



Central Nervous System Regenerative Failure: Role of Oligodendrocytes, Astrocytes, and Microglia

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Animal studies are now showing the exciting potential to achieve significant functional recovery following central nervous system (CNS) injury by manipulating both the inefficient intracellular growth machinery in neurons, as well as the extracellular barriers, which further limit their regenerative potential. In this review, we have focused on the three major glial cell types: oligodendrocytes, astrocytes, and microglia/macrophages, in addition to some of their precursors, which form major extrinsic barriers to regrowth in the injured CNS. Although axotomized neurons in the CNS have, at best, a limited capacity to regenerate or sprout, there is accumulating evidence that even in the adult and, especially after boosting their growth motor, neurons possess the capacity for considerable circuit reorganization and even lengthy regeneration when these glial obstacles to neuronal regrowth are modified, eliminated, or overcome.

The failure of injured central nervous system (CNS) axons to regenerate over long distances and reestablish connections interrupted by traumatic lesions has been known for a very long time. As early as 1890, the striking difference between central axons and the often well-regenerating peripheral nerves was experimentally studied; peripheral nerve grafts were implanted into different parts of the brain, retina, and spinal cord. The results showed that denervated peripheral nerves are excellent growth-promoting substrates for regenerating

axons, whether of peripheral or central origin. Santiago Ramón y Cajal summarized these pioneering studies in his seminal book, *Regeneration and Degeneration of the Nervous System* (1913 in Spanish; 1928 first English edition; Ramón y Cajal et al. 1991). He concluded that adult central neurons can be induced to grow long axons by attractive and trophic factors originating from peripheral nerves. He also speculated that the absence of regeneration in CNS tissue would be because of a lack of such factors in the adult brain and spinal cord. Modern tracing

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J. Silver et al.

methods and electron microscopy confirmed the old findings in the early 1980s (Aguayo et al. 1991), but the discovery of neurotrophic activities, for example, brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), or leukemia inhibitory factor (LIF), in adult CNS tissue reopened the question about molecular mechanisms. In vitro studies on the interaction of neurons confronted with CNS tissue explants or frozen sections led to a new concept of specific neurite growth inhibitory factors in the adult CNS (Schwab and Thoenen 1985; Carbonetto et al. 1987; Schwab and Caroni 1988; Fawcett et al. 1989; Rudge and Silver 1990; Mckeon et al. 1991). Surprisingly, these factors were enriched in CNS myelin and oligodendrocytes, but also in scar areas and, as found later, in perineuronal nets (PNNs) (Schwab and Caroni 1988; Sandvig et al. 2004; Pizzorusso et al. 2006; Cregg et al. 2014). Today, a detailed picture on growth inhibitory and repulsive factors expressed by different types of glial and neuronal cells at various stages of CNS development and maturation arises (Lutz and Barres 2014; Silver and Silver 2014). This article summarizes the contributions of astrocytes, oligodendrocytes, and microglia/macrophages, as well as some of their precursors to growth inhibition and regeneration failure in the adult CNS.

Although glial cells influence the growth of regenerating axons by soluble factors or membrane contacts at the level of growth cones, their influence can also regulate the growth state and programs of neurons at the transcriptional and posttranscriptional levels. Microglia and monocyte-derived macrophages (MDMs) recruited into lesioned tissue are expected to exert similar effects on axons. Thus, “extrinsic” growth regulatory cues interact with and codetermine the “intrinsic” ability of injured CNS neurons to form regenerating sprouts and elongate over long distances. Current experimental therapeutic approaches in animal models aim at manipulating all of these components, for instance, by suppressing or neutralizing growth inhibitory signals, supplying growth promoters, and enhancing neuron intrinsic growth programs (Cafferty et al. 2008; Zorner and Schwab 2010; Hollis and Tuszynski 2011; Liu et al. 2011).

OLIGODENDROCYTES AND CNS MYELIN INHIBIT NEURITE REGENERATION, COMPENSATORY GROWTH, AND PLASTICITY

Adult CNS Myelin Is Inhibitory for Neurite Growth and Regeneration

When growing dorsal root ganglion, cortical or cerebellar neurons derived from perinatal rats and mice were confronted in culture with optic nerve explants, white matter, CNS myelin, or oligodendrocytes, growth cones collapsed shortly after contact and neurite elongation stopped (Schwab and Thoenen 1985; Carbonetto et al. 1987; Schwab and Caroni 1988; Fawcett et al. 1989). Clinical and experimental observations suggested that the repair capacity of the CNS after injuries is much higher during development than at more mature stages. Using in vivo lesion experiments in embryonic chicken and newborn rodents, the switch from a regeneration-permissive to a nonpermissive property of CNS tissue seemed to be correlated in time and space with myelin formation (Reh and Kalil 1982; Keirstead et al. 1992). For a more detailed analysis of such effects, neurite growth inhibition, antiadhesive effects, and growth cone collapse were subsequently used to characterize and purify the main factors responsible for the fiber growth inhibitory effects of the adult CNS tissue. In line with the observations from the in vitro encounter assays, potent neurite growth inhibitory factors were found to be enriched in CNS myelin. The membrane proteins Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte/myelin glycoprotein (OMgp), the ephrins B3 and A3, the semaphorins 4D, 5A, and 3F, as well as chondroitin sulfate proteoglycans (CSPGs) and the myelin glycolipid sulfatide were all found to exert strong growth inhibitory effects on a variety of neuronal cells in vitro (Sandvig et al. 2004; Giger et al. 2010; Fawcett et al. 2012). The molecules are active at very low concentrations, which prompted the search for corresponding receptors. Today, the Nogo receptor family, NgR 1–3, the new Nogo-A-specific receptor sphingosine-1-phosphate receptor 2 (S1PR2), several Eph receptors, semaphorin receptors, and the CSPG-interacting proteins

LAR and protein tyrosine phosphatase PTPase- σ have been identified as functional receptors mediating growth cone collapse and growth inhibition of the corresponding ligands (Liu et al. 2006a,b; Shen et al. 2009; Giger et al. 2010; Fisher et al. 2011; Kempf 2014). Like neurotrophins or Wnt, many of the growth inhibitory ligands seem to function by activation of multisubunit receptor complexes (Schwab 2010).

Specific Growth Inhibitory Factors Acting via Neuronal Receptor Complexes Are Present in the CNS and Enriched in Myelin

Two main effects can be distinguished when growing neurites interact with growth inhibitory factors: (1) a fast local collapse of lamellipodia and filopodia of the growth cones after contact with many of the inhibitory factors, and (2) long-lasting cell-body-mediated growth inhibition, for example, by Nogo-A (Nash et al. 2009; Schwab 2010). Growth cone collapse is largely mediated by effects on the cytoskeleton, in particular, in the form of actin filament destabilization (Nash et al. 2009). Activation of the small GTPase signal transducer Rho, of the downstream Rho-associated protein kinase (ROCK), and actin regulators slingshot and cofilin seem to play major roles for Nogo-A-induced inhibition. For ephrins and Nogo-A, endocytotic uptake of ligand/receptor complexes, followed by retrograde transport to neuronal cell bodies in signaling endosomes, has been shown (Zimmer et al. 2003; Joset et al. 2010). For Nogo-A, Rho-A activation and cAMP-response element-binding (CREB) inactivation play crucial roles for the subsequent long-term down-regulation of the neuronal growth machinery (Hannila and Filbin 2008; Joset et al. 2010). Accordingly, these inhibitory effects of Nogo-A, MAG, or CNS myelin can be counteracted by elevated levels of cAMP or high concentrations of neurotrophic factors, which, in turn, elevate cAMP and P-CREB (Hannila and Filbin 2008). Interestingly, Nogo-A also down-regulates a potent cellular growth regulator, mammalian target of rapamycin (mTOR) (Peng et al. 2011). Conversely, exogenous stimulation of mTOR, either through the use of genetic tools or pharmacologically

(e.g., by rapamycin), leads to strong stimulation of sprouting and growth, even on inhibitory substrates in vitro or in vivo (Liu et al. 2011). Whether different growth inhibitory factors converge on signaling pathways, leading to growth cone collapse or cell-body-mediated growth suppression, remains to be analyzed. A first example for such a convergence is shown by the recent demonstration that the Nogo/MAG/OMgp receptor, NgR1, can also function as a receptor for the structurally very different growth inhibitory CSPGs (Dickendesher et al. 2012).

Myelin-Associated Growth Inhibitors Restrict Developmental Plasticity and Stabilize the Structure of the Adult CNS

The growth inhibitory nature of CNS myelin and the expression of several different growth inhibitory factors by oligodendrocytes and in myelin membranes was a surprising finding at first. During development, many of these factors are expressed by different cell types, including subpopulations of neurons, and they serve repulsive, negative guidance functions, or anti-adhesive or migration modulatory roles (Schwab 2010). Myelin formation in the CNS is tract dependent; it starts in a given fiber tract after the axons have reached their targets and established functional connections. Restricting any further growth and axonal branching in such a tract may be one of the important functions of myelin-associated growth inhibitory factors. A number of findings support this concept. Structural plasticity is very low in white matter, but higher in gray matter in the adult CNS, and highly plastic regions are often particularly low in myelin content. In the neocortex, a temporal coincidence exists between myelin formation in layers IV–VI and the maturation-dependent reduction of plasticity, for example, the closure of the critical window for ocular dominance plasticity (McGee et al. 2006). Importantly, developmental levels of structural plasticity could be reestablished in full adult life in Nogo or Nogo receptor (NgR1) knockout (KO) mice in the visual and the sensorimotor cortex (McGee et al. 2006; Akbik et al. 2013). Similar results were obtained by enzymatic removal of

J. Silver et al.

CSPGs (Pizzorusso et al. 2006). Furthermore, aberrant sprouting was observed after experimental or pathologic demyelination, for example, in the optic nerve (Tansey et al. 1985; Colello and Schwab 1994; Phokeo et al. 2002). Neurite growth inhibitory factors expressed by oligodendrocytes, including Nogo-A and CSPGs, therefore, appear as specific stabilizers of the highly complex structure and wiring of the CNS of higher vertebrates (Fig. 1).

Suppression of Neurite Growth Inhibitory Factors Enhances Sprouting and Regeneration of Injured Neurites and Functional Recovery after CNS Injury

A variety of methods has been used to neutralize or delete myelin-associated inhibitory factors to study axonal regeneration and repair processes after CNS injury. The most extensive literature exists for Nogo-A, for which function-

blocking antibodies or autoimmunizations, Nogo receptor–blocking peptides or fusion proteins, gene knockdowns (KOs), or receptor KOs have been used for in vivo manipulations, in particular, in the context of spinal cord injury (SCI), stroke studies, as well as autoimmune disease models (Schwab 2004; Cafferty et al. 2008; Pernet and Schwab 2012). Acute blockade of Nogo-A, the Nogo receptor complex, or of the downstream Rho/ROCK pathway led to enhanced regenerative sprouting and elongation over variable distances of injured corticospinal, rubrospinal, or aminergic axons in the spinal cord of adult rats and mice after injury. Enhanced compensatory sprouting of spared fibers is often also observed. On the level of behavior, animals frequently show significantly higher levels of functional recovery than the control reagent-treated or -untreated controls. Negative effects, which could be expected if un-directed or random growth was overstimulated,

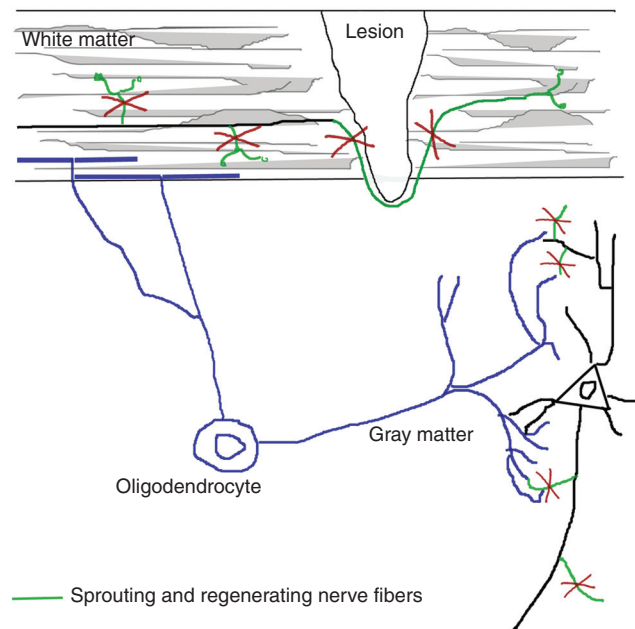


Figure 1. Oligodendrocytes express neurite growth inhibitory proteins, including the membrane protein Nogo-A, on their cell surface and CNS myelin. These proteins inhibit branch formation along the mature axon in white matter, but they also impair compensatory and regenerative fiber growth following axonal injury. In gray matter, the lower levels of these inhibitory proteins allow some structural remodeling of dendritic and axonal arbors and connections to occur, but these processes can still be potentiated by neutralization or deletion of the neurite growth inhibitors in the mature CNS.



were absent (Liebscher et al. 2005). Most remarkably, pain thresholds were not different from the ones in control animals and spasticity decreased in anti-Nogo-A antibody-treated spinal cord-injured rats (Liebscher et al. 2005; Gonzenbach et al. 2010). Absence of malfunctions was also seen in experiments with spinal cord-injured macaques, whereas skilled hand and finger movements recovered almost completely following high cervical spinal hemisection lesions and a 1-mo intrathecal anti-Nogo-A antibody infusion (Freund et al. 2006, 2009). A phase 1 clinical trial with intrathecal application of a function-blocking anti-Nogo-A antibody over 30 d in acutely and severely spinal cord-injured patients confirmed the absence of negative side effects of this treatment also in humans (Abel et al. 2011).

In stroke models, suppression of Nogo-A or NgR1 enhanced the compensatory sprouting of the spared, contralesional corticofugal system, which grew across the brain stem and spinal cord midline and reinnervated the denervated side. This process was associated with a very high degree of recovery of skilled movements, in particular, the forelimb (Cafferty et al. 2008; Tsai et al. 2011; Lindau et al. 2013). In experimental allergic encephalomyelitis, a well-studied rodent model for multiple sclerosis, Nogo-A KO, antibodies against Nogo-A or NgR1 KO led to a milder disease course and higher functional recovery (Karnezis et al. 2004; Yang et al. 2010; Petratos et al. 2012).

Whether simultaneous deletion of several inhibitory factors and/or very massive stimulation of the intrinsic neuronal growth program (Liu et al. 2011) would yield more extensive regeneration and functional repair than what has been obtained up to now requires systematic additional studies. The danger exists, however, that guidance and target interaction mechanisms of the adult CNS, which are required to establish and control new circuits and keep the CNS wiring in a stable condition, could be overrun, and chaotic connections and malfunctions, for example, epilepsies, could result. A remarkable finding has also been that (conventional) KO mice for Nogo or its receptor NgR1 have repeatedly resulted in milder fiber regrowth

effects than acute interventions with, for example, neutralizing antibodies or receptor-blocking peptides (Dimou et al. 2006; Cafferty et al. 2010; Lee et al. 2010; Schwab 2010). For a Nogo-A KO mouse, the up-regulation of several ephrins and semaphorins and their receptors has recently been shown (Kempf et al. 2013). This represents a striking example of functional compensation of the lack of a physiologically important molecule by the organism and underlines the role of myelin-associated neurite growth inhibitory factors for the homeostasis of the adult CNS.

ASTROCYTES AND GLIAL PROGENITOR CELLS PLAY CRITICAL ROLES IN REGENERATION FAILURE

The Glial Scar and PNN: Proteoglycan-Mediated Inhibition of Regeneration and Sprouting

A wide variety of injuries or diseases of the CNS, which are severe enough to cause a breach in the blood-brain barrier or overt bleeding, lead to secondary tissue damage, resulting in the encapsulation of the lesion by reactive astrocytes, which form the so-called glial “scar.” The scar is an essential part of wound healing in the brain and spinal cord because it serves to physically and molecularly wall off zones of intense inflammation to provide a measure of protection for the remaining fragile tissue (Silver and Miller 2004). This portion of the review will describe current thinking about the biological consequences of glial scarring in the spinal cord, especially, as it affects wound repair and axon regeneration; however, it is likely that similar events occur throughout the CNS.

The astroglial component of the scar wall is formed by at least five critical processes. The first is the rapid (within days) migration of astrocytes from the lesion epicenter toward its outermost edges, actively driven away by as-yet-unknown factors produced by inflammatory cells (Fitch and Silver 1997). The second is proliferation of the thin layer of reactive astrocytes (gliosis), which comes to reside just at the lesion margin (reactive astrocytes further away do not mark-

edly increase their proliferation rates, nor do they migrate extensively) (Bush et al. 1999; Faulkner et al. 2004; Wanner et al. 2013). The third is the accumulation of intermediate filament proteins, predominantly glial fibrillary acidic protein (GFAP), vimentin, and nestin, leading to cellular hypertrophy of the astroglial layer, as well as nondividing reactive astrocytes further away (Pekny et al. 1999; Xu et al. 1999; Wilhelmsson et al. 2004; Bardehle et al. 2013). The fourth involves the restructuring of the gliotic layer into a mesh-like envelope, which changes from a radial, longitudinal orientation to an alignment largely perpendicular to the long axis of the cord and is, thus, highly obstructive to any potential regrowth of the major projection axon pathways (Bardehle et al. 2013; Wanner et al. 2013). In addition, there occurs the production of a variety of potently growth inhibitory extracellular matrix (ECM) molecules, among which are the lectican family of CSPGs (McKeon et al. 1991, 1995, 1999; Davies et al. 1999; Yamaguchi 2000; Busch and Silver 2007; Alilain et al. 2011; Brown et al. 2012; Kawano et al. 2012; Li et al. 2013; Takeuchi et al. 2013). Thus, the scar presents a physical and molecular constraint against the release of intralésional inflammatory agents, but also, unfortunately, to axon regeneration.

It is now known that this entire cascade of events is triggered, in part, by TGF- β bound to fibrinogen, which pores in through the leaky or hemorrhagic blood–brain barrier and activates the SMAD2 signaling cascade. Blocking the TGF- β receptor pathway abolishes the fibrinogen-induced effects on glial scar formation and, in particular, reduces proteoglycan deposition (Schachtrup et al. 2010). The early migratory response of astrocytes appears to be, at least in part, under the control of glycogen synthase kinase-3 (GSK-3) activity because acute treatment with a potent GSK-3 inhibitor accelerates migration, resulting in better sequestration of inflammatory cells and significantly enhanced functional improvement (Renault-Mihara et al. 2011). Also, the transcription factor SOX9 appears to be a critical component of the pathway that leads to inhibitory matrix deposition in the lesion because its conditional KO leads to

reduced expression of various CSPGs and improved locomotor function (Mckillop et al. 2013). The architectural glial changes are under the control of STAT3. When this transcription activator is genetically deleted in astrocytes (or the proliferating/gliotic astrocytes are themselves deleted), the walling off phenomenon is severely perturbed and inflammatory infiltrates invade much larger regions of the cord, leading to rampant tissue destruction and further loss of function (Bush et al. 1999; Herrmann et al. 2008; Wanner et al. 2013). Another critical molecular determinant of astroglial scar building is injury-induced glial Ca^{2+} signaling, which regulates expression of the cell-to-cell adhesion molecule N-cadherin. This calcium-dependent tight adhesion-forming molecule likely plays an important role in strengthening the scar wall (Kanemaru et al. 2013). N-cadherin binds the fibroblast growth factor receptor (FGFR) and activates a FGFR-dependent signaling cascade, which, in turn, can enhance GFAP expression and is known to play a critical role in controlling the polarity of astrocytes (Goldshmit et al. 2012; Lee et al. 2013; Macaya et al. 2013). In its absence, N-cadherin KO mice display abnormal scar formation, leading to increased neuronal death (Kanemaru et al. 2013). Thus, the astrocytic response to injury is an essential component of damage control in the CNS, and the large number of molecular determinants involved with scar formation could be potential therapeutic targets.

There are also reactive changes in astrocytes much further away from the lesion, which eventually fill in the space vacated by dying oligodendrocytes and axons undergoing Wallerian degeneration (WD), but the structural changes here take a much longer time to manifest (Silver and Miller 2004; Wanner et al. 2013). Over extended periods of time, astroglial hypertrophy at the lesion edge, and in the tract beyond, leads to very dense aggregates of cells that, at the lesion and distally, especially near the pial surface, become obstructive to axonal regeneration (Silver and Miller 2004). Interestingly, denervated target regions, which are distant from the lesion, also undergo reactive glial changes, again associated with the production of sulfated proteo-



glycans that are largely contained within the PNN (Massey et al. 2006; Alilain et al. 2011; Andrews et al. 2012; Hansen et al. 2013). The molecular triggers, which instigate up-regulation of these net-associated proteoglycans far from lesions, are largely unknown, but also appear to be regulated, in part, by the SOX9 transcription factor pathway (Mckillop et al. 2013) as well as neuronal activity (Wang and Fawcett 2012). CSPG up-regulation within the PNN is extremely important because it serves to limit potential functional plasticity, which could occur via compensatory sprouting from surviving inputs (Hockfield et al. 1990; Yamada et al. 1997; Berardi et al. 2004; Massey et al. 2006; Pizzorusso et al. 2006; Gogolla et al. 2009; García-Álías et al. 2011; Kwok et al. 2011; Wang and Fawcett 2012; de Vivo et al. 2013; Xue et al. 2014).

Oligodendrocyte Progenitor Cells and the Neuroglial 2 Proteoglycan: The Role of the Lesion Core in Regeneration Failure

Although the astroglial component of scar formation and its purported role in regeneration failure has been suggested for more than a century (Ramón y Cajal 1928; Windle and Chambers 1950) and has been clearly revealed by the use of microtransplantation experiments (Davies et al. 1997, 1999), the astroglial capsule is not solely responsible for axonal regeneration failure. When one examines, precisely, the interactions that occur between dystrophic axon tips and the cells that they closely associate with over time, it was surprising to learn that, for the most part, severed axons do not interact directly with reactive astrocytes, but rather with a population of neuroglial-2-proteoglycan (NG2)-producing oligodendrocyte precursor cells (OPCs) within the core of the lesion (Busch et al. 2010; Filous et al. 2010). It had long been thought that after SCI, severed axons would retract back to sustaining collateral (Ramón y Cajal 1928), and, thus, the free segment of remaining axon within the white matter would eventually be eliminated. However, with the advent of modern labeling techniques, we now know that, following the phase of axonal retraction (which is largely the

result of an aggressive attack on the dystrophic axon tip by inflammatory blood-derived macrophages) (Horn et al. 2008; Busch et al. 2009; Evans et al. 2014), axotomized neurons often survive (Kwon et al. 2002; Nielson et al. 2010). Eventually, the cut axon stops retracting and its dystrophic tip can come to rest for many years (even decades) (Ruschel et al. 2013) within the penumbra of the lesion (Li and Raisman 1995; Guest et al. 2005; Kadoya et al. 2009). What maintains the dystrophic end of the axon chronically within the hostile environment of the glial scar? Are the mechanisms involved with long-term maintenance of the severed axon critical to regeneration failure? Although SCI results in astroglial emigration away from the lesion, inside the core of the lesion, during the first several weeks postinjury, there is a robust recruitment and proliferation of a wide variety of cell types, which all become surrounded by astroglial scar. In addition to the vast array of activated blood-derived macrophages and other inflammatory cells, which begin to invade the lesion core within the first day (Popovich and Longbrake 2008; Kigerl et al. 2009; Evans et al. 2014), the normally rarely dividing ependymal cells around the central canal become activated and rapidly proliferate (Meletis et al. 2008). Within the first week, they also move into the core of the lesion and, as they do so, they down-regulate their ependymal markers and begin to display reactive astroglial phenotypes, thus, contributing to the glial scar (Johansson et al. 1999). Additionally, after penetrating injuries that open the dura mater, but also, importantly, after contusive or ischemic injuries that leave the meninges largely intact, fibroblast-like stromal cells, which are derived from the meninges or pericytes or pericyte-like cells located around the perimeter of blood vessels, divide vigorously and slough off from the meninges or vasculature to help populate the lesion epicenter (Decimo et al. 2011; Görnitz et al. 2011; Fernandez-Klett et al. 2013; Sabelström et al. 2013; Soderblom et al. 2013). These cells interact with the astroglial component of the scar and form a fibrotic-like layer internal to the astroglial capsule. Via their interactions with astrocytes and the collagenous/proteoglycan-rich matrices that are pro-



duced, they also play a role in helping to seal the lesion, but also may play a role in blocking regeneration (Davies et al. 1999; Stichel et al. 1999; Kawano et al. 2012; Sabelström et al. 2013; Soderblom et al. 2013). Finally, there occurs a robust proliferation of OPCs, which produce the purportedly potentially inhibitory NG2 CSPG, as well as a cocktail of growth-promoting ECM molecules, including laminin and fibronectin (Zai and Wrathall 2005; Lytle et al. 2006; Busch et al. 2010). Thus, the early lesion core becomes a rich oasis of cells with a mixture of growth-inhibiting and -promoting properties.

The role of NG2⁺ cells, both in the normal CNS and after injury, remains controversial. NG2 is a member of the CSPG family of ECM molecules that is thought to contribute to regeneration failure. Because NG2 is one of the most dramatically up-regulated CSPG after CNS injury (Levine 1994), it has been suggested that NG2⁺ cells are “the” major regeneration-blocking cell type (Dou and Levine 1994; Fidler et al. 1999; Chen et al. 2002; Tan et al. 2006). In contrast, several studies suggest that NG2⁺ cells may not be repulsive at all. Indeed, the dystrophic tips of severed axons, which remain within the lesion penumbra for extended periods, reside closely among NG2⁺ glia (Zhang et al. 2001; McTigue et al. 2006; Busch et al. 2010) and NG2⁺ cells seem to facilitate growth of developing axons (Yang et al. 2006). Our laboratory suggested that the population of stem-like, NG2-producing cells in the lesion core may contribute to regeneration failure by acting as a kind of “safe haven” for dystrophic axons, stabilizing them as they are forced to retract backward into the caudal end of the lesion by activated macrophages (Busch et al. 2010). Indeed, severed axons in the lesion appear to be “addicted” to the surface of these cells and refuse to leave. However, the mechanisms that govern this tight cell–cell interaction and, in particular, whether the NG2 CSPG is involved in this close association, remained important and unresolved questions.

Recently, we sought a better understanding of the interaction between severed sensory axons and adult cord-derived NG2 glia after a dorsal column injury (Filous et al. 2012). In our studies, we observed a novel mechanism of regener-

ation failure. When combined with growth-promoting ECM molecules in critical ratios, purified NG2 and other CSPGs initially constrain axons to their territory via a GAG/LAR family CSPG receptor-mediated interactive mechanism (Shen et al. 2009; Filous et al. 2010; Fisher et al. 2011; Lang et al. 2012, 2013). NG2 glia also constrain early axonal outgrowth but, in addition, can lead to longer lasting entrapment of the neuron onto the glial cell surface through an unusual neuroglia synaptoid-mediated stabilization, both in vitro and in vivo. Given that neurons form synapses with NG2⁺ OPCs under physiological conditions throughout the CNS (Bergles et al. 2000; Chittajallu et al. 2004; Lin et al. 2005), it is possible that such synaptic-like interactions within the damaged white matter allow for long-lasting associations between the dystrophic tips of sensory neurons and NG2⁺ cells. Although these stabilizations, initially, may be beneficial to prevent further dieback (Filous et al. 2010), they may also place further limitations on the forward movements of the struggling axon tip. The idea that synaptic-like connections form between regenerating axons and reactive glia, and may serve to curtail axonal regrowth after injury, had been suggested many years ago (Carlstedt 1985), although the importance of this phenomenon in regeneration failure had largely been abandoned. After a dorsal root crush, even following a peripheral conditioning lesion, injured sensory axons can regenerate rapidly within the proximal root until they reach the dorsal root entry zone (DREZ), a transitional region between the peripheral nervous system (PNS) and the CNS, where they abruptly halt their forward progress and remain (Carlstedt 1985; Liuzzi and Lasek 1987; Di Maio et al. 2011). Early studies suggested that, as peripheral axons regenerate toward the CNS, they contact reactive astrocytes, which initiate the early stages of so-called synaptoid formations in close association with the astrocyte surface. Interestingly, our current studies suggest that, in addition to their wall-building job, reactive astrocytes may also play an indirect role in signaling for sensory axons to begin synapse formation mediated, at least in part, via thrombospondins, which are important in regulating

neuron-to-neuron synaptogenesis (Christopher et al. 2005). However, our data show clearly that dystrophic axons after DCC are actually synapsing on the NG2⁺ cell, rather than astrocytes, and this relationship is also likely to occur at the DREZ. It is also possible that reactive astrocytes can directly induce proliferation of OPCs by releasing the mitogen, Sonic hedgehog (SHH) into the injury environment (Amankulor et al. 2009). Activation of the SHH-Gli-signaling axis within the OPC population results in its dramatic expansion and the potential amplification of OPC-mediated effects on severed axons. It is also probable that inflammatory cells play a role in accelerating OPC mitosis as well (Miron et al. 2013).

This close interaction between NG2⁺ cells and injured neurons after SCI provides a new way of thinking about how CSPGs and the core of the scar “inhibit” axonal migration and helps explain how dystrophic axon tips persist within the scar-encased, hostile lesion environment. Thus, we hypothesize that in vivo within the scar core, CSPGs do not cause axon tips to cease growing because of a lack of adhesion, but rather because they create dystrophy and increasing entrapment of the growth cone via abnormally strong bonds with the substrate. Thus, the scar, with its two distinct regions (the core and the wall), can inflict a measure of inhibition that thwarts the advancement of the regenerating neuron (see Fig. 1).

Plasticity of Reactive Astrocytes?

The final question that I would like to speculate on is whether reactive astrocytes in the scar wall are permanently refractory to axonal regeneration or whether they can become plastic and promote or, at least, allow axonal growth (as they do during development) (Silver et al. 1982, 1993; Silver and Ogawa 1983). Emerging data suggests that they can be plastic and regeneration failure through the scar is the result of an imbalance between a lack of intrinsic growth machinery in the neuron (Ylera et al. 2009) and extrinsic forces (some of which are discussed above) that limit growth. Astrocytes that contribute to the scar and are derived from the ependymal tube

appear to be slightly more “immature” than astrocytes derived from self-duplication because they express less GFAP relative to vimentin (Fig. 2) (Meletis et al. 2008). It would be very interesting if the well-known regeneration-enhancing functions of ependymogial cells that are present in robustly regenerating cold-blooded species (Singer et al. 1979) are retained, at least to some extent, in the ependymal subpopulation of reactive astrocytes in scar tissue of mammals (Silver and Steindler 2009). Potential functional differences between astrocyte populations in the scar may be appearing in the rather dramatic ability of neurons to regenerate their severed axons right across and beyond carefully crafted lesions within the rodent spinal cord or optic nerve following PTEN/SOCS3 deletion (Park et al. 2010; Sun et al. 2011). Indeed, the impressive regeneration, albeit across relatively narrow lesions, when the protein products of these growth or cytokine regulatory genes are diminished or genetically deleted, is strictly confined to and dependent on astroglial bridges, which form spontaneously across the lesion core (Filous et al. 2010; Zukor et al. 2013). The appreciation of whether separate reactive astroglial subpopulations exert these guidance functions or possibly even if gliotic astrocytes in the scar wall can be plastic and made growth permissive or even promoting in response to the presence of a robustly growing axon, could be very important and therapeutically provocative (Ahmed et al. 2005). It would suggest that we consider strategies tailored toward amplifying or attenuating particular, functionally distinct astrocyte subpopulations or to further enhance the plasticity of gliotic astrocytes to help maximize functional recovery.

MICROGLIA AND MACROPHAGES

Origin of Macrophages in Injured CNS

In parallel with the injury-induced changes described above for oligodendrocytes and astrocytes, a robust and long-lasting inflammatory response is initiated, which is dominated by macrophages. These cells are mostly derived from two sources: (1) resident microglia, and

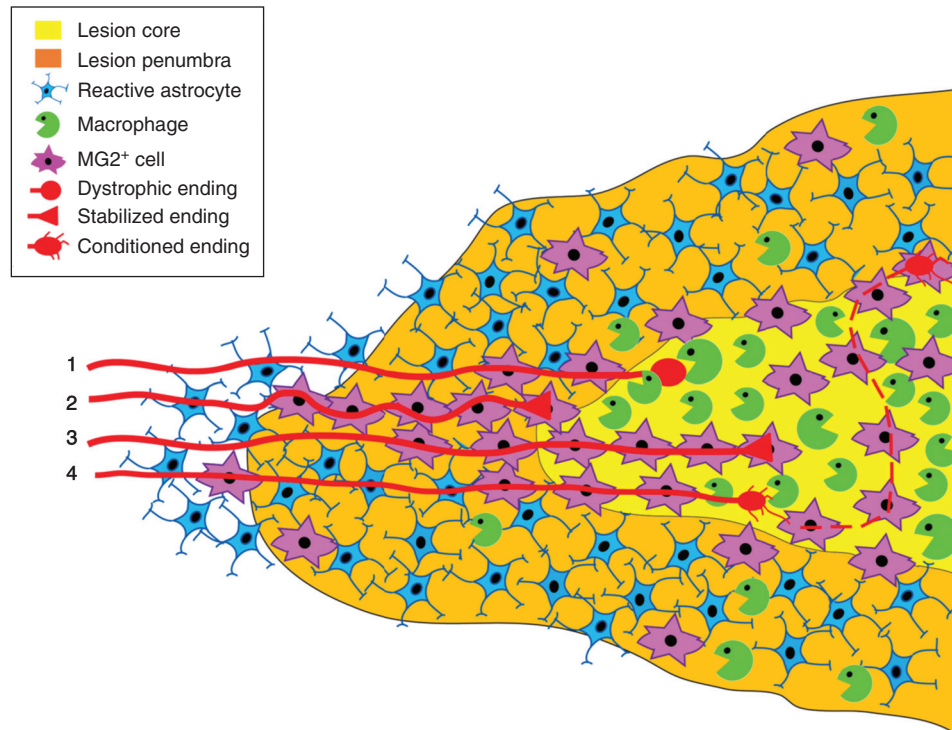


Figure 2. Schematic representation of the proximal end of a dorsal column crush lesion 7 d after injury. GFAP⁺ astrocytes (blue) have pulled away from the lesion core, which is now populated by NG2⁺ cells (purple) and phagocytic ED1⁺ macrophages (green). The fibroblastic and ependymal cell types are not displayed, but are also plentiful in the lesion core. Dorsal root ganglion neurons (red) attempt to regenerate into the lesion core. (1) Typical axon with a dystrophic growth cone that has become susceptible to macrophage attack. (2) Typical axon that has undergone macrophage-mediated retraction back to NG2⁺ cells and stabilized. (3) Atypical axon that has stabilized further distally within the lesion core on a contiguous bridge of NG2⁺ cells. (4) Growth cone of a neuron that has been stimulated or conditioned and able to overcome macrophage-induced axonal dieback and extend into the lesion core on NG2⁺ cells. (From Busch et al. 2010; reprinted, with express permission, from the *Journal of Neuroscience* and the investigators of this review.)

(2) blood monocytes, that is, macrophage precursors that emigrate from bone marrow or the spleen (Popovich et al. 1999; Popovich and Hickey 2001; Longbrake et al. 2007; Swirski et al. 2009; Blomster et al. 2013). Microglia originate from precursor cells in the yolk sac and become homogeneously distributed throughout the CNS during early embryogenesis (Ginhoux et al. 2010). Like astrocytes, microglia respond rapidly to injury, extending cellular processes or migrating toward the lesion site where they participate in scar formation (Davalos et al. 2005; Dibaj et al. 2010). Surely, this early and rapid response serves a protective role, as there is no obvious evolutionary advan-

tage for blanketing the CNS with cells that, when provoked, will mobilize and destroy delicate nervous tissue. Indeed, blocking or preventing microglial activation, via either pharmacologic or genetic means, exacerbates lesion pathology and impairs recovery of function (Lalancette-Hébert et al. 2007; Hines et al. 2009).

After a delay of ~2 d postinjury, monocytes bind to endothelial adhesion molecules and then migrate into the lesioned CNS, down chemotactic gradients established by astrocytes (Pineau et al. 2010). Shortly thereafter, monocytes differentiate into tissue macrophages. Because microglia and MDMs are both of myeloid lineage, lineage-specific markers cannot be used



to distinguish between these major CNS macrophage subsets. Equally ambiguous is the effect that CNS macrophages have on neurons and axons that survive after CNS injury.

Seemingly conflicting data implicate macrophages, regardless of their source, as effectors of both tissue repair and secondary tissue damage. Although Ramón y Cajal is often recognized as the “father of neuroscience,” he also provided some of the earliest descriptions of neuroimmune interactions in the injured CNS. Specifically, he noted that macrophages accumulated and persisted at sites of injury and concluded that their primary role was as scavenger cells (Ramón y Cajal 1991):

This leukocytic invasion of the dead neuron is not surprising. It is a general law that any mortified portion, no matter what is its character, becomes a pasture-ground for phagocytes. We believe that the protagonists of all acts of neurophagy are nothing else than the granular corpuscles which accumulate so prodigiously in the necrotic focus of the centres and in the peripheral stumps of degenerated nerves.

Because he did not have the benefit of modern-day techniques (e.g., radiation bone-marrow chimeras, transgenic mice, etc.), Ramón y Cajal and his contemporaries were unable to unequivocally determine the origin of CNS macrophages. Regardless, he accurately predicted that most phagocytes present in lesioned CNS tissue were derived from blood, that is, monocytes:

... we believe also that the phagocytes—our traumatocytes—which have penetrated into the neuronal cadaver positively represent large leucocytes with a lobulated nucleus, which have come from the host’s blood.

We now know that his predictions were correct and the biased accumulation of MDMs at the lesion center may have significant implications for the growth or retraction (“dieback”) of injured axons (see below).

Macrophage Functions in Injured CNS

Some years after Ramón y Cajal’s seminal observations (circa 1950), additional insight into CNS macrophage function was gleaned from a

serendipitous discovery. Although studying neural mechanisms of thermal regulation in dogs with SCI, Windle, Clemente, and colleagues discovered that deliberate systemic injection of pyrogens had the unintended benefit of enhancing neurologic recovery (Windle and Chambers 1950; Clemente and Windle 1954). Postmortem analysis of dogs injected with crude pyrogens revealed markedly increased numbers of intraspinal macrophages and reduced intralésional scarring as compared with injured spinal cords of untreated dogs (Clemente and Windle 1954). Almost 30 years later, Guth and colleagues extended Windle’s observations showing that systemic injections of purified endotoxin (i.e., lipopolysaccharide [LPS]) into spinal-injured rats enhanced intraspinal leukocytosis beyond that normally seen after SCI (Guth et al. 1994a). This enhanced inflammatory reaction was accompanied by more robust axon growth and quantitatively superior improvements in hindlimb locomotor function. Guth later found that the salutary effects of LPS could be further improved by simultaneously treating animals with anti-inflammatory agents, including indomethacin or steroids (Guth et al. 1994b). This combination approach, although seemingly counterintuitive, was based on keen insight regarding the divergent functions of activated CNS macrophages. Guth realized that, during maturation, macrophages become “primed” or partially activated by cytokines (and other factors) present in the injury milieu; however, to attain a greater level of functional competency, including the ability to promote axon growth or neuroprotection, macrophages likely require a second distinct signal, in this case, LPS. He also recognized that once activated, these same cells release hydrolyzing enzymes, oxidative metabolites, and arachidonic acid metabolites (e.g., prostaglandins), which can damage neurons and glia. Indomethacin and steroids were used to inhibit these destructive secretory components of activated macrophages.

Over the next 10–15 yr, data from several laboratories using rabbit, guinea pig, and rat models of SCI showed that, in the absence of a secondary stimulus, the injurious effects of in-

traspinal macrophages predominate. Regardless of species, injury type (e.g., compression, contusion), or injury severity, selective inhibition or depletion of macrophages during the first 1–2 wk postinjury consistently reduces secondary or bystander tissue injury, leading to improved recovery of sensory, motor, or autonomic functions (Giulian and Robertson 1990; Blight 1994; Popovich et al. 1999; Gris 2004).

Emerging data now indicate that in response to different combinations of factors, which are normally found in the extracellular milieu of the injured nervous system, macrophages differentiate into functionally distinct cell subsets that differentially affect neuron survival and axon growth (Stout et al. 2005; Kigerl et al. 2009). For example, cytokines, cell fragments, and nucleic acids promote differentiation of macrophages into “classically” (M1) or “alternatively” activated (M2) cells. The canonical *in vitro* model for promoting inflammatory M1 macrophage differentiation is exposure of native (unstimulated) myeloid cells to LPS and inflammatory cytokines, including interferon (IFN)- γ or tumor necrosis factor (TNF)- α . Alternatively, to promote M2 differentiation, immature myeloid cells are stimulated with interleukin (IL)-4 or -13 (Gordon and Taylor 2005). After CNS injury, signaling pathways that polarize macrophages toward an M1 phenotype predominate (Kigerl et al. 2009; David and Kroner 2011). M1 macrophages can be neurotoxic and cause axon dieback (Horn et al. 2008; Kigerl et al. 2009). Thus, the neuroprotective effects of acute macrophage inhibition or depletion in SCI models might be explained by reducing the burden of M1 macrophages at the injury site. Surprisingly, these same cells also can enhance neurite outgrowth.

In vivo injections of inflammatory stimuli (e.g., LPS, zymosan), which are needed to promote an M1 macrophage phenotype *in vitro*, enhance regeneration of injured peripheral and central axons (Yin et al. 2003; Steinmetz et al. 2005; Boivin et al. 2007; Gensel et al. 2009). In injured brain, spinal cord, and optic nerve, macrophage clusters are often associated with sprouting of injured axons (Fig. 3). This endogenous repair phenomenon is mediated by

macrophages via the release of neurotrophins and growth factors or, indirectly, by activating glia within the scar, which subsequently produces a trophic gradient. BDNF, CNTF, and glial cell line–derived neurotrophic factor (GDNF) have been implicated in this response (Batchelor et al. 2002; Yin et al. 2006; Muller et al. 2007; Gensel et al. 2009; Benowitz and Popovich 2011). The ability of transplanted microglia or macrophages to promote neurite outgrowth in different models of SCI might be explained by a similar mechanism (Prewitt et al. 1997; Rabchevsky and Streit 1997; Rapalino et al. 1998).

Compared with M1 macrophages, M2 macrophages may be less destructive and better able to repair the injured CNS. M2 macrophages promote more robust neurite outgrowth and recent data show that these cells release activin-A, which enhances oligodendrocyte progenitor cell differentiation and, subsequently, remyelination (Kigerl et al. 2009; Miron et al. 2013). Enhancing M2 microglia/macrophage differentiation in lesioned CNS tissues is associated with neuroprotection; however, limited data exist linking M2 macrophages with axon regeneration *in vivo*. Combining peripheral nerve grafts with acidic fibroblast growth factor in an injured spinal cord produces a cytokine milieu that favors M2 macrophage differentiation, polyamine synthesis with improved axon regeneration (Kuo et al. 2011). Similarly, infusion of IL-4 (M2 cytokine) into guidance channels placed into injured sciatic nerves induces an M2 macrophage response that stimulates Schwann cell migration with enhanced axon regeneration into the distal nerve stump (Mokarram et al. 2012).

Manipulating Macrophages to Promote Axon Regeneration: Future Considerations

ProCord was an experimental cell-based therapy that was developed to treat acute SCI in humans. Clinical trials were initiated by Proneuron Biotechnologies (New York, NY) based on data showing that autologous macrophages, when activated *ex vivo*, then injected into the injured spinal cord, promote axon regeneration and

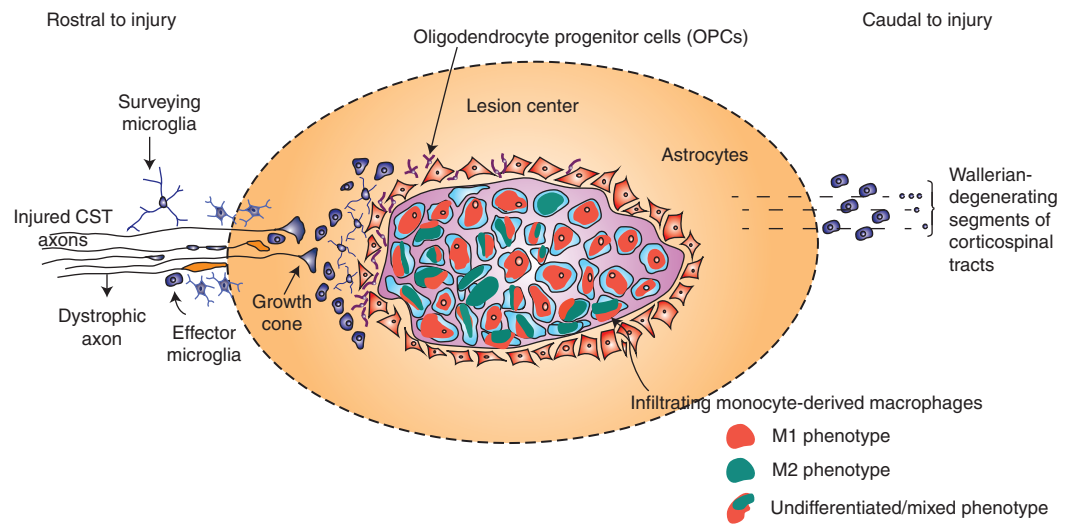


Figure 3. Schematic of microglia and MDM reactions elicited by SCI. After injury, the lesion center (also referred to as “epicenter” in contusion lesions) becomes filled with phagocytic macrophages derived from blood monocyte precursors. These cells become enlarged as they phagocytose lipid and cell debris. These and other stimuli in the lesion prime an M1 macrophage phenotype (red). Only a subset of macrophages become “alternatively” activated (i.e., M2 macrophages, green). Some cells remain undifferentiated or adopt a heterogeneous phenotype (orange/green mix). Macrophages in the lesion center are “walled off” by reactive astrocytes, which create a scar. OPCs interdigitate between scar-forming astrocytes and are drawn toward the lesion edge by undefined factors. Complete OPC differentiation into myelinating oligodendrocytes may require factors derived from (M2) microglia subsets, which often lie outside the lesion microenvironment (gradient fill). Microglia exist in intact spinal cord as sentinel cells, which continuously survey the microenvironment. After injury or in response to subtle changes in homeostasis, microglia become activated and transform morphologically and phenotypically into effector microglia. Depending on the composition of factors present in the microenvironment, microglia can become polarized to become M1 or M2 effector cells. Rostral to the site of injury, surveying and effector microglia colocalize with damaged axons, a subset of which are undergoing dieback, but also with a subset that are stabilized or attempting to grow. Caudal to the lesion, descending axons undergo Wallerian degeneration (WD). Various factors released during WD activate microglia (and macrophages). It is common to see effector microglia (and, presumably, a subset of MDMs) colocalized with WD axon segments. CST, corticospinal tract.



reduce tissue damage in two different rodent SCI models (Rapalino et al. 1998; Bomstein et al. 2003). An overview of the rationale and design for the phase I trial was reviewed previously (Kigerl and Popovich 2006). Results of the phase 2 randomized controlled multicenter trial, involving 43 participants, showed a trend for better recovery in the control group relative to patients receiving macrophage transplants, but without group differences in the number of adverse events (Lammertse et al. 2012). Although efficacy was not established, future cell-based clinical trials for SCI (and other diseases) will benefit from the ProCord experience, because this trial identified and overcame numerous logistical

and technical constraints associated with enrolling, preparing, and injecting into the spinal cord within 14 d of injury, an autologous cellular therapy (Jones et al. 2010).

Autologous macrophage transplantation remains a promising therapeutic approach; however, new preclinical data indicate that the phenotype of macrophages generated *ex vivo* may not persist after injection into lesioned CNS. When M2 polarized macrophages are transplanted into lesioned spinal cord, they differentiate into M1 macrophages. Conversely, M2 macrophages maintain their phenotype when transplanted into intact spinal cord (Kigerl et al. 2009). Accordingly, future transplantation



protocols, whether macrophages or other cell types, will need to incorporate measures that modify the lesion microenvironment. Generic immune suppressive drugs (e.g., steroids) are not practical in this context because these drugs will affect injurious and reparative macrophage subsets. Generic macrophage inhibition or depletion strategies, including intravenous injections of anti-integrin antibodies or liposome-encapsulated bisphosphonates, could be useful, especially in the acute postinjury period or if used together with neuroprotective drugs (Popovich et al. 1999; Gris 2004; Iannotti et al. 2011; Lee et al. 2011). Neuropeptides (e.g., substance P), antibodies that block cytokine signaling or stem cells, also could be used as each is able to modulate the injury milieu, creating an environment that favors polarization of endogenous macrophages toward an M2 phenotype (Busch et al. 2011; Cusimano et al. 2012; Guerrero et al. 2012).

In addition to macrophage transplantation, targeted or “precision” immunotherapies, which inhibit or stimulate one or more phenotypically distinct macrophage subsets, is an ideal approach. Along with the M1/M2 CNS macrophage subsets described above, new reagents and genetic tools have revealed the presence of other distinct intraspinal macrophage subsets (Thawer et al. 2013). For example, variations in the relative expression of the chemokine receptor CX3CR1 or maturation markers (e.g., Ly6) define functionally distinct CNS macrophages (Shechter et al. 2009; Donnelly et al. 2011; Saiwai et al. 2013). Antibodies and small molecule inhibitors can or have been designed to target these macrophages, but whether such manipulations will affect axon regeneration requires additional research. Ideally, future studies will incorporate acute and chronic CNS lesion models. Although macrophages persist indefinitely in CNS lesions, their role in the chronic injury milieu and nearby spared tissue is unknown.

The possibility that microglia and MDMs will have distinct effects on cell repair and axon regeneration after CNS injury is likely and should also be considered when designing or interpreting preclinical studies (Popovich

and Longbrake 2008; London et al. 2013). Microglia and MDMs develop by discrete transcriptional control mechanisms from unique precursor cells (Prinz et al. 2011; Schulz et al. 2012). After injury, the discrete spatial distribution of different macrophage subsets produces heterogeneous microenvironments that can differentially affect injured axons, nascent axonal growth cones, and surrounding glia. Recent data show that signals emanating from aged brain trigger a neuroprotective transcriptomic signature in microglia (Hickman et al. 2013). Whether similar neuroregenerative or neurotoxic “sensomes” exist in microglia or MDMs, respectively, is unknown, but such profiles seem likely, especially because the ratio of microglia to MDMs increases in regions remote from the injury site, along with clear evidence of anatomical and functional plasticity or endogenous CNS repair (Zhang and Guth 1997; Popovich and Hickey 2001; Zhou et al. 2003; McTigue et al. 2006; Detloff et al. 2008; Busch et al. 2010; Hansen et al. 2013).

Conversely, physical contact between axons and macrophages within the lesion core (high ratio of MDMs to microglia) causes axons to retract or “dieback” from the injury site. Both soluble factors and cell surface proteins are culpable in this degenerative response (Horn et al. 2008; Busch et al. 2011). Macrophages express numerous membrane-bound proteins, including receptors for ephrins, siglecs (sialoadhesins), and integrins (Crocker et al. 1994; Sobel et al. 1995; Tang et al. 1997; Liu et al. 2006). Axon growth and guidance may be positively or negatively affected when these proteins are bound by corresponding ligands found on axons. Given the discrete spatiotemporal dynamics of macrophages and microglia, when, where, and how much injured axons are exposed to these cells will undoubtedly affect their ability to regenerate.

Although there is a growing appreciation that macrophages are important contributors to CNS regeneration failure, we have only a rudimentary understanding of how or whether these cells influence axon regeneration. Achieving a greater understanding of CNS macrophages should improve the safety and success of

future clinical trials designed to promote regeneration or repair of the injured CNS.

CONCLUSION

Over the past several decades, there has been steady progress in understanding basic molecular mechanisms that are responsible for the poor regenerative potential of injured central nervous system axons. Indeed, there are limitations within the neuron, that is, molecular switches that impede intrinsic regeneration machinery, and there are various glial cells that create lesion barricades or “extrinsic” inhibitory cues, which curtail the relatively limited regenerative potential of injured CNS axons. In this review, we have focused on each of the major glial cell types that serve as the primary extrinsic regulators of axon regeneration with an emphasis on the injured spinal cord. We have described how the severed axon tip, struggling to advance a new growth cone, is collapsed by myelin-derived growth-inhibitory factors, made dystrophic by proteoglycans, and further attacked by the destructive actions of M1 macrophages, whose job, early on, is to phagocytose the noxious debris. The unfortunate neuron, whose axon was once enveloped by supportive oligodendrocytes and astrocytes, is left to fend for itself during the attack; oligodendrocytes die and reactive astrocytes abandon the core of the lesion as they attempt to protect and mechanically stabilize the remaining fragile tissue from an expanding inflammatory reaction, creating yet another obstacle to regeneration. But, there is some relief, even within the eye of the storm. Once neurotoxic macrophages convert into a more reparative M2 state, and various stem-like cells, including oligodendrocyte progenitors, begin to thrive within the lesion core, the retracting axon can find a safe haven and even form synaptic-like connections on the primitive glia where, unfortunately, they remain locked in place for decades. As we have acquired a more complete appreciation of the molecular mechanisms that control the untoward effects of glia, new approaches are being developed that can readily prevent axons from dying backward and also may allow them to robustly sprout

or sometimes regenerate beyond the scar toward new functional synaptic targets. A major goal for the future will be to combine the most successful glia-targeted strategies with others that drive the neuron’s intrinsic growth capacity to maximize the regenerative potential that we now know exists within the damaged adult CNS.

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J. Silver et al.

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J. Silver et al.

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J. Silver et al.

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