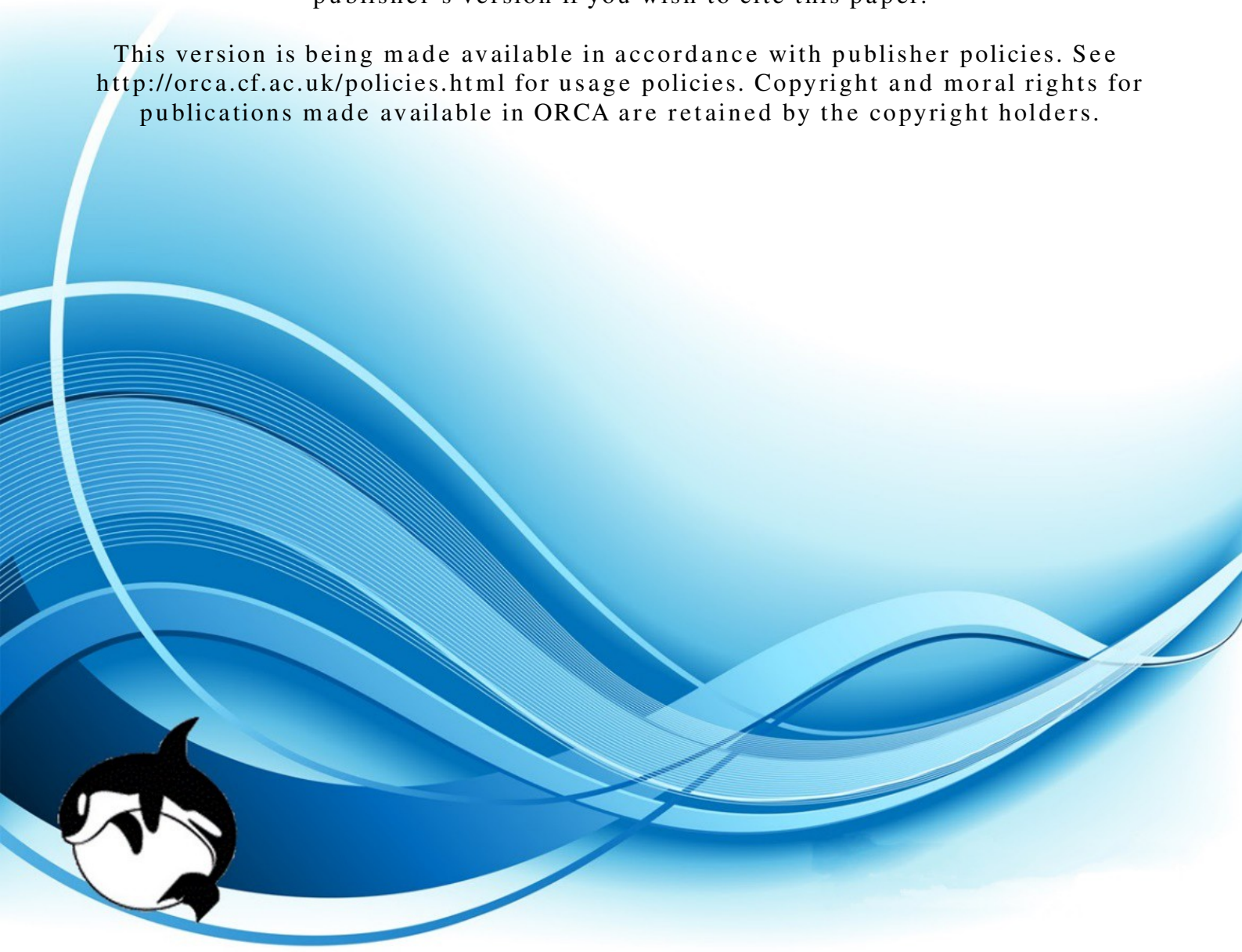

1. Introduction

Traumatic brain injury (TBI) is a global public health problem, causing significant mortality and morbidity worldwide [1]. Despite improvements in mortality rates achieved by ongoing advancements in healthcare, many survivors of TBI suffer with longstanding neuro-cognitive deficits that heavily impair their ability to function independently [2-7]. There is an unmet need for therapeutic interventions given that several pharmacological approaches to improve outcomes have remained unsuccessful to date [8]. Despite extensive neuronal death following TBI, there is evidence of an endogenous host response to promote neural regeneration [9]. Evidence from both animal models and human post-mortem studies of TBI demonstrate neural stem/ progenitor cells (NSPCs) at the site of cortical injury [10, 11], contesting the traditional hypothesis that neurogenesis is limited to particular regions of the brain, such as the subventricular and subgranular zones. However, more recent evidence demonstrates that immature newborn neurons generated following TBI do not survive and re-integrate into the injured brain [9], which may explain the lack of meaningful recovery seen in these patients.

From a pathophysiological perspective, TBI is characterised by a protracted neuro-inflammatory response to injury culminating in widespread neuronal death [12]. High Mobility Group Box protein 1 (HMGB1) is amongst the earliest pro-inflammatory cytokines released following injury, acting as the 'master-switch' of neuroinflammation [13]. Under physiological conditions, HMGB1 is a highly conserved, non-histone, DNA binding protein located in the nucleus, and involved in co-ordination of gene transcription [14]. Following injury, however, it functions as a damage-associated molecular protein (DAMP). It is released extracellularly by necrotic neurons and other immune cells recruited to the injury site before it binds to respective target receptors to upregulate the release of other pro-inflammatory cytokines including HMGB1 [15, 16]. Perpetuation of this positive feedback cycle could play a key role in the deleterious consequences of TBI. Understanding the complex relationship between neuro-inflammation, neurogenesis, and the post-injury microenvironment is vital to developing therapeutic strategies to enhance recovery post-TBI. This is of relevance to approaches targeting both endogenous neurogenesis and exogenous transplantation of stem cells, as both are heavily reliant on a permissive micro-

environment post-injury. Therefore, in this review, we discuss: (i) current perspectives on neurogenesis following TBI; (ii) the role of HMGB1 under physiological conditions and post-injury; and (iii) therapeutic strategies targeting HMGB1 for enhancing recovery post-TBI.

2. Neurogenesis after Traumatic Brain Injury

Neurogenesis is a tightly regulated process, entailing the generation of mature functional neurons from NSPCs (for review, see [17]). Conventionally, neurogenesis was thought to be confined to specific neurogenic niches, namely the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus within the hippocampus (**Figure 1**). Hence, early studies explored the neurogenic response of the hippocampus and SVZ in different animal models of TBI (**Table 1**). In their seminal study, Tzeng & Wu (1999) [18] demonstrated the presence of $BrdU^+$ cells within the SVZ of adult rats subjected to cortical stab injury, which was maximal at 24-48 hours post injury. Whilst there was no evidence of comparison to control, nor co-immunolabelling to identify the cellular subtype of $BrdU^+$ cells, this provided an early indication of the existence of neuro-proliferation post-injury. This was subsequently elaborated upon with a controlled cortical impact (CCI) injury model in adult rats, demonstrating that injured animals had significantly greater numbers of $BrdU^+$ cells in the ipsilateral dentate gyrus when compared with sham animals [19]. This was maximal at 72 hours post injury, and settled to control levels by 2 weeks. Importantly, co-localisation with TOAD-64, an immature neuronal marker, at earlier time points and calbindin, a mature neuronal marker, at later time points verified the neurogenic response to injury. Similar findings were corroborated by later studies. One study demonstrated cell proliferation in the adult rat SVZ following fluid percussion injury (FPI), comprising predominantly of immature astrocytes and microglia/ macrophages at 48 hours post injury, using ^{3}H-thymidine [20]. Another study demonstrated evidence of nestin, an NSPC marker, colocalization with BrdU at 7 days post injury, and calbindin colocalization with $BrdU^+$ cells at 60 days post injury in the granule cell layer of the dentate gyrus in a CCI model in mice; indicating longer term integration of newborn neurons [21]. Another study isolated adult rat SVZ/ hippocampus tissue at different time points following FPI, grew them in tissue culture media for 24 hours, and used flow cytometry to demonstrate a significant increase in cells double-labelled for nestin and ßIII-tubulin at 10 days following FPI [22]. This further

emphasises the potential of newly generated NSPCs to generate neurons post-TBI, when isolated from the post-injury micro-environment.

Whilst these studies demonstrated an early neurogenic response within the SVZ/ hippocampus following TBI, subsequent interest was directed towards cortical neurogenesis considering the large amount of neuronal death in this area. Braun et al. [11] used cortical contusional trauma in adult rats to demonstrate elevated expression of ßIII-tubulin expressing cells around and within the site of cortical injury at 7 days post injury, indicating that neurogenesis is not confined to the SVZ/ hippocampus following injury. Subsequent studies [23] demonstrated that isolating tissue from the site of cortical injury in adult rats could be used to grow neurospheres *in vitro*, which were capable of generating neurons (Tuj1$^+$ cells), astrocytes (GFAP$^+$ cells), and oligodendrocytes (O4$^+$ cells). However, this was only possible when tissue was extracted at 72 hours post injury, but not 24 hours or 7 days, in agreement with hippocampal counterparty studies demonstrating maximal neuro-proliferative potential at this time point [19]. Whether cortical NSPC post-injury arise from the cerebral cortex or migrate from conventional neurogenic niche, or both, remains unclear. An early study administered BrdU intraperitoneally and labelled SVZ cells with fluorescent microspheres in a mouse model of CCI to examine proliferation and migration of NSPC post injury respectively [24]. Following injection of fluorescent microspheres into the lateral ventricle prior to CCI, migrating cells were examined for BrdU and doublecortin colocalization with immunohistochemistry. Interestingly, migrating BrdU$^+$ cells were identified throughout the corpus callosum and subcortical regions at 3 days post injury and a significant number of migrating doublecortin$^+$ cells were identified at the cortical site of injury, compared with no evidence of doublecortin$^+$ cells in the cortex of control animals. This indicates a neuro-proliferative response of NSPCs originating from the SVZ post-TBI, which then migrate to the site of cortical injury. A more recent study, however, demonstrated the activation of quiescent multipotent NSPCs residing within the cortex post-injury, using an *in vitro* organotypic stretch injury model with adult mouse cortico-hippocampal slice cultures [25]. First, by growing neurospheres from mouse cortex and hippocampus at different postnatal days (PD), it was shown that cortical NSPC proliferative capacity was lost by PD15 in contrast to hippocampus. Mouse cortico-hippocampal cultures were generated from PD8 mice, subjected to stretch injury after 4 days in vitro, and

neurospheres generated from cortex and hippocampus separately after 7 days in vitro. Interestingly, following injury, proliferative capacity of cortical NSPC was restored, and neurospheres were capable of differentiating into neurons (ßIII-tubulin$^+$ cells), astrocytes (GFAP$^+$ cells), and oligodendrocytes (O4$^+$ cells). Regardless of the origin of cortical NSPCs following TBI, they are capable of generating neurons when isolated from the post-injury microenvironment and grown *in vitro*, but only during a particular time period following injury.

Despite promising evidence of cortical and hippocampal neurogenesis post-injury, the longer-term survival and functional integration of newly born neurons remained unexplored until recently. Several studies have demonstrated aberrant dendritic branching and migration patterns of newborn neurons within the hippocampus post-injury. Following CCI in a transgenic mouse model with fluorescent labelling of immature hippocampal neurons, altered dendritic morphology was identified in newly generated neurons post-injury [26]. Immunohistochemical analysis of POMC-EGFP mice at 2 weeks post injury demonstrated an increased number of GFP$^+$ cells in the ipsilateral dentate gyrus of injured mice compared to control, indicating post-traumatic hippocampal neurogenesis. Since GFP$^+$ cells could have been generated at any time between 7 and 14 days post injury, the time of generation of immature hippocampal neurons was more closely studied with the administration of BrdU at 2, 5, or 7 days post injury. Scholl analysis of BrdU$^+$/GFP$^+$ cells demonstrated more complex dendritic morphology in newborn neurons when compared to control, with shorter distance from neuronal soma to the first dendritic branch, increased number of branches, and increased dendritic length. In addition, aberrant dendritic morphology persisted as long 4 weeks post-injury, examined with the use of DexCre/TdTom mice. This was corroborated by another study [27], which examined DCX$^+$ cells within the adult mouse hippocampus post-CCI. Physiological patterns of adult hippocampal neurogenesis involve generation of new neurons in the subgranular zone and migration into the inner third of the granule cell layer (GCL) [28]. However, it was demonstrated that CCI resulted in a significantly greater number of newly generated neurons (BrdU$^+$DCX$^+$ cells) migrating beyond the inner third of the GCL into the outer two thirds at one-week post injury, representing an abnormal pattern of development. Whilst these neurons were demonstrated to develop into mature neurons (BrdU$^+$NeuN$^+$ cells) at four weeks post injury, this abnormal migration pattern

persisted. In contrast to previous findings, however, this was associated with significantly reduced dendritic branch numbers and lengths at 5 weeks post injury compared to control. Two further studies examined the localisation of DCX^+ cells within the hippocampus post-FPI in mice [29, 30]. At both 7 and 30 days post injury, there was a significant increase in the number of DCX^+ cells in the hilus of the dentate gyrus when compared to control, indicating abnormal ectopic migration of newly generated granule cells. Essentially, whilst post-traumatic hippocampal neurogenesis takes place, subsequent migration and development of newborn neurons is abnormal. Whilst several studies have examined the ongoing development of neurons generated in the hippocampus post-injury, the fate of newborn neurons generated by post-traumatic cortical neurogenesis remains comparatively less clear. Using a rat CCI model, Yi et al [9] demonstrated that NSPCs peaked at 3 days post injury in agreement with previous studies. However, despite a significant increase in newborn immature (doublecortin/ $BrdU^+$ cells) and mature ($MAP2^+$/ $BrdU^+$ cells) neurons at 7 days, both were undetectable at the site of cortical injury at 28 days. Therefore, whilst neurogenesis is activated post-injury, achieving survival and functional integration of newborn neurons remains enigmatic.

Developing a mechanistic understanding of the relationship between the post-injury microenvironment and its effects on neurogenesis may help provide therapeutic avenues for enhancing functional recovery in TBI patients. The neuro-inflammatory response that characterises the protracted injurious process post-TBI is an active area of ongoing research (for review see Wofford et al. [31]). Briefly, initial traumatic impact preferentially perturbs neuronal membrane integrity and increases neuronal permeability, with subsequent release of inflammatory cytokines and DAMPs [16, 32-34]. This results in microglial activation, increased blood-brain-barrier permeability and peripheral immune cell infiltration, and amplification of the neuro-inflammatory response [32, 35]. The resultant micro-environment, demonstrated by chronic microglial activation at time points far beyond the initial injury, is unlikely to support endogenous neurogenesis [36]. The concept of a detrimental effect of neuro-inflammation on neurogenesis has been widely studied across other neurological diseases such as stroke, epilepsy, Alzheimer's disease, and schizophrenia [37][38][39][40]. However, this relationship remains relatively unexplored in the context of post-traumatic neurogenesis. Manipulating the post-injury microenvironment to support

neurogenesis should, therefore, target factors involved in both post-traumatic neuro-inflammation and neurogenesis; of which HMGB1 is a prime example.

3. HMGB1- under physiological conditions and post-TBI

High Mobility Group Box protein 1 (HMGB1) is a 215 amino acid protein with pleiotropic functions determined by (i) intra- or extra-nuclear localisation, (ii) target receptor interactions (Receptor for Advanced Glycation End-Products (RAGE), and Toll-Like Receptor 4 (TLR4)) [41, 42], (iii) concentration, and (iv) its molecular isoform (**Figures 2-3**). It acts as a non-histone DNA binding protein to modulate gene expression and DNA repair under physiological conditions with a key role in neural development, but is released passively by necrotic neurons or secreted actively by immune cells as an alarmin under pathological insult [43]. Two DNA binding domains, known as Box A and Box B, and an acidic C-terminal tail constitute the basic structure of HMGB1 [44]. This reflects its physiological role in co-ordination of gene transcription in its natural physiological milieu within the nucleus.

The dynamics of HMGB1 release following injury has been well characterised by a study examining gene expression, protein levels, and cytoplasmic translocation of HMGB1 in neuronal cultures generated from embryonic mouse cortex [45]. Neuronal cultures were exposed to various chemical stimuli simulating chemical ischaemia, oxidative stress, and excitotoxicity, and demonstrated HMGB1 cytoplasmic translocation at 1 hour and increased protein levels at 4 hours following injury. Apoptotic stimuli could not reproduce this effect, highlighting that acute HMGB1 release following injury occurs as a result of cellular necrosis. Exposing mixed glial cultures to HMGB1 resulted in upregulation of pro-inflammatory gene expression including NOS2, COX2, and IL-1ß. Interestingly, HMGB1, at a concentration of 10µg/mL, did not significantly increase cell death in isolation, but significantly enhanced cell death in the presence of necrotic stimuli. Another study that examined differences between microglia derived from wild type and TLR4 mutant mice had similar findings [46]. Addition of HMGB1 neutralising antibodies to microglial cultures exposed to condition media derived from NMDA-treated neuronal cultures reduced microglial IL-6 production, but not when treated with control condition media. However, this effect was not found in microglial

cultures lacking functional TLR4 receptors, indicating an HMGB1-TLR4 dependent mechanism for microglial activation. This emphasises the importance of understanding the nature of injury and exploring the interaction of HMGB1 with other cytokines to refine therapeutic approaches.

Despite the relatively recent interest in the role of HMGB1 in inflammation, its role in promoting neurite outgrowth, NSPC migration, and development have also been elucidated. Early evidence demonstrated strong expression of HMGB1 amongst proliferating and migrating cortical and cerebellar granule neurons in the developing rat brain [47, 48], with subsequent decline once target locations were reached. This is in keeping with findings that HMGB1 is abundantly expressed throughout the developing mouse brain, but only found in areas of active neurogenesis at later stages [49]. The role of HMGB1 in neural development was also illustrated *in vivo* using selective manipulation of HMGB1 expression in a zebrafish model [50]. A significant reduction in NSPC survival, proliferation, brain development, and neural network formation were observed when HMGB1 gene expression was suppressed.

3.1 HMGB1 Target Receptors

Established target receptors for HMGB1 include RAGE and TLR4, with MyD88-dependent downstream signalling resulting in NFkB activation, nuclear translocation, and upregulation of target gene transcription (**Figure 4**) [51]. HMGB1-RAGE interaction has been widely studied, and involves RAGE-mediated HMGB1 endocytosis followed by direct NFkB activation [52], whilst HMGB1 interacts with MD-2 to trigger TLR-4 homodimerisation and downstream signalling [42]. Initially, RAGE was postulated to be the predominant receptor in neural tissue, and TLR-4 in immune cells [53]. Indeed, several studies characterised involvement of the HMGB1-RAGE axis in neurite outgrowth and neural migration [54-56], and TLR-4 in immune cell and microglial activation [53]. However, more recent studies have demonstrated overlapping roles of RAGE and TLR4 in glial activation and neurotoxicity. One study demonstrated that HMGB1 interaction with RAGE expressed by glial cells plays a pivotal role in mediating ischaemic brain damage using mixed glial cultures generated from neonatal mice [57]. Interestingly, the toxic effects of HMGB1 were only seen with treatment of mixed glial cultures, but not primary neuronal, pure microglial, or pure astrocytic

cultures. RAGE expression was demonstrated across neurons, astrocytes, and microglia. However, evidence that this effect was mediated by microglial RAGE expression was demonstrated by reduced cell death when neurons from wild type mice were co-cultured with microglia from RAGE knockout mice. A recent *in vivo* study examined the effects of intraventricular HMGB1 administration in mice and demonstrated disruption of memory encoding via both TLR4 and RAGE [58]. An *in vitro* study using coriaria lactone-induced activation of human microglial cultures as an epilepsy model demonstrated upregulation of HMGB1 along with both TLR4 and RAGE receptors and downstream NFkB activation, implying a role for both receptors [59]. Therefore, further studies are required to elucidate the specific roles of RAGE and TLR4 in neuro-inflammation and neurogenesis following TBI.

The role of HMGB1-RAGE interaction in neurogenesis has been well characterised in several studies. One study demonstrated that blocking RAGE receptors in postnatal mouse cerebellar slice cultures reduced both neurite outgrowth and migration of granule neurons [54]. Another *in vitro* study using N18 mouse neuroblastoma cell cultures demonstrated that HMGB1 and S100B proteins acted together to improve neuronal survival and neurite outgrowth via interaction with RAGE [55]. Importantly, it was demonstrated that S100B was capable of neurotrophic effects at lower concentrations but neurotoxic effects at higher concentrations, both via interaction with RAGE. More recently, it was demonstrated that HMGB1, at a concentration of 1ng/mL, facilitated migration of NSPCs via interaction with RAGE in cultures generated from foetal mouse cortical tissue [56]. This illustrates that the complex regulation of neuronal survival and proliferation depends on the concerted actions of multiple cytokines within particular concentration ranges (for review see Borsini et al. [60]). Particularly in the context of the heterogeneous inflammatory cytokine cascade that characterises TBI, close attention is required to ensure that potentially beneficial effects are not negated through treatment.

Although the involvement of RAGE in mediating neurogenesis post-TBI has not been specifically addressed, studies in the context of stroke and Alzheimer's disease (AD) indicate a beneficial role. A recent study, using an *in vivo* model of ischaemic stroke in mice, implicated HMGB1 in mediating the pro-neurogenic effects of exposing injured mice to an enriched environment **[61]**. When glycyrrhizin, a RAGE antagonist, was administered at one

week post injury, for a duration of two weeks, immunohistochemical studies indicated amelioration of the positive effects of enriched environment on NSPC proliferation in the SVZ. This was corroborated by poorer performance of treated injured mice on motor tasks at both 3- and 4-weeks post injury. This was consistent with *in vitro* experiments using condition media collected from primary astrocyte cultures exposed to pro-inflammatory stimuli. Primary astrocyte cultures were grown from mice at two weeks post injury, stimulated to release HMGB1 with the addition of LPS and VEGF, and condition media collected. Use of this condition media to grow neurospheres resulted in a significant increase in the number of neurospheres and percentage of $Tuj1^+$ cells, and this effect was reversed with the use of glycyrrhizin. Similar findings were demonstrated in an *in vivo* model of intracerebral haemorrhage (ICH) in rats [62]. Evidence of cortical neurogenesis was demonstrated by the presence of $BrdU^+DCX^+$ cells in the peri-haematoma region at 10- and 14-days post injury, but this was significantly reduced with administration of ethyl pyruvate at one-week post injury. This was also reflected in poorer performance on neurological assessment in the treatment group at 10- and 14-days post injury. A later study from the same group evaluated the same injury model, and observed increased numbers of $BrdU^+DCX^+$ cells in the ipsilateral striatum at 14 days post injury [63]. This effect was abolished with administration of ethyl pyruvate, which was commenced even earlier, at 3 days post injury. Interestingly, ethyl pyruvate resulted in a significant reduction in RAGE protein expression, but not TLR-4, indicating a role for the HMGB1-RAGE axis in neurogenesis post-ICH. Therefore, whilst HMGB1 is involved in the acute neuro-inflammatory response post-injury, there is evidence for a role in functional recovery at later time points. The role of the HMGB1-RAGE axis in neurogenesis has also been explored in the context of Alzheimer's disease (AD), which is known to share its clinical phenotype with the longer-term manifestations of TBI [64]. One study demonstrated a concentration-dependent, pro-neurogenic effect of HMGB1 on rat hippocampal NSPC cultures, with increased numbers of $MAP2^+$/$nestin^+$ and $MAP2^+$/$nestin^-$ cells in the presence of HMGB1 [65]. Reversal of this effect with the use of anti-RAGE antibodies and inhibitors of nuclear NFkB translocation indicated that this was dependent on the HMGB1/RAGE/NFkB signalling pathway. Interestingly, the RAGE/NFkB axis was also implicated in mediating the enhanced neurogenic potential of hippocampal NSPCs derived from TgCRND8 mice, a transgenic mouse model of AD. A more recent study examined the hypothesis that HMGB1 is

implicated in enhanced hippocampal neurogenesis in AD by evaluating outcomes following intraventricular administration of HMGB1 in 3xTg-AD mice [66]. This resulted in: (i) increased numbers of DCX^+ cells in the dentate gyrus of treated mice, (ii) increased levels of dendritic and synaptic markers, and (iii) improved performance on spatial memory tasks. Essentially, there is evidence for the HMGB1-RAGE axis in mediating neurogenesis in the contexts of both stroke and AD.

Whilst several studies demonstrate a role for the HMGB1-RAGE axis in both neural development and neurogenesis post-injury, mounting evidence also implicates TLR-4 involvement in neurogenesis post-injury, though TLR receptors are conventionally associated with the innate immune response [67]. Indeed, involvement of TLR-4 receptors in hippocampal neurogenesis has been previously elucidated using wild type and TLR-4 deficient mice [68]. Identification of an increased number of $BrdU^+$ cells at 1- and 7-days post-BrdU administration in the dentate gyrus of TLR-4 deficient mice when compared with wild type mice indicates an inhibitory role of TLR-4 on NSPC proliferation. Also, the majority of $BrdU^+$ cells were constituted by DCX^+ and $ßIII\text{-}tubulin^+$ cells, indicating an inhibitory effect on both NSPC proliferation and differentiation into the neuronal lineage. This was affirmed *in vitro*, as addition of siRNA for TLR-4 resulted in significantly greater neurosphere formation with larger numbers of $ßIII\text{-}tubulin^+$ cells grown from NSPCs derived from wild type mouse hippocampus. However, at 28 days post BrdU injection, there was no significant difference in the number of proliferating cells between wild type and TLR-4 deficient mice, indicating that other signalling pathways may be responsible for survival of newly generated neurons. Similar findings have been affirmed in the hippocampus of adult $TLR\text{-}4^{-/-}$ mice, with enhanced NSPC proliferation, indicated by Ki67 labelling, and neuronal differentiation, demonstrated by number of $NeuN^+BrdU^+$ cells, when compared to wild type mice [69]. An inhibitory role of TLR-4 was also verified in neurospheres grown from NSPCs derived from the human fetal brain, demonstrating reduced NSPC proliferation in the presence of TLR-4 antagonists [70]. Essentially, under physiological conditions, studies from multiple species implicate TLR-4 as a negative modulator of neurogenesis.

Several studies have also examined the role of TLR-4 in post-injury neurogenesis, in the context of both ischaemic stroke and TBI. One study examined cell proliferation within the hippocampus of mice post-CCI, and demonstrated enhanced co-expression of $BrdU^+TLR4^+$ cells at multiple time points post-injury, peaking at 3 days and then decreasing at subsequent time points [71]. Similar findings have been demonstrated using an *in vivo* rat model of ischaemic stroke, with increased number of $BrdU^+$ cells in $TLR4^{-/-}$ mice at 7 days post injury when comparing mice with similar infarct sizes [72]. However, wild type mice demonstrated quicker migration of neuroblast to the region of ischaemic insult than $TLR4^{-/-}$ mice, with a greater density of DCX^+ cells in the area of damage at 7 days post injury. Also, at later time points of 14 and 28 days post injury, there were a greater number of BrdU+/NeuN+ cells in wild type mice compared with TLR4-/- mice, indicating that survival and integration of newborn neurons remains greater in the area of damage despite greater SVZ proliferation in TLR4-/- mice. A recent study explored the role of TLR-4 receptors in neurogenesis following stroke, using an *in vivo* model of ischaemic stroke in wild type and $TLR\text{-}4^{-/-}$ mice [73]. Under normal physiological conditions, $TLR\text{-}4^{-/-}$ mice demonstrated a greater number of proliferating cells within the SVZ, indicated by Ki67 immunopositivity, when compared with wild type mice. The converse was true, however, following injury, with a greater number of proliferating cells at one day post injury in wild type mice compared to $TLR\text{-}4^{-/-}$ mice. This was in keeping with *in vitro* experiments using tissue derived from SVZ of wild type and $TLR\text{-}4^{-/-}$ mice, which demonstrated larger diameter neurospheres and greater NSPC migration in the presence of TLR-4 agonists such as LPS or HMGB1. This effect was abolished with the use of TLR-4 antagonists and with cultures derived from $TLR\text{-}4^{-/-}$ mice. As well as emphasising a beneficial role for TLR-4 in mediating neurogenesis post-injury, this demonstrates the importance of injury as a determinant of receptor function. This is supported by a study of TLR-4 involvement in neurological recovery post-ICH in rats [74], which demonstrated increased TLR-4 protein expression for up to 14 days post injury. Importantly, administration of a TLR-4 antagonist, TAK-242, at 3 days post injury reduced ICH-induced enhancement of $BrdU^+DCX^+$ cells within the striatum and poorer performance on neuro-behavioural assessment at 14 days post injury.

The role of TLR-4 in neurogenesis post-TBI has mainly been studied as part of the mechanism of action of various therapeutic agents. A recent study examined the effects of

microRNA-124 enriched exosomes (exo-miR-124) on microglial phenotype and hippocampal neurogenesis in a CCI model in rats [75]. Intravenous administration of exo-miR-124 at 24 hours post-injury was associated with enhanced hippocampal neurogenesis and improved performance on motor tasks at up to 28 days post injury. This effect was attributed to the TLR-4 dependent modulation of microglia to adopt an anti-inflammatory phenotype, often dubbed as M2 polarisation. This was demonstrated by: (i) enhanced gene expression of M2 markers including CD206, Arginase-1, IL-4, IL-10, and TGF-ß in injured mice treated with exo-miR-124; and (ii) reduced gene expression of TLR-4 and downstream signalling elements such as MyD88, IRAK1, TRAF6, and NFKb p65. The likely hypothesis that exo-miR-124 administration resulted in TLR-4 dependent M2 polarization was affirmed on further *in vitro* experiments that examined the effects of exo-miR-124 on gene and protein expression of M2 markers and TLR-4 signalling using BV2 microglial cultures. Whilst a focus on HMGB1 receptor expression on NSPC is important for understanding post-injury neurogenesis, this study highlights the importance of also understanding the complex interplay with other cell subtypes. This is in keeping with a PET imaging study that evaluated SVZ neurogenesis and activated TSPO+/ CD11b+ microglia in TLR-$4^{-/-}$ mice post-cerebral ischaemia [76]. Enhanced SVZ neurogenesis was demonstrated in TLR-$4^{-/-}$ mice using [18F]FLT-PET imaging at 2 days post injury, and a combination of [11C]PK11195-PET and immunohistochemical studies demonstrated reduced presence of $TSPO^{+}$/ $CD11b^{+}$ microglial cells at 2 days but not 7 and 14 days post-injury. Taken together, this suggests that microglial TLR-4 activation may negatively modulate neurogenesis post-injury. Another study examined the effect of TLR-4 signalling on hippocampal neurogenesis after electroacupuncture treatment post-CCI in mice [77]. Treatment resulted in significant increases in the number of BrdU+/NeuN+ cells in the dentate gyrus, reduced TLR-4 protein expression, and improved performance on motor tasks at up to 35 days post injury. Given that the beneficial effects of treatment were reversed with the administration of LPS, a TLR-4 agonist, this indicates that TLR-4 expression is correlated with reduced hippocampal NSPC proliferation in the context of electroacupuncture treatment post-TBI.

3.2 HMGB1 Isoforms

The modifiable redox status of cysteine and acetylation status of lysine residues within the HMGB1 peptide determine its function and localisation, respectively [78, 79]. Redox isoforms are determined by the formation of disulphide bonds between three key cysteine residues: Cys23 and Cys45 located in Box A and Cys106 in Box B. Three isoforms of HMGB1 emerge from the redox status of these cysteine residues [80]: fr-HMGB1 (fully reduced/thiol HMGB1), ds-HMGB1 (disulphide HMGB1), and ox-HMGB1 (sulphonyl/oxidised HMGB1), in order of increasing oxidation. Each is associated with different functions and reflect different underlying cellular processes. This is illustrated by studies demonstrating: (i) fr-HMGB1 is the main isoform released following necrosis, which forms complexes with CXCL12 and acts on CXCR4 receptors to recruit immune cells to the site of release [81], (ii) ds-HMGB1 is a pro-inflammatory cytokine involved in immune cell activation that interacts with TLR-4 receptors [82], and (iii) ox-HMGB1 demonstrates neither pro-inflammatory nor chemokine activity [83]. Acetylation of lysine residues in the nuclear localisation sequence (NLS) of HMGB1 triggers cytoplasmic translocation, which is the first step towards extracellular secretion and subsequent inflammation. Thus, acetylation can distinguish between the two pathways of HMGB1 release: passive release during necrosis and active secretion, the latter necessitating cytoplasmic translocation [79]. This is a molecular representation of two fundamental roles of HMGB1, as a damage associated molecular pattern (DAMP) to reflect cell death and an alarmin to represent cellular stress [13].

Despite recognition of the importance of HMGB1 redox isoforms and acetylation status in other clinical diseases, there are few studies exploring their role in neurological disease. One study evaluated the effects of different HMGB1 redox isoforms on neuro-inflammation [84] Two different HMGB1 isoforms, ds- and fr-HMGB1, were administered directly to the cisterna magna of adult rats, and the neuro-inflammatory response of the hippocampus was evaluated. Ds-HMGB1, but not fr-HMGB1, stimulated significant upregulation of pro-inflammatory cytokines' gene expressions, including TNF-a, IL-1ß, and IL-6, in the hippocampus at 2 hours and 24 hours post-injection. Furthermore, ds-HMGB1 potentiated the hippocampal inflammatory response to intraperitoneal administration of LPS at 24 hours later. This translated into altered behavioural performance, with reduced social exploration in mice treated with both LPS and ds-HMGB1 compared with ds-HMGB1 alone.

The effects on microglia isolated from whole brains were also explored, and ds-HMGB1 alone did not result in significant upregulation of pro-inflammatory cytokines including TNF-a, IL-1ß, and IL-6. However, subsequent addition of LPS revealed that ds-HMGB1 'primed' microglia and resulted in amplified upregulation of pro-inflammatory gene expression compared to LPS alone. Another study examined differences in the neuro-inflammatory response and blood brain barrier integrity following intracerebral injection of ds- or fr-HMGB1 in adult rats [85]. Both ds- and fr-HMGB1 demonstrated increased blood brain barrier permeability on MRI imaging and immunofluorescence studies, and resulted in increased numbers of IL-1 ß expressing cells. Whilst LPS administration resulted in a significant increase in the numbers of $CD68^{+}$ microglia, this effect was not seen with either HMGB1 isoforms. However, a differential effect of isoforms was observed with increased apoptosis on TUNEL assay with ds-HMGB1 but not fr-HMGB1 or LPS. The effects of HMGB1 redox isoforms on neuro-inflammation and depressive behaviour in adult mice has also been explored [86]. In keeping with previous findings, both ds- and fr-HMGB1 isoforms resulted in significant upregulation of hippocampal TNF-a, but to a greater extent with ds-HMGB1. However, a receptor specific mechanism was not identified, given that upregulation was inhibited with the use of both TLR-4 and RAGE antagonists. Whilst a differential effect of different HMGB1 redox isoforms on apoptosis and neuro-inflammation have been explored, specific receptor interactions and effects on neurogenesis under both physiological and pathological conditions remain to be elucidated. In particular, given evidence that HMGB1 redox status determines affinity to target receptors [87], and the differential roles of TLR-4 and RAGE in mediating post-injury neurogenesis, further evaluation is vital. In sum, HMGB1 is a pivotal inflammatory mediator released acutely following various forms of injury but also plays a key role in mediating neurogenesis in the developing brain. Given that cortical neurogenesis occurs following TBI, HMGB1 may provide an ideal therapeutic target for modulating neurogenesis post-injury. Several factors including type of injury, concentration of HMGB1, HMGB1 redox status, time since injury, interaction with other cytokines, and interplay between different cell types must be taken into consideration.

4. HMGB1 as a therapeutic target in TBI

Although the role of HMGB1 in neurogenesis following TBI remains to be fully elucidated, *in vivo* studies of HMGB1 antagonism in animal models of TBI may provide valuable insight (**Table 2**). Several studies over the past decade have focused on various therapeutic strategies that involve direct or indirect modulation of HMGB1 signalling to ameliorate TBI-related deficits. These studies can be broadly divided into approaches that specifically target HMGB1, and others that demonstrate an association between beneficial therapeutic agents and suppression of HMGB1 signalling. Key examples of therapeutic agents targeting HMGB1 include glycyrrhizin, Box A, ethyl pyruvate, and monoclonal HMGB1 antibodies (anti-HMGB1 mAb), which exert their effects at different stages of HMGB1 signalling. Broad themes emerging from studies of HMGB1 antagonism include reduction of cerebral oedema, suppression of pro-inflammatory cytokine release and microglial activation, reduced expression of HMGB1 target receptors, and improved neurological outcomes (**Figure 5**).

These findings are demonstrated with the use of non-specific competitive HMGB1 antagonists, such as Box A [88] and anti-HMGB1 mAb [89, 90]. Box A administration, following CCI in adult mice, resulted in improved neurological outcomes at up to 7 days post injury, reduced cerebral oedema, and enhanced integrity of the blood brain barrier [88]. Also, reduced HMGB1, TLR4, MyD88, and p65 protein expression on exposure of IL-1ß treated primary astrocyte cultures to Box A indicated a role of the HMGB1-TLR4 axis in mediating the inflammatory response of astrocytes. Later time points of up to 2 weeks post injury were evaluated with use of anti-HMGB1 mAb after FPI in adult rats [89]. Improved performance on motor and spatial memory tasks, reduced numbers of activated microglia within the cortex, and reduced neuronal apoptosis were identified. Comparatively reduced serum HMGB1 levels in the treatment group reflected the likely disruption of the positive feedback cycle triggered by HMGB1 release post-injury. In addition, the superior motor function and reduced cerebral oedema seen in the treatment group at 24 hours post injury was abolished in $RAGE^{-/-}$ mice but not $TLR4^{-/-}$ mice, indicating a pivotal role for the HMGB1-RAGE axis in TBI pathogenesis [90]. Similarly, ethyl pyruvate, which prevents HMGB1 release [91], produced beneficial effects in rats subjected to weight drop injury in two studies [92, 93]. Improved motor function, reduced gene and protein expression of HMGB1 and TLR4,

reduced pro-inflammatory cytokine (IL-1ß, TNF-a, IL-6) release, and fewer apoptotic cells were identified at 24 hours post-injury.

Three studies examined the use of glycyrrhizin in different models of TBI in adult rats, and demonstrated consistently beneficial effects on histological and neurological outcomes [94-96]. This included weight-drop injury [94], FPI [95], and a lateral head rotation model simulating diffuse axonal injury [96]. In the acute phase at 6 hours post-injury, pro-inflammatory cytokine gene upregulation (TNF-a, NOS-2, IL-1ß, and IL-6) was suppressed in treatment groups [95]. By 24 hours post injury, reduction in neuronal apoptosis [96] and reduced gene and protein expressions of HMGB1, TLR4, and RAGE were observed [94]. This translated into improved motor performance, on Rotarod testing, and spatial memory function, on Morris water maze testing, at up to 7 days post injury [95]. Surface plasmon resonance studies indicate that the mechanism of action of glycyrrhizin involves interruption of HMGB1-RAGE interaction, and the beneficial effects of treatment were not seen in $RAGE^{-/-}$ mice [95]. However, timing of glycyrrhizin administration may not be applicable to the clinical setting, as this ranged from 30 minutes pre-injury [96] to 30 minutes post-injury [94].

Other studies have demonstrated an association between disruption of HMGB1 signalling and improved neurological outcomes with the use of various therapeutic agents in *in vivo* animal models of TBI. A recent study examined the effects of omega-3 polyunsaturated fatty acid (ω-3 PUFA) administration on neurological and histological outcomes following weight drop injury in rats [97]. In the ω-3 PUFA treated group, where administration was performed 30 minutes following injury, findings included: (i) improved neurological outcomes at one week post injury, as measured by the modified neurological severity score; (ii) reduced cerebral oedema at 3 days post injury; (iii) reduced neuronal apoptosis at 3 days post injury; and (iv) reduced microglial activation at 3 days post injury. Immunohistochemistry demonstrated significantly higher HMGB1 co-localisation in neurons and microglia at 3 days post injury, but treatment with ω-3 PUFA resulted in reduction in HMGB1 cytoplasmic translocation and protein levels. Reduced protein levels of TLR4 and downstream signalling elements including NFkB, p65, and p-IkB in the treatment group implied that ω-3 PUFA may exert its effects via modulation of the HMGB1-TLR4 axis in microglia. However, findings

from a recent study examining the effects of inducible HMGB1 knockout in mice subjected to CCI demonstrate the complex relationship between HMGB1 antagonism and functional outcomes post-TBI [98]. Other than reduced contusion volume in HMGB1 knockout mice, no significant differences were identified in extent of cerebral oedema, motor or spatial memory function, or hippocampal neuron survival. Importantly, these findings were assessed up to 21 days following injury; evaluating a longer timescale than previous studies of HMGB1 as a therapeutic target after TBI. This highlights the important concept that sustained suppression of neuro-inflammatory signalling is not beneficial in the longer term.

In sum, despite several studies indicating the effects of HMGB1 blockade on dampening neuro-inflammation post-TBI, the therapeutic effects of HMGB1 antagonism on neurogenesis post-TBI remains to elucidated. Based on current evidence, it is unclear whether the potentially beneficial effects of HMGB1 antagonism are a result of neuroprotective mechanisms alone, and whether there are any unintended effects on post-injury neurogenesis. Given ample evidence of the involvement of HMGB1 and its target receptors in both physiological and post-injury neurogenesis in other disease contexts, this is an important field for future research in therapeutic approaches for TBI.

5. Conclusions & Future Directions

HMGB1 is a promising therapeutic target due to its involvement in both the neuro-inflammatory cascade post-TBI and in regulating neurogenesis. Whilst animal studies indicate that interruption of HMGB1 signalling acutely post-TBI may improve outcomes, sustained suppression is of little benefit. In order to develop effective therapeutic strategies, a fundamental understanding of HMGB1, neuro-inflammation, and neurogenesis is essential to achieve longer term functional recovery. The following aspects will require clarification in order to avoid the unintended negation of beneficial effects of HMGB1: (i) optimal timing for HMGB1 inhibition, which must also address feasibility within the clinical setting, and avoid administration of therapeutic agents before or soon after injury in *in vivo* animal models; (ii) concentration ranges that HMGB1 confers a pro-neurogenic effect versus pro-inflammatory effects; (iii) target receptors and downstream signalling pathways

responsible for mediating the different effects of HMGB1; (iv) responses of different cell subtypes to HMGB1 signalling; (v) role of HMGB1 in the aberrant neurogenesis seen post-TBI; (vi) effects of HMGB1 heterocomplexes with other cytokines released post-TBI on neurogenesis; and (vii) the possibility of differential effects of HMGB1 isoforms. Hence, with an integrated mechanistic understanding of HMGB1 signalling and neurogenesis in the post-injury microenvironment, targeted pharmacological strategies can be developed that avoid the pitfalls of previous attempts to enhance neurological recovery following TBI.

Figure Legends

Figure 1- Schematic diagram demonstrating timelines of post-traumatic neurogenesis in the cortex (highlighted in red box), subventricular zone (highlighted in red), and dentate gyrus (highlighted in red box) following animal models of traumatic brain injury (TBI) (see **Table 1** for further details).

Figure 2- Schematic diagram depicting molecular structure of HMGB1 and functions associated with different domains. (Adapted from He et al. [87]).

Figure 3- Summary of cellular sources of HMGB1, isoforms (based on acetylation status or redox status), pro-inflammatory activity, target receptors, and downstream signalling. Acetylation status of HMGB1 indicates nature of cellular source- acetylation represents active release whilst non-acetylated HMGB1 is released passively from necrotic cells. Redox status determines pro-inflammatory effects, though the role of oxidised HMGB1 remains relatively unexplored in the context of neurological injury. (Adapted from Crews & Vetreno [99]).

Figure 4- Schematic of HMGB1 signalling pathways and mechanism of action of therapeutic agents. Downstream signalling elements of RAGE and TLR4 converge on nuclear translocation of NFkB and pleiotropic effects dependent on context and cellular micro-environment. This ranges from mediating neurogenesis during development to amplifying the pro-inflammatory response post-injury. HMGB1 antagonists include Box A, glycyrrhizin, and ethyl pyruvate, and each function via distinct mechanisms. Box A is a non-specific competitive HMGB1 antagonist, glycyrrhizin is a RAGE antagonist, and ethyl pyruvate inhibits nuclear-cytoplasmic translocation of HMGB1.

Figure 5- Interplay between neurons, microglia, astrocytes, and HMGB1 signalling in post-traumatic cortical neurogenesis. HMGB1 is released passively by necrotic neurons following injury, and results in an activated microglia phenotype and facilitates increased blood brain barrier permeability via increased AQP4 expression in astrocytes. Amplification of the pro-inflammatory response results in further HMGB1 release and likely inhibition of

neurogenesis post-injury, though the exact mechanisms underlying this effect remains unclear.

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Table 1- Summary of studies demonstrating evidence of neurogenesis after TBI

Study	Species	Model	Brain region	Relevant finding (s)
Tzeng and Wu, 1999 [18]	Adult male Sprague-Dawley rats	Cortical stab injury	Subventricular zone/ ventricular zone	• Increased number of $BrdU^+$ cells within the SVZ, maximal at 24-48 hours post injury in both hemispheres (no control for comparison)
Dash et al., 2001 [19]	Male Long Evans rats	Controlled cortical impact (CCI)	Hippocampus (DG)	• Greater number of $BrdU^+$ cells within ipsilateral DG post-injury compared to control- maximal at 72 hours and settled to control levels by 2 weeks • Co-localisation of $BrdU^+$ cells with immature neuronal marker (TOAD-64) at earlier time points and mature neuronal marker (calbindin) at later time points
Chirumamilla et al., 2002 [20]	Adult male Sprague-Dawley rats	Lateral fluid percussion injury (FPI)	Subventricular zone	• Greater number of $BrdU^+$ cells at 48 hours post-injury in SVZ of injured rats compared to control • Majority of proliferating cells (^{3}H-thymidine labelled) were comprised of immature astrocytes/ microglia, with no significant increase in proliferating cells expressing mature neuronal markers (NeuN) or NSPC markers (nestin)
Kernie et al., 2001 [21]	8-week-old male 129/Sv mice	CCI	Hippocampus (DG)	• Greater number of $BrdU^+$ cells in DG at up to 60 days post-injury compared to control • Co-localisation of $BrdU^+$ cells with nestin at 7 days and calbindin at 60 days, demonstrating neurogenesis and integration of granule neurons into DG
Rice et al., 2003 [22]	Adult Sprague-Dawley rats	Lateral FPI	Hippocampus (SGZ)/ Subventricular zone	• Greater number of $BrdU^+$ cells at multiple time points between 2 and 15 days post-injury in both ipsi- and contralateral SGZ/ SVZ • Significantly greater number of nestin and ß*III*-tubulin double labelled cells on flow cytometry analysis of injured hippocampus compared with control
Braun et al., 2002 [11]	8-week-old male Wistar rats	Cortical contusion	Cortex and hippocampus (DG)	• Partial recovery of cortical and hippocampal tissue shown by Nissl staining when comparing 1 and 7 days post injury • Greater number of ß*III*-tubulin-positive neurons in the ipsilateral DG compared to control at 3 and 7 days post injury

				• High density of ß*III*-tubulin expression in/ around site of cortical lesion at 7 days post injury
Itoh et al., 2005 [23]	10-weeks-old Wistar rats	Pneumatic control injury	Cortex	• Nestin^{+} cells were observed around the damaged area at 24 hours, 72 hours and 7 days after TBI compared to no nestin-positive cells in control rats, and maximal at 72 hours • Peri-lesional tissue extracted at 72 hours could be used to generate neurospheres *in vitro*, capable of differentiation into neurons/ microglia/ oligodendrocytes
Ramaswamy et al., 2005 [24]	Adult (8 - 12 weeks old) CD1 mice	CCI	Cortex/ Subventricular zone	• Significant number of doublecortin^{+} cells at site of cortical injury, compared with no positive cells in control at 3 days post injury • Migrating BrdU^{+} cells throughout corpus callosum and subcortical regions at 3 days post-injury • Greater number of BrdU^{+} cells in the ipsilateral SVZ compared to control at 3 days post injury
Ahmed et al., 2012 [25]	Postnatal day 8 C57BL/6 or GFAP-GFP mice	Stretch injury	Cortex	• Neurospheres generated from cortical tissue 7 days post stretch injury of cortico-hippocampal slice cultures • Neurospheres capable of neurons, astrocytes, and oligodendrocytes
Yi et al., 2013 [9]	Adult Sprague-Dawley rats	CCI	Cortex	• Significant increase in newborn immature (doublecortin/ BrdU^{+} cells) and mature (MAP2^{+}/ BrdU^{+} cells) neurons at 7 days, but undetectable at 28 days post injury • No evidence of NeuN^{+}/BrdU^{+} at any time points

Table 2- Summary of studies demonstrating effects of HMGB1 antagonism post-TBI in animal models.

Study	Model	Therapeutic Agent	Findings	Referen
Yang L et al, 2018	CCI in adult male C57BL/6 mice	HMGB1 A-box fragment administered intravenously at 2 mg/kg/day for 3 days post-CCI	• Significantly reduced Evans blue release in the injured hemisphere at 24 and 72 hours after controlled cortical impact, indicating protection of blood brain barrier • Reduced brain inflammatory cytokines (IL-1B, IL-6 and TNF-a) at 24 hours after TBI. Cytokine production was evaluated through western blot and ELISA assay. • HMGB1 A-box improved wire grip scores one, two and three days after CCI, even to similar level as sham-injured mice at seven days post-surgery.	**[88**
Okuma et al, 2019	FPI in adult male Wistar rats	Anti-HMGB1 mAb (IgG2a subclass 1mg/kg) administered intravenously on adult male rats at 5 minutes and 6 hours after injury. The same mAb dose was then administered daily.	• Administering anti-HMGB1 mAb inhibited the loss of HMGB1 in neurons and inhibited neuronal death in the hippocampus two weeks after injury. • Anti-HMGB1 mAb reduced aggregation of activated microglia in the rat cortex in the ipsilateral hemisphere after TBI as evaluated two weeks after injury. • Beneficial effects of anti-HMGB1 mAb on motor and cognitive function persisted for two weeks after injury.	**[89**
Okuma et al, 2012	FPI in adult male C57BL/6 rats	Anti- HMGB1 mAb IgG2a subclass 1mg/kg administered intravenously and 5 minutes and 6 hours after injury.	• Administration of Anti-HMGB1 mAb significantly inhibited cytoplasmic translocation of HMGB1 in neurons up to 24 hours after injury. • Anti-HMGB1 mAb protected the integrity of BBB 6-9 hours after injury and suppressed inflammatory molecule expression for up to 24 hours after injury. • Experiments using $RAGE^{-/-}$, $TLR4^{-/-}$, and $TLR2^{-/-}$ mice indicate that RAGE is the responsible receptor for above findings	**[90**
Su et al, 2011	Weight-drop injury in adult male Sprague-Dawley rats	Ethyl pyruvate administered intraperitoneally (75 mg/kg) at 5 min, 1 and 6 h after TBI.	• Significantly improved beam walking balance performance of rats at 24 hours injury • Levels of HMGB1 and TLR4 mRNA in the injured cortex of treated group were significantly decreased compared to control 24 hours after injury. • Following ethyl pyruvate treatment, NF-κB binding activity was significantly downregulated in brain tissue around the injured cortex compared to control.	**[92**
Evran et al, 2020	Weight-drop injury in adult Sprague-Dawley male rats	Ethyl pyruvate (75 mg/kg) administered intra-peritoneally at 30 and 90 minutes and 6 hours post TBI.	• HMGB1 protein released from post-traumatic necrotic cells conributes to cerebral oedema by increasing expression of TLR4 and RAGE and decreasing occludin, claudin-5 and ZO-1 protein levels (BBB associated proteins) • Brain oedema reduced significantly with ethyl pyruvate administration compared to control 24 hours after injury.	**[93**
Gu et al, 2014	Weight-drop Feeney method in adult male Sprague-Dawley rats	Glycyrrhizin was a dministered intravenously at a dosage of 10 mg/kg 30 min after TBI	• Improved beam walking performance, reduced brain oedema, reduced cell apoptosis, suppressed HMGB1 translocation, inhibited mRNA and decreased inflammatory cytokines (TNF-a, IL-6 and IL-B) expression 24 hours after TBI. • Glycyrrhizin can reduce secondary brain injury and improve outcomes by down regulating TLR4 and RAGE/NF-κB -mediated inflammatory responses 24 hours after TBI.	**[94**

Okuma et al, 2014	FPI in adult male Wistar rats	Glycyrrhizin (0.25, 1.0 or 4.0 mg/kg) was administered intravenously at 5 min, 6 hours and daily for three days post injury.	• Dose-dependent inhibition of BBB permeability • Ameliorated motor neurological deficit, associated with inhibition of HMGB1 translocation in neurons at injured sites. • Beneficial effects on motor and cognitive function persisted for seven days post injury. • Reduced expression of TNF-a, IL-1b and IL-6 at injured sites • Treatment effects reduced in $RAGE^{-/-}$ mice implicating HMGB1-RAGE inhibition as mechanism of action of glycyrrhizin	**[95]**
Pang et al, 2016	Lateral head rotation device used to simulate DAI in adult male Sprague-Dawley rats	Glycyrrhizin (10 mg/kg) intravenously administered to treatment group 30 min before the induction of DAI	• Reduced activation of apoptosis-associated proteins and expression of proinflammatory cytokines 48 hours post-injury • Reduced cognitive and motor deficit, reduced neuronal apoptosis and protected integrity of the BBB after DAI in rats up to 48 hours post-injury.	**[96]**
Chen et al, 2017	Feeney DM weight drop injury in adult male Sprague-Dawley rats	Omega-3 polyunsaturated fatty acid (ω-3 PUFA) was injected intraperitoneally (2 ml/kg) 30 mins post TBI and then once per day for seven consecutive days	• ω-3 PUFA inhibited TBI-induced microglial activation and expression of inflammatory factors (TNF-α, IL-1β, IL-6, and IFN-γ) by regulating HMGB1 and TLR4/ NF-kB signalling up to seven days post injury. • ω-3 PUFA supplementation reduced brain oedema, decreased neuronal apoptosis and improved neurological function for up to seven days post-TBI.	**[97]**

Abbreviations: CCI – Controlled cortical impact; DAI- diffuse axonal injury; FPI- Fluid percussion model; BBB- Blood brain barrier