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Combination Therapies in the CNS: Engineering the Environment

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Abstract

The inhibitory extracellular environment that develops in response to traumatic brain injury and spinal cord injury hinders axon growth thereby limiting restoration of function. Several strategies have been developed to engineer a more permissive central nervous system (CNS) environment to promote regeneration and functional recovery. The multi-faced inhibitory nature of the CNS lesion suggests that therapies used in combination may be more effective. In this mini-review we summarize the most recent attempts to engineer the CNS extracellular environment after injury using combinatorial strategies. The advantages and limits of various combination therapies utilizing neurotrophin delivery, cell transplantation, and biomaterial scaffolds are discussed. Treatments that reduce the inhibition by chondroitin sulfate proteoglycans, myelin-associated inhibitors, and other barriers to axon regeneration are also reviewed. Based on the current state of the field, future directions are suggested for research on combination therapies in the CNS.

Keywords

spinal cord injury; traumatic brain injury; cell transplantation; biomaterials; chondroitin sulfate proteoglycans; myelin-associated inhibitors

1. Introduction

Over 1.4 million traumatic brain injuries (TBI) and 12,000 spinal cord injuries (SCI) occur annually in the United States [1, 44]. Causes of TBI and SCI include combat, sports-related injuries, falls, violence and motor vehicle accidents. Individuals with TBI or SCI experience disabilities that range from cognitive impairment to loss of sensation and partial to complete paralysis. Current therapies for improving clinical outcomes include limiting inflammation, preventing secondary cell death and enhancing the plasticity of spared circuits. These strategies, however, do not promote repair of neural tissue or restoration of severed axonal connections. The limited regenerative capacity of the CNS is in part due to the inhibitory extracellular environment. New therapies focused on engineering a permissive environment for regrowth of axons and restoration of neural populations are needed to improve functional recovery following TBI and SCI.

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Early work on SCI has demonstrated that CNS axons maintain an intrinsic ability to regenerate in a permissive environment [15]. In the peripheral nervous system, where regeneration is more successful, these permissive environmental cues include neurotrophic factors and growth-supporting extracellular matrices (ECM) [48]. Cell transplantation and biomaterial scaffolds have been investigated to replace damaged tissue and provide soluble or mechanical cues for regeneration. However, the multi-faceted inhibitory nature of the adult CNS has limited the efficacy of such treatments. A combinatorial approach, therefore, may be more effective. In this mini-review we discuss the barriers to neural regeneration in the CNS and highlight recent attempts to overcome these barriers using combination therapies to engineer a permissive environment for axon growth.

2. CNS Inhibitory Environment

Initial trauma after a CNS injury leads to immediate disruption of neural tissue by shearing axons, rupturing blood vessels, and causing necrotic cell death [35]. Ischemic injury and inflammation, marked by infiltration of macrophages, neutrophils, and leukocytes releasing pro-inflammatory cytokines and reaction oxygen species, initiate a secondary phase of cell death accompanied by demyelination of spared axon tracts [10, 14, 18]. Fluid-filled cystic cavities commonly form at the injury site surrounded by a glial scar composed of reactive astrocytes, glial progenitors, microglia and macrophages, fibroblasts and Schwann cells (SCs)[66]. Chondroitin sulfate proteoglycans (CSPGs) present in the scar tissue contain glycosaminoglycans (GAGs) that inhibit extending axons and prevent re-growth into the injury zone [5]. Myelin-associated inhibitors (MAIs), such as Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (Ompg), signal through a common receptor, the Nogo-receptor (NgR), to induce growth cone collapse and prevent axon regeneration through white matter [49].

This cascade of events following CNS trauma establishes a formidable barrier to regeneration and restoration of function. Replacing neuronal populations, along with promoting axonal sprouting and formation of new circuitry, are desired for repair following injury. In SCI, bridging the injury site to reconnect severed axonal pathways with their distal targets and remyelination of regenerating axons are necessary to restore function. The physical and molecular barriers of the CNS lesion, however, limit endogenous repair and remodeling. Engineering an environment suitable for regeneration is therefore crucial to enhance recovery following TBI and SCI.

3. Combination Therapies

Many strategies have been developed to address the individual aspects of CNS trauma including limiting inflammation and secondary injury, remodeling injured tissue, neutralizing inhibitory molecules, increasing trophic support and replacing neural cell populations. Functional recovery in studies targeting a single component, however, is often modest. Combining therapies may help overcome multiple barriers to regeneration and provide synergistic effects on functional recovery. Here we review the most common and recent combination therapies that include the use of two or more individual strategies to promote regeneration. While the majority of the work has been performed in SCI, successful therapies can be extrapolated to other types of CNS trauma.

3.1 Neurotrophin Release

In the developing CNS, neurotrophic factors promote the directed growth and survival of many types of neurons. The introduction of neurotrophins to the injured spinal cord can promote neuronal survival and enhance regeneration of specific ascending and descending axonal pathways. Notably, BDNF promotes growth of rubrospinal, raphespinal, cerulospinal

and reticulospinal pathways while neurotrophin-3 (NT-3) elicits sprouting and growth of corticospinal and dorsal column sensory axons (for a complete review see [48]) [6, 22, 50, 77]. NGF and GDNF also support growth of regenerating axons, however, their potential for promoting aberrant growth of pain-associated nociceptive spinal axons may reduce their desirability for SCI [4, 58, 69]. Combining neurotrophic factor delivery with cell transplantation or biomaterial scaffolds may provide synergistic effects to improve functional recovery.

3.1.1 Cell Transplantation and Neurotrophin Release—Coupling cell transplantation with neurotrophic factor delivery may enhance repair following SCI. Oligodendrocyte precursor cells (OPCs) modified to express ciliary neurotrophic factor survived to a greater extent compared to unmodified OPCs following transplantation into the contused spinal cord [9]. Survival correlated with enhanced remyelination of spared axons and recovery of locomotor function. Co-transplantation of NT-3 expressing SCs with neural stem cells (NSCs) improved locomotor recovery over transplants of unmodified SCs and NSCs [23]. Axonal growth is commonly reported in response to cellular delivery of neurotrophins [21, 22, 42, 50]; however, functional recovery is variable and often modest. Many cells endogenously express neurotrophins, thereby reducing the effect of additional secretion on locomotor recovery. Coupling enhanced neurotrophin release with other cell-type specific functions, such as remyelination by OPCs, can improve the utility of these combination therapies.

3.1.2 Scaffolds and Neurotrophin Release—Regeneration through biomaterial scaffolds is dependent on axon sprouting and growth at the interface of the spinal cord lesion. Many axon pathways will retract in response to injury and may not enter the scaffold. The addition of neurotrophic factors may provide a spatial cue for regenerating axons to prevent dieback and induce growth into a scaffold. BDNF release from collagen matrices promoted greater neural regeneration into agarose scaffolds following cervical SCI (Figure 1) [62]. Similarly, BDNF release from collagen scaffolds elicited greater axon extension into the injury zone and functional improvement in a thoracic hemisection model [24]. In addition to stimulating axon growth, BDNF release from poly-lactic-co-glycolic acid (PLGA) microspheres in an agarose scaffold may reduce glial scarring and CSPG deposition, thereby improving axon regeneration at the scaffold-host interface [31].

NT-3 is another neurotrophic factor commonly used following SCI. When injected at the caudal side of a PLGA conduit following complete transection of the spinal cord, increased neural regeneration through the scaffold and improved locomotor recovery were observed [17]. Johnson *et al.* investigated delivery of NT-3 from a heparin-based delivery system within fibrin scaffolds [33]. NT-3 delivery enhanced neural fiber sprouting at the interface of the scaffold in a 2 week sub-acute thoracic hemisection model.

In the attempt to engineer a permissive environment for axon extension, combination therapies involving biomaterial scaffolds and neurotrophins can be used to promote neural regeneration. The mode of release, neurotrophin selection, injury severity, and scaffold material can influence regeneration and further work is necessary to determine the clinical significance of these strategies. In particular, it is unclear if non-degradable materials impede neural tissue regeneration. If so, minimizing the volume of scaffolds may permit greater bridging areas and enhance recovery. The use of a degradable materials can also solve this problem, however, degradation products must be non-toxic (for a complete review see [73]). Furthermore, many studies involving scaffolds also utilize a complete transection model to provide a convenient space for scaffold implantation. Yet the prevalence of complete SCIs is low thereby limiting the practical use of such therapies. Design

considerations must be optimized in attempt to maximize the utility of material scaffolds in CNS repair.

3.2 Cell Transplantation in combination with Scaffolds

Cell transplantation and biomaterial scaffolds each have unique advantages as therapeutic strategies for SCI. Cells can provide a large repertoire of signaling molecules, including anti-inflammatory cytokines and neurotrophic factors. However, cell fail to provide topographical guidance of regenerating axons resulting in random growth [3]. Biomaterial scaffolds can guide regenerating axons but cannot replace cell populations lost due to injury. Combining cellular and material strategies may provide synergistic effects and enhance recovery following SCI. Additionally; scaffolding can serve as a vehicle for cell transplantation, enhancing survival and engraftment at the injury site.

3.2.1 Schwann Cells (SCs)—Many groups have demonstrated improved regeneration following transplantation of SCs directly into the spinal cord, however recovery is often modest [63]. SCs play important roles in peripheral nerve and spinal cord injuries including debris clearance and trophic support of regenerating axons [54, 75]. The addition of SCs to biomaterial scaffolds as a combination therapy may enhance recovery following SCI. In several studies, SCs were reported to promote neural regeneration through PLGA scaffolds implanted into a complete transection model [12, 51, 53], however; functional recovery was not improved [53]. One potential benefit of combining cellular transplantation and scaffolds is the improved survival of transplanted cells. The effect of scaffold composition on SC survival in the spinal cord lesion was tested following contusive spinal cord injury [55]. Greater cell survival, neurofilament density within the lesion, and functional recovery were observed when SCs were transplanted in Matrigel compared to no scaffold or methylcellulose (MC). Matrigel, however, is generated from a sarcoma cancer cell line and is not approved for clinical use [40, 41].

Transducing SCs to express neurotrophins prior to seeding within scaffolds can also be used in combination strategies. SCs modified to express GDNF have been shown to decrease glial scarring and increase neural regeneration [16]. When mixed with Matrigel and seeded into guidance channels of poly(acrylonitrile)/poly(vinyl chloride) (PAN/PVC) scaffolds, GDNF-expressing SCs induced migration of host astrocytes into the scaffold and reduced the presence of CSPGs at the scaffold interface. In another study, SCs expressing NT-3 improved neuronal survival and locomotor recovery when injected into collagen scaffolds with NSCs. Independent verification is necessary to determine the clinical applicability of combination therapies involving neurotrophins, SCs, and biomaterial scaffolds. Furthermore, the use of SCs is limited by the lack of suitable sources. In most studies, SCs are isolated from peripheral nerves requiring loss of function at the donor site [43]. Several weeks are needed to expand SCs to obtain a sufficient number of cells for transplantation, thus limiting their use in acute treatments. Additional work is therefore needed to generate alternative sources of SCs.

3.2.2 BMSCs—Transplantation of bone marrow stem cells (BMSCs) has been shown to reduce cavitation of the injury site, enhance regeneration, and promote functional recovery [26, 67]. Recent studies have tested the efficacy of BMSCs in combination strategies utilizing scaffolds. When seeded in chitosan conduits, BMSCs led to improved spinal cord motor evoked potential amplitude [13]. However, no significant improvements were observed in locomotor recovery. Furthermore, BMSCs reduced the lesion size and the presence of macrophages. In another study, BMSCs expressing BDNF improved GAP-43⁺ fiber regeneration in Matrigel scaffolds and greater functional recovery versus Matrigel scaffold controls, but were not statistically different from unmodified BMSCs in Matrigel

[42]. Human BMSCs transplanted in collagen scaffolds reduced the lesion size and improved spatial learning and functional recovery following TBI [47]. While BMSCs show promise for repair following SCI and TBI, inconsistencies in reported locomotor recovery currently limits their use.

3.2.3 NSCs/ESNPCs—CNS-derived NSCs and embryonic stem cell-derived neural progenitor cells (ESNPCs) have been shown to replace neural populations following SCI. NSCs and ESNPCs can improve remyelination and integrate into axonal pathways, promoting functional recovery [27, 36, 37, 71]. Early work involving murine NSCs in PLGA scaffolds demonstrated that the combination could enhance functional recovery [65]. Brain-derived NSCs seeded in guidance channels in chitosan scaffolds increased the tissue bridge area following complete transection [52]. Recently, human NSCs in PLGA scaffolds were transplanted into a non-human primate model of SCI [56]. However, sufficient animals for statistical analysis were not used, so the outcomes were inconclusive. Following cortical impact TBI, laminin-based scaffolds containing NSCs improved spatial learning. Improvements in cognitive function were not observed in scaffold only or NSC only treatment groups [64]. The transition of NSCs into the clinical setting, however, is hindered by limited differentiation of NSCs into neurons *in vivo* and lack of appropriate donor tissue for human NSCs [2, 11].

ESNPCs may provide an alternative to NSCs. When transplanted in fibrin scaffolds containing NT-3 and platelet-derived growth factor (PDGF), ESNPCs enhanced functional recovery in a sub-acute hemisection model [34]. Prolonged release of growth factors, however, increased tumor formation from transplanted ESNPCs. Methods for purification of cell populations prior to transplant are necessary before the full utility of ESNPCs can be realized. Induced pluripotent stem cell-derived NPCs may provide an alternative to ESNPCs, however, more work is needed to increase purity and determine the utility of this cell type.

3.3 Neutralizing the Inhibitory Extracellular Environment

3.3.1 Chondroitinase ABC—CSPGs associated with glial cell membranes and the ECM are upregulated in response to CNS injury [5, 20] and are a barrier to regenerating axons by inducing growth cone collapse. The bacterial enzyme chondroitinase ABC (chABC) cleaves the inhibitory chondroitin sulfate GAG chains from the core protein, reducing the inhibition by CSPGs [76]. Bradbury *et al.* demonstrated that chABC application following SCI could promote regeneration [7]. Expanding on early work by Aguayo and colleagues, Houle *et al.* demonstrated CNS axons regenerating through a peripheral nerve graft entered the caudal spinal cord following ChABC treatment [28]. When combined with GDNF delivery, chABC promoted axon extension through peripheral nerve bridges accompanied by modest functional recovery [68].

Incorporating chABC treatment into combination therapies may have several advantages. CSPG degradation can increase axon extension into scaffolds and allow regenerating axons to exit the distal end of the scaffold and continue on to distal targets. ChABC delivery can also improve migration and integration of transplanted cells. In a poly-ε-caprolactone scaffold with NSCs expressing NT-3, intrathecal (IT) application of chABC increased migration of NSCs and improved locomotor recovery [29]. IT application of chABC following implantation PAN/PVC scaffolds containing SCs increased functional recovery despite having only a modest effect on CSPG degradation [19]. In a later study, CSPG degradation was shown to promote regeneration of propriospinal interneurons through a complete transection model of SCI [70].

Delivery of chABC has been predominantly IT using osmotic minipumps. While degradation of CSPGs has been shown, the rapid deterioration of enzymatic activity may limit the efficacy of treatment. Recently, the Bellamkonda group has demonstrated thermostabilization of chABC with the sugar trehalose can reduce the temperature-dependent loss of activity [45]. When encapsulated in lipid microtubes (LMTs), thermostabilized chABC retained activity for over 2 weeks. Transplantation of LMTs containing chABC and NT-3 led to increased fiber regeneration and improved stride length over chABC treatment alone. By modifying the inhibitory nature of the injured CNS, chABC delivery in combination with other strategies can help engineer a permissive environment for regenerating axons.

3.3.2 Myelin-associated Inhibition—MAI molecules, such as Nogo, Ompg, and MAG, limit regeneration of axons in CNS white matter and in areas with myelin debris [49]. Blocking MAIs with a Nogo-A antibodies has been shown to improve functional recovery by increasing axon regeneration [61]. An NgR competitive agonist peptide, NEP1-40 has been reported to elicit axon regrowth following SCI [46]. Combination therapies targeting MAIs have recently been reported. Cross-linking the Nogo-66 receptor antibody into a hyaluronic acid hydrogel improved neural fiber regeneration into the lesion [72]. Combination of methylprednisolone and NEP1-40 resulted in increased neuronal and oligodendrocyte survival leading to functional recovery in the BBB locomotor scale [32]. NgR vaccination combined with NSC transplantation lead to increased recovery over NgR vaccination and NSC groups alone [74]. Neutralizing the NgR receptor holds promise as part of a combinatorial strategy for repair of SCI.

3.4 Other Strategies

3.4.1 cAMP—Altering the intracellular signaling events following inhibition of neurons has been investigated as a therapeutic strategy for SCI [25]. By increasing the intracellular levels of cyclic adenosine monophosphate (cAMP) and protein kinase A activity, neurons were shown to overcome myelin-based inhibition [57]. Intracellular cAMP levels can be elevated using a non-hydrolyzable analogue of cAMP, dibutyryl cAMP (dbcAMP), or rolipram, a phosphodiesterase4 inhibitor (for a complete review see [25]). Combination therapies of olfactory ensheathing cell transplantation and rolipram resulted in modest recovery [8]. Oligo (ethylene glycol) fumarate (OPF) hydrogel scaffolds implanted with dbcAMP infusion reduced glial scarring and cyst volumes [60]. When combined with BMSC transplantation, locomotor recovery was enhanced 4 weeks following injury.

Results with cAMP have been difficult to replicate and adverse effects have often been observed. When combined with transplantation of glial-restricted precursor cells, cAMP reduced the survival of transplanted cells and did not improve recovery. Conversely, pretreatment of NSCs with dbcAMP promoted differentiation into neurons and cell survival inside chitosan guidance channels [38]. These results suggest that cAMP may be beneficial for neuronal populations but not glia. Iannotta *et al.* studied the effect of rolipram combined with clodronate, a biphosphonate drug that induces selective apoptosis of monocytes and phagocytic macrophages [30]. In this study, rolipram and clodronate exerted neuroprotective effects and improved hind limb function. The combination provided the greatest axonal sparing and reduction in lesion volume leading to significant improvements in motor recovery as assessed by BBB score.

3.4.2 Angiogenesis—The loss of blood vessel integrity in the spinal cord lesion is partially responsible for cell death during the secondary phase of injury. Improving blood vessel formation may reduce cell death and promote angiogenesis within the injury zone. NSCs modified to express vascular endothelial growth factor improved white matter sparing

following thoracic contusion SCI [39]. Sprouting resulted in reduced cavitation and was accompanied by increase in new vessels marked with von Willebrand Factor. Biomaterial PLGA scaffolds loaded with NSCs and endothelial cells (ECs) showed increased vessel and neurofilament density at the injury center [59]. Co-transplantation of NSCs and ECs produced endothelial barrier antigen-positive vessels, evidence of a newly formed BBB within the scaffold.

4. Conclusions and Future Considerations

Many recent combinatorial strategies have demonstrated synergistic benefits for repair of SCI and TBI. Addressing the multiple components of CNS inhibition in a unified therapy can provide incremental improvements and help maximize recovery. As demonstrated by the body of work reviewed here, combination therapies are highly variable. It is difficult to determine optimal components, such as cell types or scaffold materials, from the current literature. Few studies have attempted to maximize the benefit of combination therapies by changing one aspect of the therapy while holding the other treatments constant. Studies of this nature are necessary to determine which combinations are best for repair of the injured CNS.

In this mini-review, we've focused on the most common combination therapies reported in the last 5 years. Modifications to cell transplantation populations and biomaterial scaffolds have shown significant improvements; particularly in the case of chABC. Blocking the inhibition associated with CSPGs at the interface of the lesion can reduce this formidable barrier to host axon regeneration, thereby enhancing the utility of biomaterial scaffolds or cell transplants located within the injury epicenter. Standard methods for localized delivery of chABC, or alternatives to chABC, are needed to provide uniformity across various combination therapies. New developments in drug delivery from MTCs, microspheres, or biomaterial scaffolds may provide the answer.

Strategies that target known barriers to regeneration, such as degradation of CSPGs by chABC, often demonstrate therapeutic potential for use in combination therapies. Anti-MAIs treatments can help regenerating host axons overcome inhibition found in white matter and myelin debris. Exposing host neurons to cAMP can increase their intrinsic capacity for regeneration. Therapies with proven and well documented functions should be considered in all combinatorial strategies for SCI and TBI repair. While many inconsistencies in functional outcomes have often been reported with cAMP and anti-MAI therapies, this is likely due to the variability in the type and effectiveness of individual therapies. Engineering consistent and effective delivery methods can improve recovery and help elucidate the role of each individual therapy.

Additional therapeutic strategies include calcium channel blockers, N-methyl-D-aspartate receptor agonists, anti-inflammatory agents, semaphorin 3A blockers, as well as many other neuroprotective agents. Many of these strategies focus on reducing secondary cell death and damage and may reduce the number and magnitude of treatments necessary to restore function. Few studies have attempted to use these treatments in combination to engineer a permissive environment for regeneration. Like chABC, consistent and effective treatment methods are necessary. Engineering combination therapies with these characteristics can help determine the overall effectiveness of each strategy.

Despite anatomical, structural, and cellular differences between SCI and TBI, the desired therapeutic strategies are similar. In each case, sprouting and regeneration of spared axons is desired to promote innervation of new tissue and distal targets. Distinct cell populations are lost and must be replaced to restore normal function. Tissue remodeling is desired in both

injuries to establish a suitable growth substrate for regeneration. Therapies that address each of these components together in one therapy may help maximize restoration of function. The ability to engineer the CNS into a permissive environment will provide new promise for recovery.

Highlights

- Multiple barriers to regeneration exist following central nervous system trauma.
- Repair may benefit from combination therapies.
- The literature on combination therapies over the last 5 years was reviewed.
- Advantages and limits of current therapies are discussed with future direction given.

Abbreviations

BMSCs	bone marrow stem cells
CNS	central nervous system
chABC	chondroitinase ABC
cAMP	cyclic adenosine monophosphate
dbcAMP	dibutyryl cAMP
ESNPCs	embryonic stem cell-derived neural progenitor cells
ECs	endothelial cells
ECM	extracellular matrices
IT	intrathecal
LMTs	lipid microtubes
MC	methylcellulose
MAIs	myelin-associated inhibitors
MAG	myelin-associated glycoprotein
NSCs	neural stem cells
NT-3	neurotrophin-3
NgR	Nogo-receptor
Ompg	oligodendrocyte myelin glycoprotein
OPCs	oligodendrocyte precursor cells
OPF	oligo (ethylene glycol) fumarate
PDGF	platelet-derived growth factor
PAN/PVC	poly (acrylonitrile)/poly(vinyl chloride)
PLGA	poly-lactic-co-glycolic acid
SCs	Schwann cells
SCI	spinal cord injuries
TBI	traumatic brain injuries

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Figure 1. Neurofilament labeling demonstrates penetration and remarkably linear growth of axons within channels of freeze-dried agarose scaffolds. (A) Scaffold lacking growth factor. (B) Scaffold loaded with 2 µg recombinant human BDNF into walls and matrix-filled lumen of individual channels. Magnitude of linear axonal growth is significantly increased. (C) Best example of linear axonal growth through complete length of channel. Scale bars = 100 µm. Reprinted with permission from [62]

Table 1

Examples of recent combination therapies for repair of SCI or TBI

Injury	Cell Type	Biomaterial	Neurotrophin	Other Treatments	Citation
Thoracic SCI	OPC		CNTF		[9]
Cervical SCI		Agarose	BDNF		[62]
Thoracic SCI	SC	PAN/PVC	GDNF		[16]
Thoracic SCI	SC	PAN/PVC		chABC	[19]
Cervical SCI	OEC			Rolipram	[8]
Cortical TBI	BMSC	Collagen			[47]
Cortical TBI	NSC	Laminin/Fibronectin			[64]

Abbreviations: spinal cord injury (SCI), traumatic brain injury (TBI), oligodendrocyte precursor cell (OPC), Schwann cell (SC), olfactory ensheathing cell (OEC), poly(acrylonitrile)/poly(vinyl chloride) (PAN/PVC), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), chondroitinase ABC (chABC)