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REVIEW

Genetic Manipulation of Cell Death and Neuroplasticity Pathways in Traumatic Brain Injury

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Abstract Traumatic brain injury (TBI) initiates a complex cascade of secondary neurodegenerative mechanisms contributing to cell dysfunction and necrotic and apoptotic cell death. The injured brain responds by activating endogenous reparative processes to counter the neurodegeneration or remodel the brain to enhance functional recovery. A vast array of genetically altered mice provide a unique opportunity to target single genes or proteins to better understand their role in cell death and endogenous repair after TBI. Among the earliest targets for transgenic and knockout studies in TBI have been programmed cell death mediators, such as the Bcl-2 family of proteins, caspases, and caspase-independent pathways. In addition, the role of cell cycle regulatory elements in the posttraumatic cell death pathway has been explored in mouse models. As interest grows in neuroplasticity in TBI, the use of transgenic and knockout mice in studies focused on gliogenesis, neurogenesis, and the balance of growth-promoting and growth-inhibiting molecules has increased in recent years. With proper consideration of potential effects of constitutive gene alteration, traditional transgenic and knockout models can provide valuable insights into TBI pathobiology. Through increasing sophistication of conditional and cell-type specific genetic manipulations, TBI studies in genetically altered mice will be increasingly useful for identification and validation of novel therapeutic targets.

Keywords Apoptosis · Growth factors · Knockout mice · Neurogenesis · Neuroplasticity · Transgenic mice.

Introduction

Traumatic brain injury (TBI), resulting from motor vehicle accidents, sports injuries, blast injuries, assaults, and falls, is a significant cause of disability and death worldwide. The neuronal damage resulting from a TBI is produced by both primary and secondary injury mechanisms. Primary injury involves mechanical impact and inertial forces that cause cellular strain and membrane damage, which leads to ionic imbalance, release of excitatory amino acids, and oxidative damage during the secondary phase of injury. Physical damage also compromises the blood-brain barrier allowing the infiltration of inflammatory cytokines and chemokines into the brain parenchyma and initiating inflammation. During the secondary injury cascade, proteases such as calpains and caspases are rapidly activated and contribute to cell death due to necrosis or apoptosis. Cell damage and death resulting from secondary injury are followed by a restoration phase during which the brain remodels itself in an effort to compensate for tissue damage. Such compensatory plasticity is believed to underlie spontaneous recovery of function that takes place after TBI, the extent of which depends on severity of injury, age, and other factors. Many therapeutic strategies for TBI tested clinically have focused on attenuating acute damage due to glutamate excitotoxicity, free radical accumulation, or calcium influx [1]. In light of the failures of past clinical trials in head injury, the identification

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of alternative therapeutic targets remains an area of intense interest. Approaches that target endogenous neuroreparative mechanisms, by promoting neurogenesis and angiogenesis or stimulating neurite outgrowth, are receiving increasing attention. Studies carried out in genetically modified mice are now a critical tool in the identification of new therapeutic targets and proof-of-concept studies establishing the importance of these targets in modifying outcome after TBI.

Most models of diffuse or focal TBI were initially developed in rats or higher order mammals, and have subsequently been adapted for use in mice, due to the advantages of exploring cellular mechanisms through genetic manipulation. By far the most commonly used TBI model in mouse studies involving transgenic and knockout approaches is the controlled cortical impact (CCI) model. In this model, the head is fixed in a stereotactic frame, and a craniotomy is created to expose the dural surface of the brain. Brain injury is then induced by rapidly and transiently impacting the brain with a rigid impactor driven at a prescribed depth and velocity. Development of a unilateral cortical contusion is accompanied by regional axonal injury and hippocampal cell death within the CA3 pyramidal layer and dentate gyrus granular layer and hilar regions [2, 3]. This contusion-type brain injury also results in neurobehavioral deficits, specifically related to motor movement and learning and memory [3–5].

Experimental studies using the CCI model, as well as other TBI models, have provided substantial information regarding secondary injury cascades, but our knowledge of therapeutic targets and their relative importance is still incomplete. Because many of these events cannot be readily and individually manipulated by pharmacological agents, and drug delivery to the brain can present significant challenges, genetically modified animals have emerged as a valuable tool to alter expression of single genes or proteins. The use of genetically altered mice in the study of TBI has increased greatly since an earlier review by Longhi et al. [6] in 2001. Here we provide an up-to-date summary and synthesis of studies targeting mediators of programmed cell death, regulators of cell cycle, and cellular and molecular events involved in neuroplasticity. Other areas of active research using transgenic and knockout mice, including the role of cytokines and the influence of TBI on chronic neurodegenerative disease pathology, are outside the scope of this review.

Regulation of Cell Death

In contusion brain injuries, rapid local brain deformation causes cell shearing and membrane rupture, resulting in irreversible cell injury and necrosis of affected tissues [7, 8]. In penumbral regions where cells undergo delayed neurodegeneration, other mechanisms can dominate, including apoptosis, which is a programmed cell death cascade regulated by effector proteins and caspase protease activation [9, 10], and autophagy, which is the lysosomal degradation of components within the cell [11–13]. These pathways are often mediated by a specific sequence of intracellular protein activation and have been heavily studied in the context of physiological cell function and also in trauma. Transgenic and knockout mice have been used to target aspects of the apoptotic cascade, inhibiting the upstream initiators or downstream cell death effectors. Other regulators of cell death, including caspase-independent mechanisms and cell cycle proteins, have been explored through studies of genetically altered mice.

Apoptosis

Apoptosis, or programmed cell death, is initiated by either extrinsic or intrinsic signals (Fig. 1). The extrinsic pathway involves Fas or tumor necrosis factor alpha (TNF-α) ligand binding and trimerization of the death receptor. Adaptor proteins are associated with the receptor through death domains, resulting in the recruitment and activation of the initiator caspase-8. Consequently, active caspase-8 initiates the activity of the effector caspase-3 through the activation of pro-apoptotic proteins and cytochrome c release from the mitochondria. In contrast, the intrinsic pathway involves activation of intracellular cues through external signals, ultimately leading to cytochrome c release from the mitochondria and its association within the apoptosome to activate caspase-3. The intracellular mediators of each pathway are often intertwined and converge onto caspase-3 activation, which causes DNA fragmentation, caspase-mediated substrate proteolysis, and cell death. The apoptotic cell death cascade is intracellularly regulated by the balance in abundance or activity of pro-apoptotic and anti-apoptotic Bcl-2 family proteins, which are localized to the mitochondria and endoplasmic reticulum of the cell. Expression of pro-apoptotic members, such as Bid, Bax, Bad, and Bak results in the release of cytochrome c from the mitochondria causing the activation of downstream effector caspases. In contrast, anti-apoptotic proteins inhibit the action of upstream pro-apoptotic signals, preventing the release of cytochrome c and, in turn, the activation of effector caspases.

Early studies in experimental TBI identified characteristic aspects of apoptotic morphology, including cell shrinkage, cytoplasmic blebs, and DNA fragmentation by terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) [10, 14–16]. Subsequent studies have used immunolabeling techniques to specifically identify the spatial and temporal expression of proteins involved in programmed cell death, noting early post-traumatic increases in apoptosis initiators, Bcl-2 family proteins, caspases, and cytochrome c [9, 17–24]. A role for apoptosis in human TBI is suggested by increased levels of Bcl-2, caspase-1, and

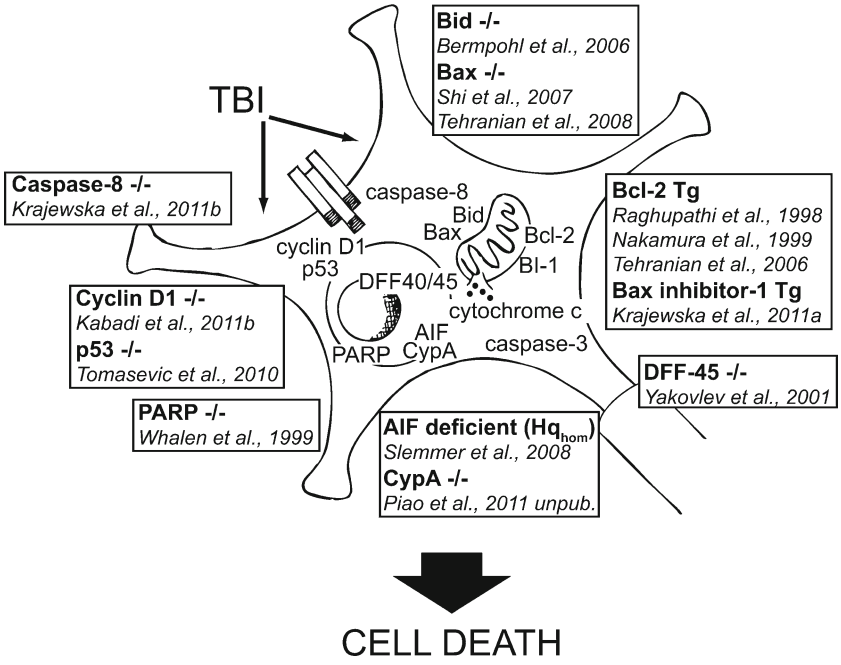

Fig. 1 Schematic of select cell death pathways following traumatic brain injury (TBI), including the activation of apoptotic and cell regulatory proteins. Boxed areas highlight studies using transgenic (Tg) or knockout (-/-) mice relevant to specific cell death-related proteins. AIF=apoptosis-inducing factor; CypA=cyclophilin A; DFF=DNA fragmentation factor; PARP=poly(ADP-ribose) polymerase

caspase-3, in concert with DNA fragmentation in brain tissue obtained from trauma patients [25]. Furthermore, Bcl-2 protein [26], cytochrome c [27, 28], and caspase-3 activity [29] have been identified in cerebrospinal fluid from severely head-injured patients.

Pharmacological targeting of post-traumatic apoptotic mechanisms has focused predominantly on the use of caspase inhibitors. Administration of either broad-spectrum or specific caspase inhibitors produces reductions in apoptotic mediator activity, cell death, or behavioral deficits [21, 30–32], which suggests the importance of apoptosis in TBI pathology. However, to better understand the balance between pro- and anti-apoptotic proteins for which no pharmacological inhibitors are available, transgenic and knockout approaches have been extremely valuable. Based on the genetic models, it is clear that a delicate interplay between pro- and anti-apoptotic mechanisms exists, and the opportunity to manipulate specific genes in the pathway could shift the balance toward cell survival in an effort to improve outcome after TBI.

Pro-Apoptotic Mediators

Although expression levels of the pro-apoptotic proteins (Bid, Bax, and caspase-8) are altered following TBI, the functional significance of these changes is unclear. The use of mice with targeted deletion of these apoptotic proteins allows their specific role to be probed in the context of TBI.

Bcl-2 Family (Bid, Bax)

Both Bid knockout (Bid-/-) and Bax knockout (Bax-/-) mice have been evaluated in TBI models using outcomes of apoptosis and more generalized cell death. Following experimental contusion, full-length Bid decreases concomitant with an increase in truncated Bid (tBid), implying post-traumatic cleavage of the Bid protein to its pathologically active form [33]. Trauma also alters the expression level and spatial distribution of Bax protein [34, 35]. Following CCI brain injury, Bid-/- mice demonstrated reduced numbers of propidium iodide-stained (i.e., dying) cells within the injured cortex acutely after injury and a smaller cortical contusion at 12 days compared to wild-type counterparts [36]. Caspase-3 activation was reduced in the dentate gyrus region of Bid-/- mice at 48 h post-CCI, but no differences were identified in the cortex. Based on this study, Bid appears to mediate the apoptotic pathway in select regions of the hippocampus, and Bid deficiency can ultimately spare neurons from cell death in the cortex. However, when tested for motor and cognitive behaviors using a wire grip test and Morris water maze paradigm, respectively, Bid-/- mice performed similarly to wild-type mice, indicating no effect on reducing injury-associated behavioral deficits following contusion injury [36].

Genetic deletion of the pro-apoptotic protein Bax also appeared to protect vulnerable cell populations. Brain-injured Bax-/- mice showed less caspase-3 activation in the dentate gyrus and improved hippocampal structure, but no decrease in numbers of hippocampal cells with DNA fragmentation detected by TUNEL staining compared to wild-type mice at 24 to 72 h after injury [37], indicating that Bax controls activation of caspase-3, but may not solely dictate a lethal outcome for the cell. Interestingly, Bax deficiency led to an enhanced stimulation of neural progenitor cells in the hippocampus after injury, which may explain overall improved hippocampal structure in injured Bax-/-

mice [37]. A second study sought to confirm acute changes in cell survival after CCI injury in Bax null mice [38]. Unlike the Bid-/- and previous Bax-/- studies, injury-induced caspase-3 expression within the cortex or hippocampus did not differ between Bax null and wild-type mice, although Bax null mice did not exhibit the post-traumatic nuclear translocation of activated caspase-3 observed in wild-type mice. Despite a reduction in numbers of TUNEL+ cells with apoptotic morphology within the dentate gyrus and CA1 regions of the hippocampus 24 h after CCI, only small, nonsignificant decreases in cortical lesion size or CA1 and CA3 neuronal death were noted [38]. Importantly, uninjured Bax null mice had significantly larger brain size and CA1 neuronal cell numbers compared to wild-type mice, consistent with abnormal developmental pruning through apoptosis. Inhibition of Bax-dependent developmental apoptosis may underlie behavioral deficits observed in Bax null mice compared to wild-type counterparts, regardless of injury [38]. Because the CCI impactor tip was not scaled for the larger Bax null mouse brain, Bax null mice likely received a milder injury than did wild-type mice. This confound might explain, in part, the apparent reductions in cell death observed in the Bax null mice. The behavioral and histological results of Bax null studies underscore the need for conditional Bax knockout mouse models, to avoid alterations in apoptosis during critical developmental periods that affect interpretation of trauma-induced outcomes.

DNA Fragmentation Factor

A primary aspect of apoptotic morphology and indication of impending cell death is the downstream fragmentation of nuclear DNA by the DNAse activity of the heterodimeric DNA fragmentation factor (DFF), composed of 40- and 45-kDa subunits. The proteolytic activity of DFF is controlled by caspase-3 cleavage of DFF-45, which results in dissociation of the 2 subunits and DNAse activity of DFF-40 [39]. The DFF-40 requires DFF-45 for activation; thus, in its absence, DFF-40 is not functional [40–42]. Cortical and hippocampal expression levels of DFF-45 decrease 2 and 24 h following lateral fluid percussion TBI, with evidence of cleavage in the cortex [43]. DFF-40 expression decreased in cortical and hippocampal cytosolic homogenates, but was increased in hippocampal nuclear fractions 2 and 24 h after injury. DFF-40 translocation to nuclear fractions may indicate a role in apoptotic cell death after brain trauma. No pharmacological inhibitors of DFF-40 and DFF-45 are currently available to suppress DNA fragmentation through this enzyme. Therefore, a genetic knockout approach may help elucidate the specific role of DFF in trauma-induced apoptosis. Mice deficient in DFF-45 were used to investigate whether the lack of DFF-40 DNAse activity reduced apoptotic cell death after trauma. In a model of CCI injury, DNA fragmentation in cortical cells was delayed in DFF-45 knockout mice compared to wild-type mice. Nevertheless, no differences were noted in cortical lesion size on magnetic resonance imaging (MRI) or in postinjury motor or learning behavior [44]. Although DFF appears to play a role in apoptosis after brain injury, other endonucleases may serve a redundant or compensatory DNAse function when DFF-45 is absent.

Studies that have used mice deficient in pro-apoptotic proteins such as Bid and Bax show only modest effects on the downstream activation of programmed cell death, and neither strategy resulted in behavioral improvements. When DNA fragmentation was interrupted by deletion of DFF-45, no neuroprotective phenotype was evident. Deletion of a single pro-apoptotic protein within the Bcl-2 family or other event that is downstream of apoptosis initiation may be too delayed to have an effect on cell survival or hippocampal mechanisms affecting behavior. Furthermore, possible compensatory mechanisms by other Bcl-2 family members could promote an apoptotic outcome in the prolonged absence of one pro-apoptotic protein. Strategies using conditional deletion are needed to avoid such potential confounds.

Caspase-8

More recently, caspase-8 knockout mice have been used in a comprehensive study to determine the role of this upstream apoptotic initiator in mediating apoptosis and neurodegeneration following TBI [45]. With ligand binding, caspase-8 cleaves pro-apoptotic protein Bid into its active form, tBid. After CCI brain injury, procaspase-8 and cleaved caspase-8 expression levels are elevated from 6 to 72 h, coincident with apoptotic-like morphology and DNA damage in injured cortical tissue [46]. Analysis of brain tissue samples from human TBI patients similarly demonstrates an increase in caspase-8 expression [47]. Selective deletion of caspase-8 in neurons (N*casp8*-/-) using a noninducible Cre-lox expression system resulted in cellular protection and reduced behavioral deficits after CCI injury [45]. Compared to injured wild-type mice, injured caspase-8-deficient mice had a lower percentage of cells with caspase-3 activation, no detection of cleaved poly(ADP-ribose) polymerase (PARP), and no increase in nuclear phospho-c-Jun at either 48 h or 21 days postinjury. Protection at the intracellular level translated into an overall reduction in post-traumatic cell death in caspase-8 knockout mice evident by higher numbers of NeuN-expressing cells at 6 to 48 h, decreased neuronal degeneration at 2 h to 21 days, and reduced cortical contusion size acutely after injury. TBI-related pathology, including immunoglobulin G extravasation due to breakdown of the blood-brain barrier and neutrophil infiltration, were also lessened in injured caspase-8 knockout mice compared to

wild-type mice. However, a higher density of activated microglia was detected at 21 days after CCI injury in knockout mice. When mice were tested for motor and cognitive behavioral deficits at 7 and 21 days following injury, caspase-8 knockout mice demonstrated better performances on hind limb flexion responses, beam balance, wire grip, and learning and memory tasks in the Morris water maze [45]. These positive results favor an upstream targeting strategy to knock out critical initiator genes and halt the apoptotic response of the cell. Furthermore, neuron-specific deletion of the pro-apoptotic protein may facilitate a neuroprotective outcome as opposed to ubiquitous deficiency, including other types of cells or tissues.

Anti-Apoptotic Mediators

An alternative strategy to target the apoptotic cascade involves transgenic mice that overexpress anti-apoptotic genes, in an effort to overwhelm and possibly prevent the cell death response after trauma. These genes are also part of the Bcl-2 family of proteins, including Bcl-2, Bcl-xL, and Mcl-1. Trauma-induced decreases in Bcl-2 or Bcl-xL pro-survival proteins in the injured cortex and hippocampus [48, 49] support the rationale for early transgenic studies testing overexpression of Bcl-2 as a strategy to attenuate the cell death cascade after trauma. Although changes in Bax-inhibitor-1 (BI-1) in TBI have not been described yet, this protein was found to confer cellular protection against hepatic ischemia-reperfusion injury in areas with high BI-1 expression, such as the liver and kidney [50]. Expression of BI-1 is a potential target to prevent the activity of Bax in mediating the apoptotic cascade and protecting against endoplasmic reticulum stress. Because pharmacological treatments designed to promote anti-apoptotic mediators have not been optimized for TBI studies, transgenic approaches allow investigations into pathways requiring an overexpression of endogenous, pro-survival proteins.

Bcl-2 Family (Bcl-2, BI-1)

Bcl-2 overexpression under the synapsin promoter resulted in high levels of Bcl-2 protein in mouse central nervous system (CNS) tissues, and conferred cortical neuroprotection after CCI injury [51]. Similarly, when controlled by either the neurofilament light chain promoter [52] or the neuron-specific enolase promoter [53], Bcl-2 overexpression resulted in reduced CCI-induced cortical contusion size and attenuated post-traumatic hippocampal cell loss in the CA2, CA3, or dentate gyrus. Surprisingly, overt cortical neuroprotection and select hippocampal cell survival with Bcl-2 overexpression was not accompanied by decreased apoptotic cell death detected by TUNEL staining in the hippocampus or by cleaved caspase-3 or -9 on Western blots [53]. Similar to contusion analyses in Bcl-2 overexpressing mice, cortical contusion size in constitutively BI-1 overexpressing mice was reduced at 2 h to 2 weeks after injury compared to wild-type mice (Fig. 2a) [54]. Here, however, cortical neuroprotection was associated with decreased post-traumatic apoptosis, as detected by TUNEL staining at 6 and 24 h (Fig. 2b) and an early reduction in markers of endoplasmic reticulum stress, including CCAAT/-enhancer-binding protein homologous protein (CHOP), phospho-Jun, and c-Jun [54]. A cellular balance in favor of the anti-apoptotic proteins Bcl-2 and BI-1 appears to promote a neuroprotective outcome at both acute and long-term time points. Although both proteins directly inhibit Bax, BI-1 may be more effective in decreasing apoptotic cell death.

When Bcl-2 overexpressing mice were assessed for injury-induced behavior deficits, no overt changes in beam balance, wire grip, or Morris water maze spatial memory

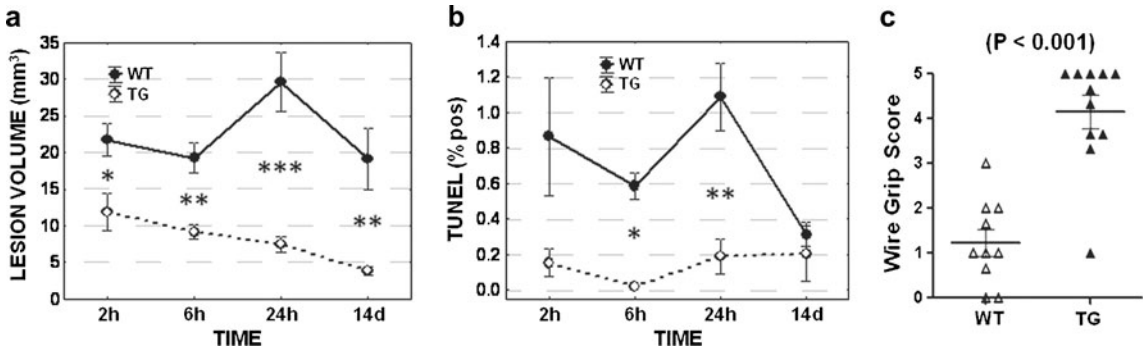

Fig. 2 Bax inhibitor-1 overexpression improves histological and behavioral outcome after controlled cortical impact (CCI) brain injury. (a) Cortical contusion lesion is larger in injured wild-type (WT) mice than transgenic (TG) mice at several time points after injury. (b) Similarly, TG mice show a lower percentage of terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL)-positive cortical cells following injury. Data are represented as mean ± standard error (*p<0.02; **p<0.002; ***p<0.008 compared to WT). (c) TG mice (▲) demonstrate improved wire grip performance at 7 days after CCI compared to WT mice (△). Each experimental animal is plotted, in addition to the mean ± standard error (p<0.001). Modified from Krajewska et al. [54] and reproduced with permission from Elsevier

performance were observed with Bcl-2 overexpression at several days past the injury [52, 53], and only select motor improvement was seen in transgenic mice with the inclined plane test [51]. BI-1 transgenic mice, however, demonstrated better wire grip performance at 7 days post-CCI, whereas their wild-type counterparts showed sustained deficits (Fig. 2c) [54]. Based on these transgenic mouse studies, increased expression of Bcl-2 and BI-1 is sufficient to reduce injury-induced cortical contusion size, presumably through suppression of apoptosis. These proteins also appear to play a protective role in select motor task performance, yet more information is required to fully understand the connection between apoptotic cell death and behavioral phenotypes.

Caspase-Independent Programmed Cell Death (PARP, Apoptosis-Inducing Factor, Cyclophilin A)

Although caspases primarily mediate apoptotic cell death, a variety of other proteins are involved in caspase-independent cell death pathways. Specifically, stressors, such as ischemia and excitotoxicity, can induce PARP activity and the release of apoptosis-inducing factor (AIF) from the mitochondria, leading to an apoptotic-like cell death [55, 56]. AIF and its carrier protein cyclophilin A (CypA) translocate to the nucleus to mediate nuclear condensation, DNA damage, and proteolysis [57, 58]. Both PARP and AIF are activated after contusion TBI, indicating an important role in trauma-induced neuronal death [59–61] and spurring studies investigating caspase-independent pathways using knockout mouse models (Fig. 1). Following CCI brain injury, PARP knockout (PARP -/-) mice showed improved beam balance performance and spatial memory compared to wild-type mice. However, this behavioral improvement was not accompanied by a genotypic difference in cortical contusion size [62]. In addition to its pro-cell death function of inducing AIF release, PARP functions in a pro-survival manner to mediate DNA repair after damage [63]. The dual roles of PARP may contribute to a lack of net effect on histological outcome. In contrast to PARP -/- mice, injured AIF-deficient harlequin mutant mice (Hq_{hom}) exhibited reduced contusion volume compared to their wild-type counterparts [64]. Increased CypA has been identified in brain vasculature after severe CCI [65] and is required for AIF nuclear translocation after cerebral hypoxia-ischemia [66]. CypA knockout mice have recently been used to investigate the synergistic effects of caspase-dependent and caspase-independent mechanisms following TBI. Brain-injured CypA-/- mice exhibited reduced AIF nuclear translocation and improved behavioral function; however, treatment with caspase inhibitor was required to reduce cortical contusion volume in CypA knockout mice [67]. The latter study underscores both the complexity of cell death mechanisms and the concomitant action of caspase-independent and caspase-dependent pathways, indicating a single transgenic or knockout strategy may be ineffective to fully prevent neuronal death and behavioral deficits after TBI.

Cell Regulatory Elements (p53, Cyclin D1)

Other strategies for targeting cell death have manipulated genes involved in cell cycle regulation and gene expression (Fig. 1). The tumor suppressor protein p53 functions to stop cell cycle progression and contributes to the expression of pro-apoptotic genes. In response to TBI, p53 mRNA is transiently elevated in the injured cortex, hippocampus, and thalamus [68], and p53 protein translocates to the nucleus at 48 h after CCI brain injury [69]. Cyclin D1 paired with its respective cyclin-dependent kinase functions in normal cells to modulate cell cycle activation, initiating the G1 phase of cell cycle entrance. Cyclin D1 is up-regulated after TBI [69, 70], specifically in neurons expressing active caspase-3 [71]. Only cell cycle inhibitor therapies selectively targeting cyclin-dependent kinases have been used in experimental TBI, demonstrating a post-traumatic attenuation of cyclin D1 expression, reduction in cortical and hippocampal cell death, and improved behavioral recovery [70, 71].

Although knockout of p53 was hypothesized to be neuroprotective in mice with severe CCI injury, the cortical contusion volume and the hippocampal or thalamic cell loss were not reduced in p53 knockout mice compared to wild-type mice [72]. Despite a lack of cellular protection, knockout mice exhibited better motor function at 7 days after injury. Recently, cyclin D1 knockout mice were shown to have reduced expression of cell cycle markers 24 h following CCI, indicative of the role of cyclin D1 in cell cycle initiation [73]. Brain-injured knockout mice also had fewer degenerating neurons in the neocortex and subcortical areas. At 21 days, brain-injured mice deficient in cyclin D1 had reduced neuronal loss within the dentate gyrus, reduced overall hippocampal damage, and a smaller cortical contusion compared to wild-type mice (Fig. 3a, b). Injury-induced, long-term cognitive deficits were also lessened in cyclin D1 knockout mice, as evidenced by a reduced latency to the platform in Morris water maze testing and a higher recognition index with a novel object recognition paradigm (Fig. 3c, d) [73].

Cell cycle regulation appears to be an important mediator of secondary neurodegeneration after TBI. Although mice deficient in the cell cycle inhibitor p53 failed to show cortical or hippocampal neuroprotection, the absence of the cell cycle initiator cyclin D1 showed both cellular protection and cognitive improvement. Aberrant increases in cell cycle proteins after TBI lead to both an initiation of apoptosis and caspase activation, and an inflammatory cell proliferation, exacerbating trauma-induced pathology. Thus, inhibition of the cell cycle after trauma may be protective.

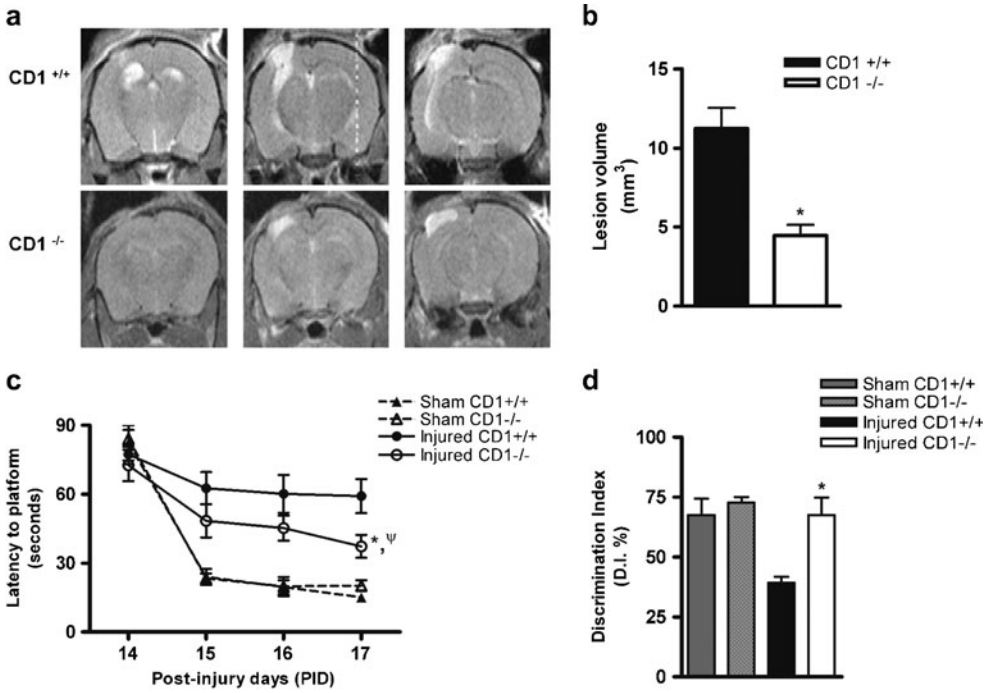

Fig. 3 Cyclin D1 knockout ($CD1^{-/-}$) mice exhibit improved histological and cognitive outcomes compared to wild-type ($CD1^{+/+}$) mice. (a) Representative T2-weighted magnetic resonance imaging (MRI) coronal brain images illustrate lesion location within three coronal planes at 21 days after controlled cortical impact (CCI) injury. (b) Lesion volume on MRI is significantly smaller in injured $CD1^{-/-}$ mice compared to $CD1^{+/+}$ mice. Data are expressed as mean lesion volume (mm^3) + standard error. (*p=0.003) (c) Although CCI injury resulted in a reduced ability to learn the location of a hidden platform in a Morris water maze for several days of training, brain-injured $CD1^{-/-}$ mice had latencies that were lower at 17 days post-injury compared to $CD1^{+/+}$ mice (*p=0.028) and significantly improved with a duration of time (ψp=0.007 *vs* post-injury day 14). Mean latency in seconds ± standard error is plotted. (d) Using a novel object recognition paradigm, injured $CD1^{-/-}$ mice showed an improved discrimination index, comparable to sham levels, at 21 days post-CCI injury. Data shown as mean + standard error (*p=0.0004 compared to $CD1^{+/+}$ mice). Modified from Kabadi et al. [73] and reproduced with permission from Mary Ann Liebert, Inc.

Summary

Collectively, the use of transgenic and knockout mice in the study of cell death in TBI has underscored the integral role of programmed cell death and cell regulatory elements, and possible cellular targets for the treatment of brain injury has identified. Models that have altered the Bcl-2 family of pro-apoptotic or anti-apoptotic proteins have shown modest and sometimes inconsistent findings with apoptosis and cell survival outcomes, and little behavioral efficacy. Although the lack of successful outcomes in these studies has limited the development of TBI therapeutics targeting apoptosis, the significant and complex nature of apoptosis in cell function must be given full consideration. These studies have offered meaningful insights into signaling cascades and cell-specific effects following the modulation of a single target within the apoptotic pathway. Given the importance of apoptosis during development, conditional transgenic strategies should be developed and investigated. Little is known regarding the functional redundancy of the Bcl-2 family of proteins in the setting of trauma. Deletion or overexpression of a single family member may be compensated for by other Bcl-2 type proteins, complicating interpretations. Therefore, development of transgenic or knockout models with multiple genetic manipulations may be required to overcome endogenous compensatory mechanisms and isolate specific pathological mechanisms. Studies that have used mice deficient in caspase-8, CypA, or cyclin D1 have demonstrated positive outcomes on post-traumatic cell survival and behavior. These results may encourage development of strategies that focus on upstream apoptotic targets, alternative cell death pathways, or cell cycle proteins to ameliorate neuronal damage or behavioral deficits after injury.

Regulation of Neuronal Repair

Brain plasticity can be considered as the ability of the nervous system to remodel itself in response to insults that alter its homeostasis. Post-traumatic plasticity may involve the modification or generation of cells through neurogenesis, gliogenesis, angiogenesis, synaptic plasticity, and axonal sprouting, events which can be stimulated by endogenous growth factors and other growth-related proteins

(Fig. 4). After a wave of early cell damage and death induced by traumatic injury, ongoing neurorestorative events that may continue for days, weeks, or even months could contribute to natural recovery of cellular function or behavior. Strategies aiming to enhance endogenous repair mechanisms may be therapeutic in the setting of TBI. For example, administration of vascular endothelial growth factor following TBI has been shown to promote functional recovery, possibly through stimulating multiple brain plasticity events, including neurogenesis and angiogenesis [74]. The use of genetically engineered animals to study neural plasticity in TBI is a comparatively recent approach. The increased availability and variety of genetically altered animals has stimulated research targeting specific plasticity events in experimental TBI.

Enhanced cell proliferation is a widely accepted hallmark of TBI. Histological staining methods using specific proliferation markers or bromodeoxyuridine (BrdU) incorporation by dividing cells have been used to identify the hippocampus as a major proliferative zone after focal or diffuse brain injury. Dividing cell populations include microglia, oligodendrocytes, astrocytes, and neural progenitors [75, 76]. Although the consequences of these proliferative responses are still being debated, the birth of new neurons may contribute to functional recovery [77, 78].

Astrocytosis

TBI induces robust gliogenesis, a response that may have both beneficial and detrimental effects on neuronal survival and brain remodeling. Following brain injury, astroglia become reactive, undergoing cellular hypertrophy and proliferation [3, 75]. Astrocytes play essential protective roles, including clearance of extracellular glutamate and potassium, water transport, production of anti-inflammatory cytokines and chemokines, and production of growth factors [79], making them a crucial part of the neurorepair process. Astrocytosis is also proinflammatory and it is detrimental to axonal growth. To better understand the role of astrocytes in promoting neurodegeneration or neuronal repair after TBI, a transgenic mouse with inducible ablation of astrocytes was investigated. In mice expressing thymidine kinase (TK) of herpes simplex virus (HSV) type 1 under the control of the glial fibrillary acidic protein (GFAP) promoter (GFAP-HSV-TK), ganciclovir administration was used to ablate proliferating astrocytes [80]. Astrocytes that express TK are able to phosphorylate the nontoxic ganciclovir to toxic ganciclovir-triphosphate, which when incorporating into replicating DNA can cause chain termination and single-strand breaks leading to apoptotic cell death. Following a moderate CCI injury, transgenic mice deficient in dividing astrocytes showed increased cortical tissue loss and inflammation, as revealed by microglial staining, indicating a neuroprotective role for post-traumatic astrocytosis. However, this effect was not observed following severe brain injury possibly due greater acute loss of astrocytes, which could minimize the effect of the ablation. Although potential toxicity of ganciclovir to nontransgenic cells and inflammatory responses stimulated by apoptotic cells killed by ganciclovir are concerns in this model, the strategy could precisely target dividing astrocytes while sparing quiescent ones. Further studies are required to understand the mechanisms by

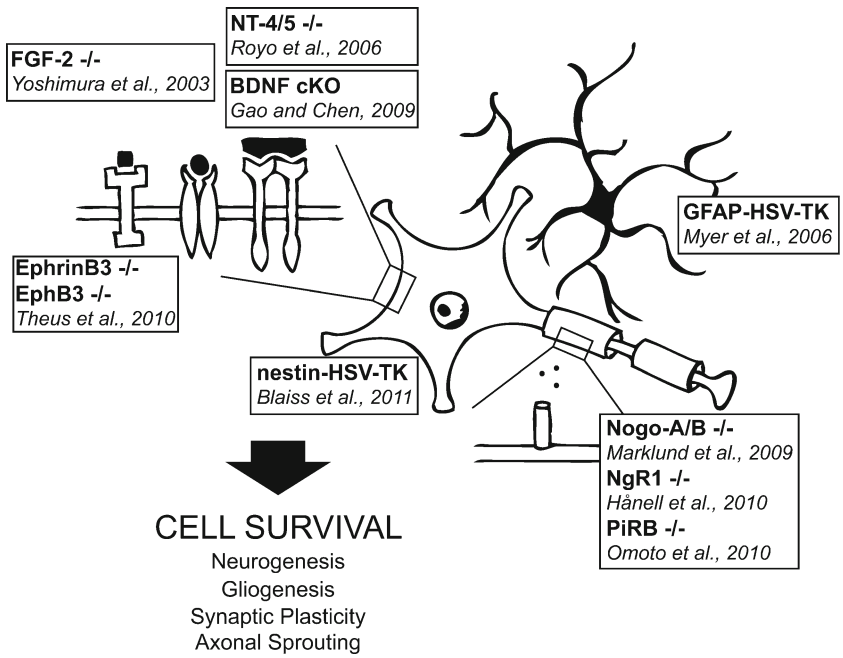

Fig. 4 Schematic representation of brain remodeling mechanisms after traumatic brain injury (TBI). Both neuron- and glial-mediated processes take part in adaptive changes that aid in neuronal repair after TBI. Boxed areas highlight current studies using genetically engineered animals to focus on plasticity responses after brain trauma. BDNF cKO=brain-derived neurotrophic factor conditional knockout; EphB3=ephrin receptor B3; FGF-2=fibroblast growth factor-2; GFAP-HSV-TK=glial fibrillary acidic protein-herpes simplex virus-thymidine kinase; nestin-HSV-TK=nestin herpes simplex virus-thymidine kinase; NgR1=Nogo-66 receptor-1; NT-4/5=neurotrophin-4/5; PiRB=paired immunoglobulin-like receptor B

which reactive astrocytes provided neuroprotection and whether these may represent potential targets for TBI therapy.

Neurogenesis

TBI and other forms of brain injuries, including ischemia, stimulate neural stem cell proliferation in the hippocampal subgranular zone (SGZ) and the subventricular zone (SVZ) [75, 81, 82]. In the uninjured brain, neurogenesis in the SGZ replenishes the dentate granule layer neurons, whereas neuroblasts originating in the SVZ migrate through the rostral migratory stream to populate the olfactory bulb. Different developmental stages for neural progenitors have been identified in the adult hippocampal SGZ: quiescent type 1 cells (putative stem cells with astrocyte morphology) and transiently amplifying type 2a (early neural progenitors expressing GFAP and nestin), type 2b (committed neuronal progenitors expressing nestin and doublecortin), and type 3 cells (committed neuronal progenitors expressing doublecortin) [83]. Precursor subtypes can be distinguished based on their morphology, proliferative ability, electrophysiological properties, and expression of markers, such as nestin, GFAP and doublecortin [83, 84]. Using a transgenic mouse expressing green fluorescent protein (GFP) under the control of a neural progenitor-specific form of the nestin promoter [85], the dynamics of neural progenitor activation were monitored following TBI. After CCI injury, GFP-positive stem cells (nestin expressing type 1 and 2a) proliferated while doublecortin-positive newborn neurons in the SGZ decreased in number at 72 h but recovered by 7 days postinjury [86]. To determine the contribution of type-1 and type-2a progenitors in replenishing the doublecortin-positive neuronal population, a nestin-HSV-TK GFP transgenic model was then used [86]. Analogous to the GFAP-HSV-TK model, which was previously described, nestin-HSV-TK GFP is used to ablate nestin-positive cells proliferating at the time of ganciclovir administration. Co-expression of GFP with TK permitted visualization of the precursor cells. Ablation of nestin-positive proliferating cells significantly reduced post-traumatic neurogenesis, demonstrating that newborn neurons formed in the hippocampal SGZ after TBI were mainly from early progenitors as opposed to doublecortin-expressing late precursor cells. The genetic strategy used in this study facilitated the finding that type-1/2a stem cell activation is necessary to repopulate the depleted immature neuron pool in the SGZ. These data raise an interesting possibility of targeting specific populations of neural stem cells to promote hippocampal repair after trauma.

To better understand the role of neurogenesis in spontaneous recovery following trauma, Kernie and colleagues again used the nestin-HSV-TK GFP transgenic model [87]. Continuous administration of ganciclovir for 4 weeks following CCI ablated 90% of injury-induced hippocampal neurogenesis by 2 months after TBI without affecting astrogliosis. Mice with pharmacogenetic ablation of trauma-induced neurogenesis displayed greater visuospatial learning impairment compared to brain-injured mice with neurogenesis when tested using a hippocampal-dependent Morris water maze paradigm for 11 consecutive days, supporting a role for neurogenesis in spontaneous cognitive recovery. Interestingly, neurogenesis ablation did not affect nonspatial learning and memory when assessed by rotarod or cued and contextual fear conditioning behaviors that may be regulated by additional regions, such as the amygdala. Compared to previous strategies that used anti-mitotic compounds or radiation to destroy dividing cells [88–90], the pharmacogenetic approach used in this study regulated neurogenesis in a more precise and temporally controlled manner. The results further strengthen the notion that targeting progenitors is a viable approach to promote post-traumatic functional recovery.

Endogenous Mitogens

Neurogenesis is supported or enhanced by endogenous molecules, such as growth factors and other mitogens. Ephrins, fibroblast growth factor-2 (FGF-2), brain-derived neurotrophic factor (BDNF), and growth-promoting compounds, such as erythropoietins have been shown to support cell survival and differentiation during early neural development [91, 92].

Ephrins

Ephrins, a class of membrane-bound growth and guidance molecules, and the ephrin receptors (Eph) are involved in brain plasticity regulation. Signaling through 1 of the ephrin family members (EphrinB3 and its receptor-EphB3) is involved in the proliferation, survival, and migration of neuronal precursor cells [93, 94]. EphrinB3 signaling maintains the number of SVZ neuroblasts in the adult brain by reducing cell death through an Ephrin receptor EphA4-dependent mechanism [94]. Because of their role in SVZ neurogenesis, it is hypothesized that Ephrins influence post-traumatic proliferation and migration of SVZ precursor cells. Following brain injury, EphB3 expression in the SVZ area decreased coincident with increased proliferation, supporting such a role for EphrinB3/EphB3 signaling in the regulation of proliferation [95]. To test the causality of this relationship, EphrinB3 or EphB3 knockout mice were subjected to CCI brain injury [95]. In the absence of EphrinB3-EphB3 signaling, BrdU incorporation and Ki67 staining increased in the SVZ after sham or CCI injury indicating that EphB3 signaling inhibits neural stem cell proliferation. Conversely, infusion of Ephrin B3-Fc suppressed post-traumatic proliferation in brain-

injured, wild-type mice. Together, these data support the notion that downregulation of EphB3 in the SVZ may be an early event stimulating post-traumatic proliferation. Decreased EphB3 levels in the wild-type SVZ after brain injury were also associated with reduced progenitor cell death compared to uninjured mice. A similar reduction in progenitor cell death was observed in uninjured EphB3 knockout mice, an effect that was not synergistic with TBI. These data point to an additional role for Ephrins in cell death regulation. However, both uninjured and brain-injured EphrinB3 knockout mice exhibited increased cell death in the SVZ area when compared to wild-type mice, raising the possibility that EphB3 may subserve other functions in the absence of EphrinB3 ligand [95]. Using genetically engineered mice, this study demonstrated a novel mechanism that controls SVZ cell proliferation and death, which may impact the development of strategies that target progenitor cells for repair and regeneration after brain injury.

Neurotrophins (NT-4/5, BDNF)

Many of the growth factor family proteins, including neurotrophins, mediate brain plasticity through multiple effects in the CNS that include neurite outgrowth, synaptogenesis, and neuronal differentiation and survival [96–98]. Brain injury elicits an increase in expression of multiple growth factors including neurotrophins, which is often transient and limited to traumatic penumbra [99–101]. Supplementing this response by exogenous growth factor administration promotes functional improvement and neuroprotection following experimental TBI [99, 101, 102]. Although no published studies in TBI, to our knowledge, have used transgenic mice to study overexpression of growth factors, 3 studies have used knockout mouse models to probe the role of endogenous growth factors in the response to TBI (Fig. 4).

In the first of these 3 studies, the effects of CCI brain injury on mice with a targeted deletion of the NT-4/5 gene were evaluated [99]. Deficiency of the neurotrophin NT-4/5 increased the vulnerability of CA2/CA3 pyramidal neurons to trauma and impeded recovery of motor function, suggesting that endogenous NT-4/5 acts to limit damage after TBI. These knockout mouse studies provide support for the usefulness of NT-4/5 therapy after trauma. Indeed, supplementation of NT-4/5 levels through post-traumatic intracerebral infusion of recombinant protein reduced hippocampal CA2/3 pyramidal neuronal loss following lateral fluid percussion brain injury in rats [99].

A second study focused on the role of BDNF in post-traumatic hippocampal neuronal survival after TBI. Experimental TBI in rodents results in early increases in BDNF mRNA levels that persist for days in the cortex but are more transient in the hippocampus [103–105]. Although BDNF infusion into the cortex or hippocampus failed to improve outcome following TBI in rats [106], transplantation of BDNF-overexpressing neural stem cells into the cortex of brain-injured rats improved sensory motor function [107]. In light of the well-established role of BDNF in hippocampal plasticity, a hippocampal-specific BDNF knockout mouse was used to study the role of BDNF signaling in TBI. Because global BDNF gene deletion in mice is lethal at 3 to 4 weeks of age [97], a conditional knockout for BDNF was developed using Cre-lox technology [108]. Pro-opiomalanocortin-Cre mice were crossed with $BDNF^{flox/flox}$ mice to knockout BDNF expression, specifically in dentate gyrus granule neurons. The absence of BDNF increased hippocampal neuronal death following CCI brain injury [108]. Many of the dying neurons co-labeled with the immature neuronal marker polysialylated-neural cell adhesion molecule (PSA-NCAM), implicating BDNF in the survival of immature neurons (Fig. 5). Studies such as this provide new insights into therapeutic targets related to post-traumatic neurogenesis. Along these lines, administration of simvastatin has been postulated to promote neurogenesis and enhance spatial learning ability following CCI brain injury in part through upregulation of BDNF expression [109].

FGF-2

A third study by Yoshimura et al. [110] used FGF-2 knockout mice to explore the role of FGF-2 in the response of the hippocampus to TBI. Following CCI injury, FGF-2 knockout mice exhibited increased hippocampal dentate gyrus cell loss and decreased granule cell layer volume. Removal of this growth factor partially suppressed brain injury-induced increases in dentate gyrus cell proliferation and neurogenesis, raising the possibility that FGF-2 supplementation may be beneficial in the setting of TBI. Administration of FGF-2 has been tested in experimental TBI with mixed results. A 3-h intravenous infusion of FGF-2 in rats reduced acute cortical damage following lateral fluid percussion brain injury [111]. However, 2 subsequent studies using acute intravenous infusion [112] or long-term intracortical infusion [113] failed to detect cortical or hippocampal pyramidal neuronal protection at 1 month or 1 week, respectively, after TBI in rats. Nevertheless, both studies demonstrated improved cognitive function in hippocampus-mediated tasks in brain-injured rats receiving FGF-2 treatment. Because neither study examined neuroprotection within the dentate gyrus, it is possible that the cognitive improvements accompanying FGF-2 administration were related to a reduction in cell death or enhancement of neurogenesis within the dentate. In a 2009 study, Sun et al. [114] demonstrated enhanced post-traumatic neurogenesis in the hippocampus and SVZ using a FGF-2 treatment paradigm that also improved cognitive function after TBI in rats. Similarly, overexpression of FGF-2 in hippocampal cells achieved through stereotactic

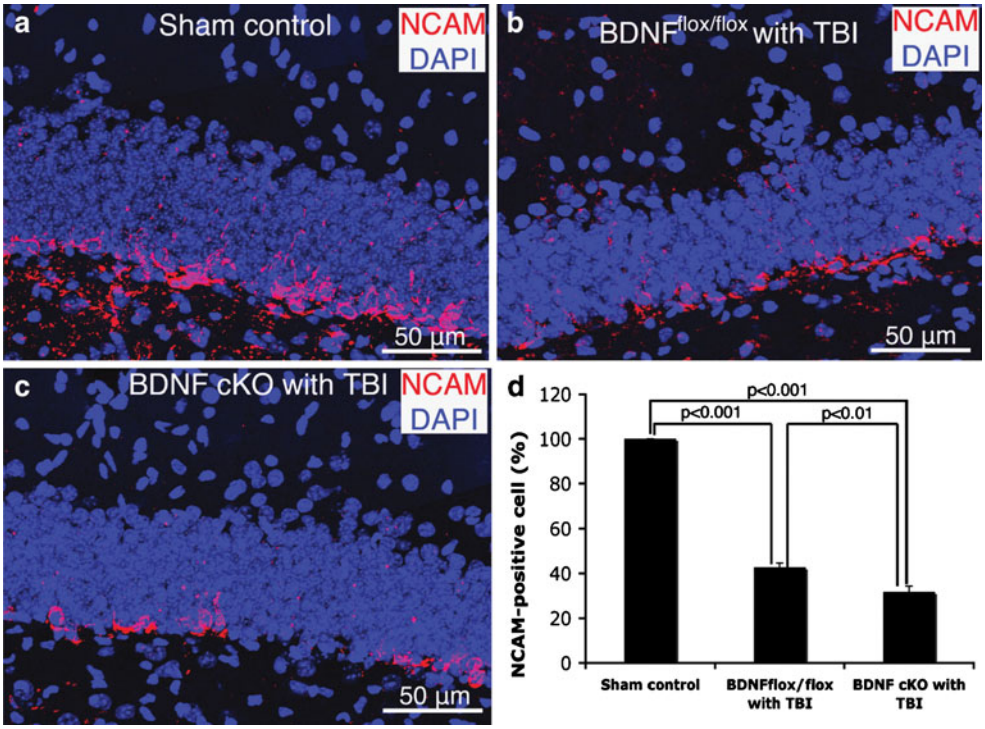

Fig. 5 Newborn immature neurons in the dentate gyrus of brain-derived neurotrophic factor (BDNF) deficient mice are more susceptible to cell death following moderate traumatic brain injury (TBI). Immature neurons labeled with polysialylated-neural cell adhesion molecule (PSA-NCAM) in (a) sham mice and (b) BDNF $^{flox/flox}$ control mice, and (c) BDNF conditional knockout (cKO) mice after controlled cortical impact (CCI) injury. (d) Quantification of PSA-NCAM-positive cells in the hippocampal dentate gyrus at 24 h after moderate CCI injury (n=5/group). Reproduced from [108] with permission from Mary Ann Liebert, Inc. DAPI = 4',6-diamidino-2-phenylindole

injection of FGF-2 expressing viral vectors enhanced neurogenesis and reduced granule cell layer cell loss in mice after CCI brain injury [110]. Collectively, knockout mouse studies and studies of FGF-2 administration suggest that FGF-2 may improve post-traumatic cognitive function through a selective enhancement of neurogenesis.

Axonal Plasticity

Brain remodeling after TBI not only involves neurogenesis and gliogenesis, but it also comprises adaptive axonal changes such as sprouting, regeneration, and remyelination (Fig. 4). White matter damage characterized by secondary axotomy and demyelination is common after diffuse and focal TBI and is believed to contribute significantly to trauma-induced behavioral impairment. Thus, the repair of damaged axons may contribute to functional recovery. In the CNS, axonal regrowth is inhibited both by the presence of a glial scar and by myelin-derived axonal growth inhibitors [115, 116]. Myelin-associated inhibitor proteins, including Nogo-A, Nogo-B, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein bind to the Nogo-66 receptor-1 (NgR1).

Myelin-Associated Inhibitor Proteins (Nogo-A/B)

The Nogo family member Nogo-A has been shown to inhibit axonal growth in CNS injury models [117, 118]. Deletion of the inhibitory proteins Nogo-A and its isoform Nogo-B was hypothesized to improve outcome after TBI through enhanced axonal plasticity [119]. However, Nogo-A/B knockout mice exhibited cortical tissue loss equivalent to wild-type littermates after CCI brain injury. Surprisingly, motor and cognitive impairments were significantly greater in homozygous knockout mice compared to heterozygous knockout mice or wild-type littermates [119]. Homozygous Nogo-A/B knockouts also exhibited hypomyelination in the corpus callosum after brain injury, which may have been related to poor behavioral performance. It is possible that long-term deprivation of Nogo-A/B impaired other vital functions controlled by Nogo-A/B. In contrast to the Nogo-A/B knockout study, pharmacological neutralization of Nogo-A protein by intracerebroventricular administration of anti-Nogo-A antibodies after TBI promoted functional recovery [120].

Myelin-Associated Inhibitor Protein Receptors (NgR1, Paired Immunoglobulin-Like Receptor B)

Because multiple inhibitory proteins interact with the Nogo-66 receptor, the deletion of NgR1 provides an alternate strategy for exploring the role of myelin-inhibitory protein signaling in plasticity in the post-traumatic brain. In NgR1 knockout mice receiving CCI brain injury, impairment of motor but not cognitive function was exacerbated compared to wild-type mice, an effect that was mimicked by pharmacological neutralization of NgR1 [121]. Worsened cognitive outcome after trauma may have been related to aberrant axonal sprouting as a result of low inhibitory protein signaling. However, the demonstration of increased hippocampal

mossy fiber sprouting in sham-injured but not in brain-injured animals with the neutralization of NgR1 [121] points to a need for further studies to fully understand this complex response. The negative outcomes of these initial myelin inhibitor protein signaling studies do not support the use of this approach for TBI therapy.

Myelin inhibitory proteins act through both NgR1 and paired immunoglobulin-like receptor B (PirB) receptors. To further study myelin inhibitor protein actions following trauma, PirB knockout mice in which sequences encoding the ectodomain and juxtamembrane domains of PirB were deleted were subjected to CCI brain injury [122]. Analyses of sprouting fibers within the corticospinal or corticorubral tracts failed to detect enhanced sprouting in brain-injured PirB knockout mice compared to wild-type mice. Motor behavior when assessed using cylinder, staircase, and grid walking tests was equivalent for wild-type and PirB knockout mice. Blocking myelin inhibitor protein signaling through PirB seems insufficient to promote axonal sprouting or improve behavioral function after TBI.

Summary

Studies with genetically engineered animals have begun to shed light on brain plasticity events that occur after TBI (Fig. 4). For years, astrocytosis has been considered an inflammatory response harmful for neuronal regeneration. Reactive astrocyte ablation achieved through a genetic approach revealed underappreciated beneficial effects of astrocytosis after TBI. Neurogenesis-targeted genetic modifications have helped to understand the contribution of different neuronal precursors in brain plasticity. Studies with growth factor-deficient mice have provided initial clues to roles for endogenous growth factors in neuroprotection, neurogenesis, and functional recovery. Genetically induced depletion of either myelin inhibitory proteins or their receptors did not improve post-traumatic behavioral function or axonal sprouting. Further studies are needed to determine whether this is due to compensatory changes in response to long-term myelin inhibition or whether antagonizing the inhibitory environment for axonal sprouting is contraindicated in TBI. Because most proteins involved in brain plasticity events are developmentally regulated, conditional knockout or overexpression systems will be immensely beneficial in avoiding potential developmental or compensatory confounds.

Conclusions

Genetically altered animals offer powerful tools to study TBI pathology and guide the design and evaluation of therapeutic interventions. Strategies that seek to manipulate a single gene product are vital to understanding the role of specific proteins in mediating functional responses. With the continued evolution of transgenic and knockout technologies, genetically altered mice will provide even greater experimental advantages. Cell- or region-specific alteration of a genetic product may help further dissect the complex interactions (e.g., between neuronal and glial cells or inhibitory and excitatory neurons). Likewise, genetic alterations in combination with reporter molecule systems can enhance understanding of affected cell types or changes in subcellular location induced by trauma. Conditional expression models further offer dynamic and temporally relevant genetic changes in which to study trauma pathogenesis while avoiding potential confounds of genetic manipulation during mouse development. Transgenic and knockout mouse studies offer a complimentary approach to pharmacological and gene therapy strategies for elucidating critical mediators in trauma-induced pathology and for validating the functional efficacy of targeting specific molecules.

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Required Author Forms Disclosure forms provided by the authors are available with the online version of this article.

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