (OMgp). Akin to the mechanisms by which neurons are lost after trauma, oligodendrocytes and their associated myelin can undergo continued damage during the subacute and chronic phases post-injury: excitotoxicity, oxidative stress, inflammatory cytokines and necrotic proteolytic enzymes. The role of lymphocytes in immune amplification is favourable for the response to pathogens; however, they form endogenous myelin-reactive lymphocytes, initiating immune-driven demyelination that potentiates CNS damage [98]. Furthermore, oligodendrocytes appear dependent on neuronal survival, and axonal degeneration and neuronal apoptosis result in further loss of oligodendrocytes [97].

4.2.4. Mitochondrial Dysfunction

Mitochondrial dysfunction after neurotrauma is a mechanism of secondary injury across cell types, with exquisite effects on the neuron due to its high metabolic demands. Rises in intracellular Ca^{2+} , typically due to excitotoxicity or oxidative stress, act as an initiator of mitochondrial crises. Mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchange channel activity permits Ca^{2+} entry into the mitochondria, with rising intra-mitochondrial calcium leading to opening of the mitochondrial permeability transition pore (mPTP), mitochondrial oedema and swelling, loss of mitochondrial membrane potential and severe disruption of ATP synthesis [99–101]. This membrane damage results in the release of mitochondrial proteins such as cytochrome c, Ca^{2+} and reactive oxygen species (ROS) into the cytosol, which in turn can trigger apoptosis [101]. Additionally, an upregulation in the activity of nitric oxide synthase (NOS) and an increase in nitric oxide (NO) production can independently impair electron transport chain (ETC) function [101].

4.2.5. Oxidative Stress

Dysfunctional mitochondrial activity results in the release of ROS, or free radicals. In states such as neurotrauma, where ROS and RNS production is confluent, production far outstrips any antioxidant/scavenger capacity [99,101]. Mitochondrial production of nitric oxide (NO) and electron leakage from the electron transport chain (ETC) to produce superoxide radicals (O^{2-}) result in the formation of peroxynitrite (PN). PN and other potent oxidising agents propagate mitochondrial damage via lipid peroxidation, leading to mitochondrial DNA damage [99]. This mediates further disruption of the mitochondrial structure, allowing the release of ROS, which results in the destruction of cellular structures, proteins and lipids, triggering apoptotic pathways, the release of pro-inflammatory mediators and perpetuating secondary injury [68]. Mitochondrial structure, function and dysfunction after neurotrauma are illustrated in Figure 6.

4.3. Consolidation Phase

4.3.1. Apoptosis

Triggered by a variety of stimuli in the post-injury tissue environment, the loss of CNS cells can persist in the chronic phase after injury due to apoptosis. Apoptosis, as a controlled process of programmed cell death, contrasts with the disordered events of necrosis, which are typical of immediate traumatic cell death in the acute phase [102]. Apoptosis may occur either as an intracellular (intrinsic) stress response, mediated by Bcl-2/Bax, or due to extracellular factors (extrinsic) in response to a variety of local cell signalling molecules [56,102–104]. Intrinsic pathways to apoptosis may be triggered via a variety of the mechanisms discussed, including mitochondrial dysfunction, oxidative stress, lipid peroxidation and excitotoxicity [91]. Intrinsic and extrinsic pathways activate a series of intracellular signalling pathways mediated by the cysteinyl aspartic proteinases (caspase) family. Caspases are grouped into “initiator” caspases (Caspase-8, -9 and -10) and “executioner” caspases (Caspase-3, -6 and -7), whilst caspase-2 shows activity across both functions (Caspase-2) [104,105].

4.3.2. Consolidation of Glial Scar

Consolidation of the glial scar during the chronic phase after injury creates a continual inhibitory environment for neurons attempting to regenerate severed axons. In SCI, cavitation, that is, fluid-filled cysts, expands over a period of months as inflammatory cells remove non-viable tissue, and an expanding zone of apoptosis, degeneration and demyelination occurs [106]. The acute and consolidation phases of glial scar formation are illustrated in Figure 7.

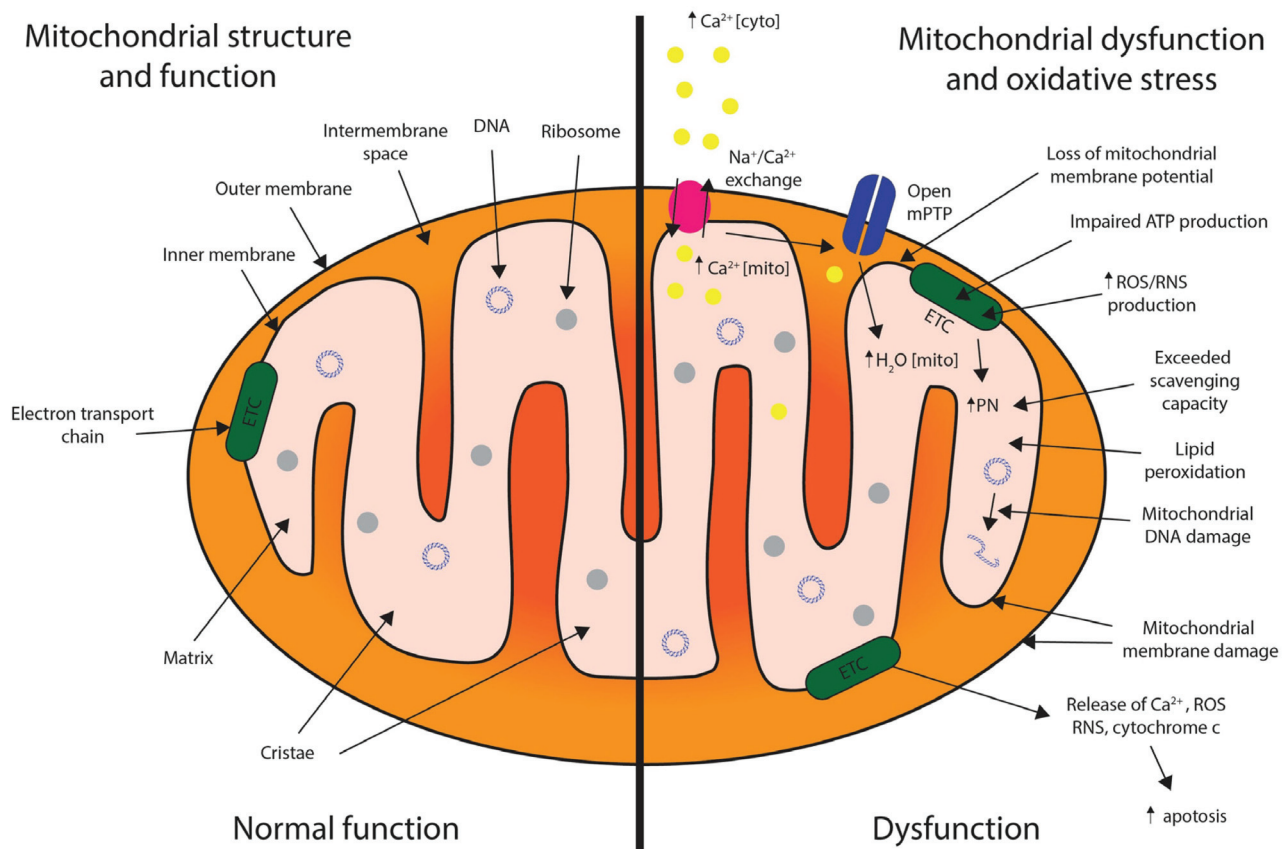


Figure 6. Schematic diagram of mitochondrial structure and normal function (**left**) and dysfunction after trauma (**right**). Dysfunction here is triggered by rising cytosolic Ca^{2+} (Ca^{2+} cyto), resulting in increased mitochondrial Ca^{2+} (Ca^{2+} mito). This opens mPTP channels and intramitochondrial oedema, loss of mitochondrial membrane potential, impaired ATP production, an increase in ROS/RNS production and the release of mitochondrial pro-apoptotic proteins into the cytosol.

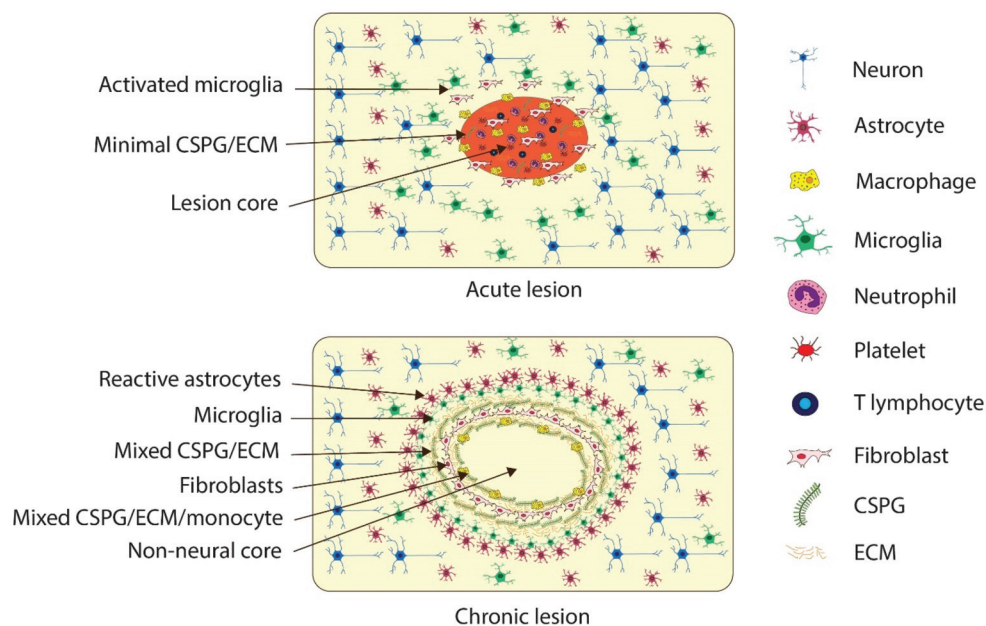


Figure 7. Schematic diagram of the acute phase (**top**) and chronic/consolidation phase (**bottom**) of glial scar formation and cavitation, with description of cellular properties of layers from the lesion core to the penumbral neural tissue.

4.3.3. Aborted Axonal Regeneration

In the non-permissive milieu of the tissue microenvironment post-injury, perpetuated by the consolidation of the glial scar, there is a combination of an abundance of inhibitory factors and a scarcity of neurotrophic factors. Low concentrations of neurotrophins are insufficient to promote or maintain axonal regeneration in the context of non-permissive factors [86,87]. Myelin-derived inhibitory factors continue to contribute to the collapse of the axonal growth cone. MAG, Nogo-A and OMgp bind to the Nogo receptor (NgR) complex (composed of toxicity and JNK inducer (TAJ)), p75 neurotrophin receptor (p75^{NTR}) and either leucine-rich repeat and immunoglobulin-like domain-containing protein 1 (LINGO-1) or amphotericin-induced gene and open reading frame-3 (AMIGO-3) [107,108] and activate an intracellular pathway mediated by Rho-A and Rho-associated protein kinase (ROCK) [16]. Via their respective receptors, non-myelin-derived signalling molecules such as ephrins [109] and semaphorins (semaphorin 3A [110]) also converge on this pathway to inhibit cofilin activity and promote growth cone collapse [16,111].

Neurotrophins (such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3/4 (NT-3/4), transforming growth factors (TGFs), fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), transforming growth factor β 1 (TGF- β 1), glial cell line-derived neurotrophic factor (GDNF) and insulin-like growth factors (IGFs)) act predominantly at the tropomyosin receptor kinase (Trk) receptor family (A/B/C) via various intracellular signalling pathways [112–115]. The transmembrane protein p75^{NTR} can interact directly with low affinity for NTFs, potentiate NTF affinity at Trk receptors or bind pro-neurotrophins at the sortilin receptor [112]. Co-activity of p75^{NTR} in the presence of NTFs mitigates its activity in the NgR complex, reducing the effect of inhibitory signals.

5. Models and Organisms Used for the Study of Neurotrauma and Regeneration

Contemporary understanding of the cascade of biological events that occur in the aftermath of trauma to the CNS is a composite of insights generated from over a century of research, derived from a broad range of in vitro and in vivo models across species, as well as observations from clinical studies. A full description and analysis of the respective advantages of these approaches is beyond the scope of the present review, but they are described briefly below as a short summary, principally to highlight key limitations in the deployment of these models in advancing understanding of neurotrauma and regeneration in humans. For further reading on these topics, the reader is directed elsewhere [116–123].

5.1. In Vitro Models

Experimental Models

In vitro models are advantageous in some respects to the study of trauma and regeneration, offering high reproducibility and throughput at a relatively low cost. Specific cell types may be studied in isolation or in combination through 2D/3D co-culture/scaffold/organoid models. The use of organotypic tissue slice cultures also offers an in vitro model that mimics the composition and cell:cell interactions of their expected state in vivo [122,123]. Application of traumatic injury modelling to these cultural paradigms allows a precise study of the specific effects of mechanical forces (or their ensuing sequelae). Common methodologies to model traumatic injury in vitro are as follows:

- Compression: direct impact via weight drop or pendular acceleration [122,123].
- Stretch: distortion of a culture membrane or other substrate, transmitted to the adhering cells or tissue. A multitude of variables are possible (uniaxial stretch, biaxial stretch, shear, etc.) [122,123].
- Transection: scratching or other sharp distortion of cells/tissue, usually perpendicular to the orientation of axons [122,123].
- Static pressure: a high-pressure chamber to replicate the conditions of raised ICP [124].

- Chemical: application of adverse biochemical conditions to simulate the post-injury microenvironment, for example, oxidative stress, oxygen-glucose deprivation, serum withdrawal, excitotoxicity, etc. [122,123].
- Whilst these methods offer some advantages, observations of cell isolates or co-culture constructs in vitro may be markedly different from those observed in vivo. As well as the general differences in behaviour of cells in vitro as compared with in vivo, this is also attributable to the roles of a broad range of cell types and contributions from a diverse array of system-level adverse conditions (e.g., raised local tissue pressure, regional ischaemia, cortical spreading depolarisation and migrating inflammatory/progenitor cells). As such, whilst in vitro investigation has a significant role in the understanding of neuroregeneration, there is an inherent risk of artefactual observations, the possibility of which must always be considered.

5.2. In Vivo Models

5.2.1. Species

The response to traumatic injury and the intrinsic regenerative capacity of the CNS varies greatly across the animal kingdom. Within vertebrates, some injurious responses differ: for example, whilst glial scarring occurs post-SCI across mice, rats and humans, only the spinal cord of the mouse does not undergo cavitation after injury and demonstrates increased post-injury angiogenesis [125]. As such, in vivo injury modelling in mammalian species has informed much of the contemporary understanding of how the human CNS responds to traumatic injury. Lower-order vertebrates, such as some species of bird, fish or amphibian, display significant contrast from mammalian species in response to CNS injury, by demonstrating capacity for significant or complete CNS repair after trauma.

Interest has been shown in the intrinsic capacity of the zebrafish (*Danio rerio*) for CNS repair [120]. In stark contrast to the events following mammalian injury, described above, in the zebrafish, ependymo-radial glial cells (ERGCs) proliferate and migrate to the lesion site and provide “bridging” support to guide regenerating axons from the ends (“stumps”) of axons cleaved during injury. Astrocyte activation and the ensuing astrogliosis are not observed. Some mechanisms, such as activation of apoptotic pathways and oxidative stress, are also common to zebrafish [126]. Owing to the vastly differing cellular populations, genetic differences and differing neuronal responses, such work carries inherent limitations. However, neuroregeneration research using zebrafish has identified novel mechanisms and elucidated detail on the role and function of some potential therapeutic targets, such as: neuropeptide Y [127], MMP-9 [128], caveolin 1 [129] and the role of lipid droplets and the TAR DNA-binding protein of 43 kDa (TDP-43) in regulating microglial activation [130].

The amphibian species *Xenopus* (*Xenopus laevis*) has been investigated due to its regenerative capacity during the larval stage, which is lost after metamorphosis [131]. During larval stages, injury results in significant proliferation of neural stem progenitor cells (NSPCs) and the absence of glial scarring, and complete regeneration is observed at 20 days post-injury. In the mature *Xenopus*, however, deposition of ECM proteins (fibronectin and collagen) and an absence of proliferation more closely represent mammalian injury responses and, similarly, result in a consolidated chronic scar without neuronal regeneration. Research has further identified a key role of JAK/STAT pathway activation within Sox2/3⁺ ependymal cells and a key role for Sox2/3⁺ NSPCs in mediating the juvenile *Xenopus* regenerative response [132,133].

Key phylogenetic differences between such species and humans may have thus far limited the translational potential of some targets ascertained through such studies, though these models offer a contrasting means to study the mechanisms of non-regeneration in mammals and may generate important insights or genetic targets through ongoing work. Furthermore, the relatively high throughput possible with such species and the possibilities of transgenics may lead to further future impact on the understanding of non-regeneration through the use of these models, in combination with studies in mammalian models.

5.2.2. Experimental SCI Models

The biomechanics of SCI in humans varies greatly owing to a complex array of variables, often in association with the type of force exerted by the varying deformation of the surrounding spinal column during (or persisting/occurring after) the injurious event. Various experimental injury methodologies have sought to replicate this *in vivo* [45,117]. These are summarised below, with examples illustrated in Figure 8.

- Compression (affecting modifiable anatomical regions, as shown in Figure 8): this is typically performed using either aneurysm clips [134], calibrated forceps [135] or an inflatable balloon catheter [136].
- Contusion: controlled impact on the spine or spinal cord by mechanical impact by a weight or driven by pressure [45,117].
- Transection: complete disconnection, usually via sharp dissection, of rostral-caudal segments, either partial (often hemisection [137]) or complete cord transection [138].
- Distraction: application of tension force along the axis of the spinal cord [45,117].
- Dislocation: displacement of one vertebra against an adjacent vertebra, resulting in shear force along the axis of the spinal cord [45,117].
- Whilst a variety of models is valuable for the study of differing responses to SCI subtypes, this can impede the relevance of findings made through the use of any one discrete model. For example, whilst cord hemisection (Figure 8) closely mirrors the Brown-Séquard syndrome described in humans (Figure 3), this phenomenon after traumatic injury is rare and usually only observed occasionally after stab injury.

5.2.3. Experimental TBI Models

A variety of methodologies for administering traumatic injury to the brain have been employed in the study of the mechanisms and therapeutics of TBI and regeneration [116,139–141]. These can be considered diffuse or focal injury models. Diffuse models, weight drop (WD) or blast injury result in damage across the brain, with severity dependent on the magnitude of the exerted force, mainly resulting in diffuse axonal injury (DAI) within white matter tracts such as the corpus callosum (Figure 9) [142]. Blast injury modelling, via sound wave propagation and differential impedance at tissue/fluid interfaces, results in a specific injury pattern distinct from WD [116]. These are both closed injury models, where the skull is left intact. Focal injury methods target a more specific area of the brain. Controlled cortical impact (CCI) is induced by a metal- or silicone-tipped rod driven by a piston into the cortical surface. Cortical stab injury, either through the skull or following craniotomy, is performed with a controlled injury delivered by typically a scalpel [141]. Lateral fluid percussion injury is delivered by a pendulum device that “percusses” a volume of sterile fluid onto the cortical surface, which somewhat diffuses the injury across a wider area than CCI [116,139–141]. Penetrating ballistic-like brain injury utilises the inflation of a balloon catheter inflated after cannulation of the brain to a subcortical depth. This is designed to mimic the cavitation effect of a ballistic injury [143].

Whilst experimental reproducibility is advantageous, TBI in humans (regardless of military or civilian populations) is often heterogeneous, and an individual case will often encompass features of both diffuse and focal injury. Direct-to-cortex methods (such as LFPI or CCI) require a craniotomy to administer, which has the disadvantage that the injury site has undergone a bony decompression prior to the brain injury. This will inherently alter the local response to injury and oedema and effectively represent a pre-emptive therapy analogous to a small decompressive craniectomy.

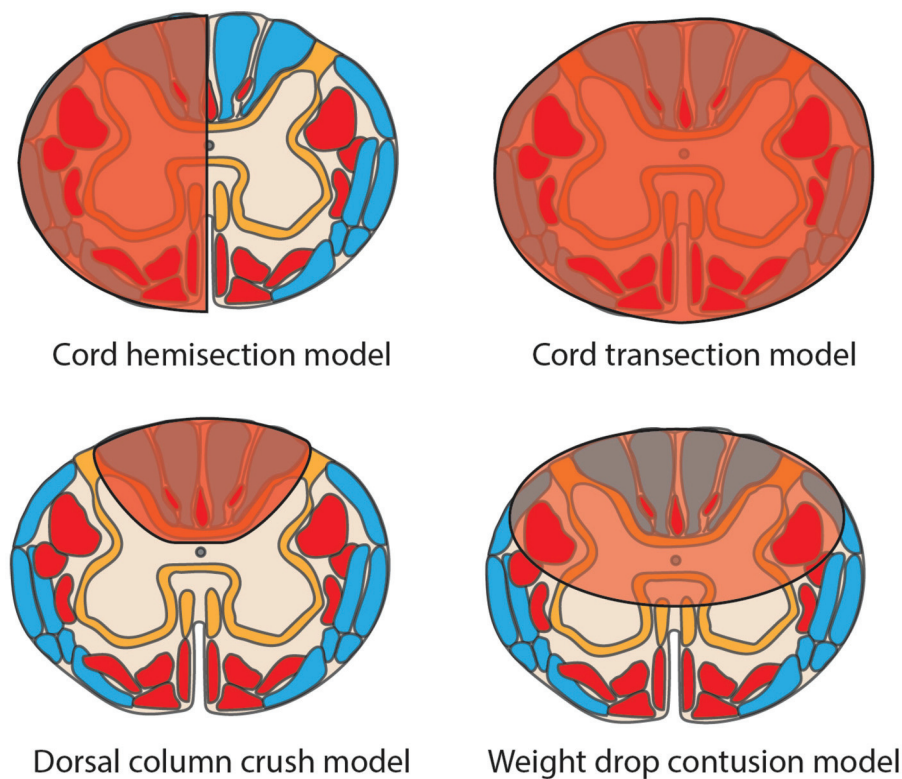


Figure 8. Schematic diagram of four pre-clinical spinal cord injury models used in the literature to replicate the conditions of traumatic injury. Damaged regions are denoted in translucent red. Motor tracts = opaque red; sensory tracts = blue.

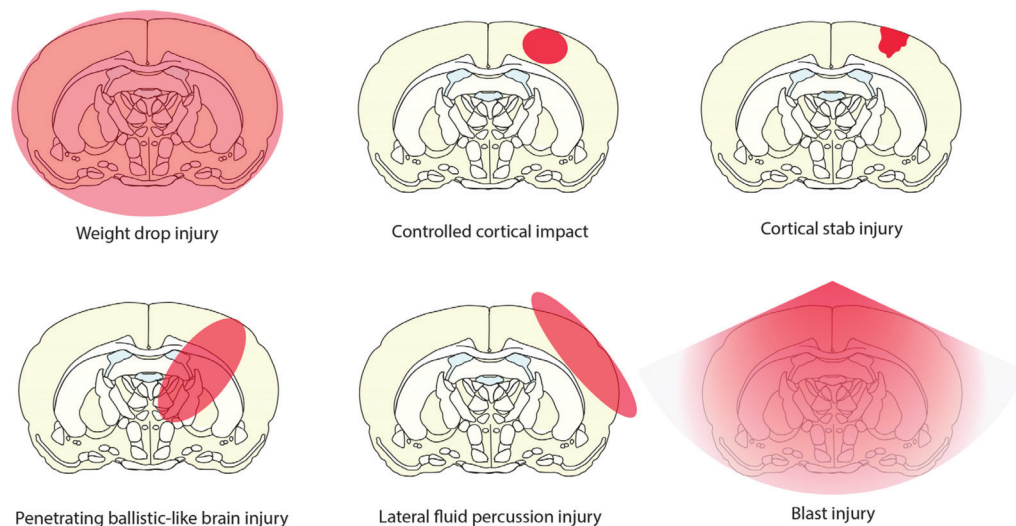


Figure 9. Schematic diagram of six pre-clinical traumatic brain injury models used in the literature to replicate the conditions of traumatic injury.

6. Approaches to Promoting Neuroprotection and Neuroregeneration

The pathophysiological mechanisms described above contribute significantly to the failure of the CNS to survive and regenerate after injury. The current understanding of neuronal regeneration describes key features of this phenomenon:

1. Insufficient and unsustained provision of neurotrophic factors after injury;
2. Neuronal/glial apoptosis;
3. Formation and consolidation of a glial scar;

4. Release of local inhibitory factors from migrating and resident immune and glial cells;
5. Collapse of growth cones of regenerating axons;
6. Rarity of establishing functional reconnections with targets distal to the injury.

Effective therapies to improve functional neurological recovery therefore need to address two broad pathophysiological mechanisms: (1) the propagation of secondary injury via the multitude of mechanisms that contribute to further cell loss and the creation of a microenvironment that is strongly inhibitory of regeneration; and (2) the promotion of axonal regeneration and the establishment of functional reconnections. A multitude of approaches have been utilised in therapeutic attempts to mitigate damage (neuroprotection) and/or promote repair (neuroregeneration) after traumatic injury to the CNS through means to intervene with the pathophysiological mechanisms described above.

The progress of potential pharmacological agents has met difficulties in recent decades. Methylprednisolone remains a controversial therapeutic option. The original publication of the National Acute Spinal Cord Injury Study (NASCIS2) in 1990 resulted in widespread implementation of methylprednisolone therapy in SCI on the basis of unclear and inconsistent results, and its inclusion in clinical guidelines has been conflicting in the period since [144,145]. More recent attempts to validate any beneficial effects of SCI have not provided conclusive evidence [145–147]. Administration of methylprednisolone in TBI has been demonstrated in the CRASH trial to increase the risk of two-week mortality [148]. There is current interest in the early administration of gabapentinoids to promote functional recovery after SCI [149,150]. Despite some encouraging results from early human studies, this is yet to be confirmed in prospective clinical trials [151–153]. Riluzole, a glutamatergic modulator approved for use in amyotrophic lateral sclerosis (ALS), was deemed promising as a neuroprotective therapy, though the trial was terminated due to enrolment challenges, and the results are awaited [154,155]. The investigation of riluzole in TBI is ongoing [156].

Research is ongoing to optimise control of ICP and intra-spinal pressure (ISP) in the acute phase after injury as an indirect means of neuroprotective therapy via mitigation of secondary injury as a consequence of pressure effects on neural structures. Following the establishment of ICP control as a therapeutic target in severe TBI [26], intraspinal pressure and perfusion have been an area of growing interest in SCI. Direct pressure monitoring with targeted therapy, perfusion pressure optimisation and dural decompression (expansion duroplasty) has been proposed to mirror the pressure-directed surgical care in TBI [157–159]. A study is presently ongoing to assess the potential therapeutic benefits of expansion duroplasty in the acute phase after SCI, with the rationale of creating additional intraspinal volume than bony decompression alone, in order to permit post-injury oedema and limit local pressure effects. Based on a similar rationale, lumbar drainage of CSF has been proposed as a strategy to achieve more favourable intraspinal pressure, with some early success in pre-clinical studies [160]. Aligned with optimisation of pressure control, ensuring appropriate perfusion of the brain and spinal cord after traumatic injury is a further area of ongoing research for neuroprotection [158,161].

7. Discussion

Whilst molecular targets and novel approaches hold some promise for promoting repair and recovery after neurotrauma, pharmacological methods typically target single receptors and affect discrete pathways within the complex and multifaceted pathophysiology of the CNS after injury. Novel therapies targeting a variety of the pathophysiological processes in neurotrauma remain a significant area of research, as comprehensively described elsewhere [16,43,69,162]. These are summarised in Table 2. Whilst a number of biological targets have proven promising in pre-clinical studies, translational success has often proven challenging [49,83,106,107,163–177]. Interventional studies continue to investigate novel targets and approaches but have thus far failed to prove efficacious in improving functional outcomes [16,69,178,179]. This may be attributable to the intrinsic limitation of targeting single pathways in a disease process mediated by a multitude of factors. Efforts to improve future outcomes from neurotrauma therefore focus upon opportunities to intervene with

the breadth of harmful cellular mechanisms, including monitoring their progression to provide targeted treatment. Combinatorial therapies may present a possible route to greater efficacy. An example of this is the success demonstrated through the combination of stem cell approaches with hydrogel scaffolds [180,181]. Exploration of targeting multiple pathways or using a multitude of approaches described in Table 2 may address this challenge. The potential for drug–drug interactions present a challenge to the potential strategy of combination therapies, which target multiple pathways and mechanisms to overcome the multitude of barriers to repair described above, or through the use of drug therapies combined with approaches such as biomaterials or CNS stimulation through devices.

The heterogeneity of TBI and SCI is itself a challenge. Compounding the enormous range of injury severities and classifications, the clinical outcomes from similar-severity injuries (based on unmeasurable variables or genomic idiosyncrasies) introduce further variability into studies that strive to improve functional outcomes. Consequently, clinical efficacy studies require large numbers of recruited patients to demonstrate benefit. The financial expense and high rate of failure of such studies have undoubtedly impacted the translational study of approaches that have proven promising in discovery science.

A broad range of biomarkers of CNS injury and injury severity have been identified, which can be readily measured in biofluids [182–185], though these are not recognised as markers of neuroregeneration. Some may be involved in neuroregeneration, for example, CSF concentrations of NGF [186,187]; however, there is insufficient evidence at present to posit these as a regeneration marker (as opposed to a marker of injury severity alone). In contrast, there are a number of specific protein markers for the identification of neuroregeneration (e.g., GAP43 [188], collapsin response mediator proteins [189] and genomic markers [190]), though these are only used in immunohistochemical analysis or next-generation sequencing of neural tissue, rendering them unsuitable for clinical applications. As such, there remains a reliance on clinical evaluations of functional outcomes, which can only be reliably measured years after injury and are subject to many other (known and unknown) variables. Advances in the availability of biomarkers of regeneration may provide much-needed early validation of the therapeutic efficacy of the interventions in clinical trials to allow real-time recognition of successfully induced neuroregeneration.

A focus on a dichotomy of favourable and unfavourable outcomes presents a challenge: therapeutic strategies must overcome a great threshold to increase the proportion of patients achieving a “favourable” outcome across a population. However, marginal gains in additional function for those severely injured can greatly improve quality of life. Short time frames of follow up compound this challenge, as recovery may continue well beyond the three- or six-month end points of typical neurotrauma clinical studies. The development of efficacy biomarkers (proxy indicators of recovery that are valid in early phases) may allow greater confidence in therapeutics to be gained in small pilot studies and is suggested as an area for further research. Other advances, for example, in patient stratification based on emerging techniques, may improve possibilities for novel study designs to improve the sensitivity of clinical studies to detect patient benefits or to personalise targeted interventions based on the individual burden of the secondary injury mechanisms discussed above [23,34,191–195].

Table 2. Summary of therapeutic approaches for neuroprotection and neuroregeneration. This is an illustrative list encompassing some common therapeutics under current and recent investigation. For further details on current clinical trials, see elsewhere for a comprehensive discussion of TBI [179] and SCI [196]. NGF = nerve growth factor; BDNF = brain-derived neurotrophic factor; IGF-1 = insulin-like growth factor 1; CS = chondroitin sulphates; PEDF = pigment epithelium-derived factor; Rho-A = Ras homolog family member A; mTOR = mammalian target of rapamycin; chk2 = checkpoint kinase 2; NgR = Nogo-66 receptor; AQP-4 = aquaporin 4; mPTP = mitochondrial permeability transition pore; ADSCs = adipose-derived stem cells; DPSCs = dental pulp stem cells; ESC = embryonic stem cells; IL-6 = interleukin-6; iPSC = induced pluripotent stem cells; NSC = neural stem cells; NPC = neural progenitor cells; MSC = mesenchymal stem cells; nNOS = neuronal nitric oxide synthase; OPC = oligodendrocyte progenitor cells; PLGA = poly (lactic-co-glycolic acid); siRNA = small interfering ribonucleic acid; HDAC = histone deacetylase; Uqcr11 = ubiquinol-cytochrome c reductase, complex III subunit XI.

Biological	Neurotrophic factors	NGF [172], BDNF [173], PEDF [135] and IGF-1 delivery via nanofibrous dural substitutes [197]
	Pathway inhibitors	Caspases [174], Rho-A [175], mTOR [176], chk2 [177], Rab [198] and transglutaminases [199]
	Cell death inhibitors	Caspases [174], Bcl-2 [200], imipramine [201], cyclosporin A [202] and statins [203]
	Receptor inhibitors	NgR [107], glutamate [163] and endothelin [204]
	Channel inhibitors	AQP-4 [83], Ca ²⁺ channel inhibitors [164] and mPTP [165]
	Inflammation	Immunomodulation [166], gangliosides [49,167], HDAC inhibitors [205] and bexarotene [206]
	Mitochondria	Mitochondria-endoplasmic reticulum contact sites [207]
	Oxidative stress	Antioxidants [168], ROS scavenger materials [170,171,208,209] and Uqcr11 overexpression [210]
Cell therapies	Glial scar	Chondroitinase ABC [169,170], decorin [106,171] and 4-methylumbelliferone [211]
	Gene therapies	Neuronal differentiation [43,212]
	Autophagy	HSPs [213]
	Endocrine	Progesterone [214], erianin [215]
	Other	Hydrogen sulphide [216], tetramethylpyrazine [217], zinc [218], probucol [219], phenserine tartrate [220] and hyperbaric oxygen [221]
	Stem cells	ESCs [222], iPSCs [43,223], NSCs/NPCs [224,225], MSCs [180,181], OPCs [226], DPSCs [216] and ADSCs [227]
	Neural cells	Olfactory ensheathing cells [228] and Schwann cells [229]
	Immune cells	Microglia [230]
Gene therapies	Advanced cell therapies	Directly reprogrammed NPCs (drNPCs) [231–233]
	Nucleic acid-based therapies	siRNA to AQP-4 [234], nNOS [235], iNOS [236], IL-6 [237], claudin-5 [238], RhoA [239,240], PLK-4 [241], PTEN [242,243], Sema3A [244], CTGF [245], combinatorial [246] and in combination with MSCs [242]
	Delivery methods	Nanoparticle-coated siRNA [247–249], polymer nanocarriers [239], exosome delivery [243,245] extracellular vesicles [250], intrathecal delivery [240], photomechanical wave [251] and intranasal delivery [242]
	Other	Chemogenetic stimulation [252]
Biomaterials	Porous polymers	Hydrogels [180,181,253,254], PLGA [255] and PLA [256]
	Natural polymers	Collagen [181,257], CS [258], silk [259,260], decellularised ECM [227], modified gelatine [261]
	Nanoscaffolds	R-GSIK [262], electrospun nanofiber nets [263] and gene scaffolds [264]
	Nerve guidance	Gold nanoparticle nerve guidance conduits [265] and collagen conduits [266]
	Other	Graphene oxide [267], IGF-1 delivery via nanofibrous dural substitutes [197] and ROS scavenger materials [170,171]

Table 2. Cont.

Physical	Stimulation	Electrical [268,269], magnetic [270,271], ultrasound [272,273], light (photobiomodulation) [274,275] and combinatorial [276]
	Neuromodulation Supportive	Spinal stimulators [277] in combination with task training [278] Exoskeletons [279,280] and neuroprosthesis [281]

8. Conclusions

Developing new, effective therapies to avert the profound and permanent functional impacts of neurotrauma is an area of urgent need. The complexities of the post-injury micro- and macro-environments are described here, which span multiple intracellular pathways and cell types and encompass phenomena on intra- and inter-cellular levels (such as metabolic) and at a system level (such as the effects of impaired perfusion and increased pressure), suggesting that multifaceted approaches to improving outcomes will be required. Exploration of methods to target multiple mechanisms of injury propagation and consolidation may yield novel, effective interventions, which may offer a step-change in opportunities to rescue and restore function of the CNS after trauma.

Author Contributions: Conceptualisation, A.R.S., A.B. and Z.A.; illustration, A.R.S.; review curation, A.R.S.; writing—original draft preparation, A.R.S.; writing—review and editing, A.R.S., A.B. and Z.A.; supervision, A.B. and Z.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no specific external funding. A.S. received funding from the Royal College of Surgeons Surgical Research Fellowship.

Institutional Review Board Statement: Ethical review and approval were waived for this study due to it being a narrative review of published literature.

Informed Consent Statement: Patient consent was waived due to this study being a narrative review of previously published studies and thus no patient or member of the public were involved in any stage.

Data Availability Statement: No new data were created in the preparation of this review.

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

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Review

Selective COX-2 Inhibitors as Neuroprotective Agents in Traumatic Brain Injury

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Abstract: Traumatic brain injury (TBI) is a significant contributor to mortality and morbidity in people, both young and old. There are currently no approved therapeutic interventions for TBI. Following TBI, cyclooxygenase (COX) enzymes generate prostaglandins and reactive oxygen species that perpetuate inflammation, with COX-1 and COX-2 isoforms providing differing responses. Selective COX-2 inhibitors have shown potential as neuroprotective agents. Results from animal models of TBI suggest potential treatment through the alleviation of secondary injury mechanisms involving neuroinflammation and neuronal cell death. Additionally, early clinical trials have shown that the use of celecoxib improves patient mortality and outcomes. This review aims to summarize the therapeutic effects of COX-2 inhibitors observed in TBI animal models, highlighting pertinent studies elucidating molecular pathways and expounding upon their mechanistic actions. We then investigated the current state of evidence for the utilization of COX-2 inhibitors for TBI patients.

Keywords: traumatic brain injury; neuroprotection; neuroinflammation; inflammation; cyclooxygenase

1. Introduction

Traumatic brain injury (TBI) is a significant cause of disability and death worldwide [1]. It is projected that globally each year, sixty-nine million individuals sustain a TBI [2]. TBI survivors may suffer life-long consequences [3], with significant socio-economic consequences [4] and high medical costs resulting from TBI [5,6]. Overall, the most common TBI mechanisms are falls and traffic accidents [7,8]. TBI incidence is unequally distributed across different sexes and ages due to the varied environments and mechanisms of injury, with a higher proportion of males than females suffering TBIs [9]. In older women, falls are the primary cause of TBIs, whereas in young men, the leading causes are traffic accidents, violence, and sports injuries [10]. The age distribution of TBI exhibits a bimodal pattern, with peaks observed in individuals under 14 years and those over 65 years [11,12].

The diverse etiology, presentation, pathophysiology, complexity, and outcomes associated with TBI lead to challenges in patient care [13]. The multifaceted pathophysiology of TBI is initiated by a primary injury, which sets off a complex cascade of secondary injuries involving cerebral autoregulation impairment, leakage of the blood–brain barrier (BBB), formation of edema, oxidative stress, disruption of calcium homeostasis, and mitochondrial dysfunction [14–17]. These interrelated mechanisms result in neuronal cell death and prolonged inflammatory responses in the brain, which can lead to a range of clinical conditions including acute seizures, chronic epilepsy, neuroendocrine dysfunction, depressive disorders, and chronic traumatic encephalopathy (CTE) [18].

2. Current TBI Treatment Options

Despite being a leading cause of mortality, effective guidelines for the acute and long-term management of TBI remain elusive [19]. Acute therapeutic strategies for patients with

moderate to severe TBI may include surgical procedures such as intracranial hematoma evacuation, decompressive craniectomy, and supportive interventions to alleviate symptoms and sustain homeostasis. Recent advancements in neuromonitoring, neuroimaging, and surgical techniques have contributed to improved outcomes and reduced mortality rates [20–24]. However, treatment trials or meta-analyses are complicated by varying and individualized treatment strategies that could be a contributor to the failure of TBI pharmaceutical interventions in clinical trials [25]. By targeting the acute injury-derived humoral sequelae with anti-inflammatory agents, the progression to more severe and chronic injury may be prevented.

3. Inflammation Following TBI

Inflammation is a fundamental component of TBI pathophysiology, irrespective of the injury's severity [26,27]. More severe brain injuries evoke a more substantial and prolonged inflammatory response [28–30] but are also characterized by significant physical trauma. Neuroinflammation functions as an immune response to facilitate debris clearance and tissue repair. However, neuroinflammation has both beneficial and harmful effects on neuronal survival and brain repair, with excessive or prolonged inflammation exacerbating neuronal damage and contributing to neurological deficits [18,31–33]. Inflammatory responses also typically overshoot their physiological requirements, which promotes excessive tissue damage and remodeling [30].

Inflammation following TBI triggers the phosphorylation of phospholipase A₂, phospholipase D, and phospholipase C, which release arachidonic acid (Figure 1). The cyclooxygenase (COX) enzymes, COX-1 and COX-2, transform arachidonic acid into prostaglandin G₂ (PGG₂). This is then further metabolized into prostaglandin H₂ (PGH₂) with the aid of the peroxidase enzyme. Subsequently, PGH₂ is transformed into various prostaglandin molecules, such as PGD₂, PGE₂, PGF₂, and PGI₂ (prostacyclin), and thromboxane A₂ (TxA₂). These resulting prostaglandins and related compounds are propagators of inflammation and contribute to a wide range of inflammation signaling processes involved in secondary injury mechanisms [34].

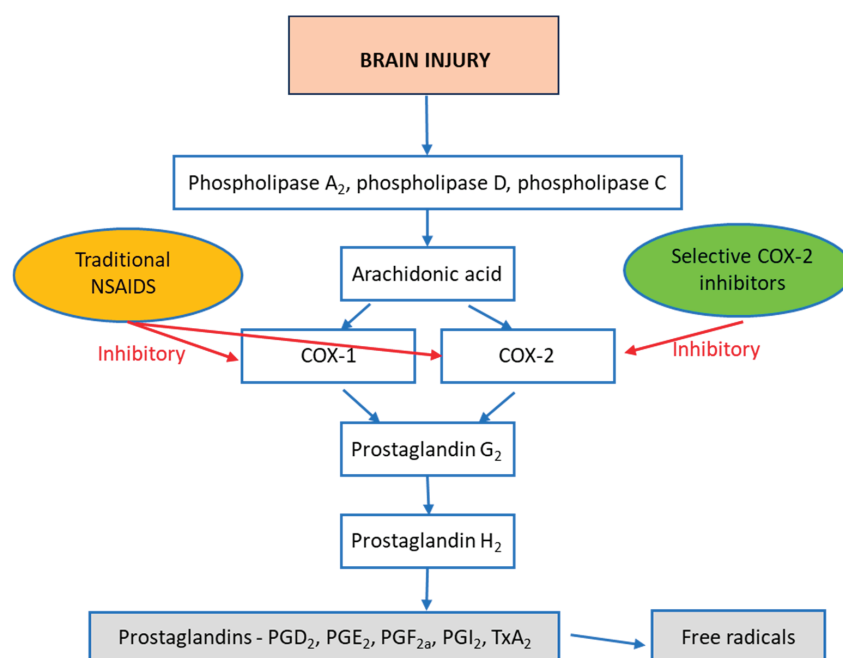


Figure 1. Generation of prostaglandins and free radicals following TBI. TBI increases the activity of phospholipase A₂, phospholipase D, and phospholipase C enzymes, resulting in arachidonic acid generation. COX-1 and COX-2 enzymes convert arachidonic acid into prostaglandin G₂, which is then converted into prostaglandin H₂. Prostaglandin H₂ is then converted into prostaglandin analogs and thromboxane A₂. These metabolites trigger physiological effects, and also result in the further generation of free radicals.

Inflammation is also intrinsically linked with oxidative stress pathways. Elevated levels of intracellular calcium ions can result in cell edema and a decrease in cerebral blood flow [35]. This damages mitochondria and leads to the increased generation of free radicals. These free radicals damage cellular membranes through lipid peroxidation, which results in an increase in arachidonic acid in the cytosol. This contributes to arachidonic acid's production of thromboxane and prostaglandin metabolites, which themselves generate more free radicals.

4. Anti-Inflammatory Drugs for TBI

A complete review of the current state of anti-inflammatory pharmacological interventions for TBI is beyond the scope of this paper, but has recently been performed [25]. One key strategy to reduce neuroinflammation involves reducing the production of prostaglandins by administering glucocorticoids or COX inhibitors. These compounds also decrease the production of free radicals, thromboxanes, and prostacyclins [36].

Glucocorticoids block COX-2 expression without affecting COX-1 expression. However, glucocorticoids may have significant adverse effects [37] and their efficacy in reducing inflammation following TBI may be limited [38]. The glucocorticoids methylprednisolone and dexamethasone have been investigated as potential TBI treatments. Early dexamethasone administration has shown promise in reducing edema through aquaporin-1 regulation [39] and microglial inhibition [40]. However, corticosteroid therapies have failed to demonstrate efficacy in clinical trials. This was exemplified by the termination of the MRC CRASH trial, which observed an elevated mortality rate in the 14 days post-TBI among patients administered methylprednisolone [37]. As a result of this evidence, corticosteroids are not being investigated as a TBI treatment [41].

Non-steroidal anti-inflammatory drugs (NSAIDs) modulate the inflammatory response by targeting COX enzyme pathways activated in response to trauma by reducing (i) prostaglandins and thromboxanes, (ii) cytokines, and (iii) proteases [42]. COX enzymes are present in two distinct isoforms, namely, COX-1 and COX-2. COX-1 is ubiquitously expressed across various tissues, indicating its non-specific distribution. However, the expression of COX-2 is specifically triggered by inflammatory products, growth factors, and hormones [43]. First- and second-generation NSAIDs were developed to non-selectively inhibit both COX-1 and COX-2 enzymes. However, a notable side effect of COX-1 inhibition is the upregulation of gastric acid production, with adverse effects including gastric ulceration, bleeding, and renal dysfunction [44,45]. In contrast, it was identified that COX-2 inhibition elicits anti-inflammatory effects without these drawbacks. Therefore, the third generation of NSAIDs were designed for selective COX-2 inhibition. Two selective COX-2 inhibitors, celecoxib and rofecoxib (Figure 2), became available on the international market in 1999. These compounds were approved for conditions such as rheumatoid arthritis and osteoarthritis, while simultaneously demonstrating a significant reduction in hemorrhagic GI complications. However, as the use of rofecoxib increased, data emerged from several studies suggesting an increased risk of cardiovascular events such as myocardial infarction and stroke. This led the FDA to instruct Merck to include rofecoxib packaging with precautionary labels. Further studies of rofecoxib suggesting cardiovascular hazards led to its withdrawal from the market, such that celecoxib is now the only selective COX-2 drug available in the United States. It has been demonstrated that celecoxib at moderate doses shows a similar risk of cardiovascular events as non-selective NSAIDs [46]. Celecoxib is thought to be associated with a lower risk of cardiovascular events than rofecoxib because it is a less selective COX-2 inhibitor.

The rationale for COX enzymes as a therapeutic strategy for addressing TBI neuroinflammation stems from studies revealing NSAID protection in Alzheimer's disease (AD) patients [47,48]. COX inhibition exhibits anticipated analgesic and anti-inflammatory effects, with emerging evidence suggesting neuroprotective properties in conditions involving neurotoxicity, neurodegeneration, and demyelination [49–53]. In an AD model, celecoxib administration led to reduced astrocyte and microglia upregulation, prevented behavioral

impairment, and normalized neurotransmitter response [54]. Other evidence of therapeutic potential has also been demonstrated in preclinical models of neurodegeneration, where celecoxib treatment has protected against learning and memory impairments [55]. Notably, neuroprotection is not solely attributed to inflammation pathways, as COX-2 inhibition has been found to invoke increased neuronal survival in the absence of decreased mRNA inflammatory signaling [56].

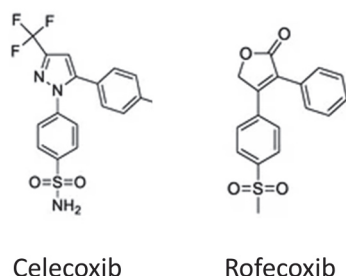


Figure 2. Structure of celecoxib and rofecoxib.

Selective COX-2 inhibitors are posited to enhance patient outcomes in TBI cases by mitigating secondary injury related to inflammation, free radicals, and edema [57,58]. However, the role of COX-2 inhibitors in TBI presents a paradox; reservations primarily revolve around the uncertainties with administration timing and dosage, and the potential repercussions of negating protective repair and recovery processes [59]. While excellent reviews of COX-2 inhibitor treatment in TBI have been undertaken previously [60,61], we aim to provide an update on the state of the literature, and to our knowledge, this is the first review of COX-2 inhibitors for TBI treatment that includes both animal and human studies. The following sections explore the preclinical and clinical evidence for the role of COX-2 inhibition in TBI.

5. Preclinical Studies

5.1. Injury Administration and Assessment

Understanding the experimental methods applied in TBI models is important for interpreting the outcomes, as a diverse range of TBI models have been employed to evaluate experimental therapeutics. TBI animal models are broadly categorized into closed head injury models, where the skull is not surgically altered, and open head injury models, where TBI is induced through a craniotomy [62]. Among closed head models, a weight drop on the skull is a common method, producing a focal injury to the ipsilateral cortex and hippocampus [62]. These weight drop methods can be further sub-categorized; for example the Marmarou weight drop model is a common method involving a helmet affixed to the rodent's head in order to produce a diffuse TBI. Open head injury models, such as lateral fluid percussion (LFP) and controlled cortical impact (CCI) yield focal injuries in the cortex, with more severe injuries potentially damaging the underlying hippocampus. The uniformity and consistency of injury can vary based on the method and the use of implements such as a stereotaxic frame.

It is important to highlight that no single injury model can replicate the wide spectrum of injury biomechanics seen in human TBI. However, preclinical models can reproduce and elucidate details of relevant pathophysiology observed in human TBI patients. This provides the opportunity to standardize preclinical studies in order to prevent the heterogeneity of etiology and clinical complexity in human TBI. For these reasons, the majority of TBI research is undertaken in animal models [63,64].

Assessing neurological outcomes to evaluate therapeutic efficacy is undertaken by testing reflexes or motor function, signs of anxiety or depression, and cognitive function or memory [27]. However, it is essential to recognize that most models induce transient impairment of reflexes and motor function that spontaneously recover in the days following TBI. Consequently, assessing the effectiveness of a drug through the measurement of

reflexes or motor impairments carries the implication that it is the rate of recovery that is being evaluated. Unlike deficits in reflexes and motor functions, impairments in cognitive and memory faculties can be permanent and provide opportunities to measure the neuro-protective potential of a drug. Models of TBI elicit measurable anxiety or depression-like responses in animals that mirror the alterations in emotional state that are frequently observed following clinical TBI. Tests of cognition or affect involve the animal moving around a maze or apparatus, making it necessary to couple the cognition test with a test confirming the absence of motor deficits to avoid false-positive cognitive deficit results.

5.2. Findings in Preclinical Studies of Selective COX-2 Inhibitors

Investigations of the modulation of TBI sequelae has targeted several categories of agents, with a collection of experimental studies examining the effects of selective COX-2 inhibitors. These COX-2-selective drugs have included carprofen, celecoxib, diclofenac, DFU, meloxicam, nimesulide, and rofecoxib, across various animal models of TBI. These agents have been administered either prior to injury or within a 30 min post-injury timeframe. Table 1 displays the details of these investigations and the pathological and functional outcomes.

Table 1. Selective COX-2 treatment in rodent models of TBI.

Drug	Animal	Mechanism	Dosage Timeframe	Inflammation	Pathology	Neurological Outcome	Reference
Carprofen	Mouse	WD	5 min post	↓ Iba-1 ↓ IL-1β ⇒ IL-4 ↓ IL-6 ⇒ IL-10	↓ Edema ↑ Gliogenesis ↓ Lesion area	↓ NSS	Thau-Zuchman et al. (2012) [65]
Celecoxib	Rat	CCI	Pre-injury	-	-	↓ Motor ⇒ MWM ⇒ Conditioned emotional response	Dash et al. (2000) [66]
Celecoxib	Mouse	M-WD	Pre-injury	↓ Gfap ↓ Iba-1 ↓ TNF	↓ MAPT	↓ MWM	Hiskens et al. (2021) [67]
Celecoxib	Rat	WD	Pre-injury	↓ IL-1β ⇒ IL-10	-	-	Khaksari et al. (2012) [68]
Diclofenac	Rat	CCI	Immediate	-	↓ Apoptosis ↓ Lesion area ⇒ Neuronal degeneration	-	Dehlaghi et al. (2019) [69]
DFU	Rat	CCI	10 min pre or 6 h post	-	↓ PGE ₂ ↓ Caspase-3 ↑ 2-AG	↓ Neuro score ⇒ Beam walk ⇒ Open-field ↓ MWM	Gopez et al. (2005) [70]
Meloxicam	Rat	M-WD	30 min post	↓ Lipid peroxidation ↓ GSH ⇒ Na K ATPase	↓ Edema ↓ BBB	↓ NSS	Hakan et al. (2010) [35]
Meloxicam	Mouse	WD	10 min post	-	↓ 6-keto PGF _{1α} ⇒ Edema	⇒ Neuro score	Girgis et al. (2013) [71]
Nimesulide	Rat	M-WD	30 min post	-	-	↓ Barnes maze ↓ Rotarod	Cernak et al. (2001) [72]

Table 1. Cont.

Drug	Animal	Mechanism	Dosage Timeframe	Inflammation	Pathology	Neurological Outcome	Reference
Nimesulide	Mouse	WD	10 min post	-	↓ 6-keto PGF _{1α} ⇒ Edema	⇒ Neuro score	Grigis et al. (2013) [71]
Rofecoxib	Rat	LFP	5 min post	-	⇒ Neuronal degeneration	-	Kunz et al. (2006) [73]
SC58125	Rat	CCI	15 min post or 24 h post		↓ PGE ₂ ⇒ Lesion area	↑ MWM	Hickey et al. (2007) [74]

Abbreviations: ↑, increased outcome measure; ↓, decreased outcome measure; ⇒, no change in the outcome measure; 2-AG, 2-arachidonoyl glycerol; BBB, blood–brain barrier; CCI, controlled cortical impact; Gfap, glial fibrillary acidic protein; IL, Interleukin; IL-1β, Interleukin 1 beta; Iba-1, Ionized calcium-binding adapter molecule 1; LFP, lateral fluid percussion; MAPT, microtubule-associated protein tau; M-WD, Marmarou weight drop; MWM, Morris water maze; NSS, neurological severity score; PGE₂, prostaglandin E₂; TNF, tumor necrosis factor; WD, weight drop.

5.3. Pathology

A diverse range of pathological outcomes has been investigated to assess the efficacy of these COX-2-selective inhibitors. Protein expression of COX-2 has been measured in a number of studies, with differing results. When injected directly into the site of focal penetrating TBI, diclofenac exhibited no reduction in COX-2 [69], and rofecoxib also had no impact on COX-2 expression [73]. In contrast, DFU treatment decreased COX-2 levels when administered 10 min prior to trauma, but not when administered 2–6 h after TBI [70].

Several measures of inflammation have been investigated. Microglial activation as measured by Iba-1 expression has been shown to be reduced at 4 h post-injury with carprofen [65] and 48 h post-injury with celecoxib treatment [67]. Carprofen and celecoxib also exhibit a notable inhibition of the proinflammatory cytokine IL-1β [65,68]. However, there were no differences in the brain IL-10 levels after TBI with carprofen or celecoxib treatment. DFU, meloxicam, and nimesulide effectively reduced prostaglandin production [35,65,66,70,71]. By reducing prostaglandin production following TBI, arachidonic acid metabolism may be directed toward a neuroprotective eicosanoid pathway.

Several studies examined the effects of treatment on tissue injury. Diclofenac-mediated COX-2 inhibition decreased apoptosis in injured rats, while modulating secondary injury mechanisms involving microglial cells and astrocytes [69]. Gopez et al. also showed that DFU was effective in reducing cell death [70]. Carprofen treatment resulted in decreased lesion size in a weight drop model of TBI [65]. Rofecoxib, when administered 5 min post-injury, did not show an effect upon hippocampal neuronal loss [73]. Nimesulide exhibited no discernible impact on edema, and the effect of meloxicam on edema remains inconclusive, with Hakan et al. [35] observing a reduction in TBI-related edema after meloxicam administration, contrasting with Girgis et al.’s findings of no change in edema with meloxicam treatment [35,71]. Hakan and colleagues proposed that the observed neuroprotective benefits of meloxicam could be partially attributed to its antioxidant characteristics, in addition to the anti-inflammatory effects of COX-2 inhibition. In this way, it could be that COX-2 inhibitors contribute to neuroprotection by curbing the excessive production of free radicals following TBI.

5.4. Functional Outcomes

There is significant variability in the performance of these selective COX-2 inhibitors in functional outcomes involving motor function. Reflexes and motor function were primarily assessed via the neurological severity score (NSS), which was improved following TBI with administration of carprofen and DFU [65,70]. There were discrepant NSS findings with meloxicam, with improvement in the study by Hakan and colleagues and no change in the study by Girgis et al. [35,71]. Nimesulide treatment effected no change in NSS; however, there was improved rotarod performance [71,72]. DFU treatment resulted in no change in

beam walk function [70], while Dash et al. revealed a worsening of motor function in rats post-TBI with celecoxib [66].

Cognitive function and memory were assessed using the Morris water maze (MWM) and the Barnes maze. Cognitive performance following celecoxib treatment did not improve MWM performance in the study by Dash et al. [66] and impaired MWM performance following repetitive weight drop mTBI [67]. Gopez et al. found that DFU improved MWM performance, while nimesulide treatment improved Barnes maze performance [70,72].

5.5. Evaluation of Preclinical Studies

Two notable concepts emerge from the findings of these preclinical TBI studies: (1) the wide range of effectiveness of the different agents across pathological and functional outcomes, and (2) the disconnect between improvements in histological neuronal damage and the lack of functional improvement, highlighting the complexity of assessing therapeutic outcomes [75,76]. The discrepancies in these animal model findings could be multifactorial. One aspect could be the role of the age of the animals, which was shown by Hickey and colleagues to play a significant role in the expression of COX-2 following injury [74]. Neonatal and 90-day-old mice showed less TBI-induced COX-2 expression than mice between the ages of 14 and 60 days [74]. This varied COX-2 expression will therefore impact the effectiveness of pharmacological intervention. Other significant factors involve the variation in the methodology of drug administration, encompassing differences in route, dosage, and timing of delivery. Another significant source of variation is the differing mechanisms and sequelae of TBI, thus contributing to differences in TBI biomechanics, severity, and outcomes, further influencing the results.

Despite the often positive effect that selective COX-2 inhibitors enact upon cellular and histological measures of injury, these have failed to yield improvements in clinical and functional outcomes. This suggests that the anti-inflammatory effect, targeted at mitigating damage induced by TBI, proves ineffective in preventing damage across secondary injury cascades, ultimately leading to functional impairment. However, our understanding of the cascade of interaction resulting from treatment with these agents, and their exact mechanisms of actions, remains limited. For example, in the diclofenac treatment study, while COX-2 expression increased at the injury site, the effects of the drug were not only attributed to COX-2 inhibition. Instead, they involved the indirect modulation of inflammatory responses in astrocytes and microglial cells [69]. This suggests a strategy for targeting TBI through selective COX-2 inhibitors, even when not specifically directing treatment at COX-2 pathways. A more complete understanding of these interactions is required for designing COX-2 inhibition studies as an effective treatment of TBI and for enacting clinical improvement.

5.6. The Role of COX-1 in Brain Injury

While COX-2 is the predominant isoform in the brain, COX-1 may also play a role in brain neuroinflammation. Data from human TBI reveal the upregulation of COX-1+ microglia and macrophages in perilesional areas affected by the acute inflammatory response to TBI [77]. This is supported by preclinical data indicating COX-1 upregulation at the site of injury [78]. This indicates that COX-1 expression in the brain exhibits distinctions from its constitutive expression in peripheral tissues.

Pharmacological inhibition of COX-1 has been investigated in a rat model of TBI via treatment by the selective COX-1 inhibitor SC560, resulting in improvements in motor, spatial learning, and memory tasks [79]. These findings imply a role for COX-1 in the cognitive deficits associated with TBI. Additionally, the dual inhibition of COX-1 and COX-2 has been investigated in the context of TBI [71,80]. The non-selective COX inhibitors indomethacin and ibuprofen were administered prophylactically or within 10 min of injury and demonstrated a robust anti-inflammatory effect by inhibiting key mediators such as IL-1 β , IL-6, IL-10, and prostaglandins in both serum and brain tissue [68,71,80]. However,

indomethacin and ibuprofen showed a limited ability to improve other outcomes such as edema [71,80].

In understanding the comparative effects of COX-1 and COX-2, the study by Girgis et al. (2013) gives insight into the selective and non-selective effects of COX enzymes. While treatment with meloxicam and nimesulide did not reduce neurological impairment following TBI, the non-selective COX inhibitor indomethacin significantly improved neurological outcomes [71].

Collectively, these studies illustrate the anti-inflammatory effect of NSAIDs in experimental TBI. However, this anti-inflammatory effect, while robust, appears insufficient to completely mitigate tissue injury and resultant functional deficits. Consequently, these investigations challenge the viability of targeting COX1 or COX2 as a standalone and effective therapeutic approach for TBI.

6. Human Studies

To date, there have been limited clinical investigations of the treatment of TBI with selective COX-2 inhibitors. Indeed, a systematic search of the US National Institutes of Health clinical trials database using the keywords traumatic brain injury, TBI, cyclooxygenase, COX-2, and the individual drug names carprofen, celecoxib, diclofenac, DFU, meloxicam, nimesulide, and rofecoxib revealed no current registered clinical trials.

In a small human trial, the COX-2 inhibitor SC-58125 was shown to reduce excitation-induced neuroinflammatory damage following moderate brain injury [81]. Twice-daily COX-2 inhibition lowered blood glutamate levels which were increased following a moderate TBI [81]. However, this investigation did not evaluate clinical outcomes.

Recently, a retrospective cohort study investigated the outcomes of TBI patients treated with the COX-2-inhibiting agent celecoxib and the non-specific COX inhibitor ibuprofen [82]. The study interrogated a United States healthcare database to analyze data from 1443 patients over the age of 18. The investigation revealed that patients receiving celecoxib within five days post-TBI exhibited a heightened 1-year survival probability in comparison to their untreated counterparts. Additionally, the celecoxib-treated cohort demonstrated a reduced likelihood of gastrostomy tube dependence, myocardial infarction, and seizures. The study also demonstrated that ibuprofen use within five days of TBI was associated with a higher 1-year survival probability and lower complication rates related to craniotomy/craniectomy, seizure, deep vein thrombosis, and ischemic stroke.

This first population-level study of acute COX inhibition in TBI provides important data on the corresponding clinical outcomes. In translating preclinical mechanistic findings, one can hypothesize that the observed findings of celecoxib treatment with reduced seizure incidence and enhanced 1-year survival probability may be attributed to a potential modulation of neuroinflammation. The details of how the effects of COX-2 inhibitors are specifically linked to pathways such as the downregulation of proinflammatory cytokines and attenuation of edema will be of interest in future prospective studies.

7. Discussion and Future Perspectives

The animal model and clinical studies undertaken to date provide a complex landscape in which to interpret the efficacy of COX-2 inhibition in TBI. The preclinical studies suggest COX-2 inhibitors provide effective treatment at a cellular and histological level, but this does not readily translate into improved functional outcomes. However, human data show improvements in key metrics of survival and comorbid outcomes. In the preclinical setting, the effectiveness of COX-2 inhibition is heavily influenced by the model of TBI, gender, and age.

Following TBI, the increase in arachidonic acid leads to the enhanced expression of COX-2, resultant prostaglandin and thromboxane production, and ultimately, the induction of inflammatory cytokines such as TNF, IL-1 β , and IL-6. The expression of these inflammatory cytokines in the brain contributes to physiological consequences such as cerebral edema, increased intracranial pressure, and neuronal dysfunction [65]. By inhibiting COX-

2, celecoxib and other COX-2 inhibitors likely disrupt this pathogenic cascade, thereby offering therapeutic benefits. The studies in this review suggest evidence of this related to several mechanisms. For example, celecoxib attenuates injury-induced prostaglandin production in the brain, which may result in the inhibition of the proinflammatory cytokine TNF, which has been demonstrated to effect blood–brain barrier integrity, edema formation, and hippocampal neuronal loss following TBI [65]. Likewise, IL-1 β mediates inflammatory responses after TBI. Thus, COX-2 inhibitors such as celecoxib, which significantly decreased brain IL1- β levels, may exert some of their effects through this mechanism.

In exploring the preclinical data, the initial inflammatory response post-injury serves a protective role and altering COX-2 release in response to injury may inadvertently lead to deleterious consequences. For instance, the rapid elevation of brain prostaglandin levels following injury, accompanied by a reflective increase in thromboxane, is thought to mitigate the risk of brain hemorrhage [70]. Additionally, blocking PGE₂ production with COX-2 inhibition has been shown to exacerbate the neuroinflammatory response [83], raising concerns that COX-2 inhibition may, in certain instances, induce detriment rather than benefit. Consequently, the introduction of COX-2 inhibitors may disrupt some adaptive processes, potentially leading to an acute exacerbation of secondary damage processes.

The early preclinical COX-2 treatment studies used pan-microglial markers such as Iba-1, meaning that the investigation could not differentiate between M1 and M2 microglial subsets [84,85]. It is noteworthy that the microglial markers utilized in these studies do not discriminate between resident microglia and infiltrating macrophages post-injury. Consequently, the conclusions drawn from studies exclusively using pan-microglial markers may not definitively establish whether a therapeutic agent exerts a proinflammatory or anti-inflammatory effect on microglia [84,86]. On the other hand, some studies measured specific inflammatory mediators associated with the M1 (iNOS) or M2 (IL-10) microglial phenotypes but did not elaborate on how the drug modulates overall microglial activation. Future examinations of microglial subsets should provide mechanistic evidence of how these COX-2 inhibitors exert their effects in limiting TBI pathology.

The time-sensitive nature of inflammation initiation after TBI necessitates immediate intervention in mitigating brain damage. Prophylactic treatment was used in some preclinical studies as an approach to mitigate this, leveraging the well-established safety profile of COX-2 inhibitors, while other preclinical studies used immediate post-injury timing. In clinical situations, administering the drug post-injury poses challenges in achieving initiation within the narrow post-injury window, and missing this critical timeframe significantly diminishes treatment efficacy [58]. Understanding the parameters for effective timing within the therapeutic window of these agents should be a key focus of future prospective studies. Additionally, other essential pharmacological parameters should be examined including dosage route, number, and timing. These details will refine our understanding of the diverse pharmacological aspects of treatment and allow for the translation of promising preclinical findings into clinically effective interventions.

Gender plays a role in the inflammatory response to TBI, with females better protected than males in experimental models, although the reasons for this are not fully known [87]. Likewise, COX-2 regulation exhibits gender-specific differences in TBI. Specifically, in a rat CCI model, males showed increased COX-2 expression, which correlated with elevated apoptotic cell death [88]. The gender-specific aspect of the secondary inflammatory response may be linked to prostaglandin regulation, potentially contributing to gender-related outcome disparities following TBI. Thus, the influence of gender on COX-2 pathways and injury is an important consideration for understanding the effectiveness of pharmacological intervention.

The safety aspect of COX-2 inhibitors is an important consideration for use. While celecoxib is a widely used agent with a well-established profile, the use of COX-2 inhibitors has been linked with adverse cardiovascular events for patients with increased risk factors [82]. Therefore, for patients who have known cardiovascular disease and elderly patients, celecoxib may be contraindicated for TBI treatment due to the higher

risk of myocardial infarction. This risk is likely related to the inhibition of endothelial COX-2-derived prostacyclin but not platelet COX-1-derived TxA₂. Interestingly, the clinical study by Bhanja and colleagues revealed an association between celecoxib use and lower rates of myocardial infarction [82]. These authors postulated that patients with known cardiovascular risk were treated more conservatively with COX2-inhibiting agents, or perhaps that celecoxib use might regulate TBI-induced coagulopathy. This risk paradox should be investigated in future treatment studies.

While not addressed in the preclinical or clinical work performed to date, the long-term effects of COX-2 inhibitor treatment for TBI will be an important consideration as a risk management tool for the development of the neurodegenerative condition CTE. While large-scale clinical trials of COX-2 inhibitors have not shown clear benefits in AD [89,90], these investigations have recruited patients with advanced disease, characterized by the presence of neurofibrillary tangles and neuritic plaques. The cellular inflammation pathways shared between AD and CTE highlight the necessity for the investigation of COX-derived CTE therapeutics, leveraging insights gained from extensive studies utilizing COX blockade in AD models [91]. An avenue for future investigation will involve exploring the potential of quenching early inflammatory cascades as a strategy for long-term protection against neurodegenerative conditions.

8. Conclusions

COX-2 inhibitors play a significant role in the context of TBI due to the role of COX-2 in producing prostaglandin metabolites and ROS that can exacerbate brain injury. The use of COX-2 inhibitors has been shown to attenuate neuropathology in animal models but is inconsistent in its ability to improve cognitive functions and motor performance. Additionally, the exact function of COX-2 in post-traumatic neuroinflammation remains unclear. In clinical use, the COX-2 inhibitor celecoxib has demonstrated improved survival for TBI patients. Given the complex but promising findings, future prospective studies are important to understand the therapeutic potential of COX-2 inhibitor treatment.

Author Contributions: M.I.H. conducted the literature search and wrote the manuscript. A.G.S. and A.S.F. reviewed the manuscript for important intellectual content. All authors have read and agreed to the published version of the manuscript.

Funding: The APC was funded by a Mackay Hospital and Health Service SERTA Grant (MIRI 2024-02).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

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

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Review

Mesenchymal Stem Cell-Derived Exosomes as a Neuroregeneration Treatment for Alzheimer's Disease

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Abstract: Background: Alzheimer's disease (AD) is the most prevalent kind of dementia and is a long-term degenerative disease. Pathologically, it is defined by the development of extracellular amyloid- β plaques and intracellular neurofibrillary tangles made up of hyperphosphorylated tau protein. This causes neuronal death, particularly in the hippocampus and cortex. Mesenchymal stem cell (MSC)-derived exosomes have been identified as possibly therapeutic and have promise for Alzheimer's disease due to their regenerative characteristics. Methods: A systematic retrieval of information was performed on PubMed. A total of 60 articles were found in a search on mesenchymal stem cells, exosomes, and Alzheimer's disease. A total of 16 ongoing clinical trials were searched and added from clinicaltrials.gov. We added 23 supporting articles to help provide information for certain sections. In total, we included 99 articles in this manuscript: 50 are review articles, 13 are preclinical studies, 16 are clinical studies, 16 are ongoing clinical trials, and 4 are observational studies. Appropriate studies were isolated, and important information from each of them was understood and entered into a database from which the information was used in this article. The clinical trials on mesenchymal stem cell exosomes for Alzheimer's disease were searched on clinicaltrials.gov. Results: Several experimental investigations have shown that MSC-Exo improves cognitive impairment in rats. In this review paper, we summarized existing understanding regarding the molecular and cellular pathways behind MSC-Exo-based cognitive function restoration, with a focus on MSC-Exo's therapeutic potential in the treatment of Alzheimer's disease. Conclusion: AD is a significant health issue in our culture and is linked to several important neuropathological characteristics. Exosomes generated from stem cells, such as mesenchymal stem cells (MSCs) or neural stem cells (NSCs), have been examined more and more in a variety of AD models, indicating that they may be viable therapeutic agents for the treatment of diverse disorders. Exosome yields may be increased, and their therapeutic efficacy can be improved using a range of tailored techniques and culture conditions. It is necessary to provide standardized guidelines for exosome manufacture to carry out excellent preclinical and clinical research.

Keywords: Alzheimer's disease; mesenchymal stem cells; drug discovery and development; neurosurgery; neurology; novel therapies

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia and is a chronic progressive disease [1]. With advanced age being the strongest risk factor, AD affects one in nine people aged 65 or older, and over 6 million people currently suffer from AD in the USA alone [2]. The term "Alzheimer's disease" (AD) dementia describes a specific neuropathology together with an age-related start and progression of cognitive and functional impairment. Alois Alzheimer initially wrote about it in 1906, describing a case he had seen in 1901. In addition to criteria designed for modern clinical diagnosis, criteria for using biomarkers to identify preclinical, or presymptomatic, phases of the illness have

also been devised. Alzheimer defined the fundamental neuropathology, which later developed into a more precise neuropathologic diagnosis in the mid-1980s that acknowledges the concomitant neuropathologies that usually lead to clinical dementia [2,3]. Given the direct relation of age to the disease, AD has rapidly become a major public health problem worldwide, and the burden of AD is expected to increase with the ageing population with an estimation of 152 million by 2050 [2]. AD is now considered the fifth leading cause of death in the United States, with deaths doubling from 2000 to 2021 [3] and reported deaths increasing by 140% [2], underscoring the urgent need for early detection and new therapeutic interventions [4]. Pathologically, AD is characterized by the presence of extracellular amyloid- β plaques and intracellular neurofibrillary tangles made up of hyperphosphorylated tau protein, which results in neuronal death, especially in the hippocampus and cortex [5,6]. This neuronal degradation is expressed clinically as memory dysfunction, frontal lobe dysfunction, aphasia, and agnosia [7,8]. Risk factors include age, diabetes, cardiovascular disease, and genetic factors such as Down syndrome [9,10]. Additionally, studies have indicated that other factors such as physical inactivity and poor diet play a role in the development of AD [10,11]. Early in the disease, there are deficits in the capacity to encode and retain new memories. The latter phases are accompanied by gradual changes in cognition and behavior. Reduced synaptic strength, synaptic loss, and neurodegeneration are the results of altered amyloid precursor protein (APP) cleavage, the APP fragment beta-amyloid ($A\beta$), and hyperphosphorylated tau protein aggregation. Important elements of the illness process include concomitant diseases, alterations in metabolism, vascular function, and inflammation [11].

To this day, there is no cure for AD, and current therapeutic drugs, like Donepezil and Rivastigmine, only aim at alleviating the symptoms and temporarily improving cognitive function without arresting the progression of AD or repairing the massive neuronal loss that defines the disease [12–14]. Additionally, these drugs may have poor outcomes and side effects in elderly patients such as syncope and reduced cardiac output that further call for the importance of developing new therapeutic approaches [15]. The present research is investigating several new strategies, such as the regulation of neuroinflammation and the prevention of amyloid beta formation, but these procedures are still in the experimental phase [16].

Recent research has highlighted the potential of mesenchymal stem cell-derived exosomes (MSCs) as a novel therapeutic approach for AD. MSCs offer several advantages that make them promising candidates for AD treatment. First, they can release anti-inflammatory substances that decrease neuroinflammation—one of the key processes in the formation and progression of AD [17,18]. MSCs may help to arrest the progression of the disease by reducing the levels of chronic brain inflammation and transforming into neuron-like cells that may help in replacing the damaged or missing neurons, thereby helping in regaining the lost memory [19].

Recent research has also demonstrated that MSCs can also help in the prevention of the formation of amyloid beta plaques, which is one of the key factors of AD, and which, if prevented, could slow the progression of the disease and enhance the results of the treatment [20,21]. Moreover, the immunogenicity of MSCs is relatively low, which means that the body's immune system will not reject the MSCs, and hence, MSC therapy is safe and effective for the treatment of AD [22,23].

Our study aims to explore and discuss the pathophysiology of AD and review recent findings on the therapeutic potential of MSC-derived exosomes, highlighting their role as a promising avenue for future treatment options.

2. Pathophysiology of AD

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive memory loss and cognitive impairment. The pathophysiology of AD is primarily caused by three main hallmarks: the accumulation of amyloid beta ($A\beta$) plaques, also known as senile plaques; the presence of neurofibrillary tangles of hyperphosphorylated tau protein;

and the marked neuronal degeneration. A β proteins are chemically “sticky” and generally build up to plaques that, if accumulated in the brain, can clump and block cell-to-cell signaling at synapses [1]. They also activate the brain’s immune system, which triggers inflammatory responses that further damage the disabled neuron cells [24,25]. Neurofibrillary tangles are tangles of the protein called tau, which is a microtubule-associated protein that stabilizes neuronal microtubules under normal physiological conditions; however, in AD, tau becomes phosphorylated, causing toxic aggregates that deposit within the neuron.

These pathological changes are associated with the loss of cholinergic neurons, synaptic dysfunction, and glial activation, contributing to widespread atrophy of the hippocampus and subsequently the cerebral cortex. While the exact pathophysiology of AD as well as its treatment remains a mystery, there are two proposed hypotheses based on these pathologic abnormalities.

The Cholinergic Hypothesis: states that reduced levels of acetylcholine caused by neuronal loss play a crucial role in the development of Alzheimer’s disease. Acetylcholine is important for several physiological processes such as memory, attention, learning, and other critical cognitive functions hence why Beta-amyloid is believed to affect cholinergic function and impair acetylcholine release negatively [26].

The Amyloid Hypothesis: the widely accepted hypothesis suggests that Alzheimer’s Disease (AD) is caused by the accumulation of amyloid beta (A β) peptides, particularly A β 42, which are derived from the amyloid precursor protein (APP) through the actions of β - and γ -secretase enzymes. Elevated levels of A β 42 lead to the formation of toxic amyloid aggregates that damage neurons [26,27].

Braak and Braak Staging

A staging method developed in the late 1980s by two scientists, T Heiko Braak and Eva Braak, divides the growth of neurofibrillary tangles into six phases. The National Institute on Aging and the Reagan Institute give generally acknowledged diagnostic criteria [28]. Neurofibrillary tangles show a better link with dementia severity in Alzheimer’s patients than amyloid plaques, yet amyloid remains a prominent hallmark of the illness. The pathogens of AD are sometimes compared to a “trigger and bullet” scenario [29]. Amyloid is thought to be the initial cause of the sickness. At the same time, tau, in the form of neurofibrillary tangles, works like a bullet that causes neurodegeneration and cognitive impairment. Accumulation of amyloid beta (A β) in cerebral blood vessels, known as cerebral amyloid angiopathy (CAA), can accelerate cognitive and memory impairment in Alzheimer’s disease patients [30].

3. Methods

The literature review was conducted in PubMed. The search terms used for this search were “alzheimers disease”, “AD”, “Mesenchymal Stem Cell”, “Exosomes”, “AD treatment”, “pathophysiology”, and “Advances in AD treatment”. This generated an initial search result of 244 articles. Articles were then screened for relevancy and then by abstract contents. These articles were filtered through to exclude keyword-mismatched articles, articles whose full texts were not available, articles not in the English language, and articles not related to AD. These came out to be 60 articles. A total of 16 ongoing clinical trials were searched and added from clinicaltrials.gov. We added 23 supporting articles to help provide information for certain sections. In total, we included 99 articles in this manuscript: 38 are review articles, 13 are preclinical studies, 28 are clinical studies, 16 are ongoing clinical trials, and 4 are observational studies.

After this, relevant information from each article was entered into an Excel sheet with each section of this manuscript as a separate sheet to ease the process of data extraction for relevant sections.

4. Neuroregeneration Therapy

4.1. Stem Cells

Stem cells are a unique type of cells with the ability to proliferate, self-renew, and differentiate into various mature cell types. Stem cells have been used for decades, especially in Parkinson's disease (PD), with significant success in numerous cell transplantation studies [23,31,32]. Therapeutic strategies involve direct cell replacement, secretion of neurotrophic and growth factors, and activation of endogenous neural precursor cells [33–35]. Regarding human embryonic stem cells' clinical use, two difficulties exist: transplant rejection following implantation and ethical dilemmas surrounding the use of human embryos. Many teams have been attempting to use nuclear transfer to create hESCs from a patient's somatic cells to get around these problems. Producing human nuclear transfer embryos is still a difficult technological task [36,37]. The ability of pluripotent stem cells (PSCs) to proliferate indefinitely has been a significant benefit, enabling the preparation of billions of diverse human cell types for transplantation. This characteristic has two drawbacks too, since tumors might develop if the cells continue to multiply even after transplantation [36]. There are three tumorigenic situations to think about. First, teratomas or tumors may develop as a result of improper patterning if undifferentiated and/or immature cells are kept in the final cell products that have been differentiated from human PSCs. Second, reprogramming factors may encourage carcinogenesis if they continue to be active in the iPS cells. Third, genetic alterations that have happened during PSC in vitro cultivation may be the source of tumorigenicity. Exosomes generated from mesenchymal stem cells (MSCs) have been shown to avert memory losses in an animal model of Alzheimer's disease (AD). Except for the intended brain locations, other tissues have shown robust tracking of intravenously delivered exosomes. Here, we suggested targeting intravenously infused exosomes produced from MSCs (MSC-Exo) to the brain of transgenic APP/PS1 mice using the central nervous system-specific rabies virus glycoprotein (RVG) peptide. RVG and MSC-Exo were conjugated via a DOPE-NHS linker [35]. After intravenous administration, RVG-tagged MSC-Exo demonstrated enhanced targeting to the cortex and hippocampus. When RVG-conjugated MSC-Exo (MSC-RVG-Exo) was delivered to the group instead of MSC-Exo, there was a significant decrease in astrocyte activation, A β levels, and plaque formation. The Morris water maze test showed that brain-targeted exosomes produced from MSCs were superior to unmodified exosomes in improving cognitive performance in APP/PS1 mice. Moreover, while the intravenous injection of MSC-Exo decreased the production of pro-inflammatory mediators such as TNF- α , IL- β , and IL-6, the anti-inflammatory cytokines IL-10 and IL-13 did not exhibit significant alterations. On the other hand, MSC-RVG-Exo treatment markedly increased the levels of IL-10, IL-4, and IL-13 and markedly decreased the levels of TNF- α , IL- β , and IL-6 [35].

Human bone marrow-derived MSCs were cultivated in three-dimensional (3D) cell culture to assess the possible therapeutic benefits of MSC extracellular vesicles (EVs). Small EVs were then extracted by differential ultracentrifugation. For 4 months, non-transgenic (NT) or 5XFAD (familial Alzheimer's disease mutation) mice received these tiny EVs intraperitoneally (IN) once every 4 days. After that, a range of behavioral tests were given to the mice to assess how their learning and memory had changed. Brain slices were then subjected to immunohistochemistry to determine the amounts of glial fibrillary acidic protein (GFAP) and amyloid beta (A β) [36]. The results showed that 5XFAD mice treated with hMSC-EV exhibited considerably improved behavior in cognitive tests when compared with 5XFAD mice treated with saline; however, there was no significant difference in behavior between EV-treated 5XFAD mice and NT animals. Furthermore, we observed a reduced A β plaque burden in the hippocampal regions of the mice treated with EV. Lastly, GFAP and A β plaque colocalization was reduced in the brains of mice treated with EV as opposed to saline [36].

There are multiple types of stem cells, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), neural stem cells (NSCs), and mesenchymal stem cells (MSCs). In our study, we focus on evaluating the mesenchymal stem cell's proposed role in

treating Alzheimer's disease. MSCs are adult multipotent cells that can be obtained from adult tissues such as bone marrow, skin, umbilical cord, adipose tissue, and spleen [36]. They can regenerate into different cell types, such as bone, cartilage, fat, lung, liver, and muscle [37]. They possess remarkable therapeutic potential, particularly in orthopedic applications. They also play roles in regenerative medicine and cancer treatment as anti-inflammatories, immunosuppressives, and vehicles for gene/protein therapy. Mesenchymal stem cells (MSCs) have been shown to promote the expression of anti-inflammatory factors like interleukin-10 and prostaglandin; however, it is important to explore the underlying mechanisms to determine if MSC transplantation directly influences inflammation or if the effects are due to tissue damage. Understanding this distinction is critical for optimizing MSC-based therapies. Further research is needed to clarify these mechanisms and their implications for treating neurodegenerative diseases [38]. In vitro, human MSCs can significantly increase the number of neurons in the hippocampus and induce neural precursor cells (NPCs) to differentiate into neurons via the Wnt signaling pathway.

Additionally, human MSCs can lower A β 42 levels by stimulating autophagy both in vitro and in vivo [39]. Figure 1 compares the neurons of a healthy cortex and those diseased by Alzheimer's. Figure 2 depicts the main pathological markers in a diseased Alzheimer's.

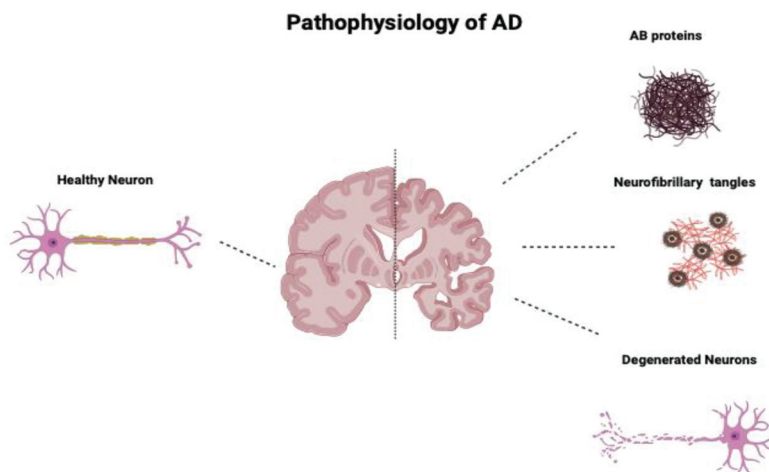


Figure 1. Comparison of neurons of a healthy cortex and those of a cortex diseased by Alzheimer's. AD's main pathologic changes are the accumulation of AB proteins, neurofibrillary tangles of tau protein, and loss and degeneration of neurons.

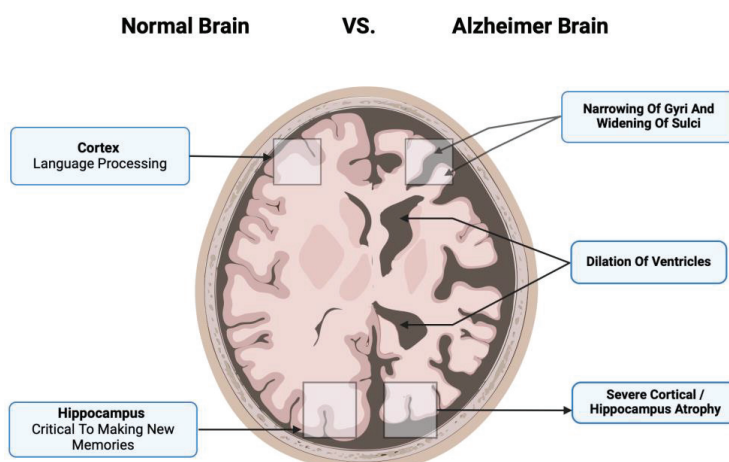


Figure 2. The main pathological markers in a diseased Alzheimer's cortex include the narrowing of gyri and sulci, significant dilation of ventricles, and severe cortical atrophy that involves important brain functions such as language processing and making new memories.

4.2. Exosomes

4.2.1. Isolation of Exosomes

Ultracentrifugation: A conventional technique frequently employed to separate exosomes generated from stem cells is ultracentrifugation. Researchers can process a huge amount of samples with this technology. Large debris is first removed with low centrifugal force; then crude exosomal fractions are pelleted with high centrifugal force. Exosomes that have been identified and deemed crude are utilized in research without additional purification or are refined using density gradient ultracentrifugation [40].

Size-Based Filtration: To exclude any further extracellular vesicles that are bigger than 150 nm or less than 50 nm, biofluid samples can be run via size-exclusion chromatography or certain pore-sized filters. Exosome enrichment is not possible with this technique. After these filtering stages, ultracentrifugation might be utilized if exosome enrichment is required [41].

Precipitation of Polymers: To collect the vesicles with an exosomal size range (30–150 nm) and decrease exosomal solubility, which permits exosomes to precipitate, biofluid samples are mixed with a polymer, such as polyethylene glycol (PEG), to use polymer precipitation techniques for exosome purification. With standard laboratory equipment, this method is possible, but it depends on the polymer net size [42].

Immunoaffinity: The ability to withstand the particular proteins (antigens) found on exosomal membranes serves as the foundation for this technique. Particular antibodies coupled to a carrier, such as agarose or magnetic beads, can be used to extract a particular subtype of exosomes with great purity [43]. This approach is extensively employed in many applications, including fundamental research and clinical investigations, such as illness diagnosis and prognosis, because it has no volume constraint and is easily carried out with ordinary laboratory instruments. However, the materials needed for this process are often pricey [44].

4.2.2. Cell Culture

Mesenchymal stem cells (MSCs) are grown under carefully controlled conditions to ensure that they develop properly for research or therapeutic purposes. Usually, MSCs are cultured in a basic growth medium like Dulbecco's Modified Eagle Medium (DMEM), which is often enriched with fetal bovine serum (FBS). Fetal bovine serum (FBS) is used to provide the nutrients and growth factors needed for the cells to grow well. To keep the cultures free from contamination, a mix of antibiotics and antifungal agents is added. These cells are usually maintained in an environment with controlled humidity and 5% CO₂ at a temperature of 37 °C, which closely resembles their natural surroundings. In clinical applications, it is crucial to decrease or eliminate animal-derived components, so serum-free media are often used. The cells are then cultured until they reach a certain level of growth, which ensures that they stay healthy and maintain their ability to develop into different types of cells [45].

4.2.3. Working Model Biogenesis, Secretion, and Uptake

To understand the process of exosome synthesis, secretion, and uptake, tremendous effort has been made. The early sorting endosomes (ESEs) are first formed by endocytosis of external components and cell surface proteins, together with the inward budding of the plasma membrane. Intraluminal vesicles (ILVs) are formed by the invagination of the limiting endosomal membrane during the maturation phase of endosomes [46]. Many molecular machinery components influence the production of ILVs, but the endosomal sorting complex needed for transport (ESCRT) machinery complex is the primary regulator of this process. About thirty proteins make up the ESCRT mechanism, which assembles into four complexes (ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III) and related proteins (including Vps4, Alix, and Tsg101) that are involved in the production of ILVs [47]. The ESCRT-I/II/III complex induces membrane deformation, ESCRT-0 sequesters ubiquitinated

cargo proteins, and the Vps4 complex facilitates vesicle scission and recycling of the ESCRT-III complex [48].

A different pathway of exosome biogenesis, including tetraspanins, ceramides, cholesterol, phosphatidic acids, and heat-shock proteins (HSPs), is produced apart from the processes of the ESCRT machinery [49]. RNA loading into exosomes via lipid mediation relies on cargo domains and self-organizing lipids. Then, cytoplasmic substances including RNA, proteins, and lipids are encased in the lumen and gathered inside the late endosome to form multivesicular bodies (MVBs) [50]. The Golgi complex and endoplasmic reticulum play a role in the process. Some MVBs are carried to lysosomes for disintegration by fusing with autophagosomes or not, while other MVBs fuse with the plasma membrane through the cytoskeletal and microtubule network of the cell, eventually releasing their vesicles into the extracellular environment as exosomes [51].

Ceramides are more abundant in secreted MVBs than in degradative MVBs. It has been suggested that the distinct outcomes experienced by MVBs might be connected to the coexistence of subpopulations inside cells. Exosomal markers include proteins including flotillin, Alix, TSG101, tetraspanins (CD9, CD63, and CD81), and the endosome pathway, which is involved in exosome creation and release [52]. Furthermore, ceramide and sphingomyelin, two components of the lipid raft, are highly concentrated in exosomes [53].

5. Exosomes as AD Biomarkers

Currently, biomarkers of AD pathology ($A\beta$ 1-42/1-40, T-Tau, and p-Tau), cognitive behavioral syndrome (CBS), and positron emission tomography (PET/CT) are the major methods used to diagnose AD. However, because AD has a latent onset, bioimaging (PET/CT) and CBS-based diagnosis are frequently delayed. Biomarkers for monitoring, particularly with CSF, are intrusive and cause harm to patients. Currently, there are no reliable techniques for diagnosing or predicting AD [54]. AD is diagnosed in the clinic using a variety of methods, such as bioimaging, biochemical analysis, and questionnaires. The results of bioimaging, such as PET or CT, might be influenced by other dementia disorders, and the procedure is expensive. Surveys are prone to subjectivity and are often influenced by the survey taker [54]. Neuron adhesion molecules and neurotransmitter receptors are two examples of the distinctive receptors found in nervous tissues present in exosomes generated from neurons. The mediating function of those receptors is essential for the interactions that exosomes have with target cells. They make it easier for exosomes to bind and be taken up selectively, which allows their “cargo” to be delivered to certain cellular targets [55].

These receptors’ presence on exosomes makes it easier to use them for diagnostic purposes in neurodevelopmental disorders (NDDS). Blood, urine, and saliva are just a few of the bodily fluids from which exosomes from AD patients may be separated. Therefore, the ease of collection and the non-invasiveness of exosomes, in addition to their stability following sample capture, further validate their usefulness in the field of AD and associated illnesses diagnoses. Ruihua Sun et al. showed that exosomes obtained from the blood of AD patients were reduced in size and number compared with those from healthy controls using transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA) [56].

Antonio Longobardi et al. discovered that AD patients’ blood had 40% fewer exosomes than that of healthy controls, which is in line with that conclusion [57]. Exosomes from AD patients, according to different research, were bigger than those from healthy controls. At the moment, there is insufficient evidence to substantiate the precise variations in exosome size between AD patients and healthy controls. Exosome size variation may be influenced by several variables, such as sample origins, methods of collection, and procedures of analysis. To confirm these variations, learn more about their significance in the pathophysiology of AD, and assess their potential diagnostic use, more research is required [58]. Exosome morphology may be one factor in the diagnosis of AD; however, standardizing the methods for extracting and examining exosomes is necessary. An essential part of exosomes is proteins. β -site APP cleaving enzyme 1 (BACE-1), soluble

peptide APP beta (sAPP β), soluble peptide APP alpha (sAPP α), γ -secretase, and A β 1-42 were detected in exosomes obtained from AD patients [59]. These findings are strongly associated with the etiology and development of AD.

Exosome lipids have the potential to be useful biomarkers for the diagnosis of AD. Su et al. discovered using semi-quantitative mass spectrometry that brain-derived exosomes from AD patients had considerably higher levels of lipids and plasmalogen glycerophosphoethanolamine (PE) molecules (p-36:2, p-38:4) on their membranes than those from the control groups [60]. Another type of “cargo” from exosomes, miRNAs, has drawn more attention because of their function in regulating gene expression. It was established that exosomes from AD patients have significantly different miRNAs compared with exosomes from healthy controls [61]. According to Liu et al., in exosomes from the serum of AD patients, 19 miRNAs (such as miR-15a-5p) were elevated while 5 other miRNAs (such as miR-15b-3p) were downregulated. Microarray analysis was used to examine the expression levels of miRNA in the CSF of AD patients [46,62]. Gamez-Valero et al. discovered that the expressions of miR-132-5p, miR-485-5p, and miR-125b-5p were up, while those of miR-16-2, miR-29c, and miR-331-5p were lower [63]. The “cargo” and amounts of biomarkers formed from exosomes changed, indicating their great potential for use in the diagnosis of AD. Exosomes obtained from diverse bodily fluids guarantee their accessibility and availability for diagnosis purposes.

Moreover, exosomes obtained from neurons and blood exhibit superior credibility in comparison with CSF biomarkers or PET/CT. Exosome markers can be combined with AD biomarkers, such as amyloid peptides or tau, and this combination can potentially improve AD diagnosis. Exosomes, which are small extracellular vesicles released by cells, carry molecular contents like proteins, lipids, and RNA, reflecting the state of their cells of origin. In Alzheimer’s, exosomes derived from neurons can contain amyloid beta and tau proteins, which are key pathological markers of the disease. By analyzing these exosomal contents in bodily fluids like blood or cerebrospinal fluid, it is possible to detect AD-related changes with high sensitivity [63]. Combining exosome markers with traditional biomarkers enhances diagnostic accuracy by providing a more comprehensive profile of the disease, potentially allowing for earlier detection and better monitoring of disease progression. Table 1 summarizes the biomarkers in diagnosing AD as reviewed by Guo et al. [17].

Table 1. Summary of biomarkers in the diagnosis of AD and their source. (↑—Increased, ↓—Decreased).

Source	Sample	Biomarker Protein Change
Neural	Plasma	P-T181-tau, P-S396-tau, and A β 1-42 ↑, NRG1, REST ↓ compared with CNC and stable MCI patients [64].
Neural	Plasma or serum	Total Tau, P-T181-tau, P-S396-tau, and A β 1-42 ↑ compared with controls [65]
Neural	Plasma	Cathepsin D, LAMP-1, ubiquitinated proteins ↑, and HSP70 ↓ compared with controls and FTD [66]
Neuronal	Plasma or serum	A β 42, T-tau, and P-T181-tau ↓ compared with a MCI and control group Intracerebroven [67]
Neuronal	Plasma	Synaptophysin, synaptopodin, synaptotagmin-2, and neurogranin ↓ compared with controls [68]
Neuronal	Plasma	NPTX2, NRXN2 α , AMPA4, NLGN1 ↓ [69]
Astrocyte	Plasma	complement proteins, IL-6, TNF- α , IL-1 β ↑; complement regulatory proteins (CD59, CD46, DAF), complement receptor type 1 ↓ compared with controls [70]
Astrocyte	Plasma	BACE-1, (s)APP β ↑, GDNF ↓ compared with controls [71]

Peripheral blood brain-derived exosomes have demonstrated significant promise as the perfect “liquid biopsy” for AD. Blood-derived exosomes are distinguished by very sensitive and specific low-invasive diagnostic techniques. A lumbar puncture, neuroimaging, cognitive testing, and symptoms all play a role in the clinical diagnosis of AD. Though their quantity is smaller than that of cerebrospinal fluid, brain-derived exosomes have the intriguing ability to cross the blood–brain barrier and enter peripheral blood circulation.

Researchers use immunoprecipitation techniques to separate brain-derived exosomes from plasma in order to get around these restrictions. Additionally, several studies indicate that CSF cannot reliably differentiate AD patients from patients with other forms of dementia due to the overlapping levels of A β 1-42, T-tau, and p-tau181 [55,56]. Interestingly, a multicenter investigation verifies the association between blood and CSF levels of AD-associated protein. According to earlier studies, neuron-derived exosomes (NDEs) have lysosomal and synaptic protein levels that can be used to predict the preclinical risk of dementia development from moderate cognitive impairment (MCI). Functionally specialized synaptic protein NDE levels may be a good indicator of how severe AD is developing. Additionally, there appears to be a correlation between the illness stage and the quantities of complement proteins in ADEs, which are exosomes generated from astrocytes. The cargo proteins found in plasma ADEs are much more than those seen in NDEs, suggesting a possible target for BACE-1 inhibitors. Large cohort studies are also required to evaluate the diagnostic usefulness of exosomes, as well as a consistent preparation and biomarker procedure [56–58].

6. Therapeutic Properties of Exosomes and Application in Alzheimer's Disease

MSCs are preferred to other stem cells, such as iPSCs or NSCs, for Alzheimer's disease treatment due to their strong immunomodulatory properties, which help reduce neuroinflammation, a key factor in AD progression. They secrete neurotrophic factors like BDNF and VEGF, supporting neuron survival, neurogenesis, and synaptic plasticity. Unlike iPSCs, MSCs have a lower risk of tumorigenicity, making them a safer option. They are also easier to source and expand from tissues like bone marrow and adipose tissue, with low immunogenicity allowing for allogeneic transplantation without extensive immunosuppression. Their established clinical safety, coupled with their therapeutic effects through paracrine signaling and extracellular vesicle release, makes MSCs particularly attractive for AD treatment compared with the more complex and risk-prone iPSCs and the more lineage-specific NSCs [21,72].

Mesenchymal stem cells (MSCs) have been presented as a potential treatment and have shown promise for AD for their regenerative properties, such as secretion of growth factors, anti-inflammatory proteins, membrane receptors, and microRNAs (miRNAs) that can block apoptosis, decrease neuronal loss, and stimulate neurogenesis, synaptogenesis, and angiogenesis [73]. Their anti-apoptotic and antioxidant qualities aid in preventing neuronal cell death. Furthermore, MSCs secrete growth factors that encourage neural progenitor cells to improve neurogenesis, such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF). MSCs produce neurotrophins, including VEGF, HGF, NGF, BDNF, and neurotrophin-3, after they migrate to injured brain regions and interact with brain cells [20,59,60,74]. These neurotrophins support neuritic formation and neurorestoration, which helps with neurological recovery.

Moreover, MSCs regulate the immune response by suppressing inflammatory microglia (M1) and activating anti-inflammatory microglia (M2), which helps in preventing tissue damage caused by chronic neuroinflammation. They also promote the accumulation of microglia around A β deposits to increase A β clearance and to stimulate autophagy that aids in the lysosomal removal of A β plaques. These actions contribute to the therapeutic potential of MSCs in neurodegenerative disease treatment [43,75].

Numerous studies reveal that soluble factors produced from MSCs can alter the neuroprotective characteristics of Alzheimer's disease (AD) models. For instance, Kim et al. reported that MSCs derived from human umbilical cord blood have a neuroprotective effect against A β toxicity in vitro by secreting galectin-3. Moreover, transplanting these MSCs into mice with AD transgenics causes microglia to produce MME/neprilysin, which improves A β clearance through soluble ICAM-1 secretion [76].

The characteristics of exosomes change depending on the cargo content, which is influenced by the extracellular environment and physiological conditions of the cell source. Therefore, it is crucial to take the effectiveness of MSC-exos into account when the en-

vironment changes. As stem cells, MSCs have a tremendous capacity for adaptability. The contents and biological activity of released exosomes are influenced by pretreatment MSCs in vitro, according to a number of recent investigations. Exosomes derived from hypoxia-preconditioned MSCs (PC-MSCs) have been shown to improve the treatment outcome in mice with AD transgenics. A pretreatment group benefits mostly in enhanced learning and memory, reduced A β buildup, elevated production of synaptic proteins, and reduced inflammatory response [35]. It is interesting to note that, under standard growth conditions, MSC-EVs did not exhibit the previously reported therapeutic benefits. It is also important to remember that MSCs probably change the properties of exosomes in pathological circumstances. Conversely, several investigations demonstrated that, by delivering physiologically active components, MSC-EVs changed the cellular metabolic milieu. Thus, the therapeutic potential of MSC-exos in AD may be improved by ongoing modifications of MSC pretreatment techniques, at least somewhat.

According to Lee et al., bone marrow-derived MSCs exert neuroprotective effects on AD models that are underpinned by cellular and molecular mechanisms, and CCL5, which is secreted from blood-derived MSCs, recruits alternative microglia to the AD brain, thereby reducing A β deposition and memory impairment through the production of IL-4 and MME [77,78]. These data indicate that MSC treatment in A β -treated cells remarkably boosts autolysosome formation and autolysosomal catabolic function, which contribute to enhanced neuronal survival. Figure 3 depicts different sources of mesenchymal stem cells and their properties of importance in AD.

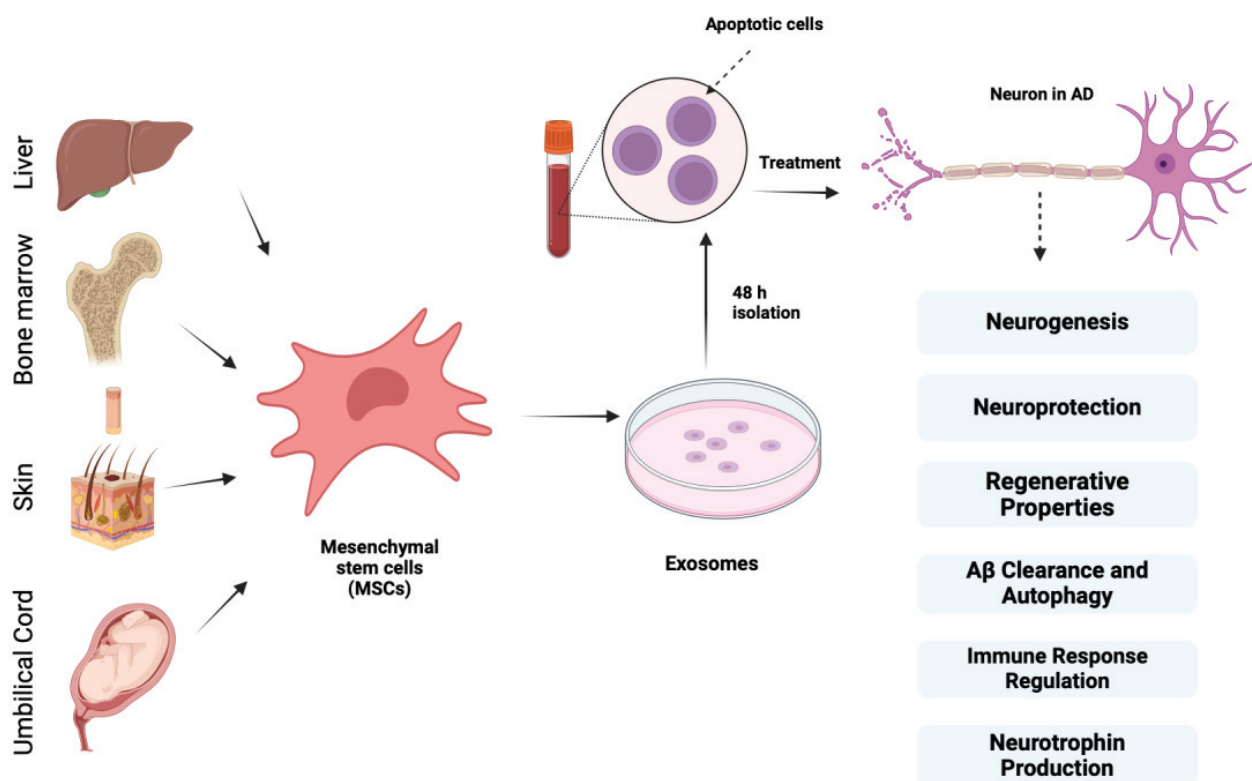


Figure 3. Different sources of mesenchymal stem cells and their properties of importance in AD treatment along with exosomes.

MSCs derived from various sources, such as bone marrow, adipose tissue, umbilical cord blood skin, and liver, exhibit regenerative properties by secreting growth factors, anti-inflammatory proteins, membrane receptors, and miRNAs that block apoptosis, decrease neuronal loss, and stimulate neurogenesis, synaptogenesis, and angiogenesis. They provide neuroprotection through their anti-apoptotic and antioxidant effects and promote neurogenesis by releasing growth factors like GDNF and BDNF. Additionally, MSCs pro-

duce neurotrophins such as VEGF, HGF, NGF, BDNF, and neurotrophin-3, supporting neuritic formation and neurorestoration and regulating immune response by suppressing inflammatory microglia (M1) and activating anti-inflammatory microglia (M2), preventing tissue damage and enhancing A β clearance through microglial accumulation and autophagy stimulation.

7. Clinical Trials

Numerous studies have been carried out to determine the effectiveness of MSCs obtained from different origins in managing Alzheimer’s disease (AD). However, there are only a few of these trials that are officially completed and have published their results. Table 2 presents a list and summary of the clinical trials registered on clinicaltrials.gov. These trials are significant for the further development of MSCs as a therapeutic target for AD, showing both the achievements and the challenges in this novel therapeutic approach.

Table 2. Completed and ongoing clinical trials on the effects of mesenchymal stem cells (MSCs) on Alzheimer’s disease patients.

	Number	Therapy	Source	Status	Pathway	N=	Study Location
1	NCT02600130 [79]	Cells	Bone marrow	Completed	Intravenous	33	USA
2	NCT03117738 [80]	Cells	Adipose tissue	Completed	Intravenous	21	USA
3	NCT02054208 [81]	Cells	UCB	Completed	Intracerebroventricular	45	South Korea
4	NCT01297218 [82]	Cells	UCB	Completed	Intracerebral	9	South Korea
5	NCT02833792 [83]	Cells	Bone marrow	Recruiting	Intravenous	40	USA
6	NCT04040348 [84]	Cells	Umbilical cord	Active, not recruiting	Intravenous	6	USA
7	NCT04482413 [85]	Cells	Adipose tissue	Not yet recruiting	Intravenous	80	USA
8	NCT04954534 [86]	Cells	UCB	Not yet recruiting	Intracerebroventricular	9	South Korea
9	NCT02672306 [87]	Cells	Umbilical cord	Unknown	Intravenous	16	China
10	NCT01547689 [88]	Cells	UCB	Unknown	Intravenous	30	China
11	NCT01696591 [89]	Cells	UCB	Unknown	Intracerebroventricular	9	South Korea
12	NCT04228666 [90]	Cells	Adipose tissue	Withdrawn due to COVID-19 pandemic	Intravenous	24	USA
13	NCT04855955 [91]	Cells	Adipose tissue	Completed	N/A	1	USA
14	NCT04388982 [92]	Cells	Adipose tissue	Recruiting	Nasal drip	9	China
15	NCT02899091 [93]	Cells	N/A	Recruiting	Intravenous	24	South Korea
16	NCT04684602 [94]	Cells	N/A	Recruiting	N/A	5000	USA

Several clinical trials have been conducted in the past years to evaluate the use of MSCs in AD, using different sources of stem cells, routes of administration, and study protocols. Some of the completed studies have given preliminary information on the safety and possibility of undertaking these therapies. The intravenous application of bone marrow-derived MSCs was investigated in a study conducted in the USA with 33 AD patients; the treatment was safe, but there was no significant improvement in the cognitive status of the patients [79]. Likewise, another trial in the USA included 21 patients who were treated with adipose tissue-derived MSCs through intravenous administration; however, the study’s small sample size limited the conclusions that could be drawn [80]. Another study conducted in South Korea used intracerebroventricular infusion of umbilical cord blood-derived MSCs (UCB-MSCs) in 45 patients and proved that this method of delivery is safe, but its effect on the progression of the disease is still ambiguous [81]. A similar trial in South Korea involved the intracerebral transplantation of UCB-MSCs in 9 patients, and while the study showed that the treatment was safe, the data to support its therapeutic use were weak [82]. Furthermore, there are active and future trials aiming at testing MSC therapies for AD employing various approaches. Another trial that is still recruiting patients in the USA plans to investigate the safety and the efficacy of intravenous delivery of bone marrow-derived MSCs in 40 patients [83]. Another ongoing trial is assessing the safety of intravenous administration of umbilical cord-derived MSCs in 6 patients, but the trial is not recruiting, nor are there any results at the moment [84]. Current and future trials include a trial in the USA recruiting 80 participants to assess the safety and anti-inflammatory

effects of adipose tissue-derived MSCs when administered intravenously and a trial in South Korea to evaluate the efficacy of intracerebroventricular infusion of UCB-MSCs in 9 patients to explore the effectiveness of a targeted delivery system [85,86]. Certain trials may have indefinite statuses or may have been affected by a reason. For instance, two trials in China are evaluating the intravenous umbilical cord-derived MSCs and UCB-MSCs in 16 and 30 patients, respectively, but their status is not known [87,88]. Another trial from South Korea aims at examining the safety and efficacy of intracerebroventricular administration of UCB-MSCs in 9 patients, but the trial's status is also unknown [89]. Moreover, a phase 1 trial in the USA aimed at assessing the intravenous injection of adipose tissue-derived MSCs in 24 patients, but it was terminated due to the COVID-19 pandemic [90]. Other researchers are using different and innovative strategies and different cell types. There is only one study conducted in the USA in which adipose tissue-derived MSCs were used in a single patient, which does not allow us to make any conclusions [91]. Currently, there is an ongoing trial in China that has 9 participants in which the researchers aim to evaluate the safety and efficacy of adipose tissue-derived MSCs delivered through nasal drip [92]. Additionally, there are other ongoing trials without the distinction of the MSC source: a trial in South Korea is testing MSC administration through the intravenous route, and a registered large-scale clinical trial in the USA plans to enroll 5000 patients to examine MSC therapy safety and efficacy [93,94].

8. Advantages and Challenges

Mesenchymal stem cells' (MSCs) ability to differentiate into various cell types, including those involved in the production of bone, cartilage, and adipose tissue, makes them highly advantageous for use in neurodegenerative diseases. Some studies have shown their anti-tumorigenic effects, such as Clarke et al., who stated that breast cancer cells cultured in an MSC-conditioned medium exhibit significant migratory inhibition compared with cells cultured in a standard medium [95,96]. Similarly, Bruno et al. showed tumor cell growth inhibition by MSCs. A human hepatocellular carcinoma cell line (HepG2), a human ovarian cancer cell line (Skov-3), and Kaposi's sarcoma cell lines co-cultured in the presence of BM-MSCs exhibited reduced in vitro growth [97,98]. MSCs can also be obtained using minimally invasive means, such as bone marrow, adipose tissue, and umbilical cord blood. They can affect the immune system function and reduce inflammation, which is very helpful for treating inflammatory and autoimmune diseases. Their therapeutic value is increased by the minimal risk of immunological rejection in transplant recipients.

While SCs have the potential to repair and regenerate damaged cells, the precise ways in which they might work are still not fully understood. Most studies show that a single transplantation of MSCs is safe and does not induce an immune response. However, repeated administration of MSCs may result in the production of alloantibodies. So far, there have been only a few clinical trials where SCs were transplanted into AD patients, and results from animal studies have not provided solid proof that these therapies are either safe or effective. Andrzejewska et al. also reported antibacterial activities and interactions of the MSC secretome with cancer cells [99]. Additionally, there are a lot of social, ethical, and regulatory issues that make research difficult and limit federal funding. In the USA, the FDA has only approved stem cells from cord blood, but many clinics are offering various unregulated treatments, often charging a lot of money. To make sure that these treatments are safe and effective, especially for complicated diseases like Alzheimer's, it is really important to have ongoing patient monitoring and clearer regulatory guidelines.

9. Conclusions

MSC-Exos play a crucial role as a mediator in the information transfer between MSCs and recipient cells, such as microglia and neurons. Improvements in cognitive function are brought about by MSC-Exo-derived miRNAs, trophic factors, enzymes, immunomodulatory agents, and pro-angiogenic chemicals, which stimulate neurogenesis and inhibit inflammation-induced damage to hippocampus neurons. Crucially, MSC-Exos generated

immunomodulation and neuroprotection that was either identical or superior to that of their parent MSCs in terms of immunomodulation. The effects of MSC-Exos are independent of the local tissue microenvironment. MSC-Exos are immunomodulatory and neuroprotective cells that do not change in response to various stimuli, unlike MSCs, which change in phenotype and function upon engraftment in different tissue microenvironments. This suggests that MSC-Exos may find clinical application in treating neurocognitive diseases. MSC-expos are a unique cell-free therapeutic agent that offers incomparable benefits over cell-based therapy, which is thought to be a potential substitute in the treatment of AD.

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

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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

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Review

Potential Applications and Ethical Considerations for Artificial Intelligence in Traumatic Brain Injury Management

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Abstract: Artificial intelligence (AI) systems have emerged as promising tools for rapidly identifying patterns in large amounts of healthcare data to help guide clinical decision making, as well as to assist with medical education and the planning of research studies. Accumulating evidence suggests AI techniques may be particularly useful for aiding the diagnosis and clinical management of traumatic brain injury (TBI)—a considerably heterogeneous neurologic condition that can be challenging to detect and treat. However, important methodological and ethical concerns with the use of AI in medicine necessitate close monitoring and regulation of these techniques as advancements continue. The purpose of this narrative review is to provide an overview of common AI techniques in medical research and describe recent studies on the possible clinical applications of AI in the context of TBI. Finally, the review describes the ethical challenges with the use of AI in medicine, as well as guidelines from the White House, the Department of Defense (DOD), the National Academies of Sciences, Engineering, and Medicine (NASEM), and other organizations on the appropriate uses of AI in research.

Keywords: TBI; artificial intelligence; machine learning; diagnostic; neuromonitoring

1. Introduction

Artificial intelligence (AI) is often praised for its potential to transform many aspects of modern life, resulting in increasing global investment in its advancement and implementation. Due to this enthusiasm, AI-driven tools have already become an integral part of daily life for many people, despite their relatively recent development. AI can be used to perform a wide variety of tasks, such as having human-like conversations, predicting online shoppers' preferences, powering voice-controlled virtual assistants like Apple's Siri and Amazon's Alexa, and deciding which posts to display on social media platforms, such as Facebook and X (formerly Twitter) [1]. In medicine, government officials, researchers, and clinicians have shown an increasing interest in expanding the use of AI techniques to help deconvolve complex, multidimensional patient data (e.g., neuroimaging, electroencephalography (EEG), genetic, and blood biomarker data) to improve the speed and accuracy of pathology detection and support clinical decision making [2]. For example, the United Kingdom government has invested GBP 50 million into the establishment of five AI Centres of Excellence in digital medicine and imaging [3]; similarly, the Japanese government has been developing an "AI Hospital System" as a method to support the country's aging population, mitigate the effects of its diminishing workforce, and improve medical care in rural settings [4].

Traumatic brain injury (TBI) is one example of a neurological condition for which the application of AI has been explored [5,6]. TBIs are particularly common among military

personnel, veterans, survivors of intimate partner violence, athletes, and the elderly [7–10], but they can affect individuals of all sociodemographic backgrounds and occupations and can be sustained through a variety of different mechanisms (e.g., motor vehicle accidents, falls, assaults, or injurious blast exposure). Thus, TBI is considerably heterogeneous, which makes the condition particularly difficult to diagnose and treat [11]. TBIs are currently categorized based on severity as mild, moderate, or severe using tools such as the Glasgow Coma Scale (GCS) in combination with neuroimaging results and the presence and duration of various clinical signs [12,13]. The majority of TBIs are classified as mild, which is characterized by a GCS score of 13–15, a confused or disoriented state lasting less than 24 h, a loss of consciousness for up to 30 min, and/or memory loss lasting less than 24 h based on criteria defined by the American Congress of Rehabilitation Medicine (ACRM), which are also used by the United States Department of Defense (DOD) [12,13]. Moderate and severe TBIs are characterized by GCS score ranges of 9–12 and <9, respectively, and typically involve longer durations of loss of consciousness, altered consciousness, or memory loss, as well as structural pathology on computed tomography (CT) imaging [12].

While these criteria are commonly used to aid triage decisions during the initial evaluation of an individual with suspected TBI, the severity of one's injury is often an insufficient predictor of the long-term outcome; even among those with mild TBI (mTBI), symptoms including somatic, cognitive, and emotional/behavioral issues have been shown to persist for months to years after the initial injury in 10–25% [14]. Current tools for evaluating TBI have a limited ability to discriminate those who will develop persisting TBI sequelae from those who will recover, since these individuals may exhibit a normal GCS score and no evidence of pathology on neuroimaging [15,16]. Tools like the GCS and CT imaging may provide more useful indicators of prognosis in individuals with moderate to severe TBI, but these tools are limited in their ability to reliably inform treatment decisions beyond surgical and other emergency interventions [17]. Individuals with moderate to severe TBI may present with a variety of structural pathologies, such as hemorrhage, diffuse punctate axonal injury, or diffuse brain swelling, that contribute differently to patient recovery and involve distinct underlying mechanisms [17,18]. The development of personalized therapeutic strategies for TBI has likely been limited because most clinical trials for TBI do not account for this heterogeneity in underlying pathological findings [17]. Thus, variability in TBI outcome and presentation has led to important challenges with its acute and long-term management.

To help refine TBI characterization and eventually enable personalized medicine for TBI, organizations such as the National Academies of Sciences, Engineering, and Medicine (NASEM) and the National Institute of Neurological Disorders and Stroke (NINDS) have endorsed increasing the collection of multimodal clinical data during initial TBI assessment and across multiple timepoints following the initial injury [19,20]. For example, an increasing number of studies have evaluated the clinical value of molecular biomarkers isolated from biofluids, including markers of brain cell damage or inflammation, in the assessment of TBI [15]. This research eventually led to the 2018 clearance of the first blood-based biomarker assay for aiding the evaluation of TBI by the United States Food and Drug Administration (FDA) [21]. Certain eye-tracking devices and neurocognitive assessment tools (e.g., the Automated Neuropsychological Assessment Metrics [ANAM] and Immediate Post-Concussion Assessment and Cognitive Testing [ImPACT]) have also recently received FDA clearance for aiding in the evaluation of TBI, and the latter have become essential components of mTBI follow-up protocols and return-to-activity decisions after mTBI in sports and the military [22–24]. In addition, more advanced neuroimaging methods, such as magnetic resonance imaging (MRI) modalities, have started to become incorporated into clinical recommendations for the evaluation of TBI to enable the detection of subtle structural and functional pathologies, thus addressing the limitations of standard CT imaging [25]. Other tools that have been increasingly applied to TBI evaluation in recent studies include EEG, genetic markers, markers of autonomic nervous system dysfunction, and vestibular function assessments [15]. With further study, the

combined assessment of these measures with sociodemographic information and patient medical history could substantially improve the clinical management of TBI and enable more personalized treatment options.

These efforts, combined with the development of TBI data repositories like the Federal Interagency Traumatic Brain Injury Research (FITBIR) informatics system indicate that clinicians and researchers will soon have access to the largest amount of data on TBI patients ever to exist [26]. Thus, some researchers have proposed the conceptualization of TBI as a “Big Data” problem, which demands more sophisticated and robust methods to help interpret this rapidly increasing volume and variety of TBI patient data [27]. Due to the complexity of these data and their extensive variability across TBI patients, it is unlikely that a single measure or tool will be able to diagnose or prognosticate TBI with sufficient accuracy or sensitivity. Additionally, because many of the underlying mechanisms and the patient and injury factors contributing to TBI progression remain unclear, it may be difficult to logically design an appropriate panel of measures for predicting the development of specific TBI symptoms or a patient’s response to a particular treatment [28]. Advanced algorithmic methods such as AI techniques have promise for addressing these challenges by quickly and efficiently reducing a large amount of patient data into clinically meaningful information with minimal human supervision [27]. For example, researchers have begun to investigate the application of AI in supporting triage decisions in emergency department settings [29], diagnosing TBI [5], identifying TBI clusters [30], and predicting post-TBI symptoms [31]. Additionally, AI techniques can allow clinical investigators to focus on the creative aspects of developing new research studies equipped with concise summaries of the results of all related published studies.

However, AI also presents new opportunities for the misuse of data and plagiarism [32–34] and can demonstrate methodological flaws with improper use, so as the technology improves there is an urgent need for some form of government regulation and guidance regarding the legitimate and appropriate use of AI. One concern is ensuring that AI-assisted clinical tools adhere to patient privacy and security standards as advancements continue. For example, large language models (LLMs) have potential applications in cybersecurity by quickly performing data encryption and automated threat detection with minimal human supervision; however, as cybersecurity threats grow more sophisticated and complex, efforts must be made to develop provisions that prevent malicious actors from compromising patients’ personal information and biometric data [35,36]. Additionally, the lack of transparency and explainability in AI systems have contributed to widespread criticism of these tools and to concerns about whether informed consent can be sufficiently obtained from patients whose data are subjected to AI-based analyses [37,38]. Other crucial ethical considerations include minimizing algorithm biases and ensuring that human oversight is maintained to monitor the safety and efficacy of AI-based medical tools [38].

Due to increasing interest in the use of AI to assist in clinical decision making for TBI evaluation and management and growing appreciation for the ethical concerns with these methods, the purpose of this narrative review is to (1) provide working definitions for various forms of AI; (2) describe evidence on the use of AI systems in TBI evaluation and management; and (3) discuss the ethical challenges with implementing AI systems in healthcare applications and the policies that have been developed to guide these efforts.

2. Search Method

PubMed was utilized to conduct the literature search for this narrative review. The search used terms related to artificial intelligence (“artificial intelligence”, “machine learning”, “deep learning”, “large language models”, “reinforcement learning”, “artificial superintelligence”, “artificial general intelligence”, “artificial narrow intelligence”, “predictive artificial intelligence”, “explainable artificial intelligence”, “artificial narrow intelligence”, “SHAP”), terms related to evaluation (“diagnostic”, “prognostic”, “clinical”, “nonclinical”), other related terms (“neuroimaging”, “evaluate”, “diagnosis”, “assess”, “etiology”, “comorbid”, “treatment”, “risk”, “risk factor”, “prevalence”, “protective factors”), and terms

related to TBI (“head injuries”, “closed”, “brain injuries”, “traumatic”, “brain concussion”, “post-concussion”, “traumatic brain injury”, “TBI”, “mTBI”, “concuss”, “brain injuries”). Articles were retrieved and evaluated for relevance to the subject. References within the identified articles were also searched, and relevant articles were retrieved.

3. AI Techniques Commonly Used in Medical Research

AI refers to technologies that involve sophisticated algorithms that learn from vast amounts of data to solve problems and perform tasks in ways that would typically require human intervention (Figure 1) [39]. AI systems can identify patterns within data to make predictions, and the incorporation of self-correcting abilities enables an AI system to improve its accuracy through feedback [2]. AI comprises a variety of computational techniques that can be selected based on the dimensionality of the data and specific application [28]. There is a growing impact of AI in healthcare, particularly in diagnostics and personalized medicine. AI systems, such as deep learning models, are achieving human-level performance in areas like medical imaging and disease detection [40]. Despite its promise, AI faces challenges such as data quality, model transparency, and ethical concerns. However, AI has the potential to reduce healthcare costs, improve patient outcomes, and increase access to care [40].

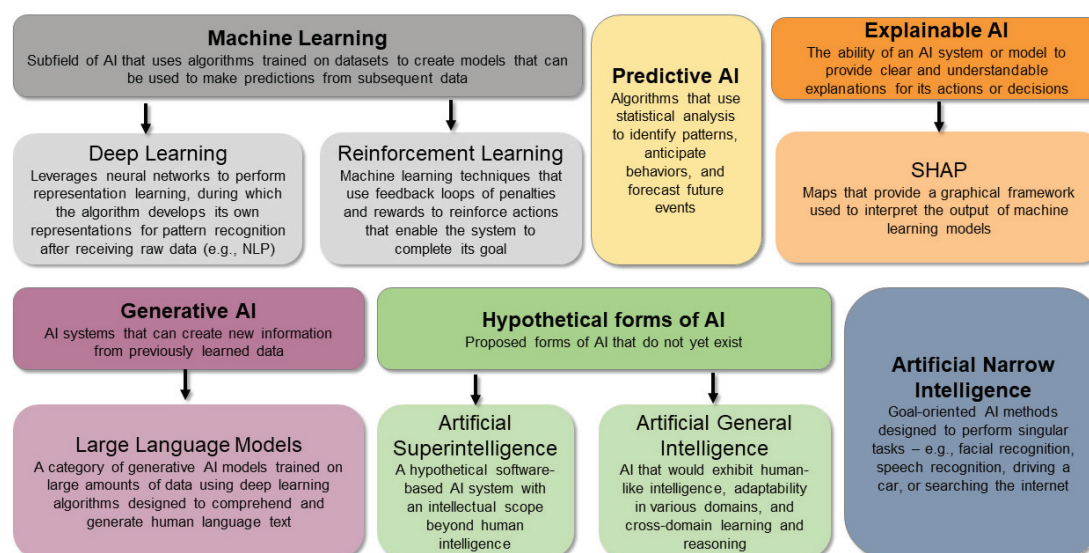


Figure 1. Artificial intelligence (AI) techniques commonly used in medical research. AI refers to technologies that involve sophisticated algorithms that learn from vast amounts of data to solve problems and perform tasks in ways that would typically require human intervention. Of note, a given AI system may involve the use of a combination of multiple methods. NLP: Natural Language Processing; SHAP: SHapley Additive exPlanations.

Artificial narrow intelligence (ANI), also referred to as weak AI or narrow AI, has been the most widely utilized and the only commercialized form of AI to date. Narrow AI is goal-oriented and designed to perform singular tasks, e.g., facial recognition, speech recognition, driving a car, or searching the internet—and is very intelligent at completing the specific task it is programmed to do [1]. ANI operates under a narrow set of constraints to perform tasks that simulate human behavior in real-time [1]. One area of medicine for which ANI is highly applicable is imaging analysis, as ANI systems are found to perform similar to or better than radiologists in detecting diseases and abnormalities [41]. However, ANI is only highly proficient in the specialized task it is designed for, meaning it cannot be generalized across broader medical contexts [41].

Predictive AI refers to a computer program’s ability to use statistical analysis to identify patterns, anticipate behaviors, and forecast future events [42]. Predictive AI goes beyond simple observations; it analyzes thousands of factors and large volumes of data

to inform predictions [42]. Predictive AI thus analyzes vast quantities of data—often referred to as “Big Data”, and the more data available, the better the predictions. AI-driven predictive analytics can improve patient care by predicting health outcomes, disease progression, and response to treatment [43]. With these systems, vast amounts of patient data can be used to provide personalized decision support, allowing healthcare providers to make more informed and timely clinical decisions [43]. By identifying patterns and risks early, predictive AI helps improve patient outcomes and optimize resource allocation in healthcare [43]. Most AI models that have been investigated for healthcare purposes, such as diagnostics and outcome prediction, involve predictive AI.

Internet of Things (IoT) refers to a network of physical devices, vehicles, appliances, and other objects equipped with sensors, software, and network connectivity [44]. These smart devices can collect and share data, enabling them to communicate with each other and with other internet-enabled devices. IoT devices, also known as “smart objects”, include simple home devices (like smart thermostats), as well as wearables (such as smartwatches) and complex industrial machinery [45]. The integration of AI into IoT devices can improve the efficiency and accuracy of patient data management. In smart healthcare systems, IoT devices continuously collect patient data in real-time, which can then be analyzed using hybrid AI techniques to optimize diagnostic processes and treatment decisions [46]. The use of these devices can not only enhance data processing but also improve the overall performance of healthcare systems by providing timely and personalized care, leading to more efficient resource allocation and improved patient outcomes [46].

Machine learning is a subfield of AI that uses algorithms trained on datasets to create models that can be used to make predictions from subsequent data without explicit instructions or human intervention [28]. Machine learning has been widely investigated for its potential role in predicting disease progression and individual responses to treatments and for its potential utility to optimize treatment strategies [47]. With successful optimization, machine learning strategies can allow healthcare providers to anticipate disease trajectories, improving clinical decision making and enabling more tailored treatment plans that improve patient outcomes [47]. Generally, machine learning can be categorized as supervised or unsupervised learning. With supervised machine learning, an algorithm is trained using data for which the state of the data is known (e.g., each input corresponds to a “TBI patient” or “healthy control”), and the developed model is used to make predictions on new data for which this information is not known [28]. Unsupervised machine learning involves using algorithms to identify patterns in unlabeled data [28]. Common forms of machine learning algorithms used in medical research include the naïve Bayes model, k-Nearest Neighbors, Least Absolute Shrinkage and Selection Operator, random forest, and support vector machine (SVM) [28].

Deep learning is a subfield of machine learning that leverages neural networks to perform representation learning, during which the algorithm develops its own representations for pattern recognition after receiving raw data as the input [48]. The term “deep” refers to the use of multiple layers in the network [49]. Deep learning models are based on neural networks, which are inspired by the human brain. These networks consist of interconnected nodes (neurons) that process and transform data [50].

Deep learning aims to transform input data into abstract and composite representations. For example, in an image recognition model, the initial layer identifies basic shapes (like lines and circles), subsequent layers encode more complex features (such as facial features), and the final layer recognizes the overall object (like a face). Unlike traditional machine learning, in which features are manually selected and incorporated into a model, deep learning models automatically learn useful feature representations from the data itself [48]. Deep learning has been successfully applied to various fields, including computer vision (image recognition, object detection, and segmentation), natural language processing (text analysis, sentiment analysis, and language translation), speech recognition (converting spoken language into text), and bioinformatics, including medical image analysis. Deep learning models like convolutional neural networks improve diagnostic capabilities by

identifying patterns and abnormalities that might be difficult to detect using traditional methods [51]. Thus, deep learning has the potential to enhance diagnostic accuracy by streamlining imaging analysis, reducing clinician workload, and allowing for faster, more reliable medical decisions [51].

Reinforcement learning refers to machine learning techniques that aim to train computational agents to achieve specific functions, and this learning can involve trial and error, expert demonstration, or a hybrid strategy [48]. With reinforcement learning, feedback loops of rewards and penalties are used to make the agent better at accomplishing its designed task [48]. When the model performs actions that move it further away from completing its objective, a penalty is mathematically applied to the model, while actions that move the model closer to achieving its objective are positively reinforced with rewards, which have the opposite sign of penalties [52]. For example, penalties can be assigned negative values and rewards can be given positive values. Robotic-assisted surgery is one healthcare application for which reinforcement learning approaches have been studied [53]. Reinforcement learning can also be applied to the optimization of medication dosages [54]. These algorithms can be integrated with pharmacokinetic/pharmacodynamic models to offer dosage recommendations that are dynamically adjusted based on patient data, with the goal of maximizing therapeutic effects and minimizing side effects [54]. This data-driven approach has the potential to improve treatment efficacy across many medical conditions such as cancer, diabetes, and neurologic diseases, for which the precision of treatment is most critical to outcome [54].

Generative AI (GAI) refers to AI systems that can create new information from previously learned data [42]. These systems “invent” new content, solutions, or concepts that did not previously exist in the data they were trained with, rather than merely analyzing or processing the data [55]. GAI systems involve algorithms that are designed to learn from large bodies of information, including scientific literature. Programs like ChatGPT and Midjourney fall under the category of GAI. These programs learn from data (such as online text and images) to create new content that feels human-made. Chatbots like ChatGPT engage in text conversations, while Midjourney generates images from simple text instructions [42]. GAI can be used for medical applications to provide new insights into complex biological processes. In a recently published study, GAI was used to describe brain amyloid load, dynamics, and progression using a cohort of 1259 subjects’ AV45 positron emission tomography (PET) images [56]. This methodology can provide invaluable information for understanding Alzheimer’s disease and could potentially inform its diagnosis and future clinical trial design.

Natural Language Processing (NLP) is a subfield of AI that focuses on the interactions between systems and human language. It involves the development of algorithms and models to understand, interpret generate, and respond to human language in a meaningful way [57]. It encompasses tasks such as text classification, sentiment analysis, machine translation, speech recognition, and language generation [57]. NLP often relies on machine learning techniques to accomplish these tasks. Some NLP systems include the original models of virtual assistants such as Siri or Alexa, and language translation services such as Google Translate. NLP can play a pivotal role in extracting valuable insights from unstructured data within electronic health records [58]. NLP techniques, particularly those powered by neural networks, are capable of analyzing vast amounts of clinical text to identify patterns, trends, and relevant medical information that might otherwise be overlooked [58]. These tools could also enhance the efficiency of clinical workflows by interpreting free-text data.

LLMs are a category of foundation model trained on immense amounts of data using deep learning algorithms designed to comprehend and generate human language text [59]. These models are a form of GAI that is based on transformer architectures and involve billions of parameters [60]. Examples of LLMs include ChatGPT-3 and GPT-4, Google’s BERT/RoBERTa and PaLM models, and IBM’s Watson Assistant and Watson Orchestrate. These models have become increasingly popular due to their ability to understand and

generate natural language, enabling them to perform a wide range of tasks. For example, LLMs can summarize lengthy text into concise versions, answer general questions, and assist in tasks like creative writing and code generation among other tasks. In the context of medicine, these models, trained on vast amounts of medical literature and clinical data, excel at understanding and generating natural language, enabling them to assist in tasks such as summarizing medical documents, generating patient reports, and even aiding in clinical decision making [61]. LLMs can process and interpret complex medical information, making them valuable tools for clinicians and researchers alike [61]. However, there needs to be careful consideration of biases and an emphasis on ensuring transparency to maintain trustworthiness when using LLMs in healthcare applications.

Explainable AI (XAI) refers to the ability of an AI system or model to provide clear and understandable explanations for its actions or decisions [62]. AI techniques like deep learning involve the detection of complex, nonlinear relationships in data that are difficult to understand, leading some to refer to these strategies as a “black box” [16]. XAI aims to make AI processes more transparent and interpretable to humans and allows users to comprehend how decisions are made. Within XAI, knowledge graphs are often utilized to increase the explainability within a model, particularly in scenarios that require the output to be linked to well-established domain knowledge, such as in healthcare and biomedical research settings [63]. In the case of biomedical ontologies, knowledge maps include relationships between diseases, treatments, and biological processes, such as with gene ontology or disease ontology [63]. The integration of AI into clinical workflows requires a high degree of interpretability. In the case of knowledge graphs, complex datasets are mapped into structured, interconnected representations to provide an intuitive framework to explain AI decision making [63]. By providing insights into how AI models make decisions, XAI techniques like saliency maps help clinicians understand the rationale behind a model’s outputs [64].

SHapley Additive exPlanations (SHAP) maps provide a graphical framework used to interpret the output of machine learning models and are thus considered one type of XAI [65]. SHAP utilizes what are known as Shapley values from cooperative game theory, which provide post hoc measures of how each feature (e.g., age, sex, blood pressure, etc.) contributes to predictions in a machine learning model [66]. This approach breaks down and attributes the impact of individual features, making complex models more transparent and interpretable [66]. In healthcare, SHAP can help clinicians and researchers identify which factors are most critical to diagnosis or treatment outcomes [67]. This approach ensures that AI outputs are accurate, interpretable, and actionable, all of which are essential for integration into clinical practice.

It is important to note that often a given AI system will often involve a combination of different AI technologies. These combinations can improve core functionality, advance capabilities, or increase transparency. For example, the ChatGPT NLP promotes its core functionality, enabling the system to interpret and generate human language and is supported by machine learning models that employ deep learning architecture [68]. The use of LLMs further enhances a system’s capacity for nuanced language processing. This specific use of AI can be classified as ANI, as it operates in a specialized domain. Additionally, XAI techniques, such as SHAP, may be integrated into an AI system to ensure transparency [69].

Hypothetical forms of AI include **artificial general intelligence (AGI)** and **artificial superintelligence (ASI)**. AGI refers to a type of AI that matches or surpasses human capabilities across a range of cognitive tasks [70]. Unlike narrow AI, which is designed for specific tasks, AGI aims to exhibit human-like intelligence and adaptability in various domains capable of cross-domain learning and reasoning. While ANI systems excel at specialized tasks such as image recognition, language translation, or playing chess, AGI would be more versatile and capable of handling novel situations. Achieving AGI remains an ongoing challenge in AI research, as it requires developing algorithms and models that can generalize knowledge, reason abstractly, and learn from limited data [71]. In summary,

AGI represents the pursuit of creating AI systems that can think, learn, and adapt like humans, making it a significant milestone in the field of AI.

ASI is a hypothetical software-based AI system with an intellectual scope beyond human intelligence [72]. Essentially, ASI would possess highly advanced thinking skills and surpass human intelligence and cognitive abilities in virtually all domains, including problem solving, creativity, and learning. However, it remains theoretical and has not been achieved yet. Developing ASI requires further advancements in technologies, such as LLMs and multisensory AI, as well as more complex neural networks [72]. According to some experts, the emergence of an ASI is highly unlikely in the near future based on current computer architectures, primarily due to energy constraints, because the amount of energy consumed by a hypothetical ASI system would likely exceed that used to power highly industrialized nations [73].

4. Potential Applications of AI in TBI Clinical Care

4.1. Applications of AI in Medicine

AI techniques have potential applications in diverse areas of evidence-based medicine, from aiding medical data management to assisting pharmacovigilance efforts and guiding personalized treatment [74,75]. GAI, for example, can be used to guide medical research planning by quickly answering queries and providing a summary of next steps for consideration (Figure 2). Additionally, AI has utility in medical education as it can be used to train students in disciplines that heavily rely on neuroimaging, such as radiology, ultrasound, echocardiography, and pathology [39]. In busy acute care settings, AI and natural language processing could aid triage efforts by generating concise and insightful medical summaries based on a patient's symptoms; this could substantially reduce the time spent examining clinical records and prevent clinicians from missing key information [76–78]. Relatedly, AI can be used to mine the large volumes of data contained in electronic records to identify patients with an elevated risk of developing certain illnesses to help select them for preventative interventions [76,79,80]. For example, AI models have high accuracy in predicting the risk of sudden cardiac arrest, allowing clinicians to intervene in a timely manner [81]. Due to these promising findings and the increasing volume of TBI patient data, clinical researchers have also begun to investigate the use of AI to improve TBI management across the continuum of care (Figure 3) [19].

EXAMPLE: PRACTICAL AND LEGITIMATE USES OF AI FOR RESEARCH	
You:	
How many subjects do I need for my clinical trial?	
Copilot:	
<p>The number of subjects needed for a clinical trial can vary based on the study's purpose, design, and specific research question. Clinical trials involve volunteers who help find answers to health-related questions. Here are some key points:</p> <ol style="list-style-type: none"> Purpose of Clinical Trials: <ul style="list-style-type: none"> Clinical trials aim to evaluate new treatments, improve existing ones, or study different ways to use approved treatments. They may involve healthy subjects or people with specific health conditions willing to try experimental treatments. Eligibility Criteria: <ul style="list-style-type: none"> All clinical trials have guidelines (eligibility criteria) about who can participate. Factors considered include age, sex, disease type, previous treatment history, and other medical conditions. These criteria help reduce variation within the study and ensure researchers can answer their questions effectively. Safety and Protocol: <ul style="list-style-type: none"> Clinical trials follow a protocol that outlines the study's plan, including patient types, tests, procedures, drug dosages, and study duration. Volunteers must agree to follow the protocol, and researchers must adhere to Food and Drug Administration (FDA) rules to ensure safety. Diversity and Health Equity: <ul style="list-style-type: none"> Ensuring diverse participation is crucial for advancing health equity. People from different backgrounds may react differently to medical products, so representation matters. Finding Clinical Trials: <ul style="list-style-type: none"> The National Institutes of Health (NIH) conducts clinical research trials for various diseases and conditions, including cancer, Alzheimer's, allergies, and neurological disorders. You can search for clinical trials on ClinicalTrials.gov. <p>Remember, the specific number of subjects needed depends on the trial's goals, statistical power, and other factors. Consult with experts to determine the appropriate sample size for your trial. Learn more at fda.gov, nih.gov, grants.nih.gov, en.wikipedia.org.</p>	

Figure 2. Example use of generative artificial intelligence (GAI) for research. GAI may be useful in guiding research study planning as shown in this example of the reasonable use of Microsoft CoPilot to help in the development of a clinical grant proposal [82].

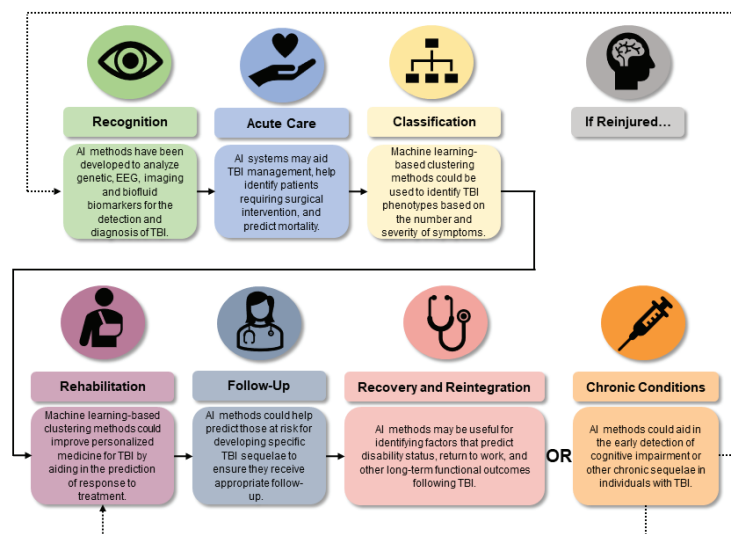


Figure 3. Potential applications of artificial intelligence (AI) throughout the traumatic brain injury (TBI) continuum of care. A recent report from the National Academies of Sciences, Engineering, and Medicine (NASEM) on accelerating progress in TBI care identified key challenges that must be addressed to improve the clinical management of TBI [19]. These challenges can be roughly aligned with specific stages in the continuum of care for TBI, which include its recognition, acute care, classification, rehabilitation, and follow-up, at which recovery and reintegration or the development of chronic conditions can be assessed. Individuals who develop chronic conditions following TBI may undergo rehabilitation to promote their recovery; in the event of reinjury, the continuum begins again with the recognition or detection of the TBI. AI-based techniques have demonstrated the potential to assist with each of these stages in the continuum of TBI care.

4.2. AI-Assisted Monitoring and Management of TBI in Acute Care Settings

In acute care settings, machine learning methods have been investigated as tools for aiding several aspects of moderate to severe TBI management, such as predicting the incidence of urgent medical events [29]. One retrospective study of over 2000 head trauma patients used 18 patient features, including age, sex, systolic blood pressure, loss of consciousness, and pupil abnormalities, to develop machine learning models to discriminate patients with traumatic intracranial hemorrhage (ICH) from those without ICH; the best-performing model had an area under the receiver operating characteristic curve (AUC) of 0.80, sensitivity of 74%, and specificity of 75% in the validation dataset [83]. Additionally, a feature importance analysis identified 5 of the 18 variables (disorientation, high-energy head trauma, head trauma scar, the eye-opening component of the GCS, and pupil abnormality) as most associated with the model's accuracy [83]. The main limitation of this study was its retrospective design and inappropriate exclusion of patients with missing data. In a recent prospective analysis of 104 patients with moderate to severe TBI who had been randomized to the control group of a clinical trial investigating tranexamic acid for TBI, models comprising machine learning-selected features (AUC = 0.78) performed better than models comprising expert-selected variables (AUC = 0.68) for predicting progression of ICH [84]. While this study did employ internal cross-validation measures, its main limitation was that the results were not validated with an independent validation dataset of TBI patients, indicating a potentially high risk of bias. Additionally, no data on model calibration were presented, which is important for determining how well the model would likely perform on new data [85]. While both studies performed analyses to determine which features were most related to the model's accuracy, another potential limitation of both these studies is that neither included certain measures related to cardiovascular function, such as anticoagulant use, which could have improved model performance.

Other studies have aimed to develop machine learning models that can be used to identify TBI patients who require surgical intervention. One study of over 2000 moderate

to severe TBI patients reported an AUC of 0.81 when using a machine learning model of 15 features to discriminate those who required neurosurgery within 24 h of hospital admission from those who did not [86]. In this study, SHAP analysis indicated the most predictive variables included GCS score, measures of pupillary abnormality, high blood pressure, and low heart rate [86]. One limitation of this study was that it did not employ appropriate internal validation measures, such as cross-validation or bootstrapping, or an independent validation dataset; instead, this study split one dataset, using 80% for model training and 20% for validation. Another study of 200 moderate TBI patients used age, sex, GCS score, and CT findings to develop an SVM-based model to predict the need for surgical intervention, demonstrating an AUC of 0.93 (82% sensitivity, 84% specificity) [87]. While this study utilized fourfold cross-validation to evaluate the developed model, the performance of the model should be confirmed in a larger, external TBI patient cohort.

Several investigators have also used machine learning methods to successfully predict the risk of mortality for severe TBI patients in the emergency room. These studies have identified features such as age, the timing of neurosurgical intervention, and clinical signs as important predictors of post-TBI mortality [31,88–90]. Notably, a recent meta-analysis including 15 studies found that machine learning algorithms outperformed traditional regression models in predicting adverse TBI outcomes [31]. However, this analysis noted extensive heterogeneity in the input variables used for model development across studies, with some studies utilizing more variables than are likely feasible to collect in a busy emergency department [31]. This heterogeneity also highlights the need for standardization in the collection of model input data across studies if AI-driven tools for mortality prediction are to be implemented in acute care settings [31]. Other applications for which AI methods have been investigated in the context of acute TBI management include identifying patients at risk of prolonged mechanical ventilation [91], predicting the lengths of hospital stays [92], and determining the need for head CTs in mTBI patients [93]. Collectively, these studies suggest broad uses for AI methods in acute TBI management if methodological concerns can be addressed.

4.3. AI-Assisted Diagnosis of mTBI

The validation of biological correlates of injury that can be used to accurately diagnose mTBI presents a unique challenge due to the mild and variable nature of brain pathology associated with mTBI. To increase the sensitivity of mTBI detection, many researchers have developed AI methods for analyzing genetic, EEG, imaging, and biofluid biomarker data from mTBI patients. For example, one group evaluated the use of a deep learning approach to predict mTBI status using DNA methylation data obtained through epigenetic analysis [94]. This study identified four methylation sites that could each be used to accurately discriminate pediatric mTBI patients from healthy controls ($AUCs \geq 0.9$ –1), and the combined analysis of these sites with clinical data using their deep learning model achieved a sensitivity and specificity of $\geq 95\%$ [94].

Many other studies have applied machine learning analysis of metrics from EEG recordings and MRI to discriminate mTBI patients from healthy controls with varying accuracy [95–102]. In one small study, the machine learning analysis of EEG data could be used to discriminate mTBI patients from healthy controls with an accuracy of 95% during model training and 70% during model validation [95]. In another study, machine learning analysis of diffusion tensor imaging metrics thought to reflect axonal pathology discriminated 50 mTBI patients from 50 healthy controls with an accuracy of 84% [96]. Other investigators have algorithmically combined functional connectivity measures with regional entropy values, achieving 75% accuracy in discriminating mTBI patients from healthy controls [100]. The primary limitations of these studies include the lack of validation of the developed models in external validation sets, the limited sample sizes of these studies, and the high dimensionality of these data, all of which could challenge the generalizability of the findings to novel datasets.

Machine learning analysis of biofluid biomarker data is a promising technique for leveraging diverse information provided by molecular markers of direct brain cell damage, inflammation, or metabolic processes to detect mTBI. One group used this approach to develop a panel of six metabolites measured in plasma that accurately discriminated athletes with mTBI from controls, and the accuracy of this panel was demonstrated when measured within six hours post-mTBI and two, three, and seven days post-mTBI [103]. Another study used unsupervised clustering analysis of blood biomarker data from athletes and military personnel in the FITBIR database to define 11 biomarker trajectories, two of which were associated with greater risk of loss of consciousness or posttraumatic amnesia at the time of injury [104]. While these findings are promising, an important consideration for the development of biofluid biomarker panels for mTBI detection is ensuring their specificity to TBI; to this end, future studies in this area should aim to include a non-CNS injury control group.

Together, these studies suggest that the use of more advanced methods for quantifying biomarker data could improve the sensitivity of mTBI detection. However, important challenges currently limit the implementation of this approach. First, these types of biomarker data are not yet part of the routine evaluation of mTBI in most clinical settings; thus, efforts to increase the utilization of multimodal mTBI assessments by clinicians are needed. Additionally, while these tools can be designed for ease-of-use, the data must be appropriately formatted to ensure consistency when used across clinicians, meaning clear common data elements must be established and adhered to. Finally, many scientists also express concerns over the interpretability of findings from machine learning models and the relationship between model findings obtained from biomarker data and actual underlying biological mechanisms. These limitations should be investigated and addressed in future studies.

4.4. AI-Based Identification of Phenotypic Clusters

Due to the complexity of TBI, many investigators are interested in identifying clinical subtypes of TBI to improve decision making and personalized treatment [105], and machine learning-based clustering methods could be valuable tools for achieving this goal. One study using this approach identified five distinct clusters of mTBI patients using measures of pain, depression, sleep disturbance, fatigue, and anxiety from the Patient-Reported Outcomes Measurement Information System, symptom measures from the ImPACT neurocognitive assessment, and other metrics [30]. These patient subtypes exhibited symptoms ranging from “minimally complex” to “extremely complex”, with complexity defined based on the number and relative severity of symptoms [30]. Another study that used a similar approach also identified five symptom clusters, with some clusters exhibiting few TBI symptoms and others that developed persisting symptoms that significantly impacted social functioning and work productivity [106].

Many other studies have aimed to use unsupervised machine learning approaches to identify TBI phenotypes and patient clusters [107]. One study used unsupervised machine learning to distinguish symptom clusters in 96 individuals with expected sports-related concussion or postconcussive syndrome, which revealed two clusters primarily characterized by the presence or absence of vestibular symptoms [108]. Clustering methods have also been used to group TBI patients based on response to rehabilitation treatments [109], as well as acute injury features and long-term functional outcomes [110]. However, in addition to the lack of external validation of findings from studies in this area, a limitation of these studies is their reliance on subjective self-report measures to identify symptoms; future studies in this area should aim to evaluate whether the addition of objective measures to clustering methods can better inform TBI phenotypes. Nevertheless, these studies collectively demonstrate the potential for machine learning methods to improve personalized medicine for TBI by enabling the identification of patient groups with persisting or severe symptoms that may benefit from targeted treatments [111,112].

4.5. AI-Based Detection of Cognitive Impairment

With increasing evidence suggesting a link between TBI and the development of neurodegenerative disorders, especially among individuals who sustain moderate to severe TBIs or multiple TBIs [113], researchers have begun to examine the use of AI methods that can aid the early detection of cognitive impairment. One study used a machine learning approach to demonstrate that features of white matter hyperintensity detected on MRI could be used to accurately predict cognitive impairment in a sample of older individuals aged 47–84 years [114]. Another study used a similar approach to develop machine learning models of brain age using white matter and gray matter features, showing that the predicted difference between the brain age and chronological age of a group of patients with TBIs of any severity was 4.66–5.97 years [115]. Additionally, this age difference could be used to predict cognitive impairment and was correlated with time elapsed since injury, suggesting TBI initiates cumulative brain atrophy over time [115]. Consistent with these findings, a different study that used machine learning analysis of white matter changes within the default mode network showed that geriatric mTBI patients exhibit functional connectivity patterns similar to those in Alzheimer’s disease patients [116]. However, unlike the former two studies, this study did not evaluate the developed model in an independent dataset. Other studies have investigated AI-based analysis of other metrics, such as eye-tracking data, as a strategy for the early detection of Alzheimer’s disease [117], which may also show promise for detecting neurodegenerative disease following TBI with further study. Collectively, these studies indicate that AI may help with the prediction of individuals at risk for developing chronic conditions following TBI.

4.6. Considerations Regarding the Use of AI Methods in TBI Research

Despite the promising potential of AI methods such as machine learning to improve the clinical management of TBI, important challenges currently limit the generalizability of findings from these studies and the implementation of AI models for predicting TBI outcomes. First, these studies often exhibit methodology concerns that contribute to a high risk of bias. In one systematic review of nine studies on machine learning strategies for predicting psychosocial outcomes after TBI, every model was found to have a high risk of bias, and none of the studies provided reliable evidence for the predictive performance of the developed models [85]. Several tools have been developed to help assess bias risk during the development of prediction model studies; these include the Prediction model Risk Of Bias ASsessment Tool (PROBAST) [118] and Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis (TRIPOD) checklist [119]. However, few studies in this area report having utilized such tools. Additionally, few studies provide a description of an a priori consideration of how appropriate their machine learning analysis was for the dataset (i.e., based on sample size and outcomes of interest) [85].

These studies exhibit other methodological concerns worthy of consideration. The majority of these studies are retrospective, single-center studies; thus, further confirmation of the findings with multicenter prospective cohorts is warranted. Some studies in this area also do not adequately evaluate the developed model or even fail to report statistics on the model’s performance or calibration [84]. Other studies do not provide a detailed description of how missing data were handled or they inappropriately exclude individuals with missing data, rather than utilizing appropriate imputation strategies [83]. Some studies lacked an independent test set of data for external validation of the developed model [84], leading to a high risk of overfitting, which occurs when a model closely mimics and performs well on one dataset (e.g., the training data) but does not generalize to new data [28]. Additionally, some studies in this area did not perform a feature selection step to minimize the inclusion of potentially irrelevant measures from the model or they did not utilize other methods such as SHAP to determine which measures were most informative. This issue poses important challenges to achieving transparency and explainability with

AI-assisted clinical tools for TBI, which may prevent their adoption and limit their use by clinicians.

5. AI Challenges, Risks, and Policies for Ethical Use

5.1. Potential Challenges with the Use of AI in Healthcare

AI has great potential, but there are also reasonable concerns and limitations regarding its use in healthcare settings. First, more complex algorithms, such as deep learning and other machine learning models, typically require high levels of computing power, especially during model training since this step must be performed using considerably large datasets [120]. Fortunately, however, applying established models to new data requires less computational power, and efforts to increase the efficiency of AI models have already started to succeed [120]. Other challenges relate to inaccurate or nonsensical outputs, often referred to as “hallucinations”, and biased content AI may generate [55]. It is not always obvious when a document is AI generated [121], and the integration of GAI tools into medical decision making, and specifically TBI diagnosis or prognosis, risks propagating errors that could lead to inaccurate diagnoses or inappropriate treatment [55].

Cybersecurity threats are another area of concern with the use of AI systems for healthcare applications. The integration of AI with electronic health record systems and other healthcare databases can create points of data vulnerability when strategies for safeguarding protected health information are not adequately considered [122]. In this context, cyberattacks have the potential to become incredibly sophisticated. For example, there is potential for attackers to reconstruct confidential and sensitive medical information for a patient from details such as the patient’s age, sex, medical history information, and lifestyle factors [123]. Some researchers have investigated the use of AI systems as one strategy for advancing scam detection and cyberattack mitigation efforts, with some success. To this end, some studies have utilized LLMs, machine learning, and deep learning methods to improve data encryption and automated threat detection [35]; for example, one study developed a neural network approach to detect deception during telecommunication [36]. However, such tools are not yet widely utilized in healthcare settings and require further investigation.

Another ethical consideration is that the often-limited transparency of AI systems presents important challenges to ensuring patient-centered care. Patient-centered care emphasizes patients as active participants in their own healing and their right to autonomy and control over medical decisions [37]. Shared decision making is integral to patient-centered care and involves conversations between the patient and clinician, during which the clinician informs the patient about the potential risks and benefits of different courses of treatment, while the patient conveys their values, preferences, and priorities for treatment [124]. These discussions rely on a clinician’s ability to understand the information they are relaying and to convey this information accurately and effectively to the patient. Ensuring the explainability of AI models is thus crucial to achieving this goal, but research in this area often instead relies on “black box” methods with unclear clinical interpretations. Increasing reliance on “black box” AI systems during clinical decision making could substantially reduce patient trust in clinicians and negatively impact doctor-patient relationships, especially if the use of such tools is not routinely disclosed [37].

The possibility that AI-based methods could amplify biases in healthcare is yet another important ethical issue. Selection bias in datasets used during model training has been shown to diminish the accuracy of machine learning algorithms when they are applied to individuals with features that are underrepresented in the training data [125]. For example, studies have demonstrated that selection bias during the development of databases used for automatic facial recognition programs has resulted in particularly low accuracy when using these programs to recognize the faces of darker-skinned women [126]. In clinical studies, where individuals from racial and ethnic minority groups and resource limited settings are often underrepresented [127,128], there is potential for a similar form of bias during the development of AI-based clinical tools. Without deliberate efforts to utilize

data that adequately and equitably reflects individuals from diverse backgrounds, it is possible that AI-based clinical decision-making tools will exacerbate barriers to healthcare for underrepresented individuals, rather than reducing them.

Finally, there is concern over maintaining human oversight and the need for developing regulatory pathways for monitoring the safety and efficacy of AI-driven clinical products. To this end, the FDA released its Artificial Intelligence/Machine Learning (AI/ML)-Based Software as a Medical Device (SaMD) Action Plan in 2021, which proposes a potential framework for regulating AI-driven clinical products that is based on current pathways for regulating medical devices [129]. However, there are limitations to applying a device-centered approach to regulating AI products. For example, it is unclear what measures would be most appropriate for monitoring the efficacy of an AI product once it has been marketed and is used in the real world [130]. Since some AI-based clinical tools will likely be able to be used with little or no human supervision, some have proposed supplementing a device-centric regulatory strategy with a scheme that considers these tools as “physician extenders”; under this scheme, the FDA review of an AI clinical product would involve consideration for whether a tool could be used independently of physician oversight [130]. This scheme is similar to the oversight of nurse practitioners and has similar potential benefits to allowing nurse practitioners to practice independently, such as increasing healthcare access at a lower cost [130].

In summary, while the accelerating development of AI-driven clinical products is undeniable and their potential benefits are clear, there are substantial ethical challenges to consider. The efficiency and accuracy of AI models must be optimized, and robust cybersecurity systems must be developed to ensure the privacy of patient data can be maintained. Improving the transparency and explainability of AI systems is key to preserving patient-centered care, and strategies for minimizing the risk of bias are required to ensure these tools are used equitably. Collectively, these challenges illustrate the importance of developing mitigation strategies and minimum standards for the ethical use of AI in medicine, as well as pathways for regulatory oversight (Table 1).

Table 1. Guidelines and policies on the ethical use of artificial intelligence (AI). A number of United States and international organizations have developed guidelines and policies for the ethical uses of AI, many of which are applicable to the use of AI in healthcare settings. The potential risks of AI to propagate biases and encourage the misuse of medical information demand the close surveillance and regulation of AI systems as they continue to develop.

Organization	Guideline/Policy	Policy Principles
Guidelines developed in the scientific community		
National Academy of Sciences	Five Principles of Human Accountability and Responsibility when Using AI in Research [131]	<ol style="list-style-type: none"> 1. Transparent disclosure and attribution 2. Verification of AI-generated content and analyses 3. Documentation of AI-generated data 4. A focus on ethics and equity 5. Continuous monitoring, oversight, and public engagement
United States government policies and guidelines		
The White House Office	Executive Order about AI: Policy and Principles [132]	<ol style="list-style-type: none"> 1. AI must be safe, secure, and trustworthy. 2. There must be responsible innovation, competition, and collaboration. 3. There should be a commitment to supporting American workers. 4. Uses of AI should be consistent with advancing equity and civil rights. 5. The interests of Americans who increasingly use, interact with, or purchase AI and AI-enabled products in their daily lives must be protected. 6. Americans’ privacy and civil liberties must be protected as AI continues to advance. 7. It is important to manage the risks from the Federal Government’s own use of AI and increase its internal capacity to regulate, govern, and support responsible use of AI to deliver better results for Americans. The Federal Government should lead the way to global societal, economic, and technological progress.

Table 1. Cont.

Organization	Guideline/Policy	Policy Principles
Department of Defense (DOD)	The 2023 Data, Analytics and Artificial Intelligence Adoption Strategy [133]	<ol style="list-style-type: none"> 1. Responsible: DOD personnel should exercise appropriate levels of judgment and care, while remaining responsible for the development, deployment, and use of AI capabilities. 2. Equitable: The Department should take deliberate steps to minimize unintended bias in AI capabilities. 3. Traceable: The Department's AI capabilities should be developed and deployed such that relevant personnel possess an appropriate understanding of the technology, development processes, and operational methods applicable to AI capabilities, including with transparent and auditable methodologies, data sources, and design procedure and documentation. 4. Reliable: The Department's AI capabilities should have explicit, well-defined uses, and the safety, security, and effectiveness of such capabilities will be subject to testing and assurance within those defined uses across their entire life cycles. 5. Governable: The Department should design and engineer AI capabilities to fulfill their intended functions while possessing the ability to detect and avoid unintended consequences, and the ability to disengage or deactivate deployed systems that demonstrate unintended behavior.
International policies and guidelines		
North Atlantic Treaty Organization (NATO)	Principles of Responsible Use for AI in Defense [134]	<ol style="list-style-type: none"> 1. Lawfulness, Responsibility, and Accountability 2. Explainability and Traceability 3. Reliability 4. Governability 5. Bias Mitigation
World Health Organization (WHO)	Guidance on the Ethics and Governance of Artificial Intelligence for Health [135]	<ol style="list-style-type: none"> 1. Protect autonomy. 2. Promote human well-being, human safety, and public interest. 3. Ensure transparency, explainability, and intelligibility. 4. Foster responsibility and accountability. 5. Ensure inclusiveness and equity. 6. Promote artificial intelligence that is responsive and attainable.
United States Food and Drug Administration (FDA), Health Canada, and the United Kingdom Medicines and Healthcare Products Regulatory Agency (MHRA)	Good Machine Learning Practice for Medical Device Development: Guiding Principles [136]	<ol style="list-style-type: none"> 1. Multidisciplinary expertise is leveraged throughout the total product lifecycle. 2. Good software engineering and security practices are implemented. 3. Clinical study participants and datasets are representative of the intended patient population. 4. Training datasets are independent of test sets. 5. Selected reference datasets are based upon the best available methods. 6. Model design is tailored to the available data and reflects the intended use of the device. 7. Focus is placed on the performance of the human–AI team. 8. Testing demonstrates device performance during clinically relevant conditions. 9. Users are provided with clear, essential information. 10. Deployed models are monitored for performance and retraining risks are managed.

5.2. Views from the Scientific Community on the Ethical Use of AI

Efforts to develop ethical standards for AI use have intensified in recent years. These efforts have involved the combined contributions of scientists, as well as experts in law, ethics, human rights, and digital technology to establish clear principles for the ethical use of AI in healthcare. In an editorial published by the Proceedings of the National Academy of Sciences, a peer reviewed journal of the National Academy of Sciences, an interdisciplinary group of experts urged the scientific community to follow five principles of human accountability and responsibility when using AI in research [131]:

1. Transparent disclosure and attribution
2. Verification of AI-generated content and analyses
3. Documentation of AI-generated data
4. A focus on ethics and equity

5. Continuous monitoring, oversight, and public engagement

For each principle, the authors identify specific actions that should be taken by scientists, those who create models that use AI, and others. For example, for researchers, transparent disclosure and attribution includes steps such as clearly disclosing the use of GAI in research—including the specific tools, algorithms, and settings employed—and accurately attributing the human and AI sources of information or ideas. For model creators and refiners, transparent disclosure and attribution means actions such as providing publicly accessible details about models, including the data used to train or refine them. The editorial emphasizes that advances in GAI represent a transformative moment for science—one that will accelerate scientific discovery, but also challenge core norms and values of science, such as accountability, transparency, equity, replicability, and human responsibility. Of these principles, ensuring the ethical and equitable use of AI in medicine is particularly important to maintain patient-centered care and ensure that current biases in healthcare are not amplified by the incorporation of AI-based clinical decision-making tools.

5.3. United States (U.S.) Policies on Ethical Uses of AI

At the national level in the U.S., a White House Executive Order about AI: Policy and Principles [132] was issued in 2023 and emphasizes several key points. First, AI must be safe, secure, and trustworthy, and there must be responsible innovation, competition, and collaboration. Additionally, there should be a commitment to supporting American workers, and uses of AI should be consistent with advancing equity and civil rights. The Executive Order further states that the interests of Americans who increasingly use, interact with, or purchase AI and AI-enabled products in their daily lives must be protected. Relatedly, the Executive Order states that Americans' privacy and civil liberties must be protected as AI continues advancing. Finally, the Executive Order highlights that it is important to manage the risks from the Federal Government's own use of AI and increase its internal capacity to regulate, govern, and support responsible use of AI to deliver better results for Americans, and that the Federal Government should lead the way to global societal, economic, and technological progress.

The 2022 National Defense Strategy further emphasizes the importance of investing in AI to help build enduring military advantages [137]. To this end, in November 2023, the Chief Digital AI Office of the U.S. DOD released its strategy, known as the 2023 Data, Analytics and Artificial Intelligence Adoption Strategy, to accelerate the adoption of advanced AI capabilities to ensure U.S. warfighters maintain decision superiority, or the ability to make and implement more informed and accurate decisions faster than adversaries, on the battlefield for years to come [133]. These principles apply to both combat and non-combat functions and are intended to assist the U.S. military in upholding legal, ethical, and policy commitments in the field of AI. The department's AI ethical principles encompass five major areas, including that the use of AI should be (1) responsible, (2) equitable, (3) traceable, (4) reliable, and (5) governable.

The U.S. Department of Health and Human Services (HHS) has also established an AI strategy, which includes establishing an AI Council that will support the governance and development of AI throughout the HHS with four focus areas, which include (1) developing an AI-ready workforce and strengthening AI culture; (2) encouraging health AI innovation and research and development; (3) democratizing foundational AI tools and resources; and (4) promoting trustworthy AI use and development [138]. Similar focus areas for ensuring the responsible use of AI are also emphasized within the Department of Veteran Affairs [139], as well as the military health system as part of its effort to advance its digital strategy [140]. The U.S. has also established a Federal Policy for the Protection of Human Subjects, also known as the "Common Rule" [141], to provide protections for the subjects of human studies that must be considered during the use of AI in clinical research. The use of AI in human studies could pose challenges to adhering with this policy as it becomes more difficult to determine what information is truly private or identifiable and what constitutes informed consent [142]. As research in this area continues, careful

consideration of these principles and potential challenges should occur at each stage of a given study.

5.4. Global Policies on Ethical Uses of AI

Several international organizations have also developed guidelines for the responsible use of AI. In July of 2024, the North Atlantic Treaty Organization (NATO) released an update to its AI strategy, which describes the desired outcomes for the incorporation of AI strategies, as well as goals for responsibly integrating AI into NATO [134]. The strategy endorses six Principles of Responsible Use for AI in Defense, which include Lawfulness, Responsibility and Accountability, Explainability and Traceability, Reliability, Governability, and Bias Mitigation [134]. Notably, the updated NATO AI strategy differs from the 2021 version, as it specifically addresses the misuse of AI and AI-generated disinformation as important areas of concern that necessitate increasing vigilance [143].

In 2021, the World Health Organization (WHO) released their Guidance on the Ethics and Governance of Artificial Intelligence for Health, which emphasizes that as the use of AI in healthcare continues to progress, consideration for human rights and ethics must be at the forefront of these developments [135]. The WHO identifies six ethical principles for the use of AI in healthcare, including: (1) protect autonomy; (2) promote human well-being, human safety, and public interest; (3) ensure transparency, explainability, and intelligibility; (4) foster responsibility and accountability; (5) ensure inclusiveness and equity; (6) promote artificial intelligence that is responsive and attainable.

Additionally, in 2021, the FDA, Health Canada, and the United Kingdom's Medicines and Healthcare Products Regulatory Agency (MHRA) developed 10 guiding principles for good machine learning practice (GMLP) [136]. These principles offer specific guidance on how to promote the development of high-quality medical products involving AI and machine learning. For example, principle four highlights that training datasets should be independent of test datasets, while principle eight proposes that AI-driven medical products should be tested in clinically relevant conditions. The principles also address several potential concerns with ethical AI use and maintaining transparency; for instance, principle three emphasizes the necessity of using study participants and data that adequately reflect the intended patient population, and principle nine states that users should be provided clear, essential information on the intended use of AI products.

Collectively, these policies lay a foundation for the ethical use of AI products that can be amended as advancements in AI development continue, and careful adherence to them at an international level will be key to promoting the ethical use of AI systems in medicine.

6. Discussion

AI and its subcategories represent a rapidly evolving field with considerable potential for facilitating and enhancing TBI research and clinical decision making with further study. With AI-driven clinical tools, important and clinically relevant conclusions can be produced in a fraction of the time it would take with conventional methods. In the context of the acute management of moderate to severe TBI, several studies have demonstrated the utility of AI in assisting with key aspects of triage, including predicting the development of ICH [83,84], identifying patients who require surgical intervention [86,87], and assessing mortality risk [88–90]. To accelerate progress in diagnosing mTBI, some researchers have leveraged machine learning analyses of the rich set of data provided by EEG, MRI, biofluid markers and other measures to accurately discriminate mTBI patients from healthy controls [96,98,103]. AI systems have also shown potential in improving the long-term management of TBI through classifying TBI phenotypic clusters and predicting the development of cognitive impairment [106,108,110,114,115]. Collectively, these studies indicate that AI-driven medical tools could allow clinicians to move beyond nonspecific, severity-based TBI classification into a new era in which patients are selected for targeted treatments and interventions based on their unique presentation of clinical signs, sociodemographic features, symptoms, and pathologic findings.

However, research in this area does exhibit several limitations that will be important to consider as work in this area continues. To develop robust AI systems that can be applied to TBI clinical care, common data elements must be identified and standardized [27]. Studies in this area must also aim to avoid methodological flaws that could undermine the quality of resultant AI products. For example, these studies must include and better describe efforts to minimize the risk of overfitting, such as by using independent training and test datasets of sufficient sample size and removing potentially irrelevant measures from diagnostic panels [85]. One potential barrier to the clinical implementation of AI products for TBI management is that some forms of AI require considerably large amounts of computing power and thus may not be applicable in resource-limited settings. Additionally, the ethical challenges with using AI-based analyses of sensitive patient healthcare data are numerous. There clearly are legitimate ways in which investigators and clinicians can and should consider using AI to improve their clinical investigation, but in all cases, that use should be disclosed, properly attributed, and developed in concert with appropriate published guidelines.

Prioritizing transparency and maintaining patient-centered care are particularly important for researchers and clinicians who aim to develop AI products that can be applied to TBI management. While there are currently no FDA-cleared treatments for TBI, many clinicians still recommend various options for managing long-term TBI symptoms in accordance with clinical practice guidelines. For example, cognitive rehabilitation programs may be beneficial for addressing cognitive deficit after TBI and are often recommended for this purpose [12,144]. Beyond these evidence-based treatments, there are many other potential treatment options ranging from neuromodulation strategies to exercise therapies that have been increasingly investigated for the management of TBI symptoms, with varying levels of evidence regarding their efficacy [145,146]. Patients often rely on medical professionals to help them navigate this complex landscape of potential treatments and make informed decisions about pursuing these alternatives. If AI products become part of the process for selecting patients for specific treatments in instances where the most appropriate treatment is not obvious, it will be important for clinicians to be able to communicate the use of these tools to safeguard patient trust. Adherence to guidelines for the ethical use of AI in healthcare, as well as close surveillance of the use of these tools, will thus be crucial for ensuring AI is used responsibly as the field rapidly continues to evolve.

Funding: This work was supported by the Traumatic Brain Injury Center of Excellence. The views expressed in this manuscript are those of the authors and do not necessarily represent the official policy or position of the Defense Health Agency, Department of Defense, or any other U.S. government agency. This work was prepared under Contract HT0014-22-C-0016 with DHA Contracting Office (NM-CD) HT0014 and, therefore, is defined as U.S. Government work under Title 17 U.S.C.§101. Per Title 17 U.S.C.§105, copyright protection is not available for any work of the U.S. Government. For more information, please contact dha.TBICOEinfo@health.mil. UNCLASSIFIED.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Review

The Relevance and Implications of Monoclonal Antibody Therapies on Traumatic Brain Injury Pathologies

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Abstract: Traumatic brain injury (TBI) is a global public health concern. It remains one of the leading causes of morbidity and mortality. TBI pathology involves complex secondary injury cascades that are associated with cellular and molecular dysfunction, including oxidative stress, coagulopathy, neuroinflammation, neurodegeneration, neurotoxicity, and blood–brain barrier (BBB) dysfunction, among others. These pathological processes manifest as a diverse array of clinical impairments. They serve as targets for potential therapeutic intervention not only in TBI but also in other diseases. Monoclonal antibodies (mAbs) have been used as key therapeutic agents targeting these mechanisms for the treatment of diverse diseases, including neurological diseases such as Alzheimer’s disease (AD). MAb therapies provide a tool to block disease pathways with target specificity that may be capable of mitigating the secondary injury cascades following TBI. This article reviews the pathophysiology of TBI and the molecular mechanisms of action of mAbs that target these shared pathological pathways in a wide range of diseases. Publicly available databases for various applications of mAb therapy were searched and further classified to assess relevance to TBI pathology and evaluate current stages of development. The authors intend for this review to highlight the potential impact of current mAb technology within pathological TBI processes.

Keywords: traumatic brain injury (TBI); monoclonal antibodies (mAbs); monoclonal antibody therapy; secondary injury cascades; pathological TBI processes; oxidative stress; coagulopathy; neuroinflammation; neurodegeneration; neurotoxicity; blood–brain barrier (BBB) dysfunction; neurological diseases

1. Introduction

Traumatic brain injury (TBI) is a public health concern with a prevalence of about 69 million worldwide per year [1]. In the U.S. alone, about 1.7 million TBIs are reported annually, which includes a range of mild to severe brain injuries [2,3]. Millions of TBI-related visits to an emergency department (ED), hospitalizations, or deaths occur annually. TBI patients can experience short-term or permanent disabilities in multiple domains of functioning, including cognition, physical health, psychological health, and social life. TBI imposes significant health, social, and economic burdens. Various therapeutic interventions are being studied in pre-clinical animal models of TBI, but effective clinical therapeutic treatments for TBI recovery are still lacking [4–11]. This problem is likely to be augmented as the TBI survivor population continues to increase [12].

Mechanistically, TBI pathology manifests from two primary mechanisms: primary and secondary damage. Primary injury occurs at the moment of initial trauma and can include skull fracture, cerebral hemorrhage, and diffuse axonal injury [4,13]. These primary mechanisms elicit complex secondary injury mechanisms that drive subsequent pathology and include cellular and molecular dysfunction, including oxidative stress, neuroinflammation, neurodegeneration, neurotoxicity, and blood–brain barrier (BBB) dysfunction, among

others [4]. These pathological processes manifest as a diverse array of clinical impairments and serve as targets for potential therapeutic intervention [4,6].

TBI-induced cerebral injury is a mixture of structural, cellular, and vascular injury. Current therapeutic interventions involve neuroprotection, neurovascular regeneration, and neurorestoration for treating TBI. These strategies often target a single factor-mediating secondary injury. These proclaimed specific factors often have additional and unexpected effects on other pathways. Monoclonal antibodies (mAbs) can bind to the cell or pathway-specific molecules and modulate injury mechanisms. MAb therapies provide a tool to influence specific cellular and molecular pathways that may be capable of mitigating the secondary injury cascades following TBI and offering a more precision medicine approach [14].

Antibodies (or immunoglobulins) are naturally occurring protective proteins that offer one of the body's main lines of defense against foreign substances. Antibodies are Y-shaped proteins. They are composed of two heavy chains and two light chains. Each chain contains a variable region and a constant region. The variable region contains a sequence of amino acids that gives each antibody its specificity towards a particular antigen and even a particular binding site on that antigen, in the case of monoclonal antibodies. This specificity maximizes the potential for harnessing monoclonal antibodies as a therapeutic strategy by inherently reducing cross-reactivity within biological systems [15].

Antibodies can defend against foreign substances through various mechanisms [16,17] (Figure 1). In some cases, antibody removal of foreign substances can be achieved without the recruitment of other molecular/cellular support. Neutralization is a process by which antibodies binding to the surface of the antigen prevent the antigen from reaching and interacting with the target cell(s) [17,18]. Alternatively, antibody binding can create aggregates by cross-linking pathogens through the use of the multiple binding sites available on the antibody. These aggregates can subsequently be more efficiently filtered from the body through the kidneys or spleen. Other mechanisms of action involve the recruitment of cellular or molecular support to remove the pathogen. Antibody binding to the pathogen can recruit and activate the complement system, facilitate the recruitment of phagocytic cells, or recruit natural killer cells, which secrete cytotoxins to kill the pathogen (antibody-dependent cell-mediated cytotoxicity; ADCC) [16,17].

Over the years, scientists have taken advantage of the natural function of antibodies in their pursuit of finding treatments for a wide range of diseases, such as many types of cancer, as well as autoimmune, infectious, and hematological diseases [19]. Hybridoma technology gave rise to the mass production of murine antibodies [20,21] and, later, chimeric and humanized antibodies [22]. The development of humanized mAbs helped launch mAbs into the commercial market through the discovery of the complementary-determining region grafting technique, which allowed for even greater conservation of human-derived regions [23]. Humanized mAbs have a human origin for the constant regions and for most of the variable regions—only the hypervariable complementary-determining regions are transplanted from a non-human source [23]. The FDA approved the first mAb for therapeutic use in 1986 [24]. Since then, the market has experienced dramatic growth. Globally, commercial companies have studied over 570 mAbs in clinical trials [25]. Over 79 mAbs have been approved for therapeutic use by the U.S. FDA over the years [19, 21]. Major technological advancements in discovery and development technologies have resulted in augmented market growth and mAbs available for therapeutic use [19,26–29]. Despite the growth, few studies have evaluated mAb potential within the TBI pathology. The authors intend for this review to highlight the potential impact and evaluate the possibilities for current mAb therapeutic technology as a relevant tool for the treatment of secondary TBI injury. To develop a firm understanding of the state of the science and literature landscape, we reviewed publicly available databases (Google Scholar, PubMed, Clinicaltrials.gov, etc.) for various applications of mAb therapy. The results were further classified to assess relevance to TBI pathology and evaluate current stages of development. A number of factors contribute to secondary TBI injury. While others exist, this work

identified and focused on several key areas of relevance, including inflammation, vascular function, coagulation, excitotoxicity, oxidative stress, and neurodegeneration (Table 1, Figure 2).

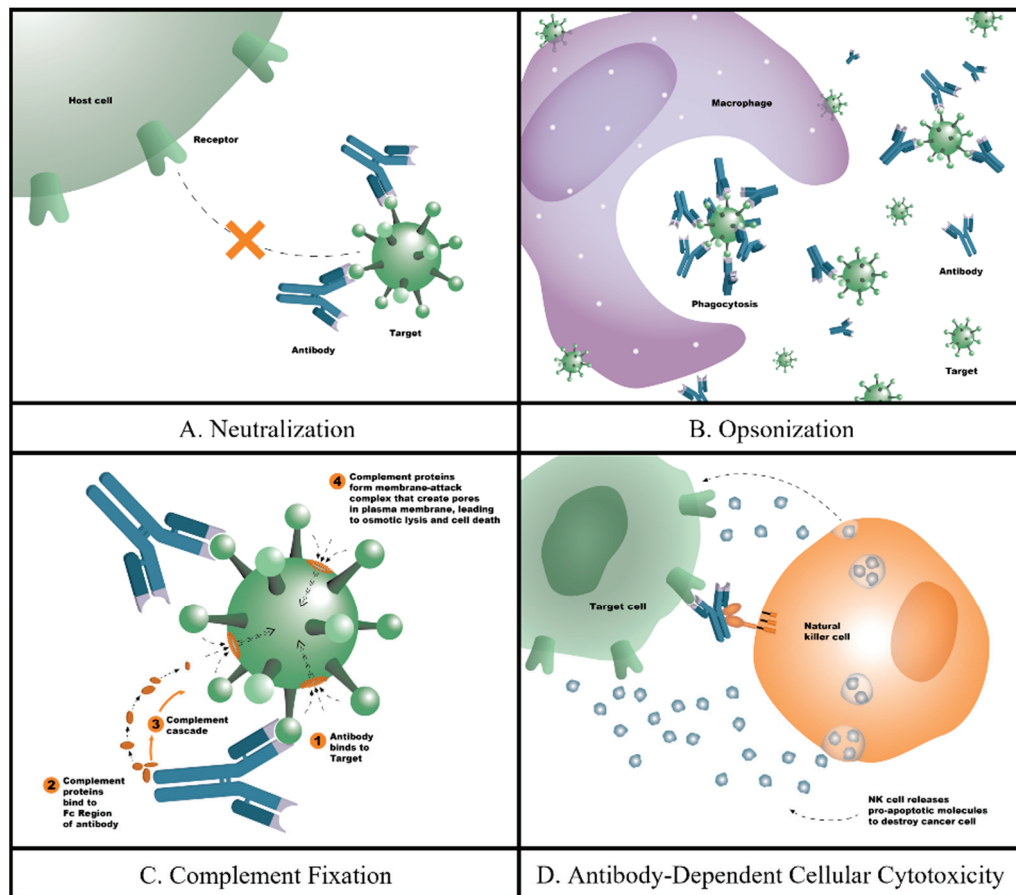


Figure 1. Four main mechanisms of monoclonal antibodies: (A) neutralization: this process can occur by allosteric inhibition, in which antibodies binding to the surface of the antigen prevent the antigen from reaching and interacting with the target cells; (B) opsonization: a process by which pathogens are coated with antibodies to increase their susceptibility for phagocytosis; (C) complement fixation: an immune reaction by which antibody binding to the pathogen can recruit and activate the complement system and facilitate the recruitment of phagocytic cells; (D) antibody-dependent cellular cytotoxicity: a process by which antibodies attach to target cells and recruit effector cells to induce target cell death.

Table 1. Pre-clinical mAbs and FDA-approved mAbs targeting secondary injury pathology in TBI.

mAb	Targeted Secondary Injury Mechanism	Stage of Development	Effects/Indications	Reference
HMGB1	Inflammation	Pre-clinical	Reduce microglial activation and neuronal death	[30,31]
CD11/CD18	Inflammation	Pre-clinical	Reduce brain edema and microglial activation	[32–36]
Tocilizumab, Sarilumab	Inflammation (IL-6/IL-6R)	FDA-approved	Rheumatoid arthritis Castleman’s disease; ovarian, prostate, and lung cancers	[19]
Siltuximab			Multiple myeloma	[37,38]

Table 1. Cont.

mAb	Targeted Secondary Injury Mechanism	Stage of Development	Effects/Indications	Reference
Canakinumab	Inflammation (IL-1 β)	FDA-approved	Cryopyrin-associated disorders	[39]
Cis-P-tau	Neurodegeneration (P-tau)	Pre-clinical	Block cis-P-tau pathology and restore neuronal dysfunction	[40]
Acetylated-tau	Neurodegeneration (acetylated-tau)	Pre-clinical	Reduce tau pathology and glia activation Improve neurobehavioral impairment	[41]
Aducanumab Leqembi Donanemab	Neurodegeneration (amyloid-beta)	FDA-approved	Alzheimer's disease	[42] [43] [44]
Remternetug	Neurodegeneration (amyloid-beta)	Phase III (Clinical Trial #:NCT05463731)	Alzheimer's disease	[45–47]
α -synuclein	Neurodegeneration	Pre-clinical	Parkinson's disease	[48–51]
Abciximab	Coagulopathy (glycoprotein IIb/IIIa)	FDA-approved	Coronary artery procedures	[52]
Caplacizumab	Coagulopathy (glycoprotein IIb/IIIa)	FDA-approved	Thrombotic thrombocytopenic purpura	[53]
Crizanlizuman	Vascular function (P-selectin)	FDA-approved	Sickle cell disease	[54]
Bevacizumab Brolucizumab	Vascular function (VEGF-A)	FDA-approved	Colorectal cancer Macular edema	[55] [56]
Ramucirumab	Vascular function (VEGFR)	FDA-approved	Gastric cancer	[57]
NMDA receptor	Excitotoxicity	Pre-clinical	Multiple sclerosis	[58]
MMP-9	Oxidative stress	Pre-clinical	Reduce fibrosis, oxidative stress, and BBB dysfunction	[59,60]
Andecaliximab	Oxidative stress (MMP-9)	Phase III (Clinical Trial #: NCT02545504)	Gastric cancer	[61]

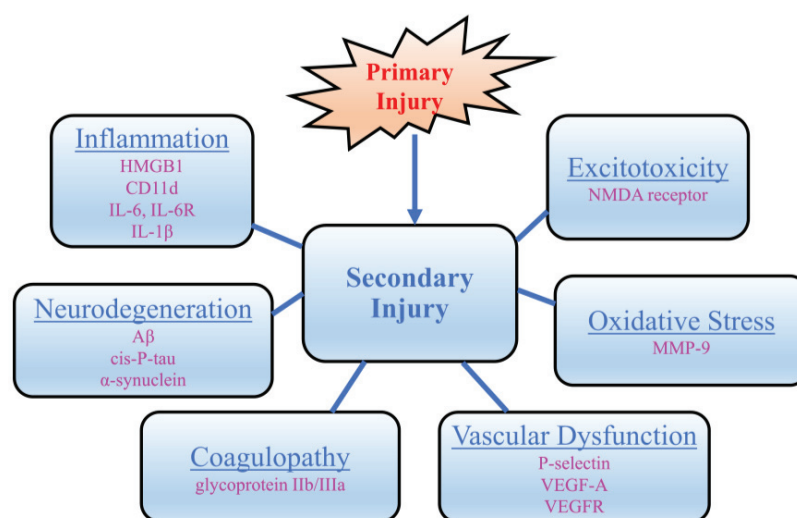


Figure 2. Secondary injury pathologies in TBI and previously studied therapeutic targets. The several key areas include inflammation (targets: high mobility group box 1 (HMGB1), cluster of differentiation

11d (CD11d), interleukin 6 (IL-6), interleukin 6 receptor (IL-6R), and interleukin 1 beta (IL-1 β)), vascular function (targets: P-selectin, vascular endothelial growth factors-a (VEGF-A), vascular endothelial growth factors receptor (VEGFR)), coagulation (targets: glycoprotein IIb/IIIa), excitotoxicity (targets: N-methyl-D-aspartate receptor (NMDA) receptor), oxidative stress (targets: matrix metalloproteinase-9 (MMP-9)), and neurodegeneration (targets: amyloid beta (A β), cis-P-tau, and α -synuclein).

2. mAbs and Inflammation

2.1. Pathophysiology Relevance

Primary damage resulting from TBI triggers a complex and heterogeneous neuroinflammatory response. This response involves various cytokines, chemokines, and inflammatory molecules that coordinate the functions of microglia, astrocytes, and infiltrating immune cells [62,63]. During the acute stages after injury, these processes are beneficial for mitigating injury progression, repairing damaged cells, and protecting against pathogen infiltration [64–68]. However, when the inflammatory response becomes persistent and unregulated, it can hinder recovery and exacerbate oxidative stress, cell death, and neurodegeneration [69,70]. As such, chronic inflammation has historically been a focus of therapeutic intervention strategies.

Upon injury, damage-associated mediator proteins, including high-mobility group protein B1 (HMGB1), are passively released from injured cells [71,72]. This release is continued by secondary injury mechanisms that create a detrimental feedback loop that leads to robust production of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF- α), interleukin 6 (IL-6), interleukin 1 beta (IL-1 β), and interferon-gamma (IFN- γ) [72,73]. The expression of these pro-inflammatory mediators initiates several downstream processes, including the increased permeability of the BBB and the upregulation of adhesion molecules. Together, these changes facilitate the infiltration of neutrophils, monocytes, and lymphocytes to cross the BBB into the brain parenchyma [74].

Once these immune cells infiltrate, they contribute to the production of inflammatory mediators and can recruit microglia, the resident immune cells of the brain. Microglia arise from macrophages and can take on a pro-inflammatory and anti-inflammatory phenotype, serving as the first line of defense to the injured brain. Studies have shown that microglia tend to favor the pro-inflammatory phenotype in the acute stages following injury, which aids initial recovery by scavenging cellular debris and coordinating restorative processes. Many of these actions are achieved by the upregulation of pro-inflammatory mediators, neurotoxic chemicals, and free radicals. While the neurotoxic chemicals and free radicals initiate cell death mechanisms, the pro-inflammatory mediators can further propagate the inflammatory response [75].

Astrocytes also play an important role following TBI [76]. They have been shown to promote axonal repair, cell proliferation, neuronal survival, and the inhibition of apoptosis. Additionally, astrocytes are involved in repairing the BBB after injury and can limit cell infiltration and blood component extravasation [77]. However, like microglia, excessive activation has been shown to be detrimental [77]. Astrocyte activation and proliferation are often associated with glial scar formation at the injury site. The scar establishes a barrier around the damaged tissue, separating healthy brain areas from neurotoxic or potentially harmful regions through the production of an inhibitory cellular matrix. While the scar can help contain the damage response, it may also compromise the repair mechanisms of the damaged tissue and hinder the reduction in glial scarring that has been associated with positive outcomes [76].

2.2. mAb and TBI Inflammatory Processes: FDA-Approved mAbs and Pre-Approval Testing

Phases Because inflammatory processes are not unique to specific pathologies, many of these mechanisms have been extensively studied in other diseases. mAbs have been utilized against a wide range of non-TBI diseases, particularly those characterized by persistent inflammation, such as irritable bowel (IBD), Crohn's disease (CD), and rheumatoid arthritis.

tis [19]. As previously mentioned, IL-6R, IL-1 β , TNF- α , IFN- γ , and HMGB1 have been implicated in TBI pathologies. These same mediators have also been associated with other somatic pathologies, leading to the development of monoclonal antibodies targeting these cytokines or their receptors, many of which have received FDA approval and demonstrate considerable efficacy. Often, multiple mAbs may be indicated for the same pathology, reflecting a variety of mechanisms of action or differences in the construction of the mAb itself [19].

2.3. Pre-Approval mAbs

2.3.1. High Mobility Group Box 1 (HMGB1)

High mobility group box 1 (HMGB1) is a significant mediator of injury-induced inflammation, making it a promising target for neuroinflammation to inhibit secondary damage post-TBI. HMGB1 is a pro-inflammatory-like cytokine released due to the activation of other cytokines and passively during cell death. It acts as a nuclear factor that enhances transcription and mediates responses to infection, inflammation, and injury. During TBI, HMGB1 mediates neuroinflammation through the activation of cytokines such as TNF- α and interleukin-1 (IL-1). The over-expression of HMGB1 has been observed in TBI [78] and other neuroinflammatory conditions, including Alzheimer's disease [79], Parkinson's disease [80], and subarachnoid hemorrhage [81]. In TBI, HMGB1 is released through the N-methyl-D-aspartate receptor [82]. Pre-clinical studies in rats have shown that anti-HMGB1 antibodies can reduce the accumulation of activated microglia in the cortex of the ipsilateral hemisphere after TBI and prevent neuronal death in the hippocampus [30]. Another rat study model of intracerebral hemorrhage (ICH) demonstrated that administration of anti-HMGB1 mAb inhibited HMGB1, brain edema, microglial activation, mRNA expression of pro-inflammatory cytokines, and apoptotic cell death in peri-hematoma areas, leading to improved neurological performance and reduced plasma levels of HMGB1 [31]. Overall, HMGB1 exhibits strong pro-inflammatory properties primarily through its interaction with the receptor for advanced glycation end products (RAGE), making its blockade a potential therapeutic strategy for brain injury [83].

2.3.2. Cluster of Differentiation 11/18 (CD11/CD18)

Cluster of differentiation 11/18 (CD11/CD18) integrins moderate the entry of leukocytes into the central nervous system. Previous research by Bao and colleagues utilized an anti-CD11d mAb to block the CD11d/CD18 and VCAM-1 interaction following experimental spinal cord injury in rats [32]. Their studies demonstrated that treatment with the CD11 mAb for up to 48 h after spinal cord injury enhanced functional recovery by decreasing the number of neutrophils and macrophages and preventing the formation of reactive free radicals, lipid peroxidation, protein nitration, and DNA damage [32–36]. Given that these mechanisms also contribute to secondary injury in TBI, researchers explored the potential therapeutic benefits of anti-CD11d for TBI. Their findings indicated that the antibody reduced neutrophil and macrophage infiltration within the injured brain, subsequently decreasing lipid peroxidation, free radical formation, astrocyte activation, amyloid precursor protein expression, and neuronal loss [84]. These effects corresponded with reduced impairments in tests of spatial cognition, anxiety, and sensorimotor function in the rats [84].

2.4. FDA-Approved mAbs

2.4.1. Interleukin 6 and Interleukin 6 Receptor (IL-6, IL-6R)

IL-6 is a cytokine that exhibits both pro-inflammatory and anti-inflammatory properties. It is produced by various cell types in response to stimuli such as trauma and infection [85]. IL-6 exerts its effects by binding to its specific receptor, IL-6R, which exists in both soluble and membrane-bound forms. The binding of IL-6 to IL-6R occurs in conjunction with a transducer protein known as glycoprotein 130 (gp130). Notably, soluble IL-6R can also initiate signaling through a process called trans-signaling. Together, IL-6,

IL-6R, and two gp130 molecules form a four-part complex at the cell surface, activating genes with IL-6 response elements via the JAK-STAT pathway. This activation commonly leads to the production of acute phase proteins [86], thereby promoting inflammation.

Currently, two IL-6R monoclonal antibodies (mAbs), Tocilizumab and Sarilumab, are FDA-approved for the treatment of rheumatoid arthritis (RA) [19]. IL-6 plays a crucial role in the pathogenesis of RA, producing extensive systemic effects. A comprehensive review by Narazaki and colleagues [87] highlights the multifaceted effects of IL-6 in RA. In summary, IL-6 influences the differentiation of T and B lymphocytes, vascular homeostasis, the acute phase response, and coagulability. It promotes the differentiation of plasma blasts into plasma cells, contributing to the hypergammaglobulinemia observed in RA. Additionally, IL-6 stimulates the differentiation of CD4⁺ T cells into Th17 cells, which negatively impacts T regulatory cell (Treg) differentiation. Furthermore, IL-6 enhances T follicular helper (Tfh) cell differentiation, promoting a T cell-dependent B-cell response. IL-6 also affects vascular homeostasis by increasing vessel permeability and influences the acute phase response by elevating levels of C-reactive protein, complement C3, fibrinogen, and thrombopoietin. Moreover, IL-6 contributes to a hypercoagulable state through two mechanisms: it increases thrombopoietin during the altered acute phase response, which enhances megakaryocyte production in the bone marrow and leads to thrombocytosis. Simultaneously, IL-6 upregulates the expression of tissue factor on monocytes. Tocilizumab and Sarilumab mitigate these effects by blocking both membrane-bound and soluble IL-6 receptors.

In contrast, a third FDA-approved mAb, Siltuximab, binds directly to IL-6, competitively inhibiting IL-6R stimulation and its downstream effects [37]. Siltuximab is indicated for Castleman's disease (CD) but is also being investigated for various cancers, including ovarian, prostate, and lung cancers, as well as multiple myeloma. In CD, IL-6 inhibition primarily targets the aberrant secretion of IL-6 by germinal center B lymphocytes and plasma cells [38].

2.4.2. Interleukin-1 β (IL-1 β)

IL-1 β is a pro-inflammatory cytokine that requires interaction with the type I IL-1 receptor (IL-1RI) and the IL-1 receptor accessory protein (IL-1RAcP) to form a heterotrimeric complex. This complex brings together the intracellular Toll-IL-1 receptor domains, ultimately activating NF- κ B and downstream transcription [39]. IL-1 β stimulation results in both localized and systemic effects, playing a crucial role in resolving infections; however, chronic stimulation can be detrimental. In the context of TBI, IL-1 β stimulation can lead to the recruitment of inflammatory cells to sites of inflammation, propagating the inflammatory response and inducing the production of reactive oxygen species through enzymes such as cyclooxygenase 2 and inducible nitric oxide synthase [88].

The FDA has approved Canakinumab for treating cryopyrin-associated disorders (CAP), including Muckle-Wells syndrome and familial cold auto-inflammatory syndrome. CAP arises from the uncontrolled over-secretion of IL-1 β due to mutations in the cryopyrin-coding gene NLRP3. These mutations lead to functional changes in the inflammasome protein cryopyrin, which regulates IL-1 β secretion [89]. Canakinumab is a human monoclonal antibody that binds free IL-1 β , preventing its interaction with IL-1RI and IL-1RAcP, thereby inhibiting the formation of the signaling complex. This blockade reduces NF- κ B activation and downstream pro-inflammatory transcription regulated through Toll-IL-1 receptor domains [39].

3. mAbs and Neurodegeneration

3.1. Pathophysiology Relevance

It is widely acknowledged that TBI is associated with increased risks of developing dementia, including Alzheimer's disease (AD) and Parkinson's disease (PD) [1,90–94]. Diffuse axonal injury is one of the most common neuropathological features of TBI, which is characterized by the mechanical deformation of axons. Its subsequent neuronal dys-

function includes abnormal glutamate release, axonal transport interruption, swelling, and accumulation of proteins, such as amyloid-beta ($A\beta$), phosphorylated tau (pTau), and α -synuclein [95–98]. These proteinopathies are also pathological hallmarks in Alzheimer's ($A\beta$ and pTau) [99–102] and Parkinson's diseases (α -synuclein) [103–105]. Plaques composed of $A\beta$ peptides are now recognized as a common pathology of both acute and chronic stages of TBI patients [94,98]. TBI-induced axonal damage leads to the accumulation of amyloid precursor protein (APP) and its cleavage enzymes, including beta secretase 1 and presenilin 1 in axonal swellings. Cleaved APP further forms pathogenic species of amyloid-beta, which are released into the brain and generate plaques within hours following TBI [97,106,107]. Toxic amyloid-beta plaques contribute directly to neuronal loss observed after TBI [97]. Widespread amyloid-beta plaques in some TBI patients persist for months or years and further trigger long-term neurodegenerative processes, which leads to progressive neurodegeneration [97,107–109].

In addition to amyloid-beta pathology, tau proteins are hyperphosphorylated and accumulated after TBI [94,97,107,109,110]. The microtubule-associated protein tau is a key constituent of axons and functions to stabilize microtubules, and it contributes to the regulation of axonal transport, neuronal development, postsynaptic scaffolding, and apoptosis [111–113]. In sports and military-related TBI patients and experimental animal models, mechanical axon deformation and impaired axonal transport during injury may induce tau disassociation from microtubules, which leads to tau hyperphosphorylation, misfolding, and aggregation. These events further produce a highly pathogenic tau species (cis-P-tau), which contributes to apoptosis, mitochondrial damage, and abnormal long-term potentiation, resulting in axonal damage and neuronal loss [40]. Interestingly, tau is also aberrantly acetylated in various neurodegenerative conditions, including AD and TBI [41].

TBI has long been associated with Parkinson's disease; α -synuclein protein aggregation is a critical pathology of PD [114]. It is a presynaptic protein that plays an important role in synaptic vesicle recycling. In a chronic TBI rat model, dopaminergic neurons in the substantia nigra were significantly decreased. In parallel, an increased abnormal accumulation of α -synuclein was detected, which suggested that α -synuclein may function as a pathological link between the chronic effects of TBI and PD-like neurodegeneration [92,115].

3.2. Pre-Approval mAbs

Pre-clinical studies in mouse models of impact and blast TBI showed increased cis-P-tau levels and related axonal damage. Administration of anti-cis-tau monoclonal antibody (mAb) blocked cis-P-tau pathology and restored neuronal dysfunction. These results suggest that cis-tau mAbs can be used as a potential therapy for preventing the development of TBI-induced tauopathy [40]. Similarly, anti-acetylated-tau treatment reduces tau pathology, rescues glial responses, and improves neurobehavioral impairment [41].

Post-traumatic proteinopathies have similarities to neurodegenerative diseases, in particular AD [98,116]. mAb therapies targeting proteinopathies have been used for the treatment of AD and PD, which also provide a promising strategy for treating TBI. Several mAbs targeting amyloid-beta have been developed to treat AD, including Aducanumab, Lemmings, Donanemab (aka N3pG or Kisunla), Leqembi, and Solanezumab [45–47,117,118]. α -synuclein-targeted mAbs for PD were developed and used to improve α -synuclein-associated neurodegeneration [48–51].

3.3. FDA-Approved mAbs

Among these mAbs, Aducanumab was the first FDA-approved (accelerated approval) mAb targeting $A\beta$ aggregates to treat AD [42]. It decreases amyloid-beta plaques, which is accompanied by a modest slowing of cognitive decline. Though post-approval phase 4 is needed to verify clinical benefit, it is a big step forward for treating and preventing AD. Leqembi was granted accelerated approval by the FDA in 2023, marking it as the second monoclonal antibody targeting $A\beta$ approved for the treatment of AD. The pivotal Phase III clinical trial, CLARITY AD, evaluated the drug in 1795 patients exhibiting symptoms of

mild dementia due to early-stage AD. Results from this trial demonstrated that after 18 months of treatment, the rate of decline in cognitive and memory function was reduced by 27% in participants receiving Leqembi compared with those given a placebo [43]. Donanemab is the third humanized IgG1 monoclonal antibody developed from mouse mE8-IgG2a. It recognizes A β (3–42), an aggregated form of A β found in amyloid plaques. Donanemab targets deposited plaque itself to clear existing amyloid burden from the brain rather than merely preventing the deposition of new plaques or the growth of existing plaques [44].

4. mAbs and Coagulopathy

4.1. Pathophysiology Relevance

Under physiologic conditions, the coagulation system is maintained in a dynamic equilibrium by a careful balance between coagulation cascades to form clots and fibrinolysis to break down clots. Hematologic aberrations are manifested by disruption of the balance between the coagulation system and the fibrinolytic system. TBI represents one of these disruptions; it causes early hypercoagulation that transitions to a subsequent hypocoagulable state and is known to correlate with worsened outcomes, including morbidity and mortality. The underlying mechanisms are poorly understood, but since the patients lack the traditional causes of coagulopathy, including significant blood loss or fluid administration, TBI-induced coagulopathy is believed to be pathogenic.

The BBB is the semi-permeable barrier of cerebral vasculature and is susceptible to shear forces during mechanical loading conditions. BBB compromise is well documented following TBI and can lead to extravasation of brain-derived molecules. Among these molecules are pro-coagulants and tissue factors that cause widespread activation of the extrinsic coagulation cascade upon reaching circulation [119]. The clotting process via the extrinsic pathway involves the production of Factor VIIa/TF complex, which generates Factor Xa. Factor Xa mediates the cleavage of prothrombin into thrombin that, in turn, acts as a protease catalyzing the conversion of soluble fibrinogen into insoluble strands of fibrin, forming a blood clot. In addition, thrombin activates platelets and Factors V and VIII, stimulating more thrombosis [120]. TBI-induced endothelial damage, including glycocalyx degradation, further accelerates coagulation through direct interaction between the circulating components of blood and the endothelial wall. This hypercoagulable state confers additional risk to TBI patients by augmenting the risk of microthrombi formation and subsequent ischemic injury [119]. In fact, TBI patients face a much greater risk of developing embolic and thrombotic complications, including deep vein thrombosis and pulmonary embolism.

Hypercoagulation causes the eventual depletion of clotting factors and platelets, likely causing the transition to a hypocoagulable state [121]. This transition has been observed clinically by prolonged prothrombin and partial thromboplastin times seen hours after injury. In addition, a clinical study reported that the level of fibrin degradation products reached a peak at 6 h post-TBI and then returned to normal within 24 h, indicating acute and transient hyperfibrinolysis. The hypocoagulation and hyperfibrinolytic state creates a particular vulnerability of the brain to bleeding diathesis and progression of intracerebral hemorrhage [3,120]. Importantly, the hyperfibrinolysis and increased fibrin degradation products were positively correlated with poor clinical outcome after TBI.

4.2. FDA-Approved mAbs

mAb therapies have proven successful in influencing several aspects of the coagulation process, including clot prevention, formation, and clearance. Drugs, including Abciximab [52] and Caplacizumab [53], have been approved by the FDA for use in individuals undergoing coronary artery procedures and thrombotic thrombocytopenic purpura, respectively. These drugs effectively target the glycoprotein IIb/IIIa–von Willebrand factor interactions to prevent platelet aggregation and elicit anti-coagulation effects. Abciximab binds directly to glycoprotein IIb/IIIa surface receptor, which plays a critical role in the

aggregation of platelets. Through steric hindrance and conformational changes, the binding of Abciximab effectively blocks fibrinogen, von Willebrand factor, and other adhesion molecules from accessing the receptor sites necessary to initiate platelet aggregation. On the other hand, Caplacizumab is able to achieve a similar effect by inhibiting the other side of the interaction. Caplacizumab is specific to von Willebrand factor, thereby compromising its ability to interact with platelets and begin the aggregation process.

5. mAbs and Vascular Function

5.1. Pathophysiology Relevance

In the normal brain, the BBB tightly regulates CNS homeostasis, which is critical for neuronal function and restricts the entry of blood-borne factors and circulating immune cells. It is composed of cerebrovascular endothelial cells (ECs) joined by tight junctions and adherens junctions. Glial cells, such as astrocytes and microglia, physically interact with ECs, which are also critical to BBB and EC integrity [122–125]. Shortly after TBI, the cerebral vasculature is damaged in the form of mechanical disruption and increased permeability of the BBB, which contributes to the pathogenesis of TBI and can lead to secondary injury, including hemorrhage, ischemia, vasogenic edema, and BBB dysfunction. BBB disruption is often associated with poor long-term outcomes and further worsens the secondary injury [74,125,126].

One major indicator of BBB disruption is the activation of ECs. In response to a surge of pro-inflammatory cytokines and increased chemokines after TBI, endothelial cells are activated, which further induces the surface expression of a number of cellular mediators [74,125]. Among these mediators are cell adhesion molecules, including selectins, integrins, intercellular adhesion molecules (ICAM-1 or CD54), and vascular cell adhesion molecules (VCAM-1 or CD106). Cell adhesion molecules are involved in the adhesion of leukocytes and immune cells to the endothelial wall and mediate leukocyte transmigration across the BBB into brain parenchyma [127–129]. During TBI, the endothelial wall is destroyed by the adhesion of leukocytes and platelets. Infiltrating leukocytes further drive the neuroinflammatory response and exacerbate secondary brain injury through the production of pro-inflammatory mediators, free radicals, and oxidative stress [74,125,127]. The selectin family of adhesion molecules, such as P-selectin, is not only expressed in endothelial cells but also in platelets [130]. In addition to promoting leukocyte infiltration into injured brain regions during TBI-induced inflammation, P-selectin also mediates platelet–platelet interactions. It facilitates platelet aggregation and platelet–leukocyte interactions, both important processes in the development of thrombosis [130]. Elevated P-selectin in TBI patients promotes multicellular aggregation in the bloodstream, which results in vaso-occlusion, further reduces blood flow, and increases the risk of brain ischemia [131].

In addition to the induction of cell adhesion molecules, numerous vascular-associated signaling molecules, including vascular endothelial growth factors (VEGFs), are elevated after TBI [74,125,132]. Among these VEGFs, VEGF-A is a potent mitogen for vascular endothelial cells, which is not only critical for vasculogenesis during embryonic development but is also a vital factor for physiological angiogenesis associated with wound healing, ischemia, and tumorigenesis at the adult stage [133]. VEGF-A is mediated by VEGF receptor tyrosine kinases, including VEGFR-1 and VEGFR-2 [133–135]. TBI-induced VEGF further promotes angiogenesis and vasculature repair in the damaged brain through interaction with its receptor. VEGF-A is also known as vascular permeability factor. In cultured brain and retinal endothelial cells exposed to VEGF-A, VEGF-A was found to downregulate the expression of tight junction proteins, including occludin and claudin5, which resulted in the increased permeability of EC monolayers and vascular leakage, thus promoting the development of edema [136–138].

5.2. FDA-Approved mAbs

mAb therapies targeting functions of cell adhesion molecules and vascular signaling molecules have been effectively used for the treatment of several disease types, including

sickle cell disease, colorectal cancer, gastric cancer, and macular degeneration. Among these FDA-approved mAbs, Crizanlizumab is designed to target P-selectin to prevent vaso-occlusion in patients with sickle cell disease [54]. Bevacizumab (Avastin) is an anti-angiogenic drug that is used to treat colorectal cancer. It targets VEGF-A and prevents the binding of VEGF-A to its receptors. Thus, it inhibits tumor vessel growth [55]. In addition, Bevacizumab treatment showed sex-specific effects in a rat mTBI model [139]. Ramucirumab is another anti-angiogenic drug and has been used to treat patients with gastric cancer. It blocks the activation of VEGFR-2, the primary receptor for VEGF-A, to reduce tumor angiogenesis involved in the development and progression of gastric cancer [57]. Brolucizumab is used to treat patients with macular degeneration. They are designed to bind to and block VEGF-A. By blocking VEGF-A, these drugs reduce macular edema caused by vascular leakage [56].

6. mAbs and Excitotoxicity

Glutamate is a key excitatory neurotransmitter that mediates many processes, including neural signaling in synapses, learning, and memory. High concentrations of glutamate are harmful. Excitotoxicity occurs when neurons are subjected to excessive stimulation by glutamate, leading to the continuous activation of NMDA (N-methyl-D-aspartate) receptors [140]. These receptors consist of multiple subunits, with GluN1 being one of them. Excitotoxicity is a pathological process that occurs after TBI, resulting in neuronal damage and cell death. During TBI, the brain experiences a heightened energy demand due to both primary and secondary injuries, which increases the need for glycolysis and subsequently elevates glucose supply, leading to overstimulation [141]. Uncontrolled release of glutamate triggers calcium influx into the cytoplasm, activating damaging signaling pathways that can result in apoptosis [142]. Consequently, blocking an NMDA receptor subunit appears to be a promising strategy for preventing excitotoxicity in TBI. Excitotoxicity is not only significant in TBI pathology but also contributes to the development of multiple sclerosis (MS) by damaging oligodendrocytes [58]. Thus, targeting NMDA receptors in an MS model may also yield beneficial outcomes. The interaction between tissue plasminogen activator (tPA) and GluN1 has been shown to promote excitotoxicity [143].

Pre-Approval mAbs

In a recent study using an experimental autoimmune encephalomyelitis (EAE) model in mice [144], the researchers used an anti-NMDAR mAb called Glunomab to block the interaction of tPA and GLUN1. The researchers employed an anti-NMDAR monoclonal antibody called Glunomab to block the interaction between tPA and GluN1. The results indicated that Glunomab effectively mitigated neurological impairments, which correlated with the preservation of the blood–brain–spinal cord barrier and reduced leukocyte infiltration. Another study [145] utilized a thromboembolic stroke model in mice to evaluate a polyclonal antibody designed to prevent the interaction between tPA and the NR1 subunit of NMDA receptors, aiming to counteract excitotoxic effects. This polyclonal antibody specifically targeted tPA receptors and successfully inhibited rtPA from increasing NMDA-induced calcium release in cortical neurons. Ultimately, it demonstrated significant neuroprotective effects from 20 min to 4 h post-clot without altering NMDA neurotransmission and improved long-term neurological outcomes [145].

7. mAbs and Oxidative Stress

TBI-induced oxidative stress results from the excessive production of free radicals, including reactive species of oxygen and nitrogen (ROS and RNS), which overwhelm endogenous antioxidant systems [146,147]. These free radical species are generated after TBI from various sources, such as dysfunctional mitochondria and impaired metabolism, as well as excitotoxic and inflammatory pathway activation [147]. They alter the physiology of lipids, proteins, and DNA, which causes dysfunction or loss of activity and eventually leads to cell apoptosis, necrosis, and neuronal damage. Free radicals react with the lipids on

cell/neuronal membranes, causing lipid peroxidative damage. This alters cell membrane fluidity, increases membrane permeability, decreases membrane ATPase activity, and disrupts the cellular/neuronal structure and integrity [148]. These ROS-induced alterations further contribute to TBI pathology and, ultimately, neurodegeneration. Antioxidant drugs targeting ROS/RNS have been used before or after TBI to mitigate oxidative injury in animal models and showed beneficial effects. However, most of them have failed in clinical trials [146,147].

Pre-Approval mAbs

There have been few studies targeting the post-traumatic oxidative environment. However, mAbs targeting MMP-9 have been tested in several rodent models, such as focal cerebral ischemia and intestinal fibrosis. MMP-9 cleaves endothelial basal lamina and the tight junction proteins of the BBB under normal conditions but has also been associated with oxidative stress by several studies [149–155]. In a rat model of focal cerebral ischemia, MMP-9 expression is increased in endothelial cells and infiltrating neutrophils after focal ischemia. Systemically, administration of the anti-MMP-9 mAb significantly reduced the infarct size, thus mitigating brain injury [59]. Similarly, increased serum MMP-9 levels have been observed in a mouse model of intestinal fibrosis, which is highly associated with the pathology. The anti-MMP-9 treatment reduced collagen deposition and hydroxyproline content in intestinal grafts, indicating reduced fibrosis [60]. These data indicate the efficacy of anti-MMP-9 mAbs in these animal models. It also suggests that selective MMP-9 inhibition using mAbs is a promising therapeutic strategy for the treatment of MMP-9-regulated processes, including TBI-associated oxidative stress and BBB dysfunction. Interestingly, andecaliximab against MMP-9 is currently being examined with a combination chemotherapy regimen in a Phase III study with gastric and gastroesophageal junction adenocarcinoma and demonstrates encouraging beneficial effects without added toxicity [61].

8. mAbs and Safety

As with most therapeutic interventions, mAb treatment is not without risk of adverse reactions/side effects. mAb therapy can elicit a diverse range of adverse effects, which are discussed and summarized thoroughly by Hansel et al. [156]. Some of the most common side effects include immune reactions, infections, platelet and thrombotic disorders, organ-specific toxicity, anaphylactic (IgE-mediated) reactions, serum sickness syndrome, cytokine storm, autoimmune diseases, and cancer [156,157]. The previous clinical testing of mAbs may provide some guidance for what to expect when testing and developing mAb therapies. However, it should be noted that the specificity of mAbs in biological systems may make them likely to yield specific and new reactions. TBI pathology is known to alter the metabolism and clearance of therapeutics, which introduces another important variable [158,159]. These adverse effects could be minimized by sound pre-clinical and clinical practice. As such, it is important that future studies evaluating the efficacy of mAbs for TBI treatment consider the whole-body safety effects as well. The development and validation of proper in vitro safety tests during pre-clinical study and the advancement of mAbs, such as the production of next-generation antibodies like bispecific antibodies, trispecific antibodies, and low molecular weight antibodies, may also facilitate the improvement of the safety of mAbs [156,160].

9. Conclusions

TBI pathophysiology is complex and multifaceted, often involving inflammation, vascular function, coagulopathy, oxidative stress, and neurodegeneration. Many of the pathological processes are conserved within other diseases, which may confer important observations in considering treating TBI. Historically, clinical heterogeneity and lack of robust and specific treatment effects have prevented the successful development of treatment strategies for TBI. However, recent advancements in mAb technology have demonstrated

success in complex neurological diseases because of their specificity in action and fewer adverse effects as compared to existing treatment options. Herein, we have explored how the current pre-clinical and clinical mAb therapies may harbor potential for the treatment of TBI through targeting specific pathological processes. As ongoing studies continue to better define the molecular underpinnings of TBI pathology and secondary injury mechanisms, the identification of novel therapeutic targets will continue. Therefore, it is imperative that we begin to consider how we can target them with therapeutic strategies, including mAbs.

Considering numerous molecular mechanisms that contribute to the complexity of TBI, future efforts for mAb therapies could target bispecific mAbs or mAb mixtures that control multiple molecular pathways in TBI. In addition, one of the limitations of therapeutic mAbs is their inability to cross the blood–brain barrier, which prevents them from reaching their target in the brain at the therapeutic level to be effective. To improve the bioavailability of mAbs in the brain, many approaches for delivery have been explored with success, including extracellular vesicles, engineered bispecific mAbs, and nanoparticles. The continuous development of brain-penetrant mAbs could be beneficial, particularly for TBI. These mAb-based therapeutics will likely facilitate drug therapy in the TBI research field and will hopefully be able to address unmet medical needs for TBI patients.

Author Contributions: Conceptualization, Z.S.B. and P.W.; writing—original draft preparation, Z.S.B. and P.W.; writing—review and editing, Z.S.B., P.W., S.O.-R. and A.H.S.; supervision, Z.S.B. and A.H.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors thank Christopher Nititham for his expertise in the creation of the antibody mechanism graphic, and Phillip Urbanczyk, Xiaofang Yang, Katherine Cardiff, and Melonie Houchins for their help with the market research and literature search.

Conflicts of Interest: This work was prepared while Zachary Bailey was employed at the Walter Reed Army Institute of Research. The opinions expressed in this article are the author’s own and do not reflect the view of the National Institutes of Health, the Department of Health and Human Services, or the United States government.

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Article

Brachial Plexus Injury Influences Efferent Transmission on More than Just the Symptomatic Side, as Verified with Clinical Neurophysiology Methods Using Magnetic and Electrical Stimulation

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Abstract: The variety of sources of brachial plexus injuries (BPIs) and the severity and similarity of their clinical symptoms with those of other injuries make their differential diagnosis difficult. Enriching their diagnosis with objective high-sensitivity diagnostics such as clinical neurophysiology may lead to satisfactory treatment results, and magnetic stimulation (MEP) might be an advantageous addition to the diagnostic standard of electrical stimulation used in electroneurography (ENG). The asymptomatic side in BPI cases sometimes shows only subclinical neurological deficits; this study aimed to clarify the validity and utility of using MEP vs. ENG to detect neural conduction abnormalities. Twenty patients with a BPI and twenty healthy volunteers with matching demographic and anthropometric characteristics were stimulated at their Erb's point in order to record the potentials evoked using magnetic and electrical stimuli to evaluate their peripheral motor neural transmission in their axillar, musculocutaneous, radial, and ulnar nerves. MEP was also used to verify the neural transmission in participants' cervical roots following transvertebral stimulations, checking the compatibility and repeatability of the evoked potential recordings. The clinical assessment resulted in an average muscle strength of 3–1 (with a mean of 2.2), analgesia that mainly manifested in the C5–C7 spinal dermatomes, and a pain evaluation of 6–4 (mean of 5.4) on the symptomatic side using the Visual Analog Scale, with no pathological symptoms on the contralateral side. A comparison of the recorded potentials evoked with magnetic versus electrical stimuli revealed that the MEP amplitudes were usually higher, at $p = 0.04–0.03$, in most of the healthy volunteers' recorded muscles than in those of the group of BPI patients, whose recordings showed that their CMAP and MEP amplitude values were lower on their more symptomatic than asymptomatic sides, at $p = 0.04–0.009$. In recordings following musculocutaneous and radial nerve electrical stimulation and ulnar nerve magnetic stimulation at Erb's point, the values of the latencies were also longer on the patient's asymptomatic side compared to those in the control group. The above outcomes prove the mixed axonal and demyelination natures of brachial plexus injuries. They indicate that different types of traumatic BPIs also involve the clinically asymptomatic side. Cases with predominantly median nerve lesions were detected in sensory nerve conduction studies (SNCSs). In 16 patients, electromyography revealed neurogenic damage to the deltoid and biceps muscles, with an active denervation process at work. The predominance of C5 and C6 brachial plexus injuries in the cervical root and upper/middle trunk of patients with BPI has been confirmed. A probable explanation for the bilateral symptoms of dysfunction detected via clinical neurophysiology methods in the examined BPI patients, who showed primarily unilateral damage, maybe the reaction of their internal neural spinal center's organization. Even when subclinical, this may explain the poor BPI treatment outcomes that sometimes occur following long-term physical therapy or surgical treatment.

Keywords: brachial plexus injury; cervical roots; Erb's point; magnetic stimulation; electrical stimulation; electroneurography; electromyography

1. Introduction

Overload forces are the leading cause of brachial plexus injuries (BPIs), causing damage to the shoulder girdle, muscle tissue, and blood vessels. The complexity of a BPI is often accompanied by severe multi-organ injuries, their associated diagnostic difficulties, and the choice of an optimal treatment [1,2]. Moreover, diagnostics after a BPI to evaluate the sensory and motor function of the arm and distal part of the upper extremity sometimes need to be postponed due to the patient's general poor health status, negatively influencing their treatment and prognosis [3,4]. The absence of a “gold standard” for the assessment of the severity of BPI lesions complicates prognostic studies of BPI [5]. The routine examination of the consequences of brachial plexus damage, as with other peripheral neuropathies, is mainly based on clinical tests [6]. This examination primarily includes a functional assessment of the shoulder girdle's muscle strength, the range of the patient's structural atrophic changes, their sensory perception deficits, and their range of motion both in the shoulder joint and in other joints of the upper extremity [7]. Both magnetic resonance imaging and ultrasound examinations enhance clinical diagnoses by analyzing the location and extent of the structural damage within injured nerves and surrounding tissues [8,9]. The variety of sources of brachial plexus injuries, the degree of their scope and severity, and the similarity of their clinical symptoms make it difficult to choose between surgery and continuing physiotherapy [10]. A differential diagnosis should be based on modern objective methods with high sensitivity; currently, clinical neurophysiology tools are preferred [11]. Neuroimaging and neurophysiological tests should be an integral part of the diagnostic process, guiding the selection of surgical or conservative treatments. Optimal diagnostic results are obtained following a comparison of the neuroimaging and functional evaluation of injured neural structures, as is the case for patients after an incomplete spinal cord injury [12]; however, this opinion has not yet been approved in cases of patients with BPI. In studies by Chanlalit et al. [13], the diagnostic value of their clinical findings, electromyography, and magnetic resonance imaging of root lesions in traumatic brachial plexus injuries was evaluated to be 60, 87, and 70%, respectively. The results of clinical neurophysiology tests of the neural conduction of motor and sensory fibers are analyzed following electroneurographic (ENG) recordings. The stimulus used to excite nerve fibers and generate motor potentials (CMAPs—compound muscle action potentials, sometimes identified as M-waves) and sensory potentials (SNAPs—sensory nerve action potentials) is an electrical pulse of known intensity and duration. The use of ENG tests in the diagnosis of nerve-damaged peripheral parts is widely described in the literature and also applied to the functional evaluation of BPIs [14]. In our previous work [15,16], we extensively studied and discussed the usefulness of a magnetic stimulus (MEP—motor-evoked potential) in the evaluation of the function of brachial plexus motor fibers. Magnetic stimulation is an advantageous addition to the diagnostic standard used in BPI cases. It allows for evaluating the function of the cervical roots, which are the origin of the peripheral innervation of the upper extremities' muscles. MEPs are crucial for the functional confirmation of the avulsion range and compression of cervical spinal roots. Magnetic cervical motor root stimulation is useful for detecting abnormal findings in the upper part of the brachial plexus, even at the acute phase of a BPI [17]. The compatibility of imaging studies and neurophysiological tests in evaluating the proximal part of the brachial plexus is essential, especially for reconstructive surgery. Moreover, extended neurophysiological diagnostics (ENG, MEP) may also include the asymptomatic side of the injury, which sometimes shows subclinical neurological deficits in basic examinations that are often ignored or neglected. Our research is driven by insufficient scientific data in the field of neurophysiological BPI diagnostics regarding the application of complementary MEP and ENG tests. Combining

both examination techniques allows us to assess the entire neuromere, which is of particular diagnostic importance in proximal BP lesions located at the level of the spinal roots or spinal nerves. So far, the diagnostic gap in the function of the brachial plexus has been caused by the lack of direct assessments of its proximal parts. MEP tests allow us to assess the function of the spinal roots, which complements the neurophysiological diagnostics performed on BPI patients. In combination with an imaging examination (MRI), this fulfills the complementary diagnosis of BPI. In this study, we answer the question of whether the use of stimulation with physically different stimuli (electrical vs. magnetic) allows for an objective and repeatable assessment of BPI patients. We also attempted to verify our hypothesis, which is that both types of stimuli can be used interchangeably for diagnostic purposes. Our next question concerns the validity of extended neurophysiological diagnostics in assessing the level, range, and type of brachial plexus damage present and whether functional changes are also present on the clinically asymptomatic side. We additionally hypothesized that an injury to one side of the brachial plexus may also cause pathological symptoms in contralateral neural structures, either as a consequence of the spinal reaction to the structural shock or due to adaptative changes, which are the reaction of the internal spinal motor centers. In this report, the “asymptomatic side” is considered to present no clearly clinically detected sensory or motor impairments, which does not mean that abnormalities cannot be observed in its neurophysiological recordings. These are the main aims of the presented study.

2. Materials and Methods

2.1. Participants, Study Design, and Clinical Evaluation

The results of single neurophysiological recordings performed in 20 patients with brachial plexus injury and 20 healthy volunteers were analyzed in this study (Table 1).

Table 1. The demographic, anthropometric, and handedness characteristics of the patients and healthy volunteers. Minimum, maximum, and mean values and standard deviations are presented.

Variable Group of Subjects	Age (Years)	Height (cm)	Weight (kg)	BMI	Handedness
Patients, N = 20					
N = 6 ♀	15–66	159–194	51.4–85.3	24.1–27.6	R = 20
N = 14 ♂	38.4 ± 13.9	174.5 ± 8.6	75.8 ± 9.4	26.1 ± 4.3	L = 0
Healthy volunteers (Control), N = 20					
N = 10 ♀	21–49	156–190	49.8–83.1	24.3–27.1	R = 19
N = 10 ♂	36.9 ± 9.8	172.5 ± 10.3	76.4 ± 11.2	25.4 ± 3.4	L = 1
<i>p</i> -value	0.045	0.06 NS	0.08 NS	0.07 NS	0.138 NS

Abbreviations: ♀—female; ♂—male; NS—non-significant; $p \leq 0.05$ determines significant statistical differences, which are marked in bold.

Subjects were examined in the Department of Pathophysiology of Locomotor Organs in the Wiktor Dega Orthopedic and Rehabilitation Hospital of Poznań University of Medical Sciences. The inclusion criterion for the patients in the research group was a diagnosis of brachial plexus injury (BPI) based on clinical examinations, including manual muscle strength testing, sensory perception evaluations with reference to the spinal dermatomes, pain evaluations using the visual analog scale, and magnetic resonance imaging (MRI) of their symptomatic and asymptomatic upper extremities. Manual muscle strength testing was performed on the proximal and distal muscle groups in the upper extremities using a Lovett scale (0–5). It consists of six grades that assess different levels of muscle strength (0—no visible voluntary contraction of the muscle, 5—normal muscle strength) [18,19]. Their sensory perception was assessed (0—analgesia, 1—normal, 2—hyperesthesia) according to the dermatomal scheme for the innervation of the brachial plexus sensory fibers, which is based on a tactile method using Von Frey’s filaments (Semmes–Weinstein monofila-

ments) [20,21]. Pain intensity was evaluated using a patient-reported 10-point visual analog scale (VAS) [22]. The time from the onset of the BPI to the clinical and neurophysiological evaluation of the patients was 4 months on average.

The exclusion criteria and contraindications for MEP examinations in the patients and healthy volunteers included pregnancy, epilepsy, cardiac disease, and the use of a pacemaker or other implanted biomedical electronic devices [23]. Moreover, for the control group of healthy volunteers, the exclusion criteria also included neck, head, and shoulder girdle injuries.

Our ethical considerations adhered to the Helsinki Declaration. Approval was received from the Bioethical Committee of the University of Medical Sciences in Poznań, Poland (including our studies on healthy people), Decision No. 554/17. Each participant signed a written consent form for their examination and the publication of their data; they were informed about the project's aims. The patients were routinely diagnosed in the hospital's clinical neurophysiology unit. A physician performed clinical studies and described the results in the patients' medical history. Three experienced clinical neurophysiologists conducted the MEP, ENG, and electromyography (EMG) studies. All of them judged the final results after the consultations. The patients' medical histories were analyzed to determine the causes of their brachial plexus injury.

The group of patients was older than the healthy volunteers (Table 1), and the difference was close to the level of statistical significance. All other differences in the anthropometric properties of the studied subjects were statistically non-significant, and the age of the healthy volunteers was within three decades of that of the patients, thus maintaining the possibility of comparing the parameters of their neurophysiological tests. Moreover, the most extreme age values in the research group, i.e., 15 and 66 years old, were limited to single cases. Both groups were homogeneous in terms of height and handedness.

2.2. Neurophysiological Examinations

The patients with BPI and the healthy volunteers were examined bilaterally using the same neurophysiological scheme. We used magnetic and electrical stimuli to evaluate the peripheral motor neural transmission in the four nerves that create the brachial plexus: the axillar, musculocutaneous, radial, and ulnar nerves. The magnetic stimulus was also used to verify neural transmission in the cervical roots according to the domain of the above-mentioned peripheral nerves and muscles (Table 2). We stimulated these nerves at Erb's point and in specific parts of the cervical segment several times, checking the repeatability of the evoked potentials. All studies were performed in the same quiet diagnostic room with constant, pleasant humidity and a temperature of 22 °C. During the ENG examination, the subjects were in a supine position; during the MEP recordings, they were in a sitting position, with the muscles of their upper extremities relaxed and their shoulders freely positioned.

The multichannel KeyPoint Diagnostic System (Medtronic A/S, Skøvlunde, Denmark) was used for the MEP and CMAP (ENG, also called M-waves) recordings. The external magnetic stimulus for the MEP studies was generated by the MagPro X100 magnetic stimulator (Medtronic A/S, Skøvlunde, Denmark) via a circular coil (C-100, 12 cm in diameter) and applied to Erb's point or transvertebrally at different cervical spine levels. During the MEP studies, the strength of the magnetic field stream was 100% of the maximum stimulus output (1.7 T for each pulse). The recordings were performed at an amplification of 20 mV/D and a time base of 5–8 ms/D. For the CMAP recording, a bipolar stimulation electrode and a single rectangular electric stimulus were used at a 1 Hz frequency for 0.2 ms. The intensity of the electric stimulus was about 100 mA, which evoked the supramaximal CMAP amplitude at Erb's point. The strength of the electrical stimulus was recommended and determined by anatomical relationships and exposure of the neural structures of the brachial plexus at the level of the supraclavicular fossa. In the ENG studies, the time base was set to 5 ms/D, the sensitivity of the recordings was set to 2 mV/D, and upper and lower filters of 10 Hz and 10 kHz were established on the recorder amplifier. A bipolar

stimulation electrode was used, and pools were moisturized with a saline solution (0.9% NaCl). The skin was disinfected with a 70% alcohol solution. This and a conductive gel reduced the resistance between the skin and the recording electrodes. The impedance did not exceed 5 k Ω . In the ENG study, bipolar stimulating electrodes were applied at Erb's point, with the orientation of the stimulus's delivery in the orthodromic direction for the excitation of the motor fibers in the nerves. The electrical stimulation generated the CMAP recording with the shortest latency value and the highest amplitude, which became the aim of magnetic stimulation at this level. The assessment of the MEP evoked in the spinal roots of the cervical segment required the magnetic coil to be placed 0.5 cm laterally and selectively below the spinous process (in pursuance of the anatomical location of the cervical spinal roots).

For the recordings of the CMAPs and MEPs, standard disposable Ag/AgCl electrodes with an active surface area of 5 mm² were used and placed in the same location when electric or magnetic stimuli were applied. The active electrode was placed over the belly muscle, and the reference electrode was placed distally to the active one, on the olecranon or the muscle tendon [24]. A list of the tested muscles, their innervation (peripheral pathway and root domain), the location of the recording electrodes, and the type of applied stimuli are presented in Table 2. This table also includes the symptoms of functional loss seen following BPI.

Table 2. Summary of the CMAP and MEP methodology (examined muscles, their peripheral innervation, and root domain) according to Ferrante [25], Leis [24], and our previous studies [15] with own modifications, as well as the symptoms of functional loss seen according to Park et al. [6].

Muscle (CMAPs and MEPs Recordings)	Electrical and Magnetic Stimulation of Nerves at Erb's Point	Brachial Plexus Trunk	Cervical Root (Magnetic Stimulation at a Significant Root Domain)	Function Loss
Deltoid (Middle part) Active electrode—belly muscle Reference electrode—olecranon	Axillary nerve	Upper	C5–C6 (C5)	Shoulder rotation and abduction
Biceps Brachii Active electrode—belly muscle Reference electrode—olecranon	Musculocutaneous nerve	Upper	C5–C6 (C6)	Elbow flexion
Triceps Brachii (Long head) Active electrode—belly muscle Reference electrode—olecranon	Radial nerve	Upper Middle Lower	C6–C8 (C7)	Elbow extension
Abductor Digiti Minimi Active electrode—belly muscle Reference electrode—muscle tendon	Ulnar nerve	Lower	C8–T1 (C8)	Ulnar intrinsic muscle stretching (ADM)

Abbreviations: CMAP—compound muscle action potential; MEP—motor-evoked potential; ADM—abductor Digiti Minimi muscle.

The same output parameters were analyzed for the compound muscle action potential (CMAP) recorded during the electroneurography examination (ENG) and the motor-evoked potential (MEP) induced by magnetic stimulation. The amplitude of the negative deflection (from the baseline to the negative peak—measured in mV) and its latency (from the visible stimulating artifact to the negative deflection of potential—measured in ms) were analyzed.

Confirmation of the axonal type of the brachial plexus injuries (axonotmesis or neurotmesis) required needle electromyography (nEMG) recordings from the muscles of the arm and the distal part of the upper extremity. We analyzed the muscles' spontaneous activity at rest (denervation potential: fibrillation, positive waves), the parameters of twenty motor unit action potentials (MUAPs), and the frequency of MUAP recruitment during maximal voluntary contraction [26]. Conducting the two last stages of the nEMG recordings was only possible when the voluntary movement of the muscle was available. Two MUAP parameters with values greater than they were at the patients' referral determined the neurogenic advancement of these patients' injury and the reinnervation process in their examined muscles. In addition to assessing the function of the motor fibers of the brachial

plexus, the ENG examination also examines their sensory component. The same diagnostic system was used for sensory nerve conduction studies (SNCS). Recording electrodes were placed over the skin of the examined nerve passage and along its anatomical course. A ring-type stimulating electrode was placed on a specific finger for the evaluation of the median and ulnar nerves and over the dorsolateral edge of the radius bone during the examination of the radial nerve.

2.3. Statistical Analysis

Data analysis was performed using Statistica, version 13.1 (StatSoft, Kraków, Poland). Descriptive statistics are reported as minimal and maximal values (range), with the mean and standard deviation (SD) given, and with the median value given for some clinical test results. The normality distribution and homogeneity of the variances were studied using Shapiro–Wilk and Levene’s tests. None of the collected data had a normal distribution or were of the ordinal scale type. All neurophysiological tests were conducted on a group of healthy volunteers to achieve the normative parameters used to compare the health status of the patients and the controls. In cases where the distribution was not normal, a Mann–Whitney U test was used. Student’s *t*-test (paired difference *t*-test) or Wilcoxon’s test (in the absence of distribution normality) was used to compare the differences between the results obtained for the patients and healthy volunteers. *p*-values of ≤ 0.05 were considered statistically significant. We also compared the differences with those calculated using the Bonferroni correction at $p < 0.05$. The results did not reveal any significant difference in the parameter values recorded in the neurophysiological tests conducted on the left and right sides of the controls. Attention was paid to matching patients’ and healthy volunteers’ demographic and anthropometric properties, including their gender, age, and height. Statistical software was used to determine the required sample size using the primary outcome variable of the MEP amplitudes recorded in the ADM muscles, with a power of 80% and a significance level of 0.05 (two-tailed) required. The mean and standard deviation (SD) were calculated using the data from the first 10 patients, and the sample size software estimated that more than 15 patients were needed for this study. This population was increased to 20 to provide the most reliable data for statistical analyses.

3. Results

Table 3 summarizes the data on the etiology of the brachial plexus damage in the patient group and distinguishes between the types of injuries according to their location.

Table 3. Characteristics of the brachial plexus injuries in the group of patients (N = 20).

Group of Patients	Injury Origin	Type of Injury				
		Multiorgan Trauma	Symptomatic Side	Preganglionic N = 5	Postganglionic N = 6	Mixed (Both Types of Injuries) N = 9
N = 20	Car/motorcycle	N = 6	R = 10 L = 10	C5 n = 10 C6 n = 9 C7 n = 7 C8 n = 6	All trunks n = 2	
	Traffic accident N = 8				Superior trunk n = 3	
	Iatrogenic injury N = 2				Superior and middle trunks n = 6	
	Obstetric brachial plexus injury N = 1				Middle and inferior trunks n = 1	
	Other causes of damage N = 9 *				Inferior trunk n = 0	
					Spinal cervical nerves/anterior triangle of the neck n = 2	
					Whiplash syndrome n = 1	

Abbreviations: * Falls from height, shoulder girdle dislocations, arm traction, whiplash syndrome; R—right side; L—left side; N—number of the patients; n—confirmation quantity of the cervical root level of damage in patients with preganglionic, postganglionic, and mixed injuries.

Brachial plexus injuries related to traffic accidents dominated. These were preganglionic injuries that mainly affected the C5 and C6 spinal roots. Magnetic resonance imaging (MRI) confirmed root avulsion at these levels. The remaining patients’ injuries involved

both the roots and trunks of the brachial plexus. Damage to the upper and middle trunks was mainly detected. One patient had iatrogenic damage associated with the surgical removal of neuromas in the anterior triangle of the neck and obstetric injuries. In general, on the symptomatic side of all patients in this study, the clinical neurological assessment made using classical evaluation methods revealed that the muscle strength of the deltoid, biceps, and triceps brachii, as well as the abductor digiti minimi, was 3–1 (mean of 2.2), the sensory perception of the spinal C5–C7 dermatomes were 1 or 0 (median of 0), and the pain experiences were rated as 6–4 (mean of 5.4) using the visual analog scale. No pathological symptoms were detected on the contralateral side using these clinical evaluation methods.

Table 4 presents the results of the neurophysiological studies (ENG, MEP) conducted on patients and healthy volunteers. In the group of healthy volunteers, there was no statistical difference between the left and right sides in terms of the parameters of the amplitudes or latencies of the potentials evoked with electrical stimulation. Following magnetic stimulation at Erb's point, only in the ADM-ulnar nerve recordings was the amplitude of the MEP higher on the right side than on the left, at $p = 0.04$. A comparison of the recording parameters evoked with magnetic versus electrical stimuli indicated that the amplitudes were higher in the left TB-axillary nerve following electrical compared to magnetic stimulation, at $p = 0.04$. Additionally, the ADM-ulnar nerve recordings on both sides were characterized by amplitudes of MEP higher than those evoked by the electrical stimulation, at $p = 0.03$. In the group of patients, recordings from all examined muscles showed that their CMAP and MEP amplitudes were significantly lower on their symptomatic side, at $p = 0.04$ – 0.009 , compared to the control group.

In the TB-radial and ADM-ulnar nerve recordings obtained following electrical stimulation at Erb's point and in the ADM-ulnar nerve recording after magnetic stimulation at Erb's point, the amplitudes of the potentials evoked in the patients were also lower on the asymptomatic side, $p = 0.04$ and $p = 0.02$, respectively, compared to the control group. The MEP amplitudes after the cervical roots were stimulated (C5–C8) were lower in the patients compared to the control group, both on their symptomatic and asymptomatic sides, at $p = 0.04$ – 0.008 . In the BPI patients' recordings, their latency parameters were longer on their symptomatic side, at $p = 0.02$ – 0.009 , in all but the DP-axillary nerve recordings. During magnetic stimulation at Erb's point, only in the BB-musculocutaneous nerve recordings on the symptomatic side was the latency longer than that of the asymptomatic side, at $p = 0.008$. Furthermore, in recordings following musculocutaneous and radial nerve electrical stimulation and ulnar nerve magnetic stimulation at Erb's point, the patients' latencies were also longer on their asymptomatic side compared to those of the control group. The above outcomes prove the mixed types of brachial plexus injury seen, which are of an axonal and demyelination nature. They lead to the conclusion that a traumatic BPI of mixed types also involves the clinically asymptomatic side. The recordings presented in Figure 1 were obtained from a patient with a superior trunk injury to their brachial plexus after a fall from height. Notice that the amplitude of the CMAP from the axillary nerve was reduced after stimulation of the cervical root C5 on the left side (the symptomatic side of their injury).

In our neurophysiological examinations, evoked potentials with lower amplitudes (CMAP, MEP) indicate an axonal loss in the nerves. This type of nerve injury may refer to axonotmesis or neurotmesis in Seddon's classification, depending on the severity of the axonal loss. A lack of recording potential implies neurotmesis or severe axonotmesis. The above results indicate predominant axonotmesis in the group of patients with BPI.

Table 4. Comparison of results from electroneurographic examinations (ENG, CMAP) and motor-evoked potential (MEP) recordings performed in 20 patients and 20 healthy volunteers (control group).

Test Parameter	Side	Control ES CMAP	Recording Side in Patients	Patient ES CMAP	Control ES CMAP vs. Patient ES CMAP	Control MS MEP	Patient MS MEP	Control MS MEP vs. Patients MS MEP	Patient ES vs. MS	Control ES vs. MS	Control Cervical- Root-Level MSr MEP	Patient Cervical- Root-Level MSr MEP	Control vs. Patient Cervical- Root-level MSr MEP
		Min.-Max. Mean ± SD		Min.-Max. Mean ± SD	<i>p</i> -value	Min.-Max. Mean ± SD	<i>p</i> -value	Min.-Max. Mean ± SD	<i>p</i> -value	<i>p</i> -value	<i>p</i> -value	Min.-Max. Mean ± SD	<i>p</i> -value
Axillar nerve and C5 roots stimulation/DP recording													
Amplitude (mV)	R	10.3–18.6 14.8 ± 1.8	Symptomatic	0–21.5 7.4 ± 3.2	0.008	10.9–19.7 14.0 ± 2.1	0–18.0 6.8 ± 5.2	0.008	0.043	0.054	6.0–23.4 13.9 ± 4.8	0–14.0 4.2 ± 3.5	0.008
	L	10.2–17.6 14.3 ± 2.1	Asymptomatic	8.4–20.0 13.2 ± 3.6	0.064	9.7–18.5 13.4 ± 2.2	9.0–19.0 13.0 ± 3.3	0.053	0.069	0.048	6.2–22.9 14.2 ± 4.3	4.1–18.9 11.0 ± 4.0	0.040
<i>p</i> -value	R vs. L	0.113	NA	0.009	NA	0.055	0.009	NA	NA	NA	0.063	0.008	NA
Latency (ms)	R	2.8–4.2 3.2 ± 0.3	Symptomatic	0–5.4 3.5 ± 1.6	0.063	2.7–3.6 3.1 ± 0.3	0–6.3 3.5 ± 1.7	0.047	0.241	0.188	4.0–5.4 4.8 ± 0.4	0–11.1 5.8 ± 3.1	0.052
	L	2.7–3.9 3.2 ± 0.3	Asymptomatic	2.8–4.2 3.6 ± 0.4	0.074	2.5–3.7 3.1 ± 0.3	2.7–4.1 3.6 ± 0.4	0.041	0.312	0.189	4.1–5.4 4.7 ± 0.4	4.3–5.9 5.0 ± 0.4	0.062
<i>p</i> -value	R vs. L	0.138	NA	0.096	NA	0.188	0.073	NA	NA	NA	0.231	0.071	NA
Musculocutaneous nerve and C6 roots stimulation/BB recording													
Amplitude (mV)	R	7.6–16.9 12.1 ± 2.8	Symptomatic	0–18.2 7.0 ± 5.1	0.009	7.2–16.7 11.7 ± 2.6	0–14 6.8 ± 4.5	0.008	0.061	0.057	7.9–21.5 12.6 ± 3.4	0–14.7 6.2 ± 4.6	0.007
	L	8.3–16.1 11.9 ± 2.2	Asymptomatic	4.3–16.2 11.2 ± 3.2	0.082	8.1–14.7 11.5 ± 2.1	6.8–15.0 11.4 ± 2.5	0.077	0.068	0.078	8.2–18.1 13.1 ± 2.9	4.5–22.9 14.4 ± 4.7	0.046
<i>p</i> -value	R vs. L	0.113	NA	0.018	NA	0.072	0.019	NA	NA	NA	0.082	0.008	NA
Latency (ms)	R	3.1–5.3 4.1 ± 0.5	Symptomatic	0–6.8 4.0 ± 1.9	0.069	3.0–4.8 3.9 ± 0.4	0–6.1 3.8 ± 1.8	0.061	0.052	0.064	4.0–6.4 5.6 ± 0.6	0–10.2 5.5 ± 2.6	0.166
	L	3.0–4.6 4.0 ± 0.4	Asymptomatic	3.4–5.6 4.3 ± 0.5	0.048	3.2–4.5 3.9 ± 0.4	3.4–5.0 4.2 ± 0.4	0.068	0.071	0.081	4.2–6.3 5.6 ± 0.6	4.8–7.7 5.8 ± 0.6	0.075
<i>p</i> -value	R vs. L	0.092	NA	0.047	NA	0.148	0.041	NA	NA	NA	0.288	0.062	NA

Table 4. Cont.

Test Parameter	Side	Control ES CMAP	Recording Side in Patients	Patient ES CMAP	Control ES CMAP vs. Patient ES CMAP	Control MS MEP	Patient MS MEP	Control MS MEP vs. Patient MS MEP	Patient ES vs. MS	Control ES vs. MS	Control Cervical-Root-Level MSr MEP	Patient Cervical-Root-Level MSr MEP	Control vs. Patient Cervical-Root-level MSr MEP	
		Min.-Max. Mean ± SD		Min.-Max. Mean ± SD	Min.-Max. Mean ± SD	p-value	Min.-Max. Mean ± SD	p-value	Min.-Max. Mean ± SD	p-value	Min.-Max. Mean ± SD	p-value	Min.-Max. Mean ± SD	p-value
		Radial nerve and C7 roots stimulation/TB recording												
Amplitude (μV)	R	6.6–12.7 10.4 ± 1.7	Symptomatic	0–14.9 6.0 ± 4.3	0.014	7.9–11.8 10.0 ± 1.2	0–15.1 6.4 ± 4.5	0.008	0.061	0.068	4.4–17.6 11.1 ± 3.9	0–14.1 5.7 ± 4.6	0.007	
	L	7.3–13.0 10.4 ± 1.7	Asymptomatic	4.3–17.0 9.2 ± 3.4	0.048	7.1–12.3 9.8 ± 1.4	6.7–16.3 9.5 ± 2.2	0.067	0.070	0.059	5.8–18.6 10.9 ± 3.3	3.0–17.6 8.8 ± 4.0	0.045	
p-value	R vs. L	0.485	NA	0.036	NA	0.051	0.038	NA	NA	NA	0.081	0.038	NA	
Latency (ms)	R	3.0–5.1 4.1 ± 0.6	Symptomatic	0–7.2 3.8 ± 1.9	0.034	3.0–5.0 4.0 ± 0.6	0–6.3 3.5 ± 1.8	0.063	0.068	0.072	4.5–7.4 5.8 ± 0.8	0–7.4 5.3 ± 2.4	0.058	
	L	3.0–5.8 4.1 ± 0.7	Asymptomatic	2.9–5.3 4.2 ± 0.7	0.041	3.0–5.5 4.0 ± 0.6	3.3–6.1 3.4 ± 0.7	0.067	0.049	0.092	4.5–7.1 5.9 ± 0.8	4.5–7.3 6.0 ± 0.8	0.073	
p-value	R vs. L	0.528	NA	0.041	NA	0.198	0.082	NA	NA	NA	0.231	0.062	NA	
Ulnar nerve and C8 roots stimulation/ADM recording														
Amplitude (μV)	R	1.6–14.2 5.6 ± 3.1	Symptomatic	0–12.5 5.2 ± 3.8	0.045	5.0–9.3 7.1 ± 1.2	0–12.0 5.2 ± 3.4	0.038	0.178	0.031	1.3–10.3 5.8 ± 2.8	0–11.2 3.4 ± 3.4	0.042	
	L	1.0–9.7 5.4 ± 2.8	Asymptomatic	4.8–14.1 10.3 ± 3.6	0.024	3.6–9.0 6.2 ± 1.4	4.8–11.8 7.3 ± 2.0	0.008	0.008	0.039	1.0–14.1 5.4 ± 3.2	0.2–12.8 4.0 ± 3.4	0.049	
p-value	R vs. L	0.074	NA	0.035	NA	0.048	0.039	NA	NA	NA	0.062	0.063	NA	
Latency (ms)	R	10.0–13.8 12.1 ± 1.0	Symptomatic	0–17.7 11.1 ± 5.1	0.063	9.7–13.3 11.7 ± 0.9	0–17.3 11.4 ± 4.3	0.127	0.153	0.071	11.8–15.1 13.5 ± 0.9	0–19.2 13.2 ± 4.8	0.081	
	L	9.9–14.4 12.1 ± 1.0	Asymptomatic	9.8–16.5 12.5 ± 1.5	0.068	9.8–13.7 11.7 ± 1.0	10.6–16.4 12.4 ± 1.5	0.040	0.211	0.073	12.0–15.1 13.6 ± 0.9	12.3–18.7 14.6 ± 1.7	0.056	
p-value	R vs. L	0.485	NA	0.043	NA	0.142	0.063	NA	NA	NA	0.119	0.052	NA	

Abbreviations: ES—electrical stimulation at Erb's point, MS—magnetic stimulation at Erb's point; MSr—magnetic stimulation of the cervical roots levels; CMAP—compound muscle action potential; MEP—motor-evoked potential; DP—deltoid posterior muscle; BB—biceps brachii muscle; TB—triceps brachii muscle; ADM—abductor digiti minimi muscle; C5–C8—cervical root levels; NA—non-applicable; NS—non-significant; $p \leq 0.05$ determines significant statistical differences marked in bold.

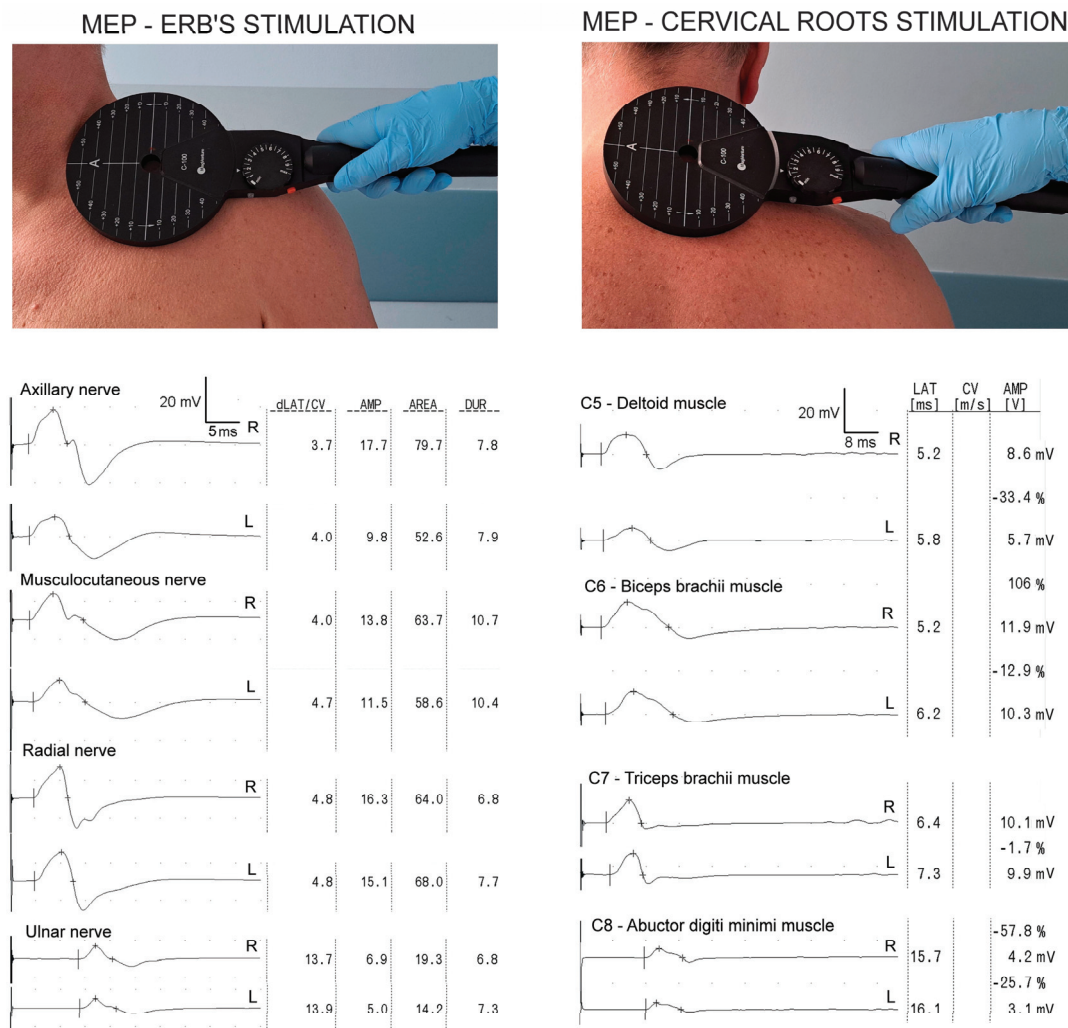


Figure 1. Photographs presenting the placement of the magnetic coil during MEP studies after stimulation at Erb's point, with transvertebral as well as MEP recordings of both sides (R—right, L—left), respectively, with the amplitude (AMP), latency (dLAT, LAT), and duration (DUR) of the evoked potentials presented.

Our sensory nerve conduction studies (SNCs) were dominated by cases presenting with median nerve injuries (Table 5). Methodologically, they were performed following the stimulation of receptors within the first finger, which corresponds primarily to the extent of innervation at the level of the brachial plexus in the upper trunk. A neurogenic injury of the deltoid and biceps brachii muscles and an active denervation process were diagnosed in 16 patients through electromyographic recordings (nEMG). The above confirms the predominance of injuries to the C5 and C6 cervical roots and the upper trunk of the brachial plexus in patients. Injuries within the sensory fibers of the other nerves examined, i.e., the radial and ulnar nerves, as well as neurogenic damage to the triceps brachii and dorsal interosseous muscles I occurred in 8 and 7 and 13 and 12 patients, respectively, out of the 20. Therefore, lesions in the middle and lower trunks of the brachial plexus and the cervical roots C7 and C8 occurred less frequently in the group of patients. Patients with damage to their entire brachial plexus, a co-occurring avulsion of their spinal roots, and a lack of evoked potentials and electrical muscle activity were included in the statistical analysis of the neurophysiological test results (Figure 2).

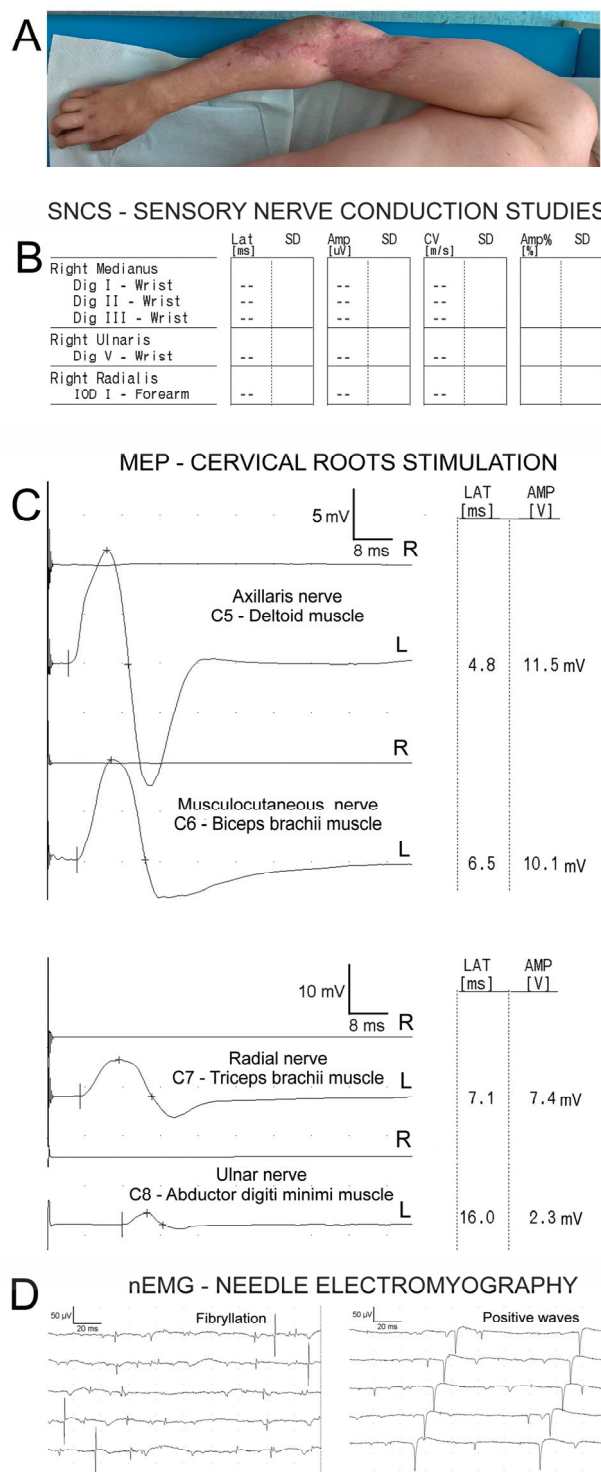


Figure 2. Examples of results from the sensory nerve conduction studies (**B**), MEP recordings after the magnetic stimulation of the cervical roots (**C**), and spontaneous activity during needle electromyography tests (**D**), showing denervation potentials (fibrillation and positive waves). These relevant results were obtained in a patient (**A**) with a total brachial plexus injury and cervical root avulsion on the right side. Abbreviations: N—nerve; Musculocut.—Musculocutaneous nerve; ADM—abductor digiti minimi muscle; C5–C8—cervical root levels.

Table 5. Results of sensory nerve conduction studies (SNCSs) and needle electromyography studies (nEMG) conducted on patients (N = 20).

Test	Score/N = 20			
SNCS				
Median nerve				
D1	0/2	1/8	2/10	
D2	0/2	1/6	2/12	
D3	0/2	1/5	2/13	
Radial nerve (snuff box)	0/3	1/5	2/12	
Ulnar nerve D5	0/2	1/5	2/13	
nEMG				
DP	DD 0/12	1/8; MUAP 0/5	1/11	2/4
BB	DD 0/11	1/9; MUAP 0/3	1/13	2/4
TB	DD 0/13	1/7; MUAP 0/4	1/9	2/7
FDI	DD 0/13	1/7; MUAP 0/2	1/10	2/8

Abbreviation: D1–D5—sensory examination of first to fifth digits; DP—deltoid posterior muscle; BB—biceps brachii muscle; TB—triceps brachii muscle; FDI—first dorsal interosseous muscle; SNCS grade: 0—lack of evoked potential, 1—pathological parameters of evoked potential, 2—normal parameters; nEMG grade: DD—denervation discharges, 0—lack of denervation potentials, 1—denervation potentials recorded; MUAP—motor unit action potentials: 0—lack of voluntary muscle activity, 1—neurogenic MUAPs, 2—normal parameters of MUAPs.

4. Discussion

This paper deals with the results of applying MEP vs. ENG diagnostic methodologies to patients with a BPI compared to healthy subjects [16] and records the results found when conducting these neurophysiological evaluations on the symptomatic versus asymptomatic side of patients.

The most important results are the CMAP and MEP amplitude changes and higher latency parameters seen in all examined nerves on patients' symptomatic side after they were stimulated at their Erb's point. Importantly, lower amplitudes of CMAP in the axillary and ulnar nerves and MEP in the ulnar nerve were recorded on the patients' asymptomatic side compared to those of the control group. Recordings following musculocutaneous and radial nerve electrical stimulation and ulnar nerve magnetic stimulation at Erb's point showed that the patients' latencies were also longer on their asymptomatic side compared to those of the control group. The sensory deficits seen in the SNCSs were dominated by cases with predominantly median nerve lesions, similar to Jones et al.'s study [27]. Interestingly, the MEP amplitudes from the recordings of all evaluated C5–C8 cervical root levels in patients were reduced bilaterally compared to the recordings from healthy volunteers. The above results indicate that the asymptomatic side of these BPI patients also demonstrates symptoms of lesions. Contemporary data may explain this phenomenon as being due to the high overloading forces that were the mechanism of injury, which were not only unilateral [28]. They may cause the direct stretch or rupture of nerves, cervical roots, or even spinal cord tissues, leading to associated edema or arterial blood flow abnormalities [29,30]. The second explanation could be related to the adaptative processes of the intraspinal neuronal connections engaged in sensory perception and movement coordination, but this theory has only been studied under experimental conditions so far [31]. Both phenomena are related to the small distance between the injured and non-injured structures on the right and left sides of the trunk and their potential interactions. The afferent impulses transmitted to the spinal cord from different receptors on the symptomatic side, mediated by interneuronal pathways, may exert crossed reflexory effects on the contralateral motor centers, a reaction that may lead to changes in motor transmission and muscle imbalances on the clinically asymptomatic side. A possible candidate that has this influence on the contralateral motor actions is the crossed disynaptic inhibitory interneuronal system [32,33]. In the available literature, there are not many neurophysiological studies that examine the consequences of BPI with results that can be compared to those presented in the current research.

In Lo and Tan's studies [34], electrical stimulation demonstrated the existence of a multilevel motor root conduction block, which reversed after a 4-month period. Motor root conduction studies are useful diagnostic and prognostic adjuncts in the management of brachial plexopathy. The importance of the nEMG recordings obtained in this study lies in confirming the denervation process of the muscle motor units innervated from certain injured brachial plexus branches and assessing the advancement of the neurogenic changes that limit the contractile properties of single muscle motor units [35]. The predominance of injuries to the C5 and C6 cervical roots and upper trunk of the brachial plexus in our patients is similar to the results reported by Dhawan [36]. Most of the reports published by other authors provide similar etiologies and ranges of brachial plexus injury as our observations [15].

The abnormalities found in the motor neural transmission on the asymptomatic side of patients following a brachial plexus injury may have important clinical consequences. Even if they are characterized as subclinical, they may explain the poor results of BPI treatments, which sometimes appear after reconstructive surgery or conventional, long-term physiotherapeutic treatment [10,37,38]. These functional abnormalities have not been detected with standard clinical methods, but there is an opportunity to detect them when using the clinical neurophysiology methods demonstrated in this study. The symptoms of these symptomatic–asymptomatic injuries have been described in a few clinical observations [39]. This leads to the conclusion that it is necessary to add expanded neurophysiological examinations to the evaluation of the level, range, and type of brachial plexus damage seen in BPIs. A similar evaluation to the one used in this study has never been presented before; additionally, clinical studies have not excluded this possibility. Moreover, it can be concluded that a peripheral upper-trunk brachial plexus injury may mimic the typical consequences of a spinal cord injury [40]. One may argue that these symptomatic–asymptomatic changes could have been related to the patient's positioning during these diagnostics or bias related to the precision of the measurements; however, the observed changes in neurophysiological parameters detected by our statistical analyses were not observed in the healthy volunteers. The subclinical changes demonstrated in our studies also draw attention to the asymptomatic side of BPIs, especially concerning prospective follow-up studies in this group of patients. Recording these changes may lead to modification of the treatment procedures and analysis of possible regeneration processes or the further degeneration of neural structures in clinical practice. In light of recent discoveries, our results also confirm a previous observation we made in healthy volunteers [16]. Using two kinds of stimuli (electrical versus magnetic) for the excitation of the brachial plexus' motor components allows for a noninvasive, objective, and replicable evaluation of a patient with BPI. They can be utilized interchangeably depending on the patient's health and ability to endure the stimulation and the requirements of the diagnostic algorithm. The currently accepted diagnostic procedures in clinical neurophysiology do not contain methodological descriptions of combining MEP tests after transvertebral cervical stimulation and at Erb's point with ENG tests for differential diagnostics in patients with BPI [41–44]. The procedure presented in the current work is unique; electrodiagnostic abnormalities on the asymptomatic side of BPI have not yet been described.

A limitation of our study could be the number of patients examined. However, a statistical tool initially estimated that the size of our sample was sufficient to detect functional motor abnormalities on patients' clinically symptomatic and non-symptomatic sides and demonstrate the differences in their CMAP and MEP parameters compared to those of the control group. Moreover, selecting the appropriate number of BPI patients who were a similar length of time from their moment of injury, had similar degrees of advancement in their pathology, and had a complete set of clinical and neurophysiological tests is always a challenge for researchers.

We will undertake new studies to further explore how age-related changes in the neuromuscular system may influence the results of neurophysiological examinations of patients with BPI. We established that an MEP examination of older patients (6 or 7 decades

old) may provide valuable information about the functional state of their cervical roots, which may be damaged through degenerative changes in their spine. The correlation between degenerative changes in the cervical roots and the possibility of injury in the proximal part of the brachial plexus structure appears to be clinically relevant.

5. Conclusions

The bilateral symptoms of neurological dysfunction detected using clinical neurophysiology methods in our BPI patients show that their unilateral injury may primarily result in the functional reorganization of their spine's internal neural connections. The electrical (ENG) and magnetic (MEP) stimuli used for the excitation of the efferent components of the brachial plexus allow for a noninvasive, objective, and repeatable evaluation of the BPI patient's functional status and can be utilized interchangeably depending on the patient's health to conduct therapeutic procedures or meet the requirements of a diagnostic algorithm. Future neurophysiological studies should focus on elucidating the phenomenon of clinically silent abnormalities on the asymptomatic side and the mechanisms that could contribute to their emergence in the absence of such tests.

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

Funding: This research received no external funding.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Bioethics Committee of Poznan University of Medical Science, decision no 554/17, dated 22 June 2017.

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

Data Availability Statement: All the data generated or analyzed during this study are included in the published article.

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

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Article

Effects of Subanesthetic Intravenous Ketamine Infusion on Stress Hormones and Synaptic Density in Rats with Mild Closed-Head Injury

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Abstract: Background: Every year, over 40 million people sustain mild traumatic brain injury (mTBI) which affects the glucocorticoid stress pathway and synaptic plasticity. Ketamine, a multimodal dissociative anesthetic, modulates the stress pathway and synaptic plasticity. However, the effects of post-mTBI ketamine administration on plasma stress hormones and brain synaptic plasticity are largely unknown. **Methods:** Adult male Sprague-Dawley rats with indwelling jugular venous catheters sustained mTBI with the Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) in a single session (3 impacts \times 1.5 J). One hour later, rats received intravenous (IV) ketamine (0, 10, or 20 mg/kg, 2 h). Catheter blood samples were collected for plasma corticosterone and progesterone assays. Brain tissue sections were double-labeled for presynaptic synapsin-1 and postsynaptic density protein 95 (PSD-95). Utilizing the Synaptic Evaluation and Quantification by Imaging Nanostructure (SEQUIN) workflow, super-resolution confocal images were generated, and synapsin-1, PSD-95, and synaptic density were quantified in the CA1 of the hippocampus and medial prefrontal cortex (mPFC). **Results:** IV ketamine infusion produced biphasic effects on corticosterone levels: a robust elevation during the infusion followed by a reduction after the infusion. CHIMERA injury elevated progesterone levels at post-injury day (PID)-1 and reduced synaptic density in the CA1 at PID-4, regardless of ketamine infusion. Ketamine infusion increased synaptic density in the mPFC at PID-4. **Conclusions:** Mild TBI and IV ketamine modulate the stress pathway and synaptic plasticity in the brain. Further research is warranted to investigate the functional outcomes of subanesthetic doses of ketamine on stress pathways and neuroplasticity following mTBI.

Keywords: ketamine; mild traumatic brain injury; stress hormone; synaptic density; rat; CHIMERA; prefrontal cortex; hippocampus

1. Introduction

Traumatic brain injury (TBI) is a leading cause of death and disability. After TBI, secondary injury processes occur that are mediated by neuroinflammation and excitotoxicity.

One downstream consequence of TBI is a disruption in synapse function [1], which may particularly affect synaptic plasticity in regions such as the prefrontal cortex (PFC) and hippocampus [2]. Mild traumatic brain injury (mTBI) is the most common form of TBI, accounting for 70–90% of reported cases [3]. Though most individuals recover, up to 15% of people develop long-term disability after mTBI [4], including emotional disturbances, cognitive deficits, and physical symptoms [5]. In preclinical studies, rodents with mTBI may experience behavioral symptoms such as motor and balance dysfunction [6], cognitive impairment [7], and enhanced fear memory [8]. These symptoms are associated with synaptic alterations commonly observed after mTBI, including reductions in synaptic proteins and dendritic length, arborization, and spine density [7,8]. Thus, mitigating synaptic alterations following mTBI may be central to therapies aimed at reducing mTBI-induced disability.

Ketamine is a non-competitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonist, commonly used as an anesthetic and analgesic drug. Ketamine is highly useful in trauma settings because it does not produce respiratory depression or cardiac instability [9–11]. The multimodal properties of ketamine, including potential immunomodulatory effects [12], may be beneficial for treatment of mTBI. Ketamine also has been shown to be neuroprotective in preclinical models of post-traumatic stress disorder (PTSD) [13], major depression [14,15], and TBI [16]. Moreover, ketamine has been demonstrated to reverse synaptic density changes in animal models of PTSD and depression, such as stress-induced alterations in dendritic branching, length, and spine density [13,17]. Ketamine may reverse the effects of TBI on synapses, as a daily ketamine injection (10 mg/kg, intraperitoneal [IP]) increased dendritic arborization, length, and spine density in the hippocampus after a moderate weight drop injury in rats [16]. However, little is known about the effects of IV ketamine infusions on synaptic density in the PFC and hippocampus after mTBI.

Another downstream consequence of TBI is disruption of the hypothalamic–pituitary–adrenal (HPA) axis, which releases hormones such as cortisol under stressful conditions [18,19]. TBI is generally considered to impair HPA axis activity [20], leading to consequences such as unchecked inflammation and psychiatric sequelae [21]. However, depending on TBI severity and timing of sampling after injury, cortisol may be reduced or elevated [22,23]. Cortisol and its rodent equivalent, corticosterone (CORT), peak and trough within two hours of an acute stressor [19], and changes in cortisol may be observed as early as 0–3 h after TBI. Similarly, preclinical studies have found elevated levels of CORT as soon as one hour post-mTBI in rats [24]. Based on these previous observations, we aim to examine the effects of mTBI on CORT at acute timepoints post-injury. Other hormones released during stress include progesterone (PROG), the biochemical precursor to cortisol [18], and a known neuroprotective agent [25]. TBI has been shown to modulate PROG levels in humans [26] and rodents [27,28]. However, endogenous PROG levels have yet to be examined, specifically after a mild TBI, perhaps because its role as a stress hormone is less well-known. To our knowledge, this is the first study to examine the effect of a preclinical model of mTBI on both basal CORT and PROG levels.

Ketamine has gained favor as a trauma drug because it provides potent analgesia and anesthesia without compromising hemodynamic stability or respiratory drive [10,11], and ketamine does not increase intracranial pressure (ICP) as was previously feared [29,30]. However, ketamine's effects on HPA axis activity in TBI patients have yet to be examined, despite the importance of the HPA axis in the management of stress and recovering normal physiologic function after TBI [21]. Ketamine is known to modulate the HPA axis, increasing circulating and salivary cortisol levels in healthy human volunteers [31–33]. Similarly, studies from our lab have demonstrated that IV ketamine increases plasma levels of CORT and PROG in rats during a two-hour infusion, which return to baseline two hours later [34–36], which is mirrored in some human studies [33]. However, it is likely

that ketamine will differentially modulate HPA axis activity in mTBI patients compared to healthy subjects. The current study is the first to examine the effects of IV ketamine on CORT and PROG at acute timepoints after mTBI.

Although many previous mTBI studies have demonstrated the importance of synaptic plasticity, conventional experimental methods used in those studies have limitations. Electron microscopy is a gold standard for synaptic evaluation, but obtaining images and manually counting synapses is labor-intensive and expensive. Dendritic spine analysis offers improved efficiency but uses dendritic spine morphology to evaluate neuroarchitectural changes. Synaptic proteins may be quantified using western blot, but this does not provide information on the spatial relationship between pre- and postsynaptic puncta. Synaptic Evaluation and Quantification by Imaging Nanostructure (SEQUIN) is a novel method to evaluate synaptic density by measuring colocalizations between pre- and postsynaptic markers [37,38] and has previously been used to find cortical synapse loss after CHIMERA injury [38]. SEQUIN produces a reproducible, quantifiable representation of synaptic density with rapid results compared to conventional methods such as dendritic spine analysis with Golgi staining or electron microscopy.

Despite the ubiquitous nature of mTBI injuries and the prevalence of ketamine as a trauma analgesic and anesthetic, the effects of ketamine on stress hormone levels and synaptic density following mTBI have not been investigated. We have established a paradigm of IV ketamine infusion in freely moving rats [36,39] and combined this technique with the Closed-Head Impact Model of Engineered Rotational Acceleration (CHIMERA) to investigate the effects of mTBI and ketamine on inflammatory cytokines and behavioral outcomes [6]. Unlike most preclinical TBI models, which utilize a craniotomy or head restraint, the CHIMERA simulates free movement in a clinical mTBI by delivering a closed-head impact to an unrestrained head. The specific aims of the current study were (1) to evaluate the effects of CHIMERA and ketamine infusion on plasma stress hormone levels and (2) to determine the effects of CHIMERA and ketamine on synaptic density in the mPFC and CA1 of the hippocampus of rats.

2. Methods

2.1. Animals

Male Sprague Dawley rats (9 weeks old) were purchased with jugular venous catheters surgically implanted at Envigo Laboratories (Dublin, VA, USA) as previously described [6]. Following three days of acclimation, animals were randomly assigned to one of six groups: Sham-0, Sham-10, Sham-20, CHIMERA-0, CHIMERA-10, and CHIMERA-20 (N = 9–10 per group). Blood and brain tissue samples used in this study were collected during a previous study that reported behavioral and neuroinflammatory effects of CHIMERA and ketamine administration [6]. None of the data collected and analyzed in this study were reported in the previous study. The animal protocol was approved by the Uniformed Services University Institutional Animal Care and Use Committee (IACUC) and followed all applicable federal regulations governing the protection of animals used in research.

2.2. CHIMERA

The CHIMERA injury procedure was performed as previously described [6]. Before the CHIMERA procedure, each animal was anesthetized using isoflurane (5% for induction and 3% for maintenance) mixed with 100% oxygen. Each animal was then placed in a dorsal position in the CHIMERA device with adhesive straps holding the body on the platform. The head was centered over crosshairs on an aluminum plate, aligning the impact piston approximately over bregma. A hole in the plate allowed a 200 g piston to impact the head (1.5 J, 5.5 m/s velocity). CHIMERA animals received three consecutive impacts

(5–10 s apart) in a single session. Sham animals underwent the same procedure except for actual impacts to the head. After the injury, animals were returned to their home cages and allowed to drink acetaminophen water (1 mg/mL) in one of the two bottles installed in each cage (the other bottle contained water) for one day.

2.3. IV Ketamine Infusion

Before the infusion, racemic (\pm) ketamine hydrochloride (100 mg/mL) (Covetrus, Dublin, OH, USA) was diluted in 0.9% sterile saline to 2 mg/mL. One hour after the CHIMERA or sham injury, animals received an IV (R,S)-ketamine (0, 10, or 20 mg/kg) infusion over a 2 h period. The ketamine doses were selected based on our previous studies showing analgesic [36] and immunomodulatory [6] effects. Animals first received a bolus of ketamine (1 mg/kg, IV) or saline before the infusion procedure (Med Associates Inc., St. Albans, VT, USA) using infusion pumps (Harvard Pump 11 Elite, Holliston, MA, USA) that used a 5 mL plastic syringe with a flow rate of 2.5 mL/kg/h. Syringes were connected to a fluid swivel (Instech, Plymouth Meeting, MA, USA) by polyurethane tubing. This tubing was encased in a metal spring-wire tether that was magnetically attached to the metal cannula on the exit port of the catheter between the rat scapulae. The tether system allowed free movement of the animals in the chambers during the infusion period. Each chamber was equipped with two infrared photobeams for real-time locomotor activity monitoring. After the infusion, animals were returned to their home cages. Catheter blood samples were collected at 1 h post-injury (immediately before the ketamine infusion), 3 h post-injury (immediately after the ketamine infusion), 5 h post-injury (2 h after the ketamine infusion), and 24 h post-injury.

2.4. CORT and PROG ELISA

Plasma CORT and PROG levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (Arbor Assays, Ann Arbor, MI, USA) as previously described [35]. Blood samples were centrifuged at 4000 rpm for 10 min at 4 °C, and plasma was collected and stored at −70 °C. For the ELISA, a serial dilution of standard samples was prepared and added to a 96-well plate. According to the manufacturer's protocol, a diluted plasma sample, antibody, and CORT or PROG conjugate were added into each well. The plate was covered with a plastic film and incubated on an orbital shaker at room temperature. After incubation, the plate was washed with wash buffer three times. TMB substrate was added to each well, and the plate was incubated for 30 min at room temperature before the stop solution was added. The optical density was read at 450 nm using an Infinite 200 Pro Microplate Reader (Tecan US, Morrisville, NC, USA).

2.5. Immunohistochemistry

Four days after the injury, rats were deeply anesthetized with isoflurane, verified by paw pinch. A trans-cardiac perfusion with 10% neutral-buffered formalin in phosphate-buffered saline (PBS) was performed using a peristaltic perfusion pump. The brain tissue was removed from the calvarium, post-fixed in 10% neutral-buffered formalin for 24 h, and cryoprotected with a 20% sucrose solution in PBS for three days. Brains were sectioned with a sliding frozen microtome (Lecia Biosystems, Nussloch, Germany), and sections (40 μ m) were stored in cryoprotectant solution at −20 °C. The CA1 and mPFC were determined based on the bregma coordinates from the rat brain atlas [40]. Four sections of brain tissue containing CA1 and four sections containing the mPFC from each animal were used for brain tissue analyses to capture representative data from the CA1 and mPFC, with the average data of the four sections being used in the analysis for each brain region. Representative sections of mPFC and CA1 are shown in Figure 1A. Immunofluorescent double-labeling was carried out as previously described [37]. Brain tissue sections were

washed with PBS on a shaker. After the final wash, samples were placed in a six-well plate filled with a blocking buffer of 20% normal goat serum (Vector Laboratories, Newark, CA, USA) diluted in PBS for one hour. After blocking, the primary antibody solution was added, consisting of rabbit anti-PSD-95 (Invitrogen, Waltham, MA, USA) and guinea pig anti-synapsin-1 (Synaptic Systems, Göttingen, Germany) in 10% normal goat serum plus 0.3% Triton X-100 (Dow Chemical, Midland, MI, USA), and was incubated for one day at 4 °C. After the primary antibody incubation, the sections were washed, then incubated in secondary antibody solution in 10% normal goat serum plus 0.3% Triton X-100 for four hours at room temperature. Brain sections were washed, mounted on clean microscope slides, and dried in a flat, dark location for approximately 10 min. While the samples were drying, mounting media were prepared by mixing AF300 and MWL488 (Electron Microscopy Sciences, Hatfield, PA, USA) in a 1:9 ratio, followed by vortexing and desktop centrifuging to remove air bubbles (5 min, 4000 rpm). Once the sections were dry, 75 µL of mounting media was placed on the sample, and high-precision 1.5 H coverslip glass (Marienfield, Lauda-Königshofen, Germany) was used to protect the samples. The prepared slides were stored and cured in a dark room for 3–7 days before imaging with a confocal microscope.

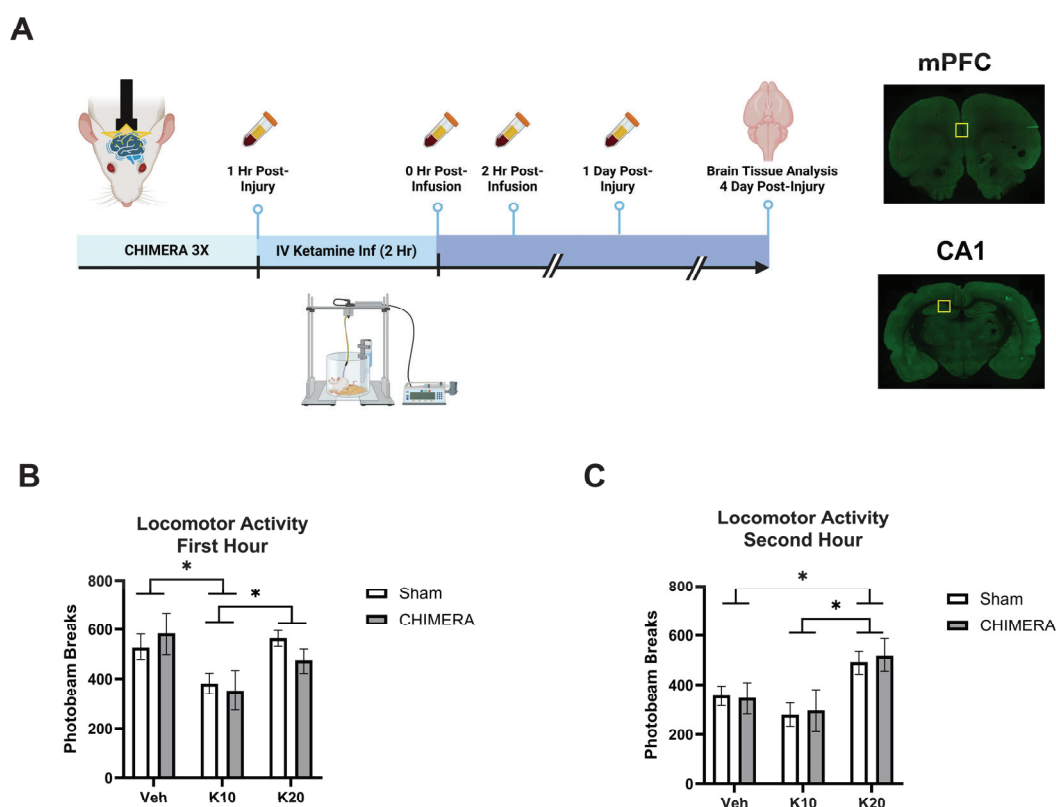


Figure 1. Experimental design and locomotor activity during ketamine infusion. (A) Experimental design indicating CHIMERA, IV ketamine infusion, catheter blood sampling, and brain tissue collection (Created in BioRender.com). Representative images of brain sections containing mPFC and CA1; the yellow square indicates the ROI. (B) Spontaneous locomotor activity during the first hour of IV ketamine infusion in rats. Ketamine 10 mg/kg (K10) infusion reduced activity compared to the saline vehicle (veh) and ketamine 20 mg/kg (K20) groups. * indicates a significant difference between the saline vehicle and K10 groups and the K10 and K20 groups ($p < 0.05$). (C) Spontaneous locomotor activity during the second hour of IV ketamine infusion in rats. Ketamine 20 mg/kg (K20) infusion increased activity compared to the saline and ketamine 10 mg/kg (K10) groups, as shown in * between the saline and K20 and the K10 and K20 groups ($p < 0.05$). $N = 8$ –10 per group due to missing data (photobeam breaks counts malfunction in two animals).

2.6. SEQUIN

Sections were imaged on a Zeiss LSM 980 confocal microscope equipped with Airyscan 2 (Zeiss Group, Oberkochen, Germany). Scan parameters were established using previously described SEQUIN techniques [37]. CA1 and mPFC regions of interest (ROIs) were targeted under $10\times$ power using an epifluorescent light source and microscope oculars. Once the ROI was targeted, Zeiss 518 immersion oil was applied, and the area was re-targeted under $63\times$ power. Once the final ROI was confirmed, confocal images were obtained via previously described experimental parameters [37]. Confocal microscope images were analyzed using Imaris software (ver. 10.0.1, Abingdon, UK). Images were converted to Imaris format and used for spot analysis of PSD-95 and synapsin-1 puncta. Source channels were set to 488 nm and 594 nm for synapsin-1 and PSD-95, respectively. Estimated XY diameter was set at $0.3\ \mu\text{m}$ based on manual measurement of typical puncta size. Point spread elongation was set at $0.65\ \mu\text{m}$ to account for the axial distortion of confocal rendered images. Background subtraction and quality filters were utilized to capture maximum puncta detection while eliminating signal noise. Parameters were saved for batch analysis across all samples. Following spot analysis, a presynaptic to postsynaptic puncta examination was conducted using the Imaris software. Imaris identified and quantified postsynaptic PSD-95 puncta center points with a Euclidean distance $< 0.55\ \mu\text{m}$ from presynaptic synapsin-1 puncta center points. Identified puncta pairs were divided by the scanned image volume to determine relative synaptic density within each volume of sample analyzed. Densities from the four brain sections for each region (one CA1 or mPFC per section) were averaged.

2.7. Statistical Analyses

Tests for normal distribution of data were performed using Shapiro–Wilk tests, and all data passed normality tests except PROG data at the 1 h post-injury time point. Thus, that dataset was analyzed using non-parametric statistics (Mann–Whitney U test), and all other datasets were analyzed with parametric statistics. Student's *t*-test was used for 2-group comparisons, and a two-way ANOVA was used for main effects and interaction between CHIMERA injury and ketamine doses. Post hoc tests (Holm–Sidak multiple comparisons tests) were used following significant ANOVA effects. For CORT and PROG time course data, a mixed model was used with between-subjects factors (CHIMERA and ketamine) and a within-subjects factor (time). All data analyses and plotting graphs were carried out with GraphPad Prism (ver. 10.4.1). Significance was determined at $p < 0.05$.

3. Results

Figure 1A represents the overall study design and specific timelines of the experiments. Animals were subjected to the CHIMERA injury and, one hour later, received an IV ketamine infusion. Catheter blood samples were collected at multiple timepoints, and brain tissue was collected at PID-4. Figure 1B shows spontaneous locomotor activity in the first hour of ketamine infusion (0, 10, or 20 mg/kg). A two-way ANOVA indicated a significant main effect of ketamine on locomotor activity ($F_{(2, 52)} = 5.702$, $p = 0.0058$). Post hoc tests revealed that 10 mg/kg reduced locomotor activity compared to the saline and 20 mg/kg groups. Figure 1C shows spontaneous locomotor activity in the second hour of ketamine infusion. A two-way ANOVA indicated a significant main effect of ketamine on locomotor activity ($F_{(2, 52)} = 7.265$, $p = 0.0017$). Post hoc tests revealed that 20 mg/kg increased locomotor activity compared to the saline and 10 mg/kg groups. The saline group showed less activity in the second hour as compared to the first hour due to habituation to the chambers over the two-hour infusion period.

Plasma CORT levels at 1 h post-injury were not statistically different between sham (mean 130 ng/mL) and CHIMERA (mean 156 ng/mL) groups ($p = 0.094$). This suggests that CHIMERA may produce mild effects on plasma CORT elevation (approx. 20%). However, the IV ketamine infusion produced robust increases in plasma CORT levels (approx. 3–4-fold) when measured immediately after the infusion (Figure 2A). A mixed model analysis indicates significant main effects of ketamine ($F_{(2, 47)} = 34.38, p < 0.0001$) and time ($F_{(1, 46)} = 287.9, p < 0.0001$) as well as an interaction between ketamine and time ($F_{(2, 46)} = 64.12, p < 0.0001$) on CORT levels. Both 10 mg/kg and 20 mg/kg groups increased CORT levels compared to the saline group based on post hoc tests. At 2 h post-infusion, CORT levels were significantly reduced in the 10 mg/kg and 20 mg/kg groups compared to the saline group. Plasma CORT levels returned to the baseline at PID-1 with no statistical differences between any of the groups at this time point (Figure 2B).

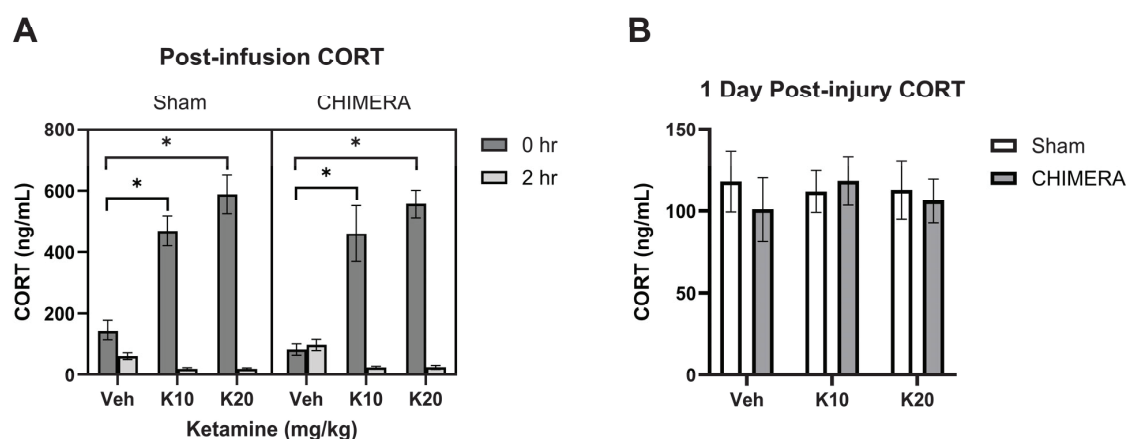


Figure 2. Plasma CORT levels following CHIMERA and IV ketamine infusion in rats. **(A)** CORT levels at 0 h and 2 h post-ketamine infusion (3 and 5 h post-CHIMERA). Ketamine produced robust effects on CORT elevation at 0 h followed by reduction at 2 h post-infusion ($* p < 0.05$). **(B)** CORT levels at 1 day after CHIMERA injury returned to normal, baseline levels in rats. $N = 7$ – 10 per group due to lack of blood samples and missing data.

PROG levels were not significantly different between sham and CHIMERA groups at 1 h post-injury based on a Mann–Whitney test ($U = 340, p > 0.05$). The time course effects of ketamine on PROG levels at 0 h and 2 h post-infusion are shown in Figure 3A. A mixed model analysis with CHIMERA and ketamine as between-subjects factors and time as a within-subjects factor indicated a significant main effect of time ($F_{(1, 47)} = 20.75, p < 0.0001$) on PROG levels. Thus, IV ketamine infusion did not elevate PROG levels after the infusion. However, CHIMERA injury significantly elevated plasma PROG levels at PID-1 compared to the sham groups (Figure 3B). There was a significant main effect of CHIMERA on PROG levels ($F_{(1, 46)} = 8.747, p = 0.0049$) at this time point. This suggests that CHIMERA injury may produce a delayed increase in plasma PROG levels, which may serve as a potential biomarker of mTBI.

Figure 4A shows a representative image of synaptic density (yellow spots) based on synapsin-1 (green) and PSD-95 (red) double-labeling in the mPFC. Synaptic density using presynaptic (synapsin-1) and postsynaptic (PSD-95) markers was quantified with the SEQUIN method. There were no effects of CHIMERA or ketamine on synapsin-1 levels in the mPFC (Figure 4B). A two-way ANOVA revealed a significant main effect of ketamine on PSD-95 levels in the mPFC ($F_{(2, 52)} = 11.83, p < 0.0001$), as shown in Figure 4C. There was a significant interaction between CHIMERA and ketamine on PSD-95 density ($F_{(2, 52)} = 3.362, p = 0.0423$). Post hoc tests revealed that the 20 mg/kg group is significantly different from the saline and 10 mg/kg groups. A two-way ANOVA indicated a significant

main effect of ketamine ($F_{(2,52)} = 3.968, p = 0.024$) and a trend of significant interaction between CHIMERA and ketamine ($F_{(2,52)} = 3.009, p = 0.058$) on the synaptic density in the mPFC (Figure 4D). Post hoc tests revealed a trend toward significance between the saline and 20 mg/kg groups ($p = 0.06$) and the 10 mg/kg and 20 mg/kg groups ($p = 0.05$).

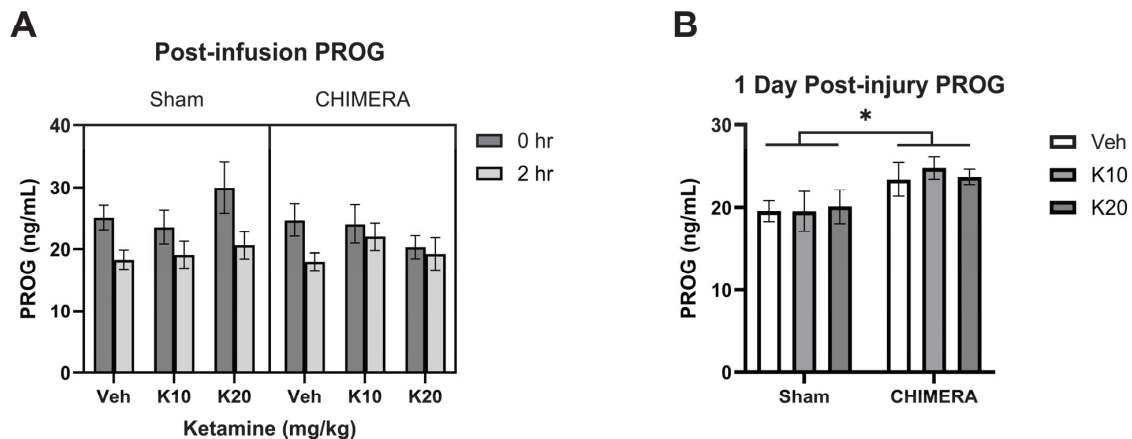


Figure 3. Plasma PROG levels following CHIMERA and IV ketamine infusion in rats. (A) PROG levels at 0 h and 2 h post-ketamine infusion (3 and 5 h post-CHIMERA). Ketamine did not alter PROG levels at these time points. (B) PROG levels at 1 day after CHIMERA injury. CHIMERA injury significantly elevated PROG levels at this time point (* $p < 0.05$). $N = 7$ – 10 per group due to lack of blood samples and missing data.

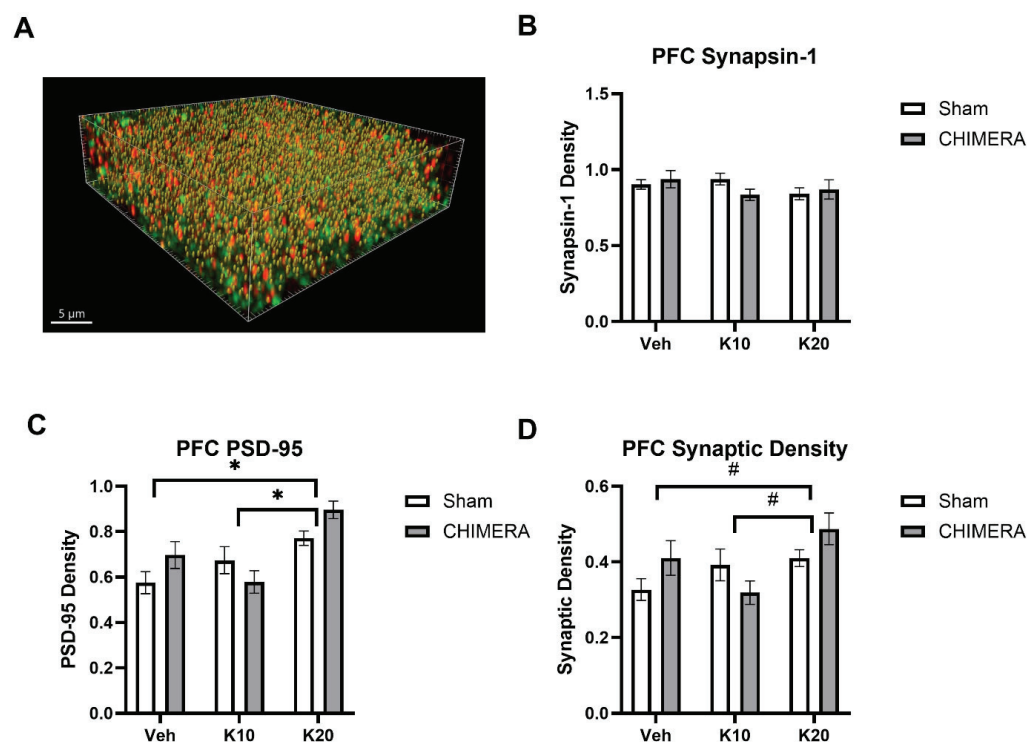


Figure 4. Synaptic density in the mPFC following CHIMERA injury and IV ketamine infusion in rats. (A) A representative Imaris image showing synapsin-1 (green), PSD-95 (red), and synaptic (gold) puncta in the mPFC. (B) CHIMERA and ketamine had no effects on synapsin-1 density in the mPFC ($p > 0.05$). (C) PSD-95 density in the mPFC. Ketamine 20 mg/kg increased PSD-95 density compared to the saline and ketamine 10 mg/kg groups (* $p < 0.05$). (D) Synaptic density in the mPFC. Two-way ANOVA indicated a significant main effect of ketamine on synaptic density in the mPFC. However, post hoc tests revealed trends of significance between saline and K20 ($p = 0.06$) and K10 and K20 ($p = 0.05$) groups, as indicated by # on the graph. $N = 9$ – 10 per group.

Figure 5A shows a representative image of synaptic density (yellow spots) based on synapsin-1 (green) and PSD-95 (red) double-labeling in the CA1 of the hippocampus. A two-way ANOVA indicated a significant main effect of CHIMERA on synapsin-1 density ($F_{(1, 52)} = 8.098, p = 0.0006$), as shown in Figure 5B. Post hoc tests revealed that the Sham-0 group was significantly different from the CHIMERA-20 group ($p < 0.05$). A two-way ANOVA indicated a significant main effect of ketamine on PSD-95 density ($F_{(2, 52)} = 5.491, p = 0.006$), as shown in Figure 5C. Post hoc tests revealed a significant difference between the CHIMERA-10 and CHIMERA-20 groups ($p < 0.05$). A two-way ANOVA indicates a significant main effect of CHIMERA on synaptic density ($F_{(1, 52)} = 4.747, p = 0.033$), as shown in Figure 5D. This indicates that CHIMERA injury reduced synaptic density in the CA1 of the rat hippocampus at PID-4.

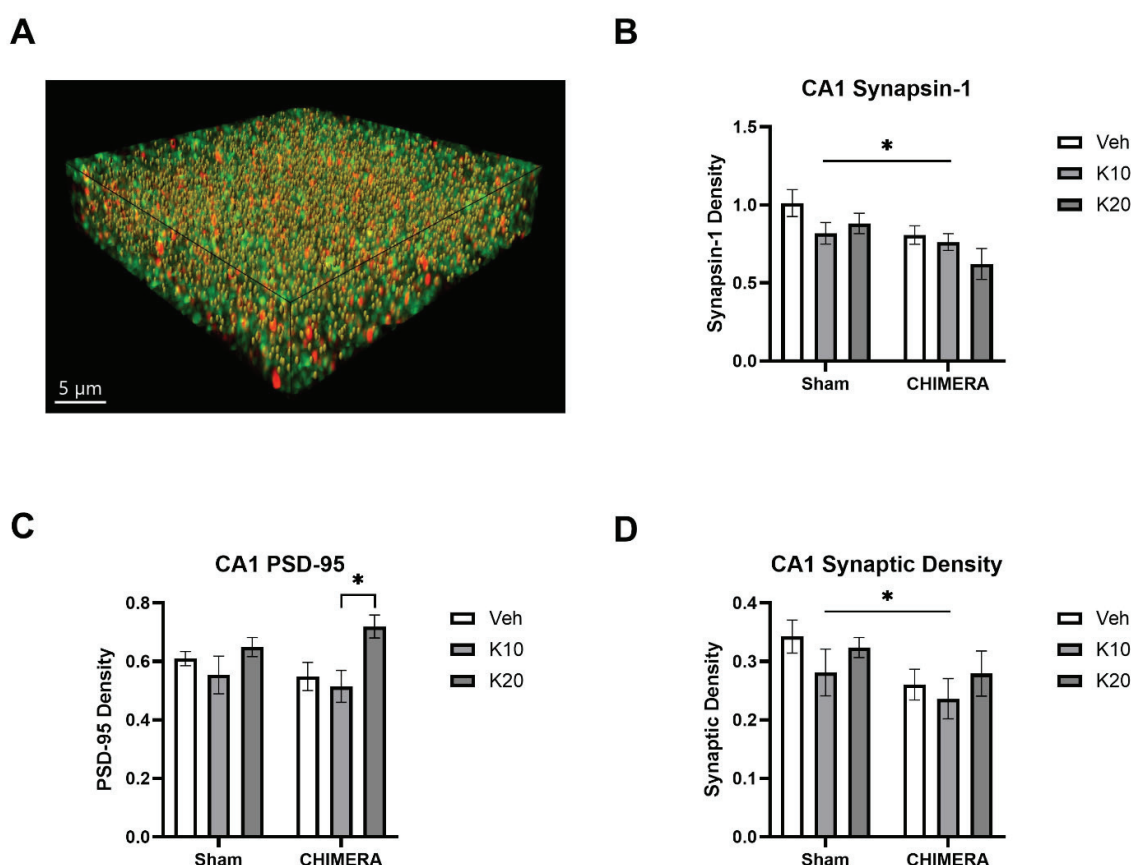


Figure 5. Synaptic density in the CA1 region of the hippocampus following CHIMERA injury and IV ketamine infusion in rats. (A) A representative Imaris image showing synapsin-1 (green), PSD-95 (red), and synaptic (gold) puncta. (B) Synapsin-1 density in the CA1. CHIMERA injury significantly reduced synapsin-1 density in the CA1 (* $p < 0.05$). (C) PSD-95 density in the CA1. Post hoc tests revealed a significant difference between K10 and K20 in CHIMERA animals (* $p < 0.05$). (D) Synaptic density in the CA1. CHIMERA injury reduced synaptic density in the CA1 region compared to the sham group (* $p < 0.05$). $N = 9$ – 10 per group.

4. Discussion

In the current study, the effects of CHIMERA and subanesthetic doses of ketamine infusion on plasma stress hormone levels and synaptic density in the mPFC and CA1 were investigated using a rat model of mTBI. A CHIMERA injury produced a delayed increase in PROG levels at PID-1, and a ketamine infusion produced biphasic effects of CORT levels: elevation immediately following the infusion and reduction two hours after the infusion. The CORT levels returned to normal at PID-1. CHIMERA injury reduced synaptic density in the CA1, while the ketamine infusion increased synaptic density in the mPFC at PID-4.

However, there was no interaction between CHIMERA injury and ketamine infusion on synaptic density. To our knowledge, this is the first study reporting the role of CHIMERA and IV ketamine infusion on stress hormones and synaptic plasticity in key brain regions that are vulnerable to mTBI.

After CHIMERA injury, CORT levels were not significantly altered but followed a pattern of slight increase at 1 h, decrease at 3 h, increase at 5 h, and return to baseline at 24 h post-injury. A similar time course was reported after a moderate controlled cortical impact (CCI), which utilized repeated blood sampling at 1.5, 6, 12, 18, and 24 h post-injury and found increased plasma CORT levels at 1.5 and 6 h, which subsequently fell to baseline and remained unaltered up to 24 h post-injury [41]. Unlike in the current study, those increases in CORT were significant, which is presumably due to a more severe and invasive injury model. However, plasma or serum CORT increases have been previously reported even after mTBI. Rats exhibited increased plasma CORT levels at 6 and 24 h after a mild midline fluid percussion injury (MFP) [42]. A repeated mild projectile concussive impact (PCI) injury increased serum CORT levels at 1 h post-injury, which returned to baseline levels at 24 h post-injury in rats [24]. Despite being framed as an mTBI, the PCI injury produced a staggering array of pathological and behavioral changes such as inflammation, neurodegeneration, gait alterations, and neurobehavioral deficits [24]. In contrast, our previous CHIMERA study only reported rotarod deficits and axonal damage in the optic tract [6]. This reinforces that the current injury is very mild, which would explain a lack of significant effect on CORT levels.

The IV ketamine infusion produced a biphasic effect on CORT levels: a robust increase consistent with previous studies [34–36], followed by a reduction below baseline at 2 h post-infusion. The observed dip in CORT below saline levels after ketamine administration in our study may be explained by negative feedback, whereby increased CORT binds to glucocorticoid receptors (GRs) in order to inhibit the HPA axis and reduce CORT secretion [43,44]. It is interesting to note that IV ketamine infusion caused CORT reduction at this time point in both sham and CHIMERA animals. This resulted in a relative suppression of CORT responses to CHIMERA injury when a normal physiological profile would increase CORT levels. CORT dysfunction, especially long-term CORT suppression, has been linked to the development of PTSD-like symptoms in preclinical studies and clinical studies [45–47]. It is unclear whether an acute reduction in CORT following mTBI could result in subsequent pathological changes. Studies have shown that basal CORT suppression, rather than acute CORT shifts, is implicated in neurobehavioral responses [45,48], including in mTBI patients [49]. Therefore, implications of biphasic CORT responses following IV ketamine infusion should be further investigated in the context of mTBI.

Unlike CORT, which had non-significant fluctuations in the first five hours post-injury, plasma PROG levels were stable following CHIMERA up to 5 h post-injury. However, CHIMERA injury significantly elevated plasma PROG levels at PID-1 in adult male rats. This finding may indicate a compensatory mechanism of PROG, which is considered to have a neuroprotective role after TBI, including inhibition of inflammation, attenuation of excitotoxicity, bolstering of myelin repair, and release of neurotrophic factors [25]. Another study reported that plasma PROG levels were elevated at 24 h post-injury with a weight drop model in male mice [27]. Thus, a delayed elevation of plasma PROG following CHIMERA injury may serve as a potential biomarker for mTBI and guide future therapeutic options for patients with mTBI.

Ketamine is known to exert synaptogenic effects through intricate signaling cascades. Its initial action is disinhibition of glutamate signaling at the NMDAR, preferentially inhibiting NMDARs on inhibitory GABAergic interneurons [50], leading to a paradoxical enhancement of glutamate release [51]. Glutamate activates postsynaptic α -amino-3-

hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA), which in turn activate tropomyosin receptor kinase B (TrkB) for brain-derived neurotrophic factor (BDNF) to stimulate downstream synaptic protein synthesis and synaptogenesis [14,52].

In the mPFC, ketamine produced robust effects on synaptic density, with a 20 mg/kg dose increasing PSD-95 and synaptic density. This effect of ketamine in the mPFC is in line with the literature demonstrating that ketamine increases spine density, synaptic markers, and synaptogenesis in the PFC and was able to reverse behavioral changes due to depression and anxiety [53–56]. As such, it would be worth examining depressive- and anxious-like behaviors in these rats to examine a connection between increased synaptic density in the mPFC and behavioral improvements. Behavioral and emotional changes after mTBI may be related to a loss of synaptic or functional connectivity in the PFC. For instance, mice sustaining mild lateral fluid percussion (LFP) injury experienced impaired fear extinction, which was concurrent with reduced complexity of dendritic arborization in the mPFC [8]. Conversely, restoration of dendritic spine length in the PFC by ketamine was concurrent with restored fear-extinction learning in rats [13]. It has been suggested that ketamine may improve fear extinction by activating synaptic signaling pathways in the mPFC, such as the mTOR pathway [57]. In humans, therapeutic effects of ketamine include upregulating functional connectivity in the PFC, which is analogous to increased synaptic connectivity in rodents [53]. Connectivity in the mPFC is inversely correlated to major depression, anxiety, fatigue, and post-concussive symptoms in patients with mTBI [58]. In depressed patients, therapeutic response to ketamine was associated with greater global brain connectivity in the PFC [59]. Taken together, the ability of subanesthetic doses of IV ketamine after mTBI to increase synaptic density in the mPFC found in the current study may have behavioral implications.

The limited effect of ketamine on synaptic density in the CA1 of the hippocampus was unexpected, as ketamine is known to produce synaptic effects in the hippocampus [17,52,60]. In the mPFC, ketamine significantly increased synaptic density along with the postsynaptic protein PSD-95 but did not affect the presynaptic protein synapsin-1. However, in the CA1, the CHIMERA injury produced a significant decrease in synapsin-1, which was unaffected by ketamine. Interestingly, ketamine increased PSD-95 in the CA1, although this effect was not strong enough to counteract the CHIMERA injury effect on overall synaptic density. The hippocampus has several factors that make it particularly vulnerable to traumatic injury. The hippocampus is situated directly below the corpus callosum, which transmits shearing forces to the hippocampus during brain injury [61]. The hippocampus is also flanked by the lateral ventricles, fluid-filled structures with large protrusions, which may contribute to the strain experienced in the hippocampus during the TBI [61,62]. Additionally, hippocampal neurons may intrinsically be more susceptible to mechanical damage than cortical neurons, experiencing greater calcium release and cell death when mechanically stretched [63]. Thus, the lack of effects of ketamine on synaptic density in the CA1 may be due to the specific nature of CHIMERA injury, along with the anatomy of the hippocampus being particularly vulnerable to this type of damage.

Ketamine produced dose-dependent effects in several metrics, namely locomotor activity during the infusion and synaptic density changes after CHIMERA injury. In the first hour of the ketamine infusion, the 10 mg/kg dose decreased locomotor activity relative to both saline and the 20 mg/kg ketamine dose due to its sedative properties at the lower 10 mg/kg dosage. However, in the second hour of the infusion, the 20 mg/kg dose increased locomotor activity compared to saline and the 10 mg/kg dose. Locomotor activity in the saline group was lower in the second hour due to habituation to the infusion chambers, and activity was higher in the 20 mg/kg group due to its dissociative effects, which likely prevented behavioral habituation. In the current study, the 20 mg/kg dose

produced synaptic effects in the mPFC, while the 10 mg/kg dose did not. This is consistent with our previous studies indicating dose-dependent effects of IV ketamine infusion on plasticity-related proteins [64,65]. However, the inability of the 10 mg/kg dose to affect synaptic density in the current study is surprising, as this dose has consistently produced behavioral and physiological effects in the previous studies [12,35,39]. In other studies, administering a 10 mg/kg dosage via the IP injection exerted synaptic effects, increasing dendritic spine density and pre- and postsynaptic markers [15,54,55,60]. In the interest of preserving the therapeutic effects of IV ketamine while minimizing its side effects (e.g., dissociation and hallucination), further research is warranted to investigate specific dose-dependent ketamine responses on synaptic density and mTBI-related behaviors.

The current study is not without limitations. Only one time point after CHIMERA injury was investigated for synaptic density (PID-4). This time point is supported by the literature: a time course study of synaptic protein levels in the rat hippocampus after cortical contusion revealed that synapsin-1 and PSD-95 reached their lowest levels at 4 days post-injury [66]. Additionally, a two-photon imaging study in mice revealed that ketamine (10 mg/kg, IP) formed nascent spines that generally disappeared within four days in the medial frontal cortex of mice [67]. This four-day time point may be significant as newly formed dendritic spines that persist past four days can form functional synapses [68]. Further, persistent dendritic spines may be implicated in the sustained antidepressant effects of ketamine [15]. Therefore, analysis of the synaptic density at PID-4 may provide insights on the lasting effects of ketamine. However, synaptic growth is a dynamic process, and using only one time point does not allow for a complete picture of ketamine's dynamic effects. Ketamine (10 mg/kg, IP) has time-dependent effects on spine density, with increases at 24 h but decreases at 7 days in the CA3 of the hippocampus in mice [60]. While ketamine induced the growth of nascent spines, it also retracted apical dendrite tuft branches the day after administration in the medial frontal cortex of mice [67]. Additionally, plasticity potential, behavioral effects, and the formation of new, persistent spines occur across different time courses after ketamine administration, with the most plasticity occurring at more acute (within 12 h) time points [69].

The main reasons for administering ketamine at the one-hour post-injury time point were (1) to adjust differences between humans and rodents. Rodents have a short lifespan (approximately 2 years), so one hour in rats can be a lot longer in humans. (2) Ketamine is often given within a few hours after injury in emergent and battlefield conditions due to its cardiovascular stability and maintenance of respiratory drive. (3) The effects of ketamine on HPA axis stimulation are fast and transient, so we aimed to overlap the effects of mild TBI and ketamine on HPA axis stimulation in rats. Similarly, we did not examine later time points of CORT and PROG levels after injury or ketamine infusion. We did not analyze these biomarkers beyond the first day because CORT typically peaks and troughs within two hours of a stressor [19], and we have previously observed these transient effects of ketamine on CORT levels as well, increasing and decreasing in the hours following infusion [34,35]. However, TBI is known to affect CORT and PROG levels several days after the injury [70–72], and follow-up studies would determine if mTBI produced persistent changes in HPA axis function. With the knowledge that PROG increased one day after injury, further testing of these later time points is warranted.

Another limitation of the current study is the use of only male rats, given the previous reports on sex-dependent effects of IV ketamine [12,73]. A study of male and female rats receiving ketamine (2.5 or 5 mg/kg, IP) after isolation stress found that ketamine reversed spine density loss in the mPFC and restored synaptic protein levels in male rats only [56]. Ketamine had more robust effects on spine density and synaptic marker expression in the hippocampus and PFC of male mice compared to female mice [60]. In particular,

ketamine produced glutamatergic bursting in the mPFC of male mice only, which may be one explanation for greater synaptic protein upregulation [60]. Thus, future studies should examine the synaptic effects of ketamine in both sexes at multiple time points after mTBI to allow for greater clinical translation.

In conclusion, subanesthetic doses of an IV ketamine infusion produced dose-dependent effects on locomotor activity and increased postsynaptic PSD-95 proteins and synaptic density in the mPFC. Additionally, ketamine produced biphasic effects on plasma CORT levels after the infusion. The CHIMERA injury produced a delayed elevation of plasma PROG levels at PID-1, which can serve as a potential biomarker for mTBI. CHIMERA injury also produced a significant reduction in synaptic density in the CA1, which was not restored by ketamine infusion. As some of the therapeutic effects of ketamine are thought to be mediated by restoration of synaptic plasticity in the brain, increased synaptic density in the mPFC following IV ketamine infusion may have clinical significance. As the mPFC is one of the major target regions of ketamine, this finding has significant implications for its therapeutic potential following an mTBI. Overall, the current investigation demonstrates the utility of combining a clinically relevant mild closed-head injury in rats, IV ketamine infusion, and super-resolution confocal microscopy with an efficient synaptic data analysis workflow for mTBI research.

Author Contributions: Conceptualization, M.B., K.R. and K.C.; Methodology, M.B., R.B., H.S., O.R. and E.M.; Software, M.B., R.B., H.S. and O.R.; Validation, H.S., O.R. and E.M.; Formal analysis, M.B., R.B. and O.R.; Investigation, M.B., R.B., H.S., E.M. and K.R.; Resources, K.C.; Data curation, R.B., H.S., O.R. and E.M.; Writing—original draft, M.B., R.B. and K.C.; Writing—review & editing, M.B., R.B., H.S., O.R., E.M., K.R. and K.C.; Visualization, R.B.; Supervision, K.R. and K.C.; Project administration, K.C.; Funding acquisition, M.B. and K.C. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the TriService Nursing Research Program (TSNRP) and the Center for the Study of Traumatic Stress (CSTS).

Institutional Review Board Statement: This research protocol (PSY-21-057) was reviewed and approved by the IACUC in accordance with all applicable federal regulations governing the protection of animals used in research.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be available upon reasonable request to the corresponding author.

Conflicts of Interest: The opinions and assertions expressed herein are those of the author(s) and do not reflect the official policy or position of the Uniformed Services University of the Health Sciences or the Department of Defense in the U.S.

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ISBN 978-3-7258-4626-9