Endogenous and Exogenous CNS Derived Stem/Progenitor Cell Approaches for Neurotrauma

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Abstract: Neural stem/progenitor cells capable of generating new neurons and glia, reside in specific areas of the adult mammalian central nervous system (CNS), including the ependymal region of the spinal cord and the subventricular zone (SVZ), hippocampus, and dentate gyrus of the brain. Much is known about the neurogenic regions in the CNS, and their response to various stimuli including injury, neurotrophins (NFs), morphogens, and environmental factors like learning, stress, and aging. This work has shaped our current views about the CNS's potential to recover lost tissue and function post-traumatically and the therapies to support the intrinsic regenerative capacity of the brain or spinal cord. Recently, intensive research has explored the potential of harvesting, culturing, and transplanting neural stem/progenitors as a therapeutic intervention for spinal cord injury (SCI) and traumatic brain injury (TBI). Another strategy has focused on maximizing the potential of this endogenous population of cells by stimulating their recruitment, proliferation, migration, and differentiation *in vivo* following traumatic lesions to the CNS. The promise of such experimental treatments has prompted tissue and biomaterial engineers to implant synthetic three-dimensional biodegradable scaffolds seeded with neural stem/progenitors into CNS lesions. Although there is no definitive answer about the ideal cell type for transplantation, strong evidence supports the use of region specific neural stem/progenitors. The technical and logistic considerations for transplanting neural stem/progenitors are extensive and crucial to optimizing and maintaining cell survival both before and after transplantation, as well as for tracking the fate of transplanted cells. These issues have been systematically addressed in many animal models, that has improved our understanding and approach to clinical therapeutic paradigms.

Key Words: Neural stem/progenitors, traumatic brain injury, spinal cord injury, transplantation, neurotrophins, endogenous, regeneration.

INTRODUCTION

The goal of regenerative medicine is to utilize the body's intrinsic population of cells to repair or grow cells, tissues, and organs, and includes strategies such as stem cell therapy. The regenerative potential of stem cells in neurological disorders or injuries is being investigated in a variety of therapeutic contexts. Among the illnesses that could benefit from stem cell therapy are traumatic brain injury (TBI), stroke, spinal cord injury (SCI), and other neurodegenerative conditions such as Parkinson's Disease and Alzheimer's Disease. Devising innovative stem cell strategies to enhance regeneration of the nervous system holds great promise for millions of individuals.

Neural stem cells have the capacity to proliferate in an undifferentiated state, and to self-renew, and they are multipotent, giving rise to neurons, astrocytes, and oligodendrocytes. Neural stem cells can be isolated from embryonic or adult brain or spinal cord tissue, and can also be derived from more primitive embryonic stem cells cultured from the blastocyst [1-4]. Neural stem cells induced from embryonic stem cells will not be reviewed here, as this has been done recently by others [5, 6].

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The ideal source of stem cells for repair of the damaged central nervous system (CNS) is an issue of ongoing debate, with some arguing the side that all stem cells are alike and that all can be triggered to differentiate into the appropriate phenotype in response to cues from the local microenvironment. On the other hand, the impetus to transplant region specific stem cells is based on studies that suggest that the local microenvironment, or niche, that a stem cell is surrounded by dictates its behaviour and fate. Since there is evidence to support either side of the argument, discriminating between the innate capability of neural stem cells to determine their own phenotypic fate and the environment-specified differentia tion of these cells is crucial [7, 8].

The environmental signals governing the survival and differentiation of neural stem cells may be region-specific within the CNS and may influence neural stem/progenitor cells in the immediate surroundings to differentiate into very specific phenotypes. In particular, adult neural stem/progenitor cells transplanted into the hippocampus differentiate into neurons, whereas the same stem/progenitors transplanted into the spinal cord produce astrocytic progeny [9]. Moreover, it was shown that hippocampal astrocytes instruct the transplanted stem cells to differentiate into neurons *in vitro*. Exposure to the ideal microenvironment *in vivo* allows transplanted stem cells to differentiate in a region appropriate manner and integrate into the spinal cord circuitry. As such, an important consideration when transplanting stem cells is to influence differentiation in a way that is appropriate to the local

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CNS region. Among the niche cues that may regulate stem cell fate are those that influence survival, migration, adhesion, self-renewal, and differentiation. For this reason, adult stem cells may have advantages over embryonic or fetal cells for transplantation studies, firstly because they are the most similar to the cell types they are designed to replace, secondly because they may have less oncogenic potential than embryonic stem cells, and thirdly because of the avoidance of some of the ethical issues surrounding the use of embryonic or fetal stem cells.

There are at least two possible strategies involving stem/progenitor cells for repair and regeneration of the injured brain and spinal cord: stimulation of endogenous stem/progenitor cells or transplantation of exogenous stem/progenitors, both of which have their respective advantages and disadvantages. The first strategy is to enhance endogenous stem/progenitor cell proliferation and influence their differentiation by providing extrinsic cues. In contrast to transplantation studies, there have been very few studies of endogenous neural stem/progenitors for repair or regeneration of the brain or spinal cord, and all have involved the use of neurotrophic or growth factors. Much of the knowledge in this area is based on the investigation of the normal role of endogenous neural stem cells, progenitors, and precursors, their differentiation capacity, and their response to cellular and molecular cues. The major advantage of augmenting the endogenous stem cell response is that it obviates the need for an external cell source. However, the endogenous neural stem/progenitor cell response to trauma may be too weak and the number of cells too low, even for augmentation by external cues. Determining and administering the exact combination of molecular signals in the appropriate sequence required to induce proliferation and differentiation of endogenous stem/progenitors are also major obstacles to the implementation of a therapeutic strategy based on endogenous stem/progenitors. Moreover, enhancing the endogenous response may be limited to specific regions, especially in the case of TBI, based on the limited regional distribution of the multipotent cells in the hippocampus and the subventricular zone (SVZ).

On the other hand, transplantation therapies aim to restore neurological function post-injury by introducing a new population of cells that have the capacity to differentiate and reintegrate into the host tissue in a manner that appropriately replaces lost neuronal tissue and function. There are two main mechanisms that can account for such regenerative support [10, 11]. Firstly, differentiated neurons and/or glial cells can reintegrate into the host brain or spinal cord in the region of trauma or other disease, and make appropriate connections with the surrounding tissue thereby either replacing lost neurons or supporting surviving and/or regenerating neurons. The second possible mechanism is that transplanted stem cells may secrete neurotrophic or growth factors into the host tissue to support neuronal survival and regeneration of host tissue. The disadvantages of exogenous neural stem cell transplantation include limited tissue availability, the requirement for immunosuppression, impaired cell survival, political and ethical considerations of stem cell procurement, and the risk of tumorigenicity.

This review will encompass both endogenous and transplanted stem/progenitor cells in the adult CNS and strategies to stimulate their response to trauma *in vivo* and/or maximize their potential to repair the injured brain or spinal cord after transplantation. Although there has been extensive research using non-CNS derived stem/progenitor cell sources such as bone marrow, skin, olfactory ensheathing glia (OEG), schwann cells and embryonic stem cells for neurotrauma, we will be focusing exclusively on neural stem cells derived from the adult, fetal, and early post-natal brain and spinal cord. We will begin with an introduction of the current status of SCI and TBI treatment in humans, followed by an overview of the current knowledge of stem/progenitor cells in the brain and spinal cord. This will be followed by a discussion of the use of neurotrophic, growth factors, and other morphogens for stimulating the endogenous stem cell response after experimental SCI and TBI. Finally, we will review the experimental and human studies involving transplantation of neural stem/progenitor cells derived from the CNS for SCI and TBI, and will discuss strategies for manipulating neural stem/progenitor cells *in vitro*, labeling strategies for identifying transplanted cells, and grafting parameters.

CURRENT STATUS OF SCI AND TREATMENT IN HUMANS

In most countries acute SCI occurs at an annual rate of 20-40 per million, and the main causes are motor vehicle crashes, sports and recreation, accidents at work, violence, and falls at home [12]. Each complete cord injury costs society several million dollars for medical costs and lost earning in addition to the great personal loss sustained by the victims and their families [13]. There is evidence that a secondary injury of the cord occurs after the initial mechanical trauma, and therapy can interrupt this secondary process and lead to improved recovery. The improved neurological recovery in SCI patients in randomized, prospective clinical trials of pharmacotherapy with either methylprednisolone or GM-1 ganglioside supports the concept of a treatable secondary injury [14, 15]. Unfortunately, the improvements were minimal. In general, there is greater potential for recovery in incomplete injuries, although in many instances little recovery occurs. Studies of spinal cord repair by enhancing the endogenous regenerative stem/progenitor cell response or by transplanting neural stem/progenitors have great relevance to patients with complete SCI and also those with incomplete SCI who may not have sufficient residual multipotent cells to respond to stimulation by exogenous agents.

CURRENT STATUS OF TBI AND TREATMENT IN HUMANS

Epidemiological data in many countries shows that almost 2 million people a year incur TBI. TBI is defined as externally inflicted brain trauma and as with SCI, most often occurs as a result of falls, motor vehicle crashes, sports, and violence [16]. Almost 100,000 individuals suffer a TBI annually that results in long-term, severe deficits in function. TBI can impair a person's physical, cognitive, psychological, emotional, and social functioning [17]. As with SCI, the losses in potential earnings, the large medical and rehabilitative expenses, and the disruption in family life are staggering [16]. Although there have been substantial improvements in the acute care of TBI, including quicker emergency care, faster, safer, and more effective transportation to special facilities, and innovations in medical management of acute TBI, there are currently no

effective long-term clinical therapies to regenerate injured brain tissue and function [17]. The focus of current treatments includes physical, cognitive, and behavioral rehabilitation, drug therapy, environmental manipulation, psychotherapy, education, and assistive technology.

Over the past decade, there have been many clinical trials focusing on the potential of a variety of neuroprotective agents to improve the outcome of TBI patients. However, none of the trials that has reached phase III has demonstrated significant efficacy in treating clinical TBI, despite the encouraging pre-clinical data [18-20]. Most of the effort to date has been towards minimizing the secondary damage that occurs after the initial trauma, based on new information that has emerged about the mechanisms of secondary injury. More recently, the focus of pre-clinical studies has shifted to the potential of enhancing the endogenous response of stem cells in the brain and/or supplementing this response with an exogenous source of neural stem/progenitor cells (see below). Both of these strategies have the potential to significantly impact the acute and long-term management of clinical TBI.

STIMULATING THE ENDOGENOUS NEURAL STEM CELL RESPONSE

Endogenous Stem/Progenitor Cells in the Brain and Spinal Cord

Neural stem cells can self-renew and are multipotential, and are found in embryonic, fetal, and adult mammalian brain [1-4]. It is now widely accepted that the adult mammalian brain and spinal cord, including that of primates and humans, contains endogenous stem/progenitor cells, supporting the concept of plasticity in the mature CNS. In adult brain, the subependymal layer of the lateral ventricles and the dentate gyrus of the hippocampus retain proliferative and neurogenic activity [21]. The subependyma of the forebrain contains a rapidly dividing population of precursors that generate neurons and glia *in vitro* [22] and migrate rostrally to the olfactory bulb *in vivo*, where they differentiate into neurons [22]. The adult mammalian CNS contains both multipotent and lineage restricted stem cells [23] which are regulated by trophic factors [24-29]. Cells with somewhat less potential have also been demonstrated in the adult mammalian spinal cord by our laboratory and others [9, 30-34]. Frisen and others [31, 35, 36] hypothesized that nestin expressing precursors exist around the central canal and proliferate and migrate to the site of SCI to contribute to the glial scar. Nestin is found in neuroepithelial precursors [26, 37-40] both *in vivo* and *in vitro* in the adult CNS [27, 34, 41-43]. During spinal cord development, nestin is observed in stem cells lining the central canal, and is progressively reduced with maturation [44]. However, nestin is not a specific marker for stem/progenitor cells, and is also found in reactive astrocytes associated with injury [36]. Recently, neural stem cells in the spinal cord were shown to reside close to the central canal since multipotential, self-renewing neurospheres were generated only when the cultured tissue included parts of the central canal [45, 46]. However, Yamamoto generated spheres from both medial and lateral sections of the adult rat spinal cord [47], although the spheres derived from peripheral spinal cord regions were more limited in their self-renewal and multipotential properties. This suggests that the peripheral spinal cord contains neural progenitors that are more restricted in terms of proliferation and differentiation than the neural stem cells that can be isolated from the ventricular neuraxis of the brain and spinal cord [46].

Injury as a Stimulus

In lower vertebrates such as amphibians and lizards, the ependyma in the spinal cord plays a significant role in neuronal regeneration [48-51]. In these animals, the ependymal cells of the transected spinal cord rapidly proliferate, migrate and differentiate to regenerate the cord. The proliferating ependymal cells extend long processes to guide the caudally regenerating central axons, and subsequently, these cells differentiate into neurons which send axons rostrally within the regenerated cord [52-54]. Proliferation of ependymal cells is common during embryonic and early postnatal periods of development in most species, however, ependymal cell turnover declines significantly postnatally [55]. In immature [56, 57] and adult [30, 55, 58-60] mammals, there is proliferative activity in the ependyma of the normal and injured spinal cord [61-65].

Ependymal cell proliferation has been observed in response to several types of spinal cord trauma, including compression injury [33, 64, 65], contusion injury [61], dorsal funiculus incision [31], and spinal cord transection [62]. In the adult rat it has been shown that multipotent ependymal cells can divide and proliferate according to the severity of SCI. Electron microscopic (EM) observations have been made by Matthews (1979) who describe the relationship between regenerating axons and ependymal cells; clusters of axons became engulfed by the ependymal cells, and in some instances, mesaxons were formed suggestive of and “attempt to support a regenerative process” [62].

The capacity of adult endogenous stem/progenitor cells to migrate and differentiate after activation by injury depends upon the type of lesion and the germinative zone from which they arise. Several animal models have been utilized to determine the involvement of subependymal cells in the repair of acute or chronic injury in the brain. These studies have demonstrated that subependymal cells are reactivated in response to different insults. The proliferation rate of subependymal cells increased following brain injury [66, 67], seizure [68], transection [66], ischemia [69-72], and demyelination [73, 74]. Lateral fluid percussion injury was shown to stimulate an increase in the proliferation of endogenous neural stem/progenitor cells in both the subgranular zone of the dentate gyrus and the subependymal zone under the injury site [67]. These studies demonstrate the ability of neural stem/progentor cells in the adult brain to undergo increased proliferation, ectopic migration, and multipotential differentiation. This differentiation appears to be lesion specific, since trauma primarily induces the differentiation of astrocytes, a loss of neurons favors neuronal differentiation, and oligodendrocyte differentiation occurs essentially in response to demyelination [75]. Thus, it seems that local cues originating either from the lesion or from the environment in which cells migrate are important to direct their differentiation into the appropriate cell fate.

Thus, injury to the CNS leads to proliferation of normally quiescent endogenous neural stem cell populations and the resultant cell types are predominantly of glial origin. For example, most endogenous neural stem cells that reside in the

adult rodent brain differentiate into astrocytes following traumatic injury [66, 76]. Following a dorsal funiculus lesion of the spinal cord, ependymal cell proliferation increased, ependymal cells migrated to the lesion site and expressed GFAP [31]. We have recently shown that even a minimal needle injury adjacent to, but not directly involving the central canal, induces an ependymal cell response where endogenous ependymal stem/progenitor cells proliferate and express nestin, migrate from the region of the central canal, and differentiate to an astrocytic phenotype (Mothe and Tator, in press).

Our laboratory has previously reported extensive proliferation and centrifugal migration of ependymal cells into the gray and white matter of the injured cord after clip compression SCI in adult rats [65]. More recently we have reported that the growth factors epidermal growth factor (EGF) and fibroblast growth factor 2 (FGF2), enhanced the proliferation of these cells in normal [77] and injured rats [78]. Moreover, we found a small but significant improvement in clinical neurological recovery after intrathecal administration of these agents by osmotic minipump for 14 days after clip compression SCI of moderate severity [78]. We hypothesized that the improvement was due to enhancement of neuroprotection and regeneration. However, it is still unclear whether these actively proliferating ependymal cells in adult mammals can be stimulated to exert the same major beneficial post-traumatic regenerative effects as those in lower vertebrates.

Although stem/progenitor cells in the adult mammalian brain and spinal cord are stimulated to proliferate, migrate and differentiate after injury, it is clear that they are unable to reconstitute the normal cellular architecture of the CNS. Supplementation of these endogenous neural stem/progenitor cells with neurotrophins or other agents or transplanted neural stem/ progenitor cells will likely be required, especially for treatment of major SCI or TBI.

Environmental Effects on Neurogenesis

Neurogenesis in the adult CNS, specifically the dentate gyrus, can be modulated by environmental factors. Adult hippocampal neurogenesis is an ideal example of the effect of behavioural and physiological processes on the endogenous stem cell response. Adult hippocampal neurogenesis has been shown *in vivo* in adult rodents, monkeys, and humans [79-83]. Neurogenesis begins in the dentate gyrus of the adult hippocampus, specifically the subgranular zone, which constitutes the innermost region of the granule cell layer. The proliferating cells migrate into the granule layer and extend processes into the molecular and CA3 regions [84-86]. This maturation process occurs quickly- as soon as 4 days after cell division has occurred [86]. Interestingly, these newly formed neurons have the same morphology as the existing neurons in the granular layer of the hippocampus [87], and exhibit the same electrophysiological characteristics as mature neurons [88].

Studies investigating the differentiation potential of adult hippocampal multipotent precursors transplanted into regions outside of the dentate gyrus have shown that these cells can adopt a variety of fates. For example neural precursors isolated from the adult rat hippocampus and transplanted into the rostral migratory stream, migrate to the olfactory bulb and differentiate into a neuronal phenotype unique to the olfactory bulb, but not the hippocampus [89]. However, adult hippocampal precursors are limited in their differentiation potential, as demonstrated by their ability to express a functional neuronal phenotype when transplanted into the retina, but not any of the photoreceptor phenotypes [90,91]. *In vitro*, neural stem/progenitors isolated from the hippocampus behave like those isolated from the SVZ of the brain. They grow as free-floating neurospheres, exhibit a proliferative response when cultured in the presence of the mitogens EGF and FGF-2, and are self-renewing. Upon removal of the mitogens and adherence to a substrate, they exhibit multipotentiality by differentiating into neurons, astrocytes, and oligodendrocytes [87].

The rate of neurogenesis in the mature hippocampus changes in relation to large and small scale environmental fluctuations. Signals expressed by the adult hippocampus influence the proliferation, survival and differentiation of endogenous stem cells, which are augmented by a wide variety of extrinsic factors including growth factors [92-94], physical activity [95, 96], learning [97], vitamin E deficiency [98], caloric restriction [99], seizures [68, 100-103], enriched environment [104-106], and antidepressants [107]. These have all been shown to enhance hippocampal neurogenesis, whereas aging [108-110], stress [111-113], opiates [114], reduced serotonin [115, 116], and methamphetamines [117] have all been shown to inhibit neurogenesis. Moreover, these environmental factors influence one another to achieve a balance in neurogenesis. For example, it has been shown that an enriched environment [110] or a reduction in corticosteroid levels [108] alleviates the age-associated decline in neurogenesis in the adult hippocampus.

The age-related decrease in hippocampal neurogenesis may be due to changes in multipotent precursor cell properties, a decline of precursors over time, or a reduction in overall levels of molecular signals that instruct neurogenesis. The literature investigating the causes of this age-related decline is extensive, and is highly relevant to the understanding of the possibilities of manipulating the endogenous neural stem/progenitor population to devise strategies for neuronal replacement therapies.

Neurotrophic and Growth Factors in Experimental Spinal Cord and Brain Injury

The neurotrophins are a group of polypeptide growth factors comprising nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and others that regulate neuronal survival, division, and differentiation, and promote axonal growth [118-128]. In this review, agents that influence the growth and differentiation of stem/progenitors cells are referred to collectively as neurotrophic factors (NFs) and thus include EGF and FGF2. During development, the presence of target derived NFs can prevent apoptosis in receptive neurons, and thus promote the survival of cells that have made appropriate connections [126]. *In vitro*, NFs promote axon growth, synaptic rearrangement and dendritic sprouting, and the survival and proliferation of neuronal precursors [129]. *In vivo*, many NFs exert neuroprotective effects [118, 130-136] and many including NGF, BDNF, NT-3, FGF1 and FGF2 improve recovery after experimental SCI, both in the early stage as a neuroprotective agent possibly to prevent secondary injury mechanisms, and in the later stage by processes such as axonal regeneration and myelination [119, 137-144]. Current

views on the lack of regeneration after SCI in adult mammals stress a deficiency of NFs [138, 145-147]. Intrathecal FGF2 has been shown to improve recovery after SCI in rats [143]. Injury can cause upregulation of gene expression of growth factors including FGFs and their receptors [123, 148], and the low affinity NGF receptor [145, 149, 150]. For example, FGF2 expression was elevated specifically in astrocytes after SCI [148]. Injury to a peripheral nerve by axotomy, or direct contusion of the spinal cord causes upregulation of the low affinity NGF receptor [145, 149, 150]. FGF1 has also been detected in ependymal cells *in vivo* [151].

There have been several *in vitro* studies examining the effects of growth factors on progenitor cells from the fetal rodent brain [152-154], and a small number of *in vivo* studies of this type in the adult brain [24, 25]. In contrast, there is a paucity of either *in vitro* [34, 155, 156] or *in vivo* [155, 157] studies of these factors on stem/progenitor cells in the rodent spinal cord. There have been no studies examining the effect of NFs on stem/progenitor cells in the adult human spinal cord. Although little is known about the *in vivo* response of brain subependymal cells to TBI [21, 29], it has been suggested that adult subependymal cells may migrate and generate new cells to replace damaged neural tissue, as described above. The precursor cells isolated by Weiss'group from the embryonic or adult mouse brain striata proliferated *in vitro* in response to EGF to produce cells, which differentiated into neurons and glia [34]. Importantly, Weiss' group reported that the combination EGF and FGF2, although ineffective alone, induced CNS stem cells isolated from the adult mouse spinal cord to proliferate and differentiate *in vitro* into neurons, astrocytes, and oligodendrocytes [34]. Also, O'Hara and Chenoff reported that migration and proliferation of injury-reactive ependymal cells in the axolotl spinal cord is dependent on EGF [158]. In contrast, Gage's group reported recently that *in vitro* FGF2 alone is sufficient to generate progenitors in the adult rat spinal cord [156].

Our laboratory's study with EGF and FGF2 was the first *in vivo* therapeutic trial of NFs for the purpose of enhancing the response of endogenous ependymal stem cells after SCI [78], although this approach had been tested on the ependymal/ subependymal stem/progenitor cells in the normal adult brain [24, 159, 160], especially with respect to neurogenesis as a therapeutic strategy for degenerative disorders. We found that ependymal region cells in the spinal cord behave like stem cells after SCI, and intrathecal EGF and FGF2 enhance their proliferation and migration [78]. Van der Kooy's laboratory recently reported that EGF and FGF2 infusion into the fourth ventricle of adult mice caused *in vivo* ependymal cell proliferation in the cervical spinal cord and neurosphere formation *in vitro* from the dissected ependymal zone [46]. Thus, ependymal cells in the adult mammalian spinal cord proliferate, migrate and assist regeneration after SCI, and these functions can be enhanced by NFs such as EGF and FGF2. However, in contrast to the adult forebrain subependyma, there have been few *in vivo* studies of the precursor cell qualities focussed on the ependyma of the adult spinal cord, especially afer SCI, and the role of transplantation in augmenting their response to injury. Differentiation of pluripotent neural stem cells transplanted into the adult rat normal and injured spinal cord appears to be mainly restricted to the glial lineage [161]. This suggests that either *in vitro* induction toward a neuronal lineage prior to transplantation or manipulation of the microenvironment in the injured spinal cord may be necessary for neuronal differentiation. It is likely that NFs or other differentiating agents will be necessary to direct these cells along the appropriate path.

With respect to TBI, it has been shown that under normal conditions, growth factor infusion into the brain can increase proliferation, migration, and differentiation of neural stem cells in the SVZ [24, 25, 93]. Under pathological conditions, growth factor infusion improves the contribution of resident neural stem cells to regenerative and repair mechanisms in the brain. For example, Lachapelle showed that a single injection of FGF-2 prior to transplantation primes SVZ cells and enhances their myelin forming capabilities [162]. In the normal hippocampus, IGF-1 infusion increases neural stem cell proliferation and neurogenesis [94]. Under pathological conditions, IGF-1 and glia-derived neurotrophic factor (GDNF) enhance neural stem cell proliferation and survival time [163]. Shingo showed that erythropoietin (EPO) regulates the production of neural progenitors both *in vitro* and *in vivo* by neural stem cells in the mouse forebrain. Infusion of EPO into the lateral ventricles of adult mice resulted in a decrease in the numbers of neural stem cells in the SVZ, an increase in new cells migrating to the olfactory bulb, and an increase in new olfactory bulb interneurons [164]. *In vitro*, neural stem cells can be induced to differentiate by mitogen removal [42, 165]. Moreover, the proportions of neurons, oligodendrocytes, and astrocytes can be manipulated by adding various NFs to promote differentiation along a particular lineage [166]. It has been shown that astrocytic differentiation can be enhanced with ciliary neurotrophic factor (CNTF) [165, 167-172]. Astrocytes and gliosis appear to have a permissive or non-permissive role on axonal regeneration [125, 173-178], depending on cell maturity [179]. Platelet derived growth factor-AA (PDGF-AA), FGF2, IGF-1, CNTF, and NT3 have all been shown to enhance the oligodendrocyte lineage [180-182]. PDGF-AA has shown a more pronounced effect on oligodendrocyte precursors *in vivo* in mice overexpressing PDGF-AA [182] and in rats after intracerebral administration [180]. PDGF-AA from recombinant fibroblasts injected intrathecally in rats increased the number of O2A progenitors in the spinal cord [183]. PDGF-AA knockout mice showed a significant decrease in the number of O2A progenitors and mature oligodendrocytes in the spinal cord during development [182]. The neuronal lineage has been shown to be enhanced by the administration of IGF-1 [94, 184, 185]. IGF-1 stimulated mouse CNS neural precursors to differentiate into neurons *in vitro* [184], and IGF-1 knockouts had reduced numbers of neurons *in vivo* [185]. The systemic infusion of IGF-1 increased progenitor proliferation and selectively induced neurogenesis in the progeny of adult neural progenitors in the rat hippocampus [94]. Interestingly, Benraiss recently found that intraventricular adenoviral BDNF induced neurogenesis from endogenous progenitors in the ependyma/subependyma of the rat brain [159].

Regulatory Mechanisms in Lineage Determination

Several regulatory mechanisms have been identified that play a role in neuronal lineage determination and the developmental potential of stem cells from the CNS. The decision to generate neuronal versus glial progenitors is a critical step during neural stem cell differentiation [186]

and NFs play an important role in this process. Because the proliferation and differentiation of individual stem cell colonies can be monitored in culture, this has permitted studies to be conducted on the role of growth factors on individual lineage determination. More recent studies have focussed on the regulation of neurogenesis and gliogenesis through differential gene expression. It has been shown that cell-intrinsic changes in gene expression can influence the fate of CNS stem/progenitor cells regardless of any opposing effects of added neurogenic growth factors. These intrinsic changes affect cell sensitivity to lineage determination factors.

Neural stem cells from different stages in development respond very differently to extracellular factors. Notch is a transmembrane receptor that is involved in the inhibition of neuronal differentiation and maintenance of mammalian neural stem cells [187]. It terminates neurogenesis and initiates gliogenesis mainly through inhibition of proneural gene expression [188]. Proneural basic helix-loop-helix (bHLH) transcription factors (i.e. Ngn2, Mash1, Math1, and NeuroD) drive neurogenesis and inhibit gliogenesis [189-193]. Notch1 expression is enhanced in the adult rat after SCI, and neurogenic bHLH factors Ngn2, Mash1, and NeuroD1 could not be detected in injured tissue [194]. This HLH gene family also included Id genes which have been implicated in astrocyte proliferation and are upregulated in neural progenitor subpopulations after SCI [195], Hes1 and Hes5 which suppress neurogenesis [189], and oligo1 and oligo2 for oligodendrocyte differentiation [196, 197]. There is evidence that NFs stimulate neuronal differentiation by altering the balance of expression of various bHLH transcription factors [189]. Investigations of Notch and specific bHLH factors and their role in NF effects on neural stem cells in the brain and spinal cord are important in order to optimize potential future NF therapy.

For example, the LIF- and Notch-signalling pathways maintain the multipotentiality of stem cells in the early stages of embryogenesis. In contrast, the induction of these same pathways promotes gliogenesis in neural stem cells at later developmental stages [186]. Paradoxical evidence shows that CNTF and LIF both enhance gliogenesis and self-renewal in adult stem cells [165, 166, 198]. Similarly, bone morphogenetic proteins (BMPs) enhance neural differentiation during the early stages of CNS development, but at later stages enhance astrocytic differentiation [199, 200]. Moreover, BMPs and noggin control the balance between neurons and astrocytes in the adult CNS [31, 201]. Thyroid hormone also instructs neural stem cells to adopt an oligodendrocytic phenotype, as well as promoting astrocytic differentiation [165]. Sonic Hedgehog (Shh) is another morphogen that has been shown to enhance the proliferation and maturation of adult neural stem cells [202]. Machold showed that oral administration of a Shh agonist enhances proliferation in the adult SVZ and hippocampus, and that this process is mediated by an upregulation of Gli1 signalling [203].

Overall, these studies suggest that a combination of NFs, morphogens, and other factors could be infused into the injured brain or spinal cord at specific times and/or used to prime cultured neural stem/progenitor cells *in vitro* prior to transplantation. Such strategies could enhance proliferation, migration, and stimulate differentiation into the appropriate cell types required for tissue and functional regeneration. It should be noted that studies of pre-differentiated or post-differentiated transplanted stem/progenitor cells in either the brain or spinal cord have only recently been considered, and thus there is very little published data to suggest which lineage would optimize post-traumatic recovery. Furthermore, it is highly likely that there will be major regional specificity within specific sites of the brain or spinal cord with respect to the optimal differentiation pathway, and the mechanisms to achieve it. For example, in the spinal cord the ingredients required to restore long tract function in the thoracic cord and hence locomotion will be very different from those required to repopulate the conus medullaris with neurons subserving bowel and bladder function.

NEURAL STEM CELL TRANSPLANTS FOR SCI AND TBI

As noted above there are a number of reasons why neural stem cells are appealing candidates for cell replacement therapy for treating CNS trauma. Neural stem cells can be propagated in culture to produce large numbers of progeny. They are multipotent and can generate both neurons and glia. Neural stem cells naturally secrete a variety of neurotrophic factors which can contribute to brain and spinal cord repair. They have the ability to migrate through the CNS and adopt the phenotype of surrounding cells after transplantation. Therapeutic genes could also be introduced into neural stem cells so that upon transplantation they can migrate to damaged areas and deliver the gene product. Also, there is some evidence that region-derived stem cells may prove to be therapeutically more effective, such that stem cells derived from the spinal cord would be most effective for treating SCI, and brain-derived stem cells for TBI.

Labeling and Identifying Transplanted Neural Stem Cells

In transplantation studies, it is important to be able to unequivocally identify the transplanted cells and demonstrate that they have survived and integrated into the host CNS. A number of different methods can be used to label neural stem cells so that they can be identified after transplantation *in vivo*. Cells can be pre-labeled *in vitro* prior to transplantation with cell compartment specific labels or reporter genes, or intrinsic markers of grafted cells can be detected in the host in cases of allografts, xenografts, and gender-specific grafts, or if cells are derived from transgenic animals expressing a reporter gene. Determining the most suitable approach to label stem cells for subsequent transplantation depends on a number of considerations, such as the length of time the cells will need to express the label *in vivo* until visualization, whether the protocol to detect the label is compatible with other analyses such as phenotypic determination of grafted cells, and whether the label is stably incorporated or has the potential to transfer to endogenous cells. The advantages and disadvantages with each approach will be discussed. A number of excellent reviews and experimental studies have examined different labeling methods for many cell types [204-207].

Nuclear Labels

Stem cells can be pre-labeled *in vitro* with nuclear, cytoplasmic, or membrane labels, or reporter genes. Nuclear, cytoplasmic, and membrane labels can be utilized to label different cellular compartments. For example, the nuclear label bisbenzimide (Hoechst) is a fluorescent nucleophilic dye that

can be directly visualized *via* UV fluorescence. Hoechst has been widely used to label cells prior to transplantation [208-210]. However, a major drawback to pre-labeling of grafted cells with Hoechst is the tendency for the dye to leak out of cells into host cells over time. A better option for nuclear labeling in chronic grafting experiments is the use of tritiated [^{3}H]-thymidine or bromodeoxyuridine (BrdU) which show long-term stability in donor cells [89, 157, 211-213]. ^{3}H-thymidine and BrdU are stably incorporated into the DNA of dividing cells. Transplanted cells labeled with ^{3}H-thymidine are detected *via* autoradiography, while BrdU labeling is visualized with an anti-BrdU antibody *via* immunohistochemistry and can yield a colorimetric reaction product or fluorescent signal depending on the secondary antibody conjugate. Double-immunolabeling can also be performed to examine the phenotype of labeled cells. However, if grafted cells continue to divide *in vivo* the label will be diluted with continued proliferation. Also, a major disadvantage of nuclear labels is the lack of visualization of cell morphology.

Cytoplasmic Labels

The morphology of grafted cells can be visualized *in vivo* without additional immunostaining if cytoplasmic or membrane labels are used. Examples of cytoplasmic labels include fluorescent microspheres or beads, fluorescent dyes, Fluoro-Gold and gold particles, and iron oxide nanoparticles. Most of the cytoplasmic labels are internalized *via* endocytosis and the amount of label within a cell depends on the concentration of the label in the culture medium. After internalization, usually into intracellular vesicles and/or lysosomes, cytoplasmic labels may be degraded which will diminish their visibility over time [206]. Latex microspheres or beads are coated with fluorescent dyes such as fluorescein or rhodamine and are taken up by dissociated cells. Fluorescent microspheres and beads have been used to label cells in a number of studies but appear to be useful only for short term labeling and show considerable variability in uptake between cell types [206]. Another disadvantage is that the beads were shown to be toxic for a neural cell line [205], and debris and dead cells also bind the label [205, 214], and thus the label can potentially be transferred to host phagocytic cells.

Cytoplasmic fluorescent dyes that have application for stem cell labeling include Fast Blue, rhodamine-dextran-amine (RDA), carboxyfluorescein esters, and Thiol-reactive Cell Tracker™ probes (Molecular Probes Incorporated, Eugene, OR). In general, these dyes appear to be useful only for short-term studies due to fading of the fluorescent signal. Fast Blue is known to leak to host cells and has been shown to bind with cellular debris *in vitro* [204, 205]. In contrast, RDA, carboxyfluorescein esters, and CellTracker™ probes label live cells in culture. The CellTracker™ probes and carboxyfluorescein ester derivatives are retained in cultured cells after a number of cell divisions and the dye does not leak to adjacent cells because once the dye is internalized it is modified to produce a membrane-impermeable reaction product. For example, CFDA-SE (carboxyfluorescein diacetate, succinimidyl ester) passively diffuses into metabolically active cells and is colorless and nonfluorescent until its acetate groups are cleaved by intracellular esterases, yielding succinimidyl ester groups which react with intracellular amines forming highly fluorescent conjugates that are intracellularly retained (Molecular Probes). However, we have found that CFDA-SE also appears to be retained in dead or dying cells (unpublished observations), and these fluorescent dyes can be toxic to cells at high concentrations, indicating that optimal labeling parameters need to be determined for each type of cell. Fluoro-Gold, commonly used as a retrograde tracer in neuroanatomical studies, has also been used to pre-label cells for transplantation but loss of fluorescent label in grafted cells and leakage to host cells have been reported [215]. Most of the cytoplasmic labels are internalized *via* endocytosis and the amount of label within a cell depends on the concentration of the label in the culture medium. After internalization, usually into intracellular vesicles and/or lysosomes, cytoplasmic labels may be degraded which will diminish their visibility over time [206].

Dissociated cells labeled with gold particles have the advantage of both light and electron microscopic (EM) identification but transfer of gold particles to host macrophages has also been detected, as noted by Harvey [206]. Recently, superparamagnetic iron oxide nanoparticles have been used for long-term labeling of transplanted stem cells [216]. A unique advantage of this cytoplasmic label is that noninvasive magnetic resonance imaging can be used to track transplanted cells *in vivo*, in addition to histology and EM [216, 217]. However, the potential concern of retention of iron oxide nanoparticles in dead cells and transfer to phagocytic host cells has not yet been excluded.

Membrane Labels

Membrane labels such as DiI and PKH26 are advantageous for stem cell labeling in that they are highly fluorescent labels and allow visualization of cell processes. DiI is a carbocyanine lipophilic dye that is incorporated irreversibly into the outer leaflets of the plasma membrane and diffuses laterally along the membrane. DiI is not transferred from labeled to unlabeled cells unless the membrane of the labeled cell is physically disrupted [218]. The fluorescent DiI signal can be photoconverted to a stable oxidized form so it can be visualized with both the light and EM. DiI has been successfully utilized for labeling grafted cells in short-term studies [205, 214]. PKH26 is a lipophilic chromophore similar to DiI, that has been used to label fetal subventricular zone cells before transplantation, and at least in short-term studies, transfer of the PKH26 label to host cells was not observed [219]. However, membrane labels are not suitable for long-term studies since membranes turn over rapidly. The membranous labeling initially observed with DiI is largely replaced by a granular cytoplasmic labeling as the tracer becomes internalized by endocytosis [218].

For all these labels, it is important to assess any possible toxic effects and perturbations in normal cellular physiology. Many of these labels are toxic to cells at high concentrations, thus optimal labeling parameters need to be empirically determined prior to transplantation. Also, the sensitivity of a cell to a label can vary depending on the cell type or stage of maturation. For example, a proliferating neural stem cell may be less sensitive to a particular label that is toxic to that cell when it has differentiated [205], or vice versa. Therefore, it is essential to establish the stability of the label and potential toxicity in differentiated cells *in vitro* and *in vivo* [161]. In addition, since these labels have the potential to be transferred to endogenous cells, it is important that appropriate controls

such as lysed or dead cell transplant controls also be performed [205]. When using either cytoplasmic or membrane labels it is essential to thoroughly wash the cells prior to transplantation to prevent the transfer of any unincorporated label into the host. Also, as cells divide in culture, nuclear, cytoplasmic, and membrane labels, unlike reporter genes which are discussed below, will be progressively diluted so it is important to label the cells close to the time of transplantation to maximize the strength of the signal.

Reporter Genes

The distinct advantage of reporter genes is that they can be stably integrated into the stem cell genome so that they cannot be transferred to endogenous cells [207]. In addition, reporter gene expression does not undergo dilution as a result of cell proliferation, and because of their continual expression they are suitable for long-term transplantation studies. Transplanted cells have been labeled with a variety of reporter genes, the most often used are lacZ and enhanced green fluorescent protein (EGFP). The lacZ reporter gene encodes the *E. coli* enzyme -galactosidase (-gal), which has the advantage of both histochemical detection using the X-gal reaction that can be visualized at both the light and EM levels, or immunohistochemical detection using anti- -gal antibodies. However, this method has major disadvantages because the mammalian CNS contains endogenous -gal activity so the pH of the histochemical detection solutions needs to be very carefully controlled to eliminate staining of endogenous host cells [220]. Likewise, *E. coli* specific anti- -gal antibodies need to be used with appropriate controls to differentiate non-specific staining. The major advantages of EGFP include the following features: it is constitutively fluorescent and so is readily detected; the signal can also be amplified immunohistochemically; it is detected in both cultured and fixed cells; and GFP-expressing cells can be enriched or sorted with other markers using fluorescence-activated cell sorting. Reporter genes can be cloned into eukaryotic expression vectors along with selected markers, transfected into proliferating stem cells, and then transfectants are selected. However the rate of transfection is often very low, so reporter genes are more commonly introduced into stem cells *via* infection with viral vectors. Replication-deficient retroviral vectors are used most often but only infect dividing cells, which would not be a concern with stem cells, otherwise adenoviral or lentiviral vectors can be used to infect postmitotic cells. The detection of the reporter gene product depends on the stable and sustained expression of the transgene in transplanted cells. A major disadvantage is that reporter gene expression in transplanted cells can be significantly down-regulated over time [205, 213], and we have experienced this with lacZ containing mouse brain ependymal region derived stem/progenitor cells. In our laboratory stem/progenitor cells generated from the adult rat spinal cord of EGFP expressing rats have retained their fluorescence for several weeks after transplantation into normal or injured rat spinal cord and have been very useful for studying migration and differentiation of transplanted cells.

Utilization of Intrinsic Markers of Transplanted Cells

The necessity to pre-label stem cells prior to transplantation can be avoided, or used in conjunction with situations where intrinsic markers of the grafted cells can be detected. For example, a reporter gene product can readily be visualized when transplanted cells are derived from transgenic animals, and it also allows more sustained expression of the transgene in grafted cells *in vivo*. Transgenic animals expressing EGFP and other reporter genes under the control of constitutive, cell-specific, or inducible promoters continue to be developed [221, 222]. Intrinsic markers of transplanted cells can also be detected in the host in cases of allografts, xenografts, and gender-specific grafts. Donor cells can be identified in allograft or xenograft transplants using strain or species-specific antibodies, such as Thy-1.1-expressing donor mouse cells into Thy-1.2 mouse hosts [223] and human donor cells expressing human-specific hNuc protein grafted into mouse [224], respectively. When donor stem cells derived from male tissue are grafted into female CNS, the transplanted cells can be detected with *in situ* hybridization using Y-chromosome-specific DNA probes. However, the morphology of grafted cells will not be evident so this technique will need to be performed in combination with immunohistochemistry which may not be compatible.

In summary, determining an optimal approach to label neural stem/progenitor cells prior to transplantation depends on a number of factors, but labeling is essential for the accurate identification and tracking of grafted cells *in vivo*. Given the limitations with many types of labels, it may be prudent to employ more than one labeling method to optimize detection.

Considerations for Neural Stem Cell Transplantation into the CNS

The efficacy of neural stem cell transplantation into the injured brain or spinal cord depends on a grafting method that will optimize the survival of the transplanted cells and minimize the graft-induced lesion. The mechanical process of grafting into the CNS itself creates damage that could diminish the viability of the transplanted cells and exacerbate the lesion site. To optimize the grafting procedure a number of issues need to be considered.

Neural Stem Cell Viability

First, it is essential to ensure excellent viability of the stem cells prior to transplantation. Viability is routinely assessed with trypan blue exclusion or propidium iodide. Single cell suspensions are counted in a hemocytometer to obtain a cell count and viability using trypan blue dye. Live cells exclude the dye and appear phase bright, while dead cells take up the dye and appear dark blue. Viability can also be determined with fluorescent propidium iodide which is added to single cells suspensions and is taken up by cells whose membranes are compromised. Therefore, live cells exclude the dye and remain unlabeled, while dead cells take up the dye and fluoresce red under the rhodamine filter.

Routes of Administration

Most of the transplantation studies with neural stem/progenitor cells have involved intraparenchymal or direct injection into the CNS [9, 89]. In many injury models, cells are grafted directly into the lesion cavity, adjacent to, or near the lesion which ensures that the cells are in the vicinity of the injury in order to exert potential beneficial effects [137, 225, 226]. In addition, studies suggest that neural stem cells are

able to migrate to an adjacent damaged region of the host brain or spinal cord [89, 227, 228], similar to other stem cells. For example, bone marrow stromal cells show homing to the injury site in the brain or spinal cord after systemic delivery [216, 228, 229], although only a small fraction of the injected cells reach the injured target zone. Stem cells can also be delivered intraventricularly, intrathecally, or intravenously. Neural stem/progenitor cells derived from the hippocampus of the fetal green fluorescent protein (GFP) rat were injected into the fourth ventricle and were transported through the cerebrospinal fluid into the subarachnoid space of the spinal cord and attached to the surface of the cord as cell clusters [230]. Interestingly, grafted cells only invaded the spinal cord in rats with contusion injuries but not in normal or intact controls [230]. Introducing stem cells through the vascular system allows the cells potential access to all organs or to restricted targets depending on the method used. The carotid artery, or femoral, jugular, and tail veins are all potential sites to introduce stem cells into the vascular system, the latter being most convenient and least invasive. Stem cells such as bone marrow stromal cells and embryonic stem cells have been delivered systemically and show some potential for migration to the site of injury in the CNS [216, 228, 229], although this has only recently been demonstrated for neural stem cells. Neural progenitor cells derived from the embryonic hippocampus of transgenic rats transplanted intravenously one day after contusion injury were shown to migrate to the lesion site, survive, and differentiate [231]. A recent study tested the possibility of delivering bone marrow stromal cells and lineage restricted neural precursor cells derived from embryonic rat tissue in a partial cervical hemisection injury by different routes including intravenous, intraventricular, and intrathecal *via* lumbar puncture [228]. The authors found that both bone marrow and lineage restricted precursor cells home toward the injured spinal cord. The intrathecal lumbar puncture and intraventricular routes allowed more efficient delivery of the cells to the injured cord compared with the intravenous route [228].

Grafting Parameters

For transplantation into the brain or spinal cord the animal should be securely positioned in a stereotaxic frame. When cells are injected, it is important to minimize the extent of the graft-induced lesion. Therefore, use of a smaller diameter micropipette or smaller gauge needle will reduce the extent of the damage to host tissues. However, this will also depend on whether dissociated cell suspensions or intact neurospheres are transplanted. It is essential to prevent clogging of the needle when cells settle. Thus, the size of the stem cells to be engrafted or the average size of the neurospheres to be injected needs to be determined. Micropipettes are pulled to minimize the tip diameter and the tip can be beveled to aid penetration into the tissue. Needles can be custom made with a slight bevel for the same purpose. It is generally agreed that the best way to inject cells or spheres is to slowly deliver a fixed volume at a constant rate in a preset time period. This can be successfully accomplished with a motorized syringe pump. If cells are transplanted too quickly, they may leak out along the micropipette or needle track. The volume of cells transplanted will depend on the size of the transplant site and whether the cells will be injected into a lesion cavity that will accommodate a larger volume. Nevertheless, it is advantageous to inject as small a volume as possible in both the brain and spinal cord. Average volumes of 0.5-5 μl are commonly used. The cell density and total number of cells transplanted needs to be established to ensure an adequate number of cells for grafting and optimal survival. After the cells have been injected, the micropipette or needle tip should be left in the same position for at least two minutes before withdrawing slowly to prevent any backflow. Immunosuppressive agents such as cyclosporine are often used even in cases of syngenic transplants where the extent of graft rejection on the survivability of the cells is unknown. Host immunosuppression in allograft and xenograft transplants is usually required although even this is controversial depending of the type of grafted cells [232, 233].

Experimental and Clinical Studies of Neural Stem Cell Transplantation for SCI and TBI

As noted above, neural stem/progenitor cells have unlimited therapeutic potential for restoring CNS function lost as a result of trauma. Neural stem cells can be propagated in culture without losing their multipotentiality, and when transplanted back into the CNS, these stem cells have the capacity to migrate, integrate with the host tissue, and to respond to local cues for differentiation. Considerable progress has been made in developing efficient methods for culturing neural stem cells for subsequent transplantation into animal models of brain and spinal cord injury.

Embryonic CNS tissue transplants integrate into the adult mammalian brain and spinal cord [157, 230, 234-243] and enhance functional recovery after traumatic or degenerative lesions [226, 244-246]. Improved locomotor recovery after SCI in adult rodents treated with fetal spinal cord cell transplants was first documented by Stokes and Reier [247]. Transplantation of adult mammalian spinal cord derived stem cells into the adult mammalian spinal cord after SCI has only been attempted several times. Gage's group demonstrated that clonally expanded multipotent progenitors from the adult rat spinal cord undergo region-specific differentiation following engraftment into the normal adult spinal cord and hippocampus [9]. Teng *et al.* showed improved post-SCI functional recovery in the rat after implantation of a scaffold seeded with neural stem cells [248]. Vroemen transplanted these cells and found that they survived, differentiated and integrated along injured axonal pathways in the spinal cord [213].

In humans, stem cells have also been identified in the brain [249] and fetal spinal cord [250], and like those from other species, human neural stem cells respond to mitogenic stimulation [251]. Adult human subcortical progenitors survive and differentiate into oligodendrocytes after transplantation in demyelinated lesions of the rat brain [252, 253]. Akiyama transplanted clonal neural precursor cells derived from adult human brain, and found that these cells elicited extensive remyelination with peripheral myelin in demyelinated regions of the adult rat spinal cord created by x-radiation and ethidium bromide [254].

Recently, cultured human neuronal cells derived from a human embryonic carcinoma cell line have been transplanted into the brain of patients with stroke [255, 256]. Although long term survival of the transplanted cells has been reported, there is no definite evidence of clinical improvement. With

respect to human SCI, there have been several Phase 1 trials of cell or tissue transplantation directly into the damaged spinal cord in patients with SCI or other myelopathies. Fetal human spinal cord tissue has been transplanted into a small number of patients with post-traumatic syringomyelia [257]. However, there is no definite evidence of long term survival of the cells or beneficial functional effects on the patients. The only stem cell trial of CNS derived neural stem cells in human SCI has been the heterotransplantation of porcine stem cells into the spinal cord of a small number of patients (unpublished report), but there have been no reports of cell survival or long term clinical benefit. In at least two countries there are trials underway involving the transplantation of autologous OEG cells into humans with SCI [258], but these are not stem/progenitor cells (see below). Fetal human brain-derived neural stem cells were transplanted into the brain and spinal cord of TBI and SCI rats, and differentiated in a region specific manner by acquiring a cholinergic neuronal phenotype [259]. Adult human spinal cord stem cells have never been transplanted into the spinal cord of laboratory animals or humans. Our laboratory is currently harvesting and culturing adult human spinal cord stem cells for transplantation into rat SCI in order to explore this potentially beneficial stem cell therapy for humans.

Clinical Trials of Humans Receiving Non-CNS Derived Stem Cells for SCI, TBI, etc.

As noted above, there have been numerous experiments in laboratory animals on the use of endogenous or transplanted CNS derived neural stem/progenitor cells for neurotrauma. We have excluded OEG cells from this review because they are certainly not brain or spinal cord derived stem cells, although olfactory epithelium contains cells that underlie regeneration of olfactory sensory neurons, even in adults, and the glial component of the olfactory bulb is capable of expansion *in vitro* with some potential for generating cells with either schwann-like or oligodendroglia-like properties. Currently, SCI patients who have received transplants of autologous OEG cells represent the largest numbers of SCI patients who have received transplants, and these transplants have taken place in China, Portugal, and Australia according to media reports and personal information from patients. To our knowledge there are no scientific reports to date of these human experiments with OEG cells.

Other non-CNS derived neural stem cells transplanted into humans with neurotrauma include autologous bone marrow derived stem cells, and these have been administered intravenously into the spinal cord of SCI patients in the Czech Republic (personal communication). These cells have also been administered into the spinal cord of patients with amyotrophic lateral sclerosis with minimal side-effects [260]. The segments of fetal spinal cord transplanted into humans with syringomyelia in Sweden [261] and the USA [257] noted above undoubtedly contained some stem cells, but as noted above only a small number of patients received this treatment, and there is uncertainty about the survival of the cells and the possible clinical benefit. There are some studies of the transplantation of various progenitor like lines of cells of human origin into animals or humans with SCI, TBI, or stroke. For example, neurons derived from a human teratocarcinoma have been transplanted into rats with SCI [262] or rats with TBI [263], and these cells have also been administered directly into the brain of humans who have suffered a stroke [264]. Human umbilical cord blood generated stem cells have been injected into rats with SCI [265]. Fetal pig spinal cord derived stem cells have been transplanted into humans with SCI in the USA (personal communication), but there have been no reports of this in the scientific literature. Fetal human cerebral cortex derived stem cells have been studied extensively in experimental animals after implantation into the brain or spinal cord with impressive survival and differentiation [259], but to our knowledge these cells have not been transplanted into humans with SCI or TBI.

Regional Specification of Cell Fate of Transplanted Neural Stem Cells

As noted above, transplantation studies have demonstrated that neural stem cells react to environmental cues within the host environment and differentiate accordingly [9, 89, 225]. Shihabuddin demonstrated that clonally expanded multipotent progenitors from the adult rat spinal cord undergo region-specific differentiation following engraftment into the adult spinal cord and hippocampus. After transplantation into the dentate gyrus, the neural stem cells differentiated into neurons but were unable to exhibit neurogenic potential when transplanted back into the adult spinal cord [9]. Similarly, stem/progenitor cells derived from the adult hippocampus can generate olfactory bulb neurons when implanted into the SVZ or rostral migratory stream of the adult rat and express a neurotransmitter phenotype characteristically found in the olfactory bulb [89]. Therefore, these studies suggest that the local environment primarily determines the cell fate of engrafted cells.

Neural stem cells derived from both embryonic and adult tissue differentiate primarily into glia when transplanted into nonneurogenic regions of the adult CNS, such as the striatum or spinal cord, either in the normal uninjured animal or after injury [9, 137, 225, 266]. When embryonic hippocampal neural stem cells were transplanted into the injured striatum, the majority of grafted cells became astrocytes [266]. Similarly, embryonic rat cortical neural stem cells differentiated primarily into astrocytes but not neurons after engraftment into the normal or contused spinal cord [225]. Vroemen transplanted adult spinal cord derived neural progenitors into acute dorsal column transection of the rat cervical cord and found that the grafted cells survived, differentiated into astrocytes and oligodendrocytes, and integrated along injured axonal pathways [213]. Embryonic neural stem cells engrafted 6 days after a cortical lesion differentiated mainly into astrocytes and a few oligodendrocytes [267]. Ogawa transplanted *in vitro*-expanded fetal neural progenitor cells 9 days after spinal cord contusion injury and found that the grafted cells produced mainly astrocytes but also observed a small percentage of neurons [226].

Neurospheres derived from the embryonic rat hippocampus injected into the fourth ventricle attached to the pial surface of the spinal cord, but only invaded host cord tissue and nerve roots in rats with a contusion injury producing primarily astrocytes but no neurons [230]. In comparison, when embryonic hippocampal neural stem cells were delivered intravenously one day after contusion injury of the spinal cord, they migrated to the lesion site and differentiated into neurons,

astrocytes, and oligodendrocytes [231]. These studies suggest that the mode of delivery of transplanted cells may also influence their fate.

Riess transplanted the neural stem cell clone C17.2 into the cortical-hippocampal interface following a lateral controlled cortical impact brain injury. The authors found that the engrafted cells differentiated into both neurons and glia and attenuated motor dysfunction after TBI [263]. Neural stem cells have also been genetically modified *in vitro* to produce specific proteins. For example, the neural stem cell line C17.2 was transfected to constitutively secrete NT-3 and grafted into cystic dorsal column lesions in the cervical cord [268]. The transplanted cells supported extensive growth of host axons, and intrinsic growth factor production by the neural stem cells corresponded to observed patterns of axonal growth [268]. Gene modification of neural stem cells can also be used to modulate cell fate. Setoguchi engrafted fetal neural precursor cells expressing the BMP inhibitor noggin into the lesioned cord. Since BMPs promote an astrocytic lineage commitment, the authors found that the transplanted cells differentiated not only into astrocytes, but also neurons and oligodendrocytes due to the expression of noggin [269].

There are several variables associated with transplantation into the injured CNS that require further study. For example, it is not know whether region-specific cells such as the ependymal region stem/progenitor cells would produce better functional recovery compared with non region-specific cells such as brain derived subventricular region cells. This issue has never been addressed in SCI, but there is some data available from cerebral [270] and retinal transplantation [271] that suggests that regional cells are lineage restricted, and may respond better to region specific cues [272]. Also requiring further study is the relationship between the survival of the cells, functional recovery and the interval after trauma at which the cells are transplanted. It has been suggested that survival and differentiation conditions for neural stem cells transplanted immediately following injury are unfavourable due to the acute inflammatory response in the post-traumatic environment [273]. The small amount of available evidence suggests that survival of transplanted cells is increased after subacute or chronic transplantation [274, 275], although in the chronic stage after injury, glial scars surrounding the lesion inhibit the regeneration of axons.

Seeding Neural Stem Cells on Artificial Scaffolds for Implantation

Recently, tissue engineering techniques have been used to transplant neural stem/progenitor cells into damaged neural tissue after CNS injury. One of the most interesting strategies in the field of biomaterials is the implantation of synthetic three-dimensional biodegradable scaffolds seeded with neural stem/progenitors into CNS lesions. Artificial three-dimensional scaffolds have been used for repair of CNS lesions where bridging is necessary to promote axonal regrowth into the lesion site [276-279]. Artificial scaffolds are also advantageous for allowing the combination of neural tissue implantation with additional therapeutic strategies for enhancing axonal regeneration [280-284].

For seeding neural stem/progenitor cells on solid scaffolds, the scaffolds have been composed of nonbiotoxic and biodegradable polymers, such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymers poly(lactic-co-glycolic acid) (PLGA), which support the growth of the seeded cells [285]. One of the benefits of utilizing a scaffold seeded with neural stem/progenitors for repairing CNS lesions is that scaffolds can provide a platform for the stem/progenitor cells in a wide variety of neural defects. Park reported that implantation of a PGA-based scaffold seeded with mouse neural stem/progenitor cells into the massive cavity following brain hypoxic injury dramatically reduces parenchymal loss of the injured brain and facilitates reconstruction of the neuronal network between host- and donor- derived neurons [286]. Furthermore, transplanted neural stem/progenitors transplanted into CNS lesions have been known to promote host-axonal regrowth by constitutive secretion of several neurotrophic factors [268]. Therefore, the implantation strategy with neural stem/progenitors on a scaffold can be expected not only to replace lost tissue by the donor cells but also to promote more extensive host-axonal regrowth compared with utilizing the scaffold alone. Teng reported that implantation of a PLGA-based scaffold seeded with mouse neural stem/progenitors improves functional recovery after rat spinal cord hemisection and promotes host-axonal regrowth without any differentiated donor cells at the transplanted site [248]. They also suggested that the scaffold may impede astrogliosis and subsequent cyst formation following SCI.

Remarkably, some of the biomaterials may stimulate the seeded cells on a scaffold to selectively differentiate into neurons. Silva reported that an artificial nanofiber scaffold induced rapid differentiation of mouse neural stem/progenitor cells into neurons, while discouraging the development of astrocytes [287]. Scaffolds can also contribute to the selective differentiation of seeded neural stem/progenitor cells by providing an environment for combination therapy with neurotrophic factors [288]. There have been no reports concerning the implantation of a solid degradable scaffold seeded with adult rat neural stem/progenitors.

CONCLUSION

This review of the literature in the field of endogenous and exogenous CNS stem/progenitor cell therapies for neurotrauma indicates that such techniques have major potential. However, given the paucity of clinical studies that exist, these therapies have not yet realized their full potential. Nevertheless, the extensive pre-clinical studies provide compelling evidence to support the concept that the endogenous proliferative response of neural stem/progenitor cells to trauma can be enhanced by a combination of exogenous factors including NFs and transplanted CNS derived neural stem/progenitors that have been expanded *in vitro*. An integrated approach such as this may maximize the innate potential of CNS neural stem/progenitor cells to regenerate lost tissue and function following injury. In major injuries with extensive tissue loss, the transplantation of neural stem/progenitor cells offers promise of retoration of lost tissue and improvement of function.

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ABBREVIATIONS

-gal = -galactosidase
µl = Microliter
BDNF = Brain-derived neurotrophic factor
bHLH = Basic helix-loop-helix
BMP = Bone morphogenetic protein
BrdU = 5-bromo-2'-deoxyuridine
CFDA-SE = Carboxyfluorescein diacetate succinimidyl ester
CNS = Central nervous system
CNTF = Ciliary neurotrophic factor
DG = Dentate gyrus
DiI = 1,1'-dioctadecyl-3,3,3',3'-tetramethylindo-carbocyanine perchlorate
DNA = Deoxyribonucleic acid
EGF = Epidermal growth factor
EGFP = Enhanced green fluorescent protein
EM = Electron microscopy
EPO = Erythropoietin
FGF2 = Fibroblast growth factor 2
GDNF = Glial derived neurotrophic factor
GFAP = Glial fibrillary acidic protein
GFP = Green fluorescent protein
Hes1 = Hairy and enhancer of split 1 gene
Hes5 = Hairy and enhancer of split 5 gene
hNuc = Human nuclear antigen
Id = Inhibitor of DNA binding gene
IGF = Insulin-like growth factor
lacZ = -galactosidase gene
LIF = Leukemia inhibitory factor
Mash1 = Mammalian homologs of achaete-scute complex 1 gene
Math1 = Atonal protein homolog 1 gene
NeuroD1 = Neurogenic differentiation 1 gene
NFs = Neurotrophins
NGF = Nerve growth factor
Ngn2 = Neurogenin 2
Notch1 = Translocation-associated NOTCH homolog 1 gene
NT-3 = Neurotrophin-3
O2A = Oligodendrocyte-type-2 astrocyte
OEG = Olfactory ensheathing glia
PDGF-AA = Platelet derived growth factor-AA
PGA = Poly(glycolic acid)
pH = Potential of Hydrogen
PLA = Poly(lactic acid)
PLGA = Poly(lactic-co-glycolic acid)
RDA = Rhodamine-dextran-amine
SCI = Spinal cord injury
Shh = Sonic Hedgehog
SVZ = Subventricular zone
TBI = Traumatic brain injury

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