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Axonal growth and connectivity from neural stem cell grafts in models of spinal cord injury

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Spinal cord injury (SCI) damages both gray matter and white matter, but white matter damage is responsible for the vast majority of the subsequent functional loss. Neural stem cells (NSCs) have been investigated as a means of improving outcomes after SCI, either through neuroprotective properties that limit secondary damage or by direct cell replacement. This review will focus on cell replacement strategies, and the ability of multipotent NSCs to form new functional synaptic relays across sites of even severe SCI. The ability of these early stage neurons to extend axons from the lesion site in large numbers and over long distances constitutes an important mechanism underlying their potential to promote neural repair.

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Introduction

Spinal cord injury (SCI) results in the death of neurons at the injury site and the loss of axons that carry signals to and from the brain. Axons of the injured adult central nervous system (CNS) exhibit little ability to spontaneously regenerate, often resulting in permanent functional deficits below the level of injury [1]. For more than a decade, neural stem cells (NSCs) or neural precursor cells (NPCs) have been an attractive cell source for the treatment of SCI, because they have the potential to replace lost neurons and glia, and to restore disrupted connectivity at lesion sites. Recently we have come to appreciate that grafts of NSCs to spinal cord lesion sites are able to extend numerous new axons into the host spinal cord for long distances, and to receive inputs from injured host axons. On the basis of this bi-directional growth of axons and establishment of reciprocal synapses, NSCs form new relays between separated segments of the spinal cord after injury (Fig. 1).

NSCs or NPCs can be isolated from the developing CNS, or derived from pluripotent stem cells, including embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. Here, we systematically review NSC transplantation studies in SCI research, with an emphasis on axonal growth and connectivity between transplanted and injured host neurons. We conclude by discussing future challenges in the field of NSC transplantation for SCI.

Axonal projections arising from neural stem cell grafts

There is an extensive history of CNS tissue grafting in models of SCI [2•,3,4]. Early studies demonstrated axonal projections from grafted tissue into host for distances as long as 4–5 mm, using both anterograde and retrograde axonal labeling techniques [3]. Intrinsic cellular markers and reporters, such as green fluorescent protein (GFP) and alkaline phosphatase (AP), may be more reliable methods for labeling axons emerging from neural implants than tracer injections. These cell-intrinsic reporters are often readily visualized, and are less prone to errors and misinterpretation arising from tracer mis-targeting or spread [5]. Fischer's group was among the first to use cell reporters to trace axons arising from NSC implants, and observed axonal outgrowth from grafts of rat embryonic day 14 (E14) fetal spinal cord tissue expressing alkaline phosphatase for distances of ~5 mm in the injured spinal cord [6•], although there was considerable variation among animals. They also found that cells isolated from the developing spinal cord at the neuronal restricted precursor (NRP) stage failed to extend appreciable numbers of axons into the host spinal cord 3–5 weeks post-transplantation [6•]. Axons will, however, emerge in greater numbers from NRP cell implants when attracted by the neurotrophic factor brain derived neurotrophic factor (BDNF), injected adjacent to the implant [7••].

Many studies have examined the role of NSCs in rat models of SCI. Some studies have also utilized mouse models, a potential advantage when considering the array of genetic models available in mice. One early study in a mouse model reported that embryonic mouse hippocampal neurons grafted into the *intact* rat cervical corticospinal tract extended long, straight, and uniform axons into host spinal cord white matter for distances as long as 8 mm (using a mouse specific marker, M6) [8•]. The extension of the donor axons was confirmed by retrograde labeling with horseradish peroxidase. The graft-derived axons were intermingled with the host myelinated axons. Thus, this study was an early indicator that adult myelin is

Fig. 1

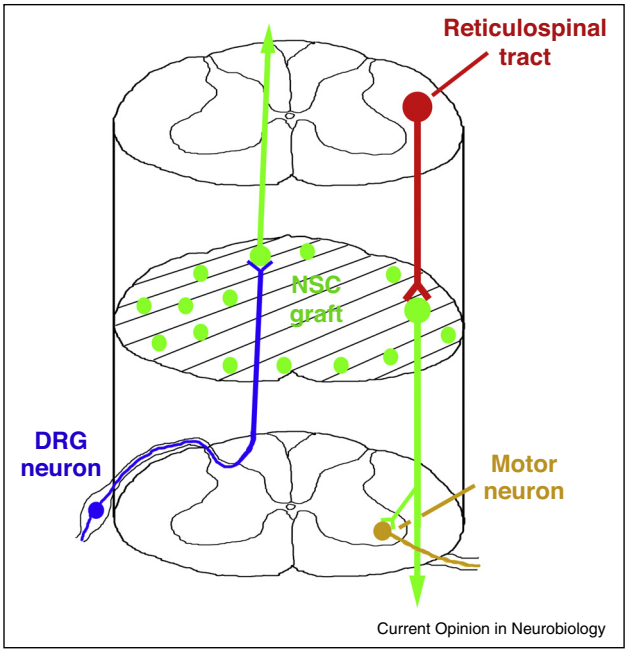


Diagram of a neuronal relay formation by transplanted NSCs after SCI. Descending supraspinal axons (a reticulospinal axon as an example, red) regenerate into and make synaptic connections with grafted neurons (green) in the lesion site. Grafted neurons extend their axons (green) into caudal host spinal cord and make synaptic connection with host neurons (a motor neuron as an example, yellow). Similarly, grafted neurons can make functional relay for ascending sensory system (a dorsal root ganglion (DRG) neuron as an example, blue).

not necessarily inhibitory to the extension of developing axons. Other studies grafting mouse NSCs into spinal cord lesion models yielded less clear evidence of axonal growth, however, possibly due to limited graft survival, glial differentiation of implanted cells [9], cell immortalization [10], or inhibitory effects of glia or myelin surrounding the lesion site [11].

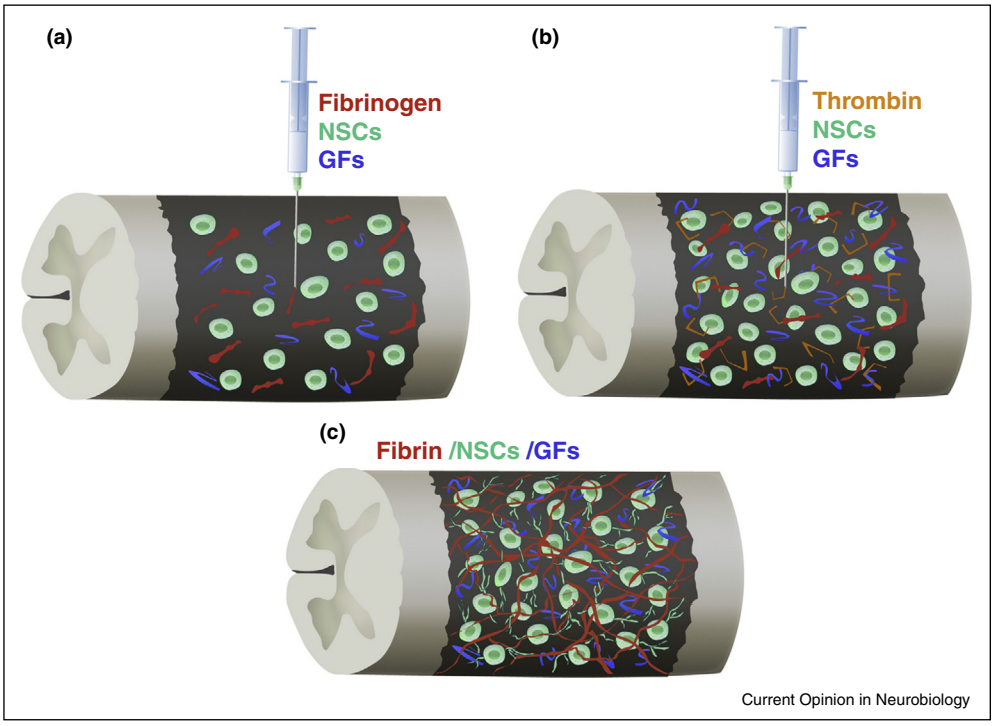
Recently, we initiated a series of studies to more thoroughly explore the hypothesis that early stage neurons would exhibit an ability to extend axons in the lesioned adult CNS, even after severe forms of injury [12••]. To approach this question, we adopted a few changes from previous studies. First, we used NSCs or multipotent NPCs that robustly expressed enhanced GFP (EGFP), to allow unequivocal tracking of cell survival, fate and axonal extension *in vivo*. Second, because we were grafting cells into a severe injury model, we directed considerable effort toward developing methods to enhance graft cell survival, retention and filling of even severe lesion sites. This was accomplished by grafting cells in a fibrin matrix that retained cells in the large lesion site, and by grafting cells with a protein cocktail of growth factors to support cell survival and vascular ingrowth (Fig. 2) [12••]. Without the cocktail, graft filling of the lesion site was incomplete and did not allow the formation of neural relays from host to graft, and graft to host.

We began these studies by using donor cells from the E14 spinal cord, which consists of a mixture of NSCs and cells committed to either neuronal fates (NRPs) or glial fates (glial restricted precursors, GRPs) [6•,12••]. Cells at the NPC stage are capable of continuing to divide for several times to generate additional cells, but they do not continue to propagate a NSC population. The NPCs were isolated from fresh E14 spinal cords, dissociated gently with trypsin, and immediately grafted into sites of *complete* T3 spinal cord transection, in a fibrin matrix with growth factor cocktail (Fig. 2). We did not culture the cells before grafting, because additional culture time results in an attenuation of the ability of grafted neurons to extend axons after grafting *in vivo*. Cells were isolated from Fischer 344 rat embryos and grafted into adult Fischer 344 rats; because this rat strain is inbred, immunosuppression was not needed. This study was one of the few in the SCI literature that used a complete transection model; the model has generally been avoided because of the difficulty in achieving cell survival and altering injury-associated anatomical and functional losses.

When examined 7 weeks later, grafted rat NSCs and NPCs consistently filled the complete transection site. The implanted cells differentiated into neurons, astrocytes and oligodendrocytes in roughly equal proportions. Most importantly, grafted neurons extended very large numbers of axons into the host spinal cord both rostral and caudal direction to the lesion [12••]. Remarkably, the progenitor cell-derived axons grew for distances of more than 20 mm (7 spinal segments) in the rostral direction and 27 mm (9 segments) in the caudal direction. We conservatively quantified 29.000 GFP-labeled axons *emerging* from the graft in the caudal direction, at a distance 0.5 mm caudal to the lesion. In addition, *22%* of progenitor cell-derived axons became myelinated by host oligodendrocytes at a distance 3 mm caudal to the graft (unpublished data). A time course study demonstrated that graft-derived axons grew at a rapid rate of 1-2 mm/day along host white matter at early stages [12••]. These findings established very clearly that developing neurons are able to extend axons over very long distances in the adult CNS, even when intimately associated with adult myelin membranes from host axons.

Other studies have demonstrated that adult-derived NSCs also survive grafting to sites of SCI. The subventricular zone and hippocampal fissure are sites of ongoing neurogenesis throughout life, providing neurons to the olfactory bulb and hippocampus, respectively [13]. NSCs were harvested from both of these structures and grafted to the spinal cord [14–17]. While some cells survive grafting, almost all grafted cells adopted glial fates, and

Fig. 2



Transplantation of neural stem cells with fibrin and growth factors to retain and support their survival, growth, and differentiation after spinal cord injury. **(A)** Neural stem cells (NSCs) are first re-suspended into a *Fibrinogen* matrix containing *a growth factor* cocktail (*GFs*) and are injected into the spinal cord lesion site. **(B)** A separate companion batch of *NSCs* is re-suspended into a *Thrombin* matrix containing the same growth factor cocktail (*GFs*), and injected into the spinal cord injury site immediately following the first injection. **(C)** The mixture of *Fibrinogen* and *Thrombin* forms a solid gel of *Fibrin* with the growth factor cocktail (*GFs*) to retain and support graft survival and growth.

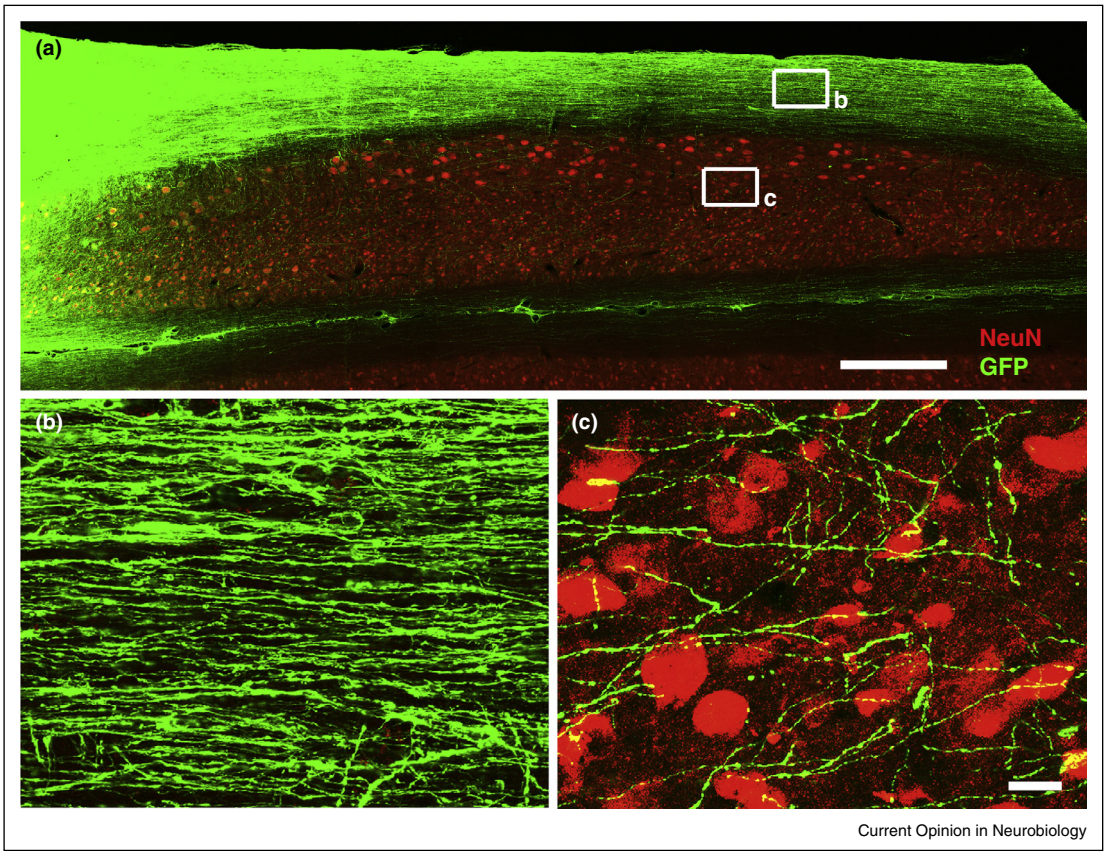
many cells expressed oligodendroglial markers and in some cases myelinated host axons. When comparing findings from these studies to studies that grafted developing rather than adult stem cells, it is evident that the age, source and method of cell grafting to sites of SCI has a profound effect on cell survival, differentiation and ability to extend axons.

The preceding studies used NSCs isolated from rodents. Human NSCs have also been grafted to the rodent nervous system in an effort to determine whether human NSCs can also be harvested and grafted, and whether they exert detectable effects in the nervous system. Wictorin and Bjorklund [18] grafted human fetal spinal cord-derived cell suspensions from weeks 6 to 7 of human development into the spinal cords of adult rats. Grafts were placed both one spinal cord segment above or below a partial lesion site. They found partial graft survival and extension of human axons into host white matter; most axons grew for lengths of 3–4 mm, but a few axons extended for distances as long as 10 mm, 3–4 months post-grafting. However, human cells were implanted into undamaged spinal cord parenchyma surrounding the lesion site, and cells were not implanted in the actual lesion site [18]. Wictorin and colleagues also reported long distance growth of human forebrain-derived axons that were grafted into the rat brain [19••].

Giovanini and colleagues [20] conducted a more in-depth characterization of human fetal spinal cord transplantation into rat adult SCI. In contrast to the previous findings, human graft-derived axons rarely extended into host white matter. Instead, human dendrites and some axons projected into host gray matter over short distances [20]. The rare growth of human axons into the host may have been related to poor graft survival and integration into the lesion site, since immunosuppression was not reported in this xenograft study.

Recent studies have grafted human fetal nervous system-derived NSCs in rodent and primate models of SCI [21,22,23,24]. These studies focused on the differentiation of the grafted human cells, and did not label cells with markers that could sensitively detect axonal outgrowth from grafts. Graft survival was relatively modest, and labeling for human-specific neurofilament disclosed only short distance growth of human axons from grafts in the lesion site [23].

Fig. 3



Growth of human ES cells-derived neural stem cells in sites of spinal cord injury. **(A)** GFP-labeled human ES cells-derived NSCs were grafted into sites of C5 hemisection SCI. Horizontal section immunolabeled for GFP and NeuN demonstrate remarkably large numbers of GFP-labeled axons (co-localized with Tuj1) extending caudally into the host spinal cord. **(B and C)** Higher magnification view from boxed area of panel A showing human axonal growth in host **(B)** white matter and **(C)** gray matter (region of NeuN labeling). Scale bar: A, 600 μm; **(B and C)**, 22 μm.

Given our findings that rat NSCs/NPCs could extend many axons for very long distances in the severely injured spinal cord [12••], we also examined whether human NSCs exhibited similar properties when grafted in a fibrin matrix and supporting growth factor cocktail. Human NSCs were isolated from two sources: the human embryonic spinal cord [24] and the ES cell lines HUES7 [25]. These cells were grafted into severe spinal cord lesion sites in immuno-deficient rats. Robust long-distance axonal growth was evident 7–12 weeks post-grafting (Fig. 3) [12••]. Indeed, the amount and distance over which human axons extended exceeded observations from grafts of donor rodent NSCs, perhaps because of the prolonged developmental period of the human compared to the rat. Axons extended over a distance of 50 mm into the spinal cord caudal to the lesion site, and 30 mm rostral to the lesion site. Indeed, axons densely extended into the brainstem from grafts placed at the mid-cervical level. Recent studies by others confirmed that human spinal cord-derived NSCs extend axons from a spinal cord lesion site in rats [26,27]. Thus, the ability of developing neurons to extend axons in large numbers and over long distances through inhibitory white matter extends across species, including humans.

The recent development of iPS cells can serve as an alternative stem cell source for generation of NSCs [28•]. The advantage of iPS cells over ES cells is that iPS cells are adult-derived and can be transplanted autologously to avoid both ethical concerns and, potentially, immune rejection. Human iPS cell-derived NSCs have been transplanted into rodent models of SCI, where they have been reported to differentiate into both neurons and glia in several recent studies [29•,30,31]. However, axonal growth and connectivity from human iPS cell grafts has not reported. We recently grafted human NSCs derived from iPS cells into mid-cervical spinal cord lesion sites in adult immunodeficient rats. We found, once again, extensive axonal outgrowth from the injury site into virtually all regions of the spinal cord caudal and rostral to the injury

site (Lu *et al.*, unpublished result). Indeed, human axons traveled virtually the entire rostral-to-caudal length of the rat CNS.

Connectivity and function of axons arising from neural stem cell grafts

Several studies have reported connectivity and functional recovery after grafting various types of NSCs to sites of SCI, although the strength of evidence supporting these reports varies across studies. The first study that grafted mouse ES cell-derived NSCs to a site of contusive rat SCI reported functional recovery, but provided no detailed mechanism [32•]. However, only 8% of grafted cells became neurons in this study, and there was no labeling of neuronal processes. Another study also reported a slight improvement in motor function after grafting human NSCs to a moderately severe contusive injury, and this recovery was abolished by eliminating the grafted cells by diphtheria toxin administration [23]. However, cell survival in this study was modest and axonal outgrowth from the graft was not assessed. Neuroprotection may have been a mechanism supporting the functional outcome. Several studies focused on grafting cells derived from human ES cells and driven to more specific fate, such as oligodendrocyte precursor cells (OPCs) [33,34,35•]. Modest functional benefits were reported and grafted cells were shown to partially remyelinate host axons spared by after partial injuries. These studies led to the first clinical trial of human ES-derived cells for SCI in the United States [36]. After enrolling four patients, the clinical trial was abandoned by the sponsoring company, Geron.

In our own work with grafts of either rodent or human NSCs described in the preceding section, we found that graft-derived axons grew in highly organized linear trajectories through host white matter. Axons in white matter gave off collateral branches that penetrated host gray matter, forming bouton-like terminals around some host neurons. These bouton-like structures co-labeled with the pre-synaptic marker synaptophysin, suggesting synapse formation. Indeed, immunoelectron microscopy confirmed synapse formation between graft-derived axons and host neurons and dendrites [12••]. Thus, it is clearly the case that grafted NSCs derived from several different sources, including developing CNS tissue or ES cells or iPS cells, can connect with host neurons. Yet return of function is not always observed, suggesting a need to further shape the topography and specificity of projections from grafted cells (more on this topic below).

Axonal projection and connectivity from host neurons into neural stem cell grafts

The outgrowth and connectivity of grafted NSCs is only one component of neuronal relay formation. A complete neuronal relay also relies on ingrowth and connectivity of host axons with grafted neurons. Several studies have reported regeneration of *adult* host axons, including rubrospinal, reticulospinal, raphespinal, propriospinal, and sensory axons, into grafts of embryonic spinal cord tissue or ES cell-derived NSCs placed in sites of SCI [3,4,7••,10,37••,38]. However, the distance and relative density of ingrowth of adult host axons into the grafts is typically fairly modest. Moreover, several older studies used retrograde labeling techniques to assess axonal growth into grafts, rather than the more clear and less artifact-prone technique of anterograde labeling [2,3]. Our recent study showed results similar to that in the published literature, indicating that ingrowth of host supraspinal axons, including anterogradely labeled reticulospinal axons and immunolabeled serotonergic axons [12••], is relatively modest. While host supraspinal axons usually penetrated the rostral host/graft interface, they are substantially reduced in number in the middle of NSC grafts. We never observed host supraspinal axons crossing over the entire NSC graft and re-entering the caudal spinal cord. Compared to axonal outgrowth arising from NSCs, therefore, host axonal *ingrowth* into grafts is limited. This appears to be at least partially due to the refractory growth state of adult neurons. Nonetheless, host axons entering NSC grafts form bouton-like structures with grafted NSCs in the lesion site and express the pre-synaptic marker synaptophysin, suggesting synaptic connections with grafted neurons [12••].

This reciprocal growth of axons from grafted NSCs into the host, and from host neurons into the grafted NSCs, establishes a mechanism whereby novel relay circuits across an injury can be established. Indeed, we observed behavioral improvement in rats that received NSC grafts even in sites of severe, complete spinal cord transection [12••]. Electrophysiologcal measures confirmed the formation of neural transmission across the lesion site, with responses detected that exhibited prolonged latency and altered waveforms compared to intact animals. Moreover, both functional recovery and electrophysiological transmission were abolished by re-transection of the spinal cord *above* the NSC implant [12••], indicating that host inputs into the graft are essential in mediating functional recovery. Other studies have also suggested that novel relays can be established across sites of SCI to support improvement of function [7••,11,39,40]. Collectively, these findings indicate that NSC grafts to sites of SCI have the potential to interpose a new neural relay to act as a substrate for functional improvement.

Future perspectives

Axonal growth from NSCs and the potential formation of new functional neuronal relays across lesion sites offers new hope for SCI treatment. Several challenges remain. The modest growth and regeneration of adult host axons into the NSC grafts is one challenge, since the intrinsic growth capacity of adult neurons is greatly reduced [41]. Enhancing the intrinsic adult neuronal growth state [42•,43•] or provision of neurotrophic factors in regions

of lesioned axon terminals [44•] could enhance host axonal regeneration and strengthen the functional neuronal relay. The seemingly random organization of the NSC graft in the lesion cavity is another challenge. Grafting NSCs within bioengineered scaffolds that segregate the lesion site into specific rostral-caudal channels may provide a means of organizing the lesion site: host axons could be directed into the graft along specific linear trajectories, and graft-derived axons could exit the scaffolds in matching linear trajectories mimicking the organization of the intact spinal cord [45]. In addition, the optimal neuronal phenotypes that might optimize relay formation are unknown. Most likely, a combination of different spinal cord neuronal phenotypes will be needed to optimize functional recovery. Furthermore, the very large numbers of axons extending from NSC grafts may project to ectopic targets, making undesirable connections with host neurons due to a lack of guidance molecules [46]. Exogenous provision of guidance molecules may direct newly growing axons toward proper host target neurons. Finally, intensive rehabilitation may re-shape newly generated circuits and enhance functional recovery, since training and activity influence new circuit formation during development, particularly by activity-dependent stabilization of new connections and pruning of weak connections [47].

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