Mol Neurobiol (2012) 46:227–241
DOI 10.1007/s12035-012-8290-9

Neural Regeneration: Lessons from Regenerating and Non-regenerating Systems

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Received: 29 December 2011 / Accepted: 7 June 2012 / Published online: 21 June 2012

Abstract One only needs to see a salamander regrowing a lost limb to become fascinated by regeneration. However, the lack of robust axonal regeneration models for which good cellular and molecular tools exist has hampered progress in the field. Nevertheless, the nervous system has been revealed to be an excellent model to investigate regeneration. There are conspicuous differences in neuroregeneration capacity between amphibia and warm-blooded animals, as well as between the central and the peripheral nervous systems in mammals. Exploration of such discrepancies led to significant discoveries on the basic tenets of neuroregeneration in the last two decades, identifying several positive and negative regulators of axonal regeneration. Implications of these findings to the comprehension of mammalian regeneration and to the development of spinal cord injury therapies are also addressed.

Keywords Axonal regeneration · Biocurrents · Therapies

Introduction

> "I'd give my right arm to know the secret of regeneration"—*Oscar Schotté*

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Regeneration has puzzled great thinkers since antiquity. Thomas Morgan is known for having said "since I have been unable to solve the problem of regeneration…I have decided to try something easier such as the problem of heredity." The regenerative process entails not only the recognition and restoration of missing structures, but also the integration of the newly formed tissues into the organism [1]. Some creatures populate the collective imagination for their mastery in this regard. Planarians (non-parasitic flatworms) can generate an entire animal from a minute piece of their bodies [2]. In the vertebrate universe, adult salamanders (urodele amphibians) can regenerate the tail, jaws, heart, limbs, lens, retina, brain, and spinal cord. Curiously, the closely related frogs (anuran amphibians) lose this capacity after a certain point in development. The same appears to happen in birds and mammals, including ourselves [1, 3, 4].

The nervous system is involved in receiving, processing, and transmitting information to all the other tissues in the body. It comprises two broad cell categories, neurons and glial cells. The latter play a myriad of roles, some of them only recently appreciated, from nourishing neurons and insulating axons to modulating synapses and serving as precursors in adult and embryonic neurogenesis [5, 6]. The brain, spinal cord, and retina constitute the central nervous system (CNS). Coupling of the CNS to limbs and organs is mediated by nerves composing the peripheral nervous system (PNS). Despite the significance of the nervous system, efficient neuroregeneration in adult mammals is restricted to the PNS [3]. The nervous system is an outstanding model from a regenerative biology standpoint: not only is there a marked difference between the regenerative capacity of the CNS and PNS in adult mammals [7, 8], as well as between mammals and amphibia, but nerves are also deeply involved in appendage regeneration [1, 9]. Studying neuroregeneration in amphibia and warm-blooded animals can shed light on the basic mechanisms of vertebrate regeneration and

contribute to develop much-needed treatments to CNS damage in humans.

CNS Regeneration

Amphibians as Robust Models of Brain and Spinal Cord Regeneration

After resection of a massive part of the telencephalon, which comprises the cerebrum and olfactory bulb, frog (*Xenopus laevis*) larvae completely regenerate this structure and restore its function [4]. This process relies on proliferation, migration, and differentiation into neurons of a subtype of glial cells located at the ventricular zone (VZ) and subventricular zone (SVZ) of the brain, the ependymal cells [10]. This mirrors normal development, where pioneer olfactory axons penetrate the forming telencephalon and induce cell division in the incipient olfactory bulb [11].

In stark contrast, in adult frogs, the telencephalon fails to regenerate due to a reduced migration ability of ependymal cells. Following partial removal of the telencephalon of larval and adult frogs, proliferation of VZ ependymal cells increased substantially in both larvae and froglets. However, gradual coverage of the surgically opened lateral ventricles by a mass of mainly ependymal cells, followed by reconstitution of the lost portion of the brain, was observed only in larvae. Remarkably, injection of either larval or froglet brain cells in the cavity of operated froglets led to the restoration of the telencephalon. Although this new structure was slightly deformed and its function was not tested, it was derived from donor cells, well integrated into the host telencephalon and connected to olfactory nerves [8]. Hence, adult frog ependymal cells retain the potential to participate in regeneration if located in the injury site.

Regeneration of the tail spinal cord in newts is an example of regeneration recapitulating development. Classical observations divided this process into several stages. After amputation, the severed end of the tail undergoes fast sealing by a thick wound epidermis. An ependymal tube, redolent of the neural tube that arises during embryogenesis, is assembled from cells that migrate from the stump. It then elongates through intense cell division and gradually differentiates into neurons and glia in the direction of the emerging tail tip. This is accompanied by reconstitution of vertebrae, muscle, and cartilage, coupled with growth and reconnection of axons to these structures [12].

Mammals Possess Neural Stem Cells

Unlike cold-blooded animals, such as fish, amphibia, and reptiles, mammals do not experience brain growth throughout adulthood and have very poor CNS repair capacity. In adult mammals, there are only two regions in the telencephalon that have been unequivocally shown to harbor neurogenesis. These were established by Joseph Altman in the 1960s using tritiated thymidine neuron labeling in adult rat brains: the subgranular zone (SGZ) of the hippocampus [13] and the SVZ, where migration of dividing neurons to the olfactory bulb was also observed [14]. Despite having faced much controversy at the time [15], these findings bore scrutiny, and SGZ and SVZ still persist as the only neurogenesis foci of the adult mammalian brain. In humans, adult neurogenesis has been established in SGZ and is seen as an essential part of memory formation in the hippocampus [16]. In contrast, SVZ neurogenesis, as well as immature neuron migration to the olfactory bulb and prefrontal cortex, appears to be restricted to infants younger than 18 months, decaying to extinction through adulthood [17].

Neuron generation is now thought to be the result of neural stem cells (NSCs) residing in these locations. NSCs are characterized by two essential properties: self-renewal and capacity to give rise to neurons, astrocytes, and oligodendrocytes in vitro. Yet, their cellular origin and exact identity in vivo remain hotly debated, due to technical difficulties and lack of stringent markers, existing reports identifying astrocytes [18], and ependymal cells [19] as putative NSCs. Interestingly, a NSC niche was recently found in rat spinal cord meninges. These cells were able not only to differentiate into functional neurons and glial cells when isolated and cultured in vitro, but also to proliferate, migrate, and contribute to glia production after spinal cord trauma [20]. However, the basic properties of NSCs and their increasing relevance as targets for CNS injury therapies have been reviewed elsewhere [21].

PNS Regeneration

The PNS comprises motor (or efferent) neurons, which stem from the brain or the spinal cord and convey information from the CNS to muscles, and sensory (or afferent) neurons, whose cell bodies reside in ganglia along the spinal cord and transmit information to the CNS. During development, assembly of neuronal circuits is achieved through outgrowth and guided migration of axons. The axon tips of developing neurons sprout into growth cones, which emit numerous fingerlike protrusions, philopodia, to pull the elongating axon along its path. Guidance is provided by signals that growth cones find in their way, which can be either attractive or repulsive [22]. Diffusion gradients of soluble molecules provide long-range cues. Contact interactions with either glia or other neurons' surface molecules or extracellular matrix (ECM) molecules provide short-range cues. Hence, axon outgrowth, pathfinding, and ultimately synapse formation are fine tuned by four different flavors of signals,

as both attraction and repulsion can be either short range or long range [22, 23]. PNS regeneration is remarkably efficient in mammals and closely mimics neurodevelopment. When a peripheral neuron is severed, the tip of the injured axon develops a growth cone, which then samples its environment for growth signals emitted by its target cells and extends towards them. PNS axons can regrow several centimeters in this fashion [24]. However, axonal regeneration is not always flawless, and peripheral neurons often form synapses with erroneous target cells (misrouting) after injury. Experimentally, increasing the intensity of growth factor gradients from target cells, such as nerve growth factor (NGF, a neurotrophin), significantly augments the fraction of correctly reconnected sensory neurons after transection of the femoral nerve [24], suggesting that learning the contrivances of PNS regeneration may inform therapies for CNS trauma. In this section, we will address the cell autonomous mechanisms as well as the glial cues employed during PNS axonal regeneration.

Neuron Intrinsic Pathways

Dorsal root ganglia (DRGs), which are at the frontier between the PNS and CNS, have been used as a model to study axonal regeneration. Indeed, DRG neurons show a strong regenerative response when their peripheral branches are damaged, but not when the central branches are lesioned [25, 26]. Interestingly, the limited regenerative capacity of the central branch can be rescued when the peripheral axon is damaged prior to, at the time of, or following the injury of the central one, and this is known as the conditioning effect [25–28]. The first molecule to be implicated in this phenomenon was cyclic adenosine monophosphate (cAMP), after studies showing that injection of a cAMP analog in DRGs leads to an improvement in dorsal column axon regeneration after CNS injury comparable to the conditioning effect [29–31]. These observations strongly suggest that sensory neurons have an intrinsic regenerative capacity, and the DRG model can be used to investigate the molecular and genetic factors driving axonal regeneration. Costigan and colleagues compared gene expression profiles of DRGs following axotomy of the sciatic nerve to naive conditions, identifying 240 genes involved in cellular organization, immunity, inflammation, and neurotransmission that were associated with DRG axonal regeneration [32]. In order to induce this pro-regenerative gene expression pattern, injury-dependent signals must propagate from the injury site and reach the cell body of the DRG neurons to sustain the elongation of the damaged axons [33]. Our current understanding of the mechanisms responsible for the transport of injury-dependent signals is still very limited, and three non-exclusive models have been proposed: an action potential is activated by the injury and quickly and transiently communicated to the soma; the regular retrograde transport of trophic and tropic signals is interrupted due to the axonal discontinuity induced by the damage (negative injury signals); axoplasmic protein expression is changed by the injury and transported to the cell body via the vimentin–importin-β system to the sensory neurons (positive injury signals) [34–36].

In the last years, a number of transcription factors have been associated with axonal regeneration, such as KLF4, p53, STAT3, NFAT, RARß, c-Jun, ATF3, and Sox11 [37–39]. Common aspects to all these transcription factors are their association to regulation of cell death and survival in several cell types, including neurons, and their involvement in neuronal development, strongly supporting the idea that successful axonal regeneration after CNS trauma in adults can be achieved by targeting transcriptional activity and molecular mechanisms associated with development [39, 40]. Further, a number of neurotrophins, including NGF, brain-derived neurotrophic factor (BDNF), and NT3/4, have been shown to initiate and contribute to the pro-survival and pro-growth response of axotomized peripheral or central nervous system neurons, as reviewed elsewhere [41, 42]. Of note, TrkB viral overexpression in the sensorimotor cortex was able to promote axonal sprouting and regeneration of the corticospinal tracts following experimental spinal cord injury [43].

Schwann Cells' Plasticity in Axonal Regeneration

Important players in PNS regeneration are the peripheral nerves' supporting cells, the Schwann cells. These glial cells execute the combined functions of astrocytes and oligodendrocytes, myelinating axons and encasing synapses in the PNS [5]. Upon injury of myelinated nerve fibers, these cells revert to an immature state and divide intensively. Growth, first transversally to the direction of the fibers and then along them, is followed by either production of plasma membrane to myelinate the regenerated fiber or cytoplasmic encircling of single axons in the case of non-myelinated fibers. Such remarkable plasticity for an adult specialized cell was first demonstrated by Aguayo in the 1970s. He cut and grafted unmyelinated nerve fibers into myelinated leg nerves in mice, leading to active proliferation of Schwann cells within a few days. Some months later, the previously unmyelinated fibers became myelinated. In order to discern Schwann cell migration from actual plasticity, resection of only the myelinated leg nerves was followed by radioactive labeling of Schwann cells. The unmyelinated nerves were severed, and the stumps from the leg nerves were then grafted to them. Remarkably, no migration of the leg nerves' Schwann cells was observed, indicating that every Schwann cell can be reprogrammed between non-myelinating and myelinating states [44]. Interestingly, classical experiments

demonstrated that Schwann cells' integrity is not important in the early stages of axon regeneration. Peripheral nerves were isolated from mice, and Schwann cells associated with them were killed through successive freeze-thawing cycles. Upon grafting into their original places, dead Schwann cells were promptly cleared by the immune system, leaving their basal laminae, and the regenerating axons grew exclusively along the inner side of those basal lamina scaffolds [45]. Subsequent experiments showed that these scaffolds also provide support for the recruitment of Schwann cells and myelination of the elongating nerves [46].

Neurons possess a membrane-bound form of neuregulin-1, NRG1 type III (also known as HRG1), which interacts with myelinating Schwann cell ErbB receptors and triggers their division. In normal physiology, this mechanism ensures that the number of myelin-producing cells associated with an axon is appropriate for its caliber [47]. Myelinating Schwann cells dissociated from nerves and cultured spontaneously revert to an immature state [48]. Some genes involved in Schwann cell dedifferentiation have been identified. For instance, expression of inducible NO synthase (iNOS) was found to be upregulated in Schwann cells after peripheral nerve injury in mice [49]. Ablation of iNOS leads to persistently myelinated fibers after nerve damage and retardation of axon regeneration [50]. At the transcriptional level, c-Jun appears to be the principal regulator of Schwann cell dedifferentiation both in vitro and in vivo [51].

Nerve Signals: Molecular Basis of Salamander Regeneration

The role of peripheral nerves in salamander limb regeneration is now fairly well understood. Early studies showed that resection of nerves at the shoulder level ablates limb regeneration after amputation in salamanders [52]. Strikingly, axolotls form an ectopic blastema if an extra nerve supply is provided to a lesion on the torso. This blastema can differentiate into a well-defined limb when dermal fibroblasts are grafted to the wound [53], suggesting that nerve signals induce dedifferentiation of dermal fibroblasts into blastemal cells [54].

A blastema is a mass of undifferentiated mesenchymal cells that form at the limb stump following amputation. Such amorphous appearance beguiles its incredible mhorphogenetic potential. The blastema contains information about its position in the anterior–posterior axis. If a blastema from a wrist-level amputation is implanted into the stump of a limb cut more proximally, the regenerated limb will miss the intercalary portion between the stump and the wrist [55].

The molecules involved in this process were discovered by Jeremy Brockes and colleagues. Blastemal cells express Prod1, a cell surface protein whose expression level correlates with the position of the cell along the limb proximo-distal axis. In culture, cells from proximal newt limb blastemas engulf their counterparts from distal blastemas. Treatment with blocking antibodies against Prod1 abrogated this phenomenon, suggesting that this protein is involved in establishing positional identity through cell–cell recognition [56]. Ensuing studies by the same group discovered a soluble protein ligand of Prod1, the newt anterior gradient protein (nAG). Immediately after amputation, nAG is secreted by Schwann cells. As blastema formation proceeds, a specialized structure forms at the limb stump, the wound epidermis. This tissue contains glands which become the major source of nAG. Importantly, expression of nAG, first at the nerve sheath and then at wound epidermis glands, is induced by the regenerating axons. Denervation ablates nAG expression and impairs limb regrowth; regeneration can be rescued after denervation by electroporating nAG DNA into the limb stump cells [57]. Importantly, nAG induces blastemal cell proliferation in vitro [57] through activation of endothelial growth factor receptor signaling downstream of Prod1 binding in cultured limb cells [58]. Unfortunately, such remarkable mechanism seems to have evolved in salamanders independently from other animals. Structure-based phylogenetic analysis of Prod1 revealed that its function is most likely restricted to salamanders [59], curbing the hope of enhancing epimorphic regeneration in humans.

Peripheral nerve regeneration is a sine qua non condition for limb regeneration. The dependence on innervation appears to have been selected in various species across the animal kingdom to guarantee that regenerated appendages are properly innervated and thus functional [1, 60]. Hence, albeit regeneration might have evolved independently from developmental mechanisms, it is still subject to constraints imposed by ontogeny.

Reemerging Concept: Bioelectricity and Neuroregeneration

Biocurrents Orchestrate Cell Division and Migration

Neuroscience has been classically devoted to explore electrical phenomena in neurons. Albeit we have addressed axonal regeneration from a cellular and molecular standpoint so far, bioelectricity is also known to play a role in the process for a long time. Steady-state bioelectric fields arise from ion flow and pH differences across cells and tissues [61, 62]. Two main mechanisms through which cells might sense electrical fields and migrate (electrotaxis) have been proposed. One states that electrical fields exert forces on cell surface receptors, guiding the cells towards one direction, while the other is based on asymmetric activation of voltage-gated channels, which would then create an

intracellular gradient of calcium ions and direct cell growth and migration [63]. Still, both of them remain mostly theoretical and have been challenged by experimental data, namely that blocking cell surface receptor movement does not affect electrotaxis and that the small potential differences used as electrical cues cannot activate membrane channels [63]. In mammals, the direction and greatness of injury currents are independent of wound size, with the flow of positive charges always going toward the center of the lesion [64]. Nevertheless, ectopic electric fields determine cell migration in in vitro wound models consisting of a monolayer of epithelial cells where a cell-free stripe is created. Electric fields with a polarity opposing the direction of healing lead to "wound" opening, whereas electric fields with the normal orientation enhance "wound" closing. The effects were directly proportional to field strength within the physiological range [64]. Moreover, electric fields have also been shown to direct neurite growth and neuron migration in amphibia [65] and rats [66].

In the 1960s, Robert Becker found that the bioelectric field pattern of salamanders closely matches the anatomical distribution of their nervous system. Positive sink areas correlated with the optic lobes and the brachial and pelvic aggregates of spinal neurons, while negativity increased distally along the limbs and tail [61]. He then compared the bioelectrical parameters of limb regrowth in regenerating (salamanders) and non-regenerating (frogs) amphibia. In both species, amputation was ensued by a reversal of the normal polarity of the body's electric field, with the limb stump becoming positive relative to anterior regions. However, the changes in electrical potential during regeneration were distinct. In salamander limbs, voltage dropped to negative values abruptly, reaching its minimum (−30 mV) in the onset of blastema differentiation, and then shifted to normal values (−10 mV) during limb regrowth. In stark contrast, the initial reversal in potential in non-regenerating forms was followed only by a slow decrease toward 0 mV, reacquiring negative values only days after the healing was complete [67]. The fact that the initial change in voltage at the stump is similar in both species suggests that it is due to the surgical procedure itself. Severing of axons and breakage of the skin short circuits the trans-epithelial potential difference, which exists across layers of cells connected by tight junctions in epithelia, creating a local current at the injury site [62, 68].

Whether the larger amplitude of currents of injury in salamanders as compared to non-regenerating species is a cause or a consequence of blastema formation and regeneration is still debated. Notwithstanding, experimental enhancement of regeneration via the use of external currents has been reported. In one study by Becker, exacerbation of the limb stump negative polarization of frogs by daily application of small currents visibly increased the growth of the limb regenerate [67]. Astonishingly, insertion of a bimetallic electrogenic device at the stump of limbs amputated at the mid-humerus level resulted in the formation of a blastema, nerve and blood vessel sprouting, and restoration of the humerus in rats [69, 70]. The exact molecular mechanisms behind Becker's achievements remain a mystery. Recent advances in the long-neglected field of biocurrents unveiled a signaling protein that may provide a link between electricity and neuroregeneration.

PTEN Integrates Cell Proliferation, Migration, and Axonal Regeneration Signaling

The PI3K/Akt pathway is mainly involved in cell growth and survival. Growth factor binding to receptor tyrosine kinases activates phosphatidylinositol-3-OH kinase (PI3K), which in turn phosphorylates phosphatidylinositol bisphosphate (PIP2), a membrane glycolipid, to phosphatidylinositol trisphosphate (PIP3). This leads to Akt activation and downstream apoptosis inhibition, protein synthesis, and proliferation [71]. Penninger and colleagues observed that external currents induce kinase phosphorylation in mouse cells. In particular, they found that, during electrotaxis, activated Src kinase and PIP3 become localized at the leading edge of migrating cells, similarly to what takes place during chemotaxis. Consistently, pharmacological inhibition of PI3K repressed keratinocyte electric field-driven motility, whereas genetic deletion of phosphatase and tensin homologue protein (PTEN), a PI3K signaling inhibitor [72], improved it. Moreover, stratified epithelial cells from PI3K-deficient mice cornea explants failed to respond to external electric fields, highlighting the potential relevance of electrotaxis to organ regeneration [64]. Importantly, PTEN has also been shown to be involved in axonal regeneration in rodents. Deletion of PTEN enhances regeneration of retinal ganglion cells (RGCs) following optic nerve crush [73], as well as sprouting of corticospinal tract (CST) neurons after spinal cord injury (SCI) [74]. This phenotype was associated with mTOR signaling inhibition by PTEN in both instances. mTOR is a protein kinase which acts downstream of insulin and growth factors to increase glucose metabolism and protein biosynthesis [75]. The mTOR pathway has been shown to promote RGC axonal regeneration by activating growth-associated genes [76]. Furthermore, the decay in CST neuron regrowth capacity parallels loss of mTOR activity in adult mice [74, 76].

PTEN converts PIP3 back to PIP2, antagonizing Akt signaling [72]. Yet, it also dephosphorylates Shc (Src homology domain-containing protein) and focal adhesion kinase (Fak), blocking random and directional cell motility, respectively [77]. Both Shc-dependent and Fak-dependent migration rely on organization of actin filaments [77]. It is thought that proton transport by cytoskeleton-associated Na^+/H^+ exchangers (NHE1) at the leading edge of migrating

cells orients cell motility [78]. In line with this model, NHE1 inhibition ablates Akt activation and impairs directionality in electric field-driven cell migration [64].

Structurally, PTEN is composed by an N-terminal domain with both tyrosine and phosphoinositide phosphatase activity and a C-terminal domain, involved in phosphoinositide recognition and membrane anchorage [79]. Interestingly, a voltage sensor-containing phosphatase (VSP) possessing four transmembrane segments (N-terminal), akin to voltage-gated channels, and a phosphatase-like domain (C-terminal) highly homologous to PTEN has been recently identified in *Ciona intestinalis*. VSP uses the same catalytic mechanism as PTEN to dephosphorylate PIP3 when activated by changes in membrane potential, being the first protein transducing electrical signals via known second messengers described in the literature [80]. A recent report showed that membrane depolarization activates the voltage-sensing domain of VSP, prompting a linker region between the membrane and the cytoplasmic domain to assume a conformation that induces enzymatic activity. The PIP2 produced from PIP3 by VSP stabilizes the active conformation, seemingly by interacting with the linker. Unlike PTEN, VSP can also dephosphorylate PIP2. Gradual depletion of PIP2 eventually shuts down phosphatase activity, representing a built-in negative feedback mechanism [81].

One may envisage that a protein with a membrane voltage-gated domain impinging on PTEN phosphatase activity of a cytosolic domain would couple the extracellular electrical signals to PI3K signaling, which is involved in cell proliferation and migration as well as in axonal extension, hallmark processes of cellular and axonal regeneration. Indeed, *Xenopus* VSPs have been characterized in the plasma membrane of cells from the kidney, testis, ovary, and liver with a voltage sensitivity within physiological values [82]. However, the mammalian counterpart of VSP is yet to be found. So far, described mammalian proteins with PTEN phosphatase activity and intermembrane segments do not contain a voltage sensor and have intracellular localizations: PTEN2, a mouse protein found in the Golgi apparatus [83], and TPIP, a human protein localized in the endoplasmic reticulum [84]. Discovery of such molecule, if it exists, would be an utmost valuable piece in the puzzle of mammalian regeneration, providing missing mechanistic links in mammalian cell electrotaxis, which relies on the PI3K/Akt pathway [64], and the conspicuous effect of exogenous currents on rat limb regeneration [69]. A summary of the molecular pathways known to be involved in transducing electrical signals into mammalian cellular reponses is provided in Fig. 1.

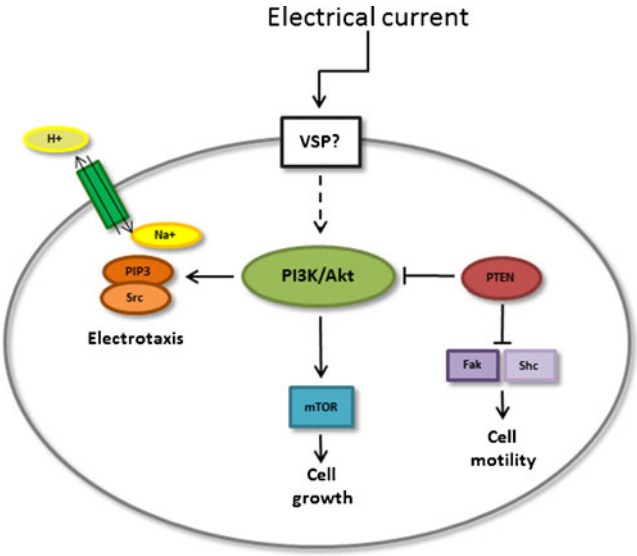

Fig. 1 Signal transduction of electrically stimulated mammalian cell growth and motility. The PI3K/Akt pathway appears to have a central role in regulating cell responses downstream of external electrical currents. During electrotaxis, PIP3 and activated Src localize to the leading edge of the cell, together with NHE1. PTEN inhibits PI3K signaling, halting mTOR-induced cell growth of neurons after injury. In addition, PTEN also dephosphorylates Fak and Shc, blocking directed and random cell motility, respectively. It is not clear yet how the information contained in biocurrents is transduced into cell responses. The discovery of a VSP in *C. intestinalis* suggests that a similar receptor molecule could exist in mammalian cells

Inhibition of Regeneration by the CNS Environment

CNS axons cannot regenerate and possess only a limited sprouting capacity after injury, leading to the idea that CNS neurons are intrinsically different from PNS neurons. This is only partially true as suggested by seminal experiments carried out during the 1970s and 1980s by Albert Aguayo. He cut and grafted optic nerves (CNS) to peripheral nerves in order to enshroud the latter with CNS myelin. Replacement of Schwann cells with astrocytes and oligodendrocytes led to a visible diminution in axonal regeneration, strongly suggesting that differences in the microenvironment were responsible for the distinct restoration capacities of CNS and PNS [85]. A spectacular demonstration of such hypothesis was provided by studies where peripheral nerves were grafted to the brain stem and spinal cord of rats, providing "bridges" between these two portions of the CNS. One month after the surgical procedure, CNS axons at the injury site had extended centimeters along "bridges" covered by Schwann cells that infiltrated the CNS [86]. In order to discern between axon regeneration and sprouting of extant axons, CNS nerves were allowed to grow along the "bridges," as previously, and then crushed at two points. Axons cut in this way were anterogradely labeled with a blue fluorescent dye. After 2 weeks, a cut at midpoint between the two crush spots was performed, and cut ends of axons were retrogradely labeled with a yellow fluorescent dye to assess axon regrowth. This allowed discrimination between lateral sprouting of uninjured axons (yellow only) or actual regeneration after damage (blue and yellow).

Indeed, double-stained brain stem and spinal cord axons were found, proving that CNS neurons can be stimulated to regenerate [87].

Indeed, the limited regenerative potential of CNS neurons is also due to a lack of intrinsic neuronal potential for axonal regeneration, as opposed to what occurs in the PNS, as well as to the CNS microenvironment, which actively represses axonal regeneration after injury. Similarly to neurodevelopment and adult PNS regeneration, neuron–glia interactions play a pivotal role in the process.

Myelin Proteins Inhibit Neuroregeneration

In the CNS, myelin is produced by oligodendrocytes and has been identified as a strong inhibitor of axonal outgrowth in vitro and in vivo following CNS trauma [88]. Myelin's inhibitory effect was first uncovered by Martin Schwab and collaborators. First, it was found that CNS myelin inhibition on the outgrowth of neurites could be abrogated by protease treatment. Analysis of myelin fractions for inhibitory properties revealed two membrane-bound protein fractions (35 and 250 kDa) capable of repressing neurite sprouting when reconstituted into liposomes [89]. Intracerebral implantation of tumor cells producing an antibody against those inhibitory proteins allowed young mice to regrow axons for more than 1 cm within 3 weeks after resection of a major spinal cord nerve, compared with approximately 1 mm in control animals [90]. This work led to the identification, 10 years later, of "Neurite outgrowth inhibitor" (Nogo), the archetypical class of myelin inhibitory proteins. Nogo-A, one of three isoforms generated by alternative splicing, is highly expressed in oligodendrocytes [91]. Various CNS myelin inhibitory components have been identified since then. We will focus on three particularly interesting myelin proteins, Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp).

MAG, a cell surface molecule present in myelin produced by oligodendrocytes, was initially described as mainly responsible for inhibiting neurite outgrowth of neurites in cultured neurons using CNS myelin protein fractionation [92]. Interestingly, MAG inhibits neurite growth in cerebellar neurons (CNS) twice as effectively as in DRG neurons (PNS) [93]. However, *MAG* knock-out mice are not more proficient in spinal cord regeneration [94]. Demyelination is known to cause not only a drastic decrease in the speed of propagation of electrical nerve impulses, but also axonal atrophy. Strikingly, absence of MAG, but not other myelin structural proteins, sufficed to provoke atrophy of myelinated nerves in the PNS and even small levels of neurodegeneration without demyelination [95]. Later experiments confirmed this effect in PNS and CNS neurons and found that MAG (both in soluble and membrane-bound forms) halts pharmacologically induced neurodegeneration in vitro [96]. This suggests that MAG not only inhibits nerve regeneration but also protects nerves from toxic insults, admonishing us that therapies blocking myelin-associated inhibitors of axonal regeneration might have highly deleterious side effects.

Nogo-A, MAG, and OMgp do not show homology, but bind to the same receptor complex expressed on axons, formed by Nogo receptor (NogoR), p75, TROY, and Lingo-1. The first component identified from this complex was NogoR, a brain-specific leucine-rich repeat protein that binds Nogo-A with high affinity [97]. NogoR requires and interacts with p75, a transmembrane protein that binds neurotrophin factors, transducing inhibitory signals in neurons after activation of the complex by Nogo, MAG, or OMgp [98]. Furthermore, p75 directly interacts with Rho GDP, resulting in activation of RhoA and axon growth cone collapse [99]. Subsequent studies showed that NogoR and p75 alone are not sufficient to activate the RhoA pathway, suggesting that other proteins are required to make the Nogo receptor complex functional, such as Lingo-1, a CNS-specific transmembrane protein [100]. Based on the observation that p75 is expressed only by a limited number of neurons in adulthood after CNS trauma, the presence of another protein with similar function but more broadly expressed in adult neurons was proposed [101]. This missing piece was TROY, a TNF receptor family member that interacts with NogoR and Lingo-1 as part of the Nogo receptor complex, resulting in the activation of RhoA [101].

The inhibitory properties of Nogo, MAG, and OMgp have been tested for the single molecules in vitro and in vivo in SCI models [90, 92, 102–105], indicating Nogo-A as the major actor in myelin-dependent CNS repair failure. The potential synergistic inhibitory effect of these three proteins on axonal regeneration in injured adult CNS has been recently tested. Triple knock-out (TKO) mice for Nogo, MAG, and OMgp were independently generated in two laboratories [106, 107]. The axonal regenerative capacity of differentially myelinated fibers (CST and 5-HT), as well as the motor functional recovery of the TKO mice compared to wild-type and single mutant (Nogo−/−, OMgp−/−, or MAG−/−) mice, was evaluated after SCI [106, 107]. Surprisingly, the results reported in these two studies are notably different. In one report, the TKO led to an improved CST and 5-HT sprouting and functional recovery compared to any other single mutant tested, suggesting that Nogo, MAG, and OMgp play a synergistic effect and that counteracting their actions may be a potential therapeutic way to cure paralysis [106]. On the other hand, the study by Lee and colleagues did not find significant differences, neither in CST and 5-HT sprouting and regeneration, nor in motor functional recovery, therefore suggesting that these myelin components do not synergize to hamper axonal sprouting and regeneration [107]. Differences in the mouse

strain, the injury model, and the methods to evaluate sprouting vs. regeneration used in these two studies ought to be analyzed in depth and complemented by independent studies to shed light on the individual and combined roles of Nogo, MAG, and OMgp.

The Glial Scar

The glial reaction is a hallmark of CNS injury. Upon damage of nerve fibers, myelin sheaths are destroyed and immune cells, oligodendrocyte precursors, and astrocytes migrate toward the injury site. Astrocytes hyperproliferate and become "reactive," releasing ECM molecules, such as heparan, dermatan, and chondroitin sulfate proteoglycans (CSPGs) and laminin, that contribute to the reorganization and remodeling of the injured tissue [108]. These events, together with the release of myelin-associated inhibitors from myelin degradation [109], also culminate in a glial scar, which can be thought of as a physical and chemical barrier to axonal regrowth, sprouting, and regeneration [109]. The proliferation and recruitment of reactive astrocytes around the lesion site start a few hours following the injury, peaking at 3–7 days, and result in chronic astrogliosis [108]. Recent provocative reports demonstrate that stromal cells derived from pericytes, which control the vasculature in the central nervous system, constitute a substantial portion of the cells found at the glial scar. Indeed, genetically modified animals with severely reduced populations of pericytes failed to insulate spinal cord lesions with glial scar tissue [110].

However, numerous beneficial effects of reactive astrocytes on neuroregeneration and functional recovery have been reported, such as scavenging of neurotoxic agents (e.g., glutamate and potassium), production of neurotrophic factors (e.g., glucose, BDNF, and NT-3), and axonal regeneration permissive molecules (e.g., laminin), contributing to lesion sealing and capacity to enhance the revascularization of the spinal cord [111–115]. Still, this beneficial astrocytic effect on CNS repair seems to be limited to the acute phase after the lesion. In the chronic phase, the proteoglycan deposition in the scar results in the inhibition of axonal outgrowth and functional recovery [115].

The inhibitory effect of extracellular proteoglycan deposition in a CNS lesion on regeneration is very robust [116, 117]. As more extensively explained below, therapeutic dissolution of the CSPGs-rich matrix has proved beneficial to axonal regeneration and functional recovery after SCI in preclinical studies in rodents [118]. The initial identification of the inhibitory role of chondroitinase and heparin sulfate proteoglycans (CSPGs and HSPGs) in axonal outgrowth was performed in vitro. In these studies, RGCs were plated on glial scar explants from naive, chondroitinase ABC, or heparinase-treated rat injured spinal cords. The neurite elongation of the RGCs plated on chondroitinase ABC or heparinase-digested scar explants was significantly enhanced compared to naive explants [112, 116]. In vivo, CSPG-rich matrix persists chronically around the lesion site and coincides with the axonal regrowth failure after SCI [117, 119]. Albeit it is well characterized that astrocytes produce several different CSPG family members that are differentially expressed after SCI [120–122], the molecular mechanisms through which CSPGs activate growth cone collapse, contributing to CNS repair failure are not fully understood yet. Recently, the first specific and with high binding affinity receptor for CSPGs has been identified: PTPσ, a transmembrane tyrosine phosphatase receptor [123]. Subsequently, LAR, another member of the PTPR subfamily, was shown to bind with high affinity to CSPGs. Treatment with LAR-targeting peptide led to improved axonal regeneration and motor functional recovery after SCI in rodents [124]. Altogether, these observations paved the way to a more clear understanding of the possible molecular pathways and signaling transduction cascades activated by CSPGs. The principal molecules involved in axonal regeneration addressed here are shown in a schematic in Fig. 2.

Spinal Cord Injury Therapies

The response of the CNS to SCI is complex and involves neurons and their axons as well as glia. Approaches targeting the different components of such response have been developed and preclinically tested. Here, we address some meaningful studies in this regard using cell-based, molecular, pharmacological, and combinatorial strategies.

Cell Therapies

Irving Weissman and colleagues succeeded in isolating stem cells from human fetal brains (hCNS-SCs) able to self-renew and produce neurons, astrocytes, and oligodendrocytes in culture. Severe combined immunodeficiency (SCID) mice, which are genetically engineered to accept foreign tissue grafts, were then used to test hCNS-SCs in vivo [125]. Remarkably, hCNS-SCs transplanted to the brain lateral ventricles of SCID mice proliferated, migrated, and differentiated for at least 7 months. Their behavior closely paralleled that of the endogenous mouse SVZ and SGZ NSCs [126]. Such notable integration of human stem cells in mouse nervous tissue prompted the use of hCNS-SCs in SCI mouse models. The spinal cord of SCID mice was damaged by contusion, greatly impairing their locomotion. hCNS-SCs injected at the injury site differentiated into neurons, which formed synapses with a few of the mice neurons, and oligodendrocytes, which myelinated both human and mouse axons. Significant locomotor recovery was

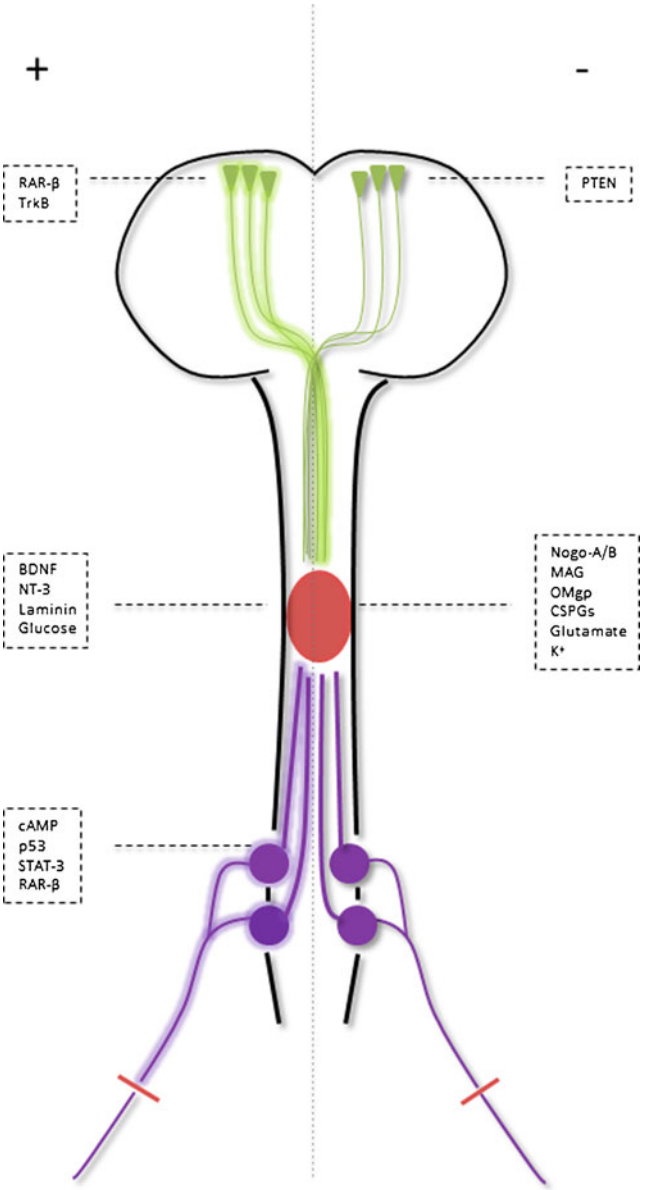

Fig. 2 Main molecules involved in central and peripheral nervous system regeneration discussed in this review. Layer V neurons and the corticospinal tract are labeled *green*. Dorsal root ganglia, dorsal column, and sciatic nerve are labeled *purple*. *Red lines* and the *circle* represent injury of the peripheral nerves and spinal cord, respectively. Molecular players in neuroregeneration are listed in *boxes* on the left and right side of the diagram, corresponding to positive (+) and negative (−) effects on neuroregeneration, respectively. *Glow* and *shadow effects* indicate regenerating and non-regenerating neurons and axons, respectively

observed 16 weeks after treatment; selective killing of the injected hCNS-SCs with diphtheria toxin ablated the positive effect. Curiously, hCNS-SCs neither produced astrocytes nor contributed to the glial scar [125].

Similar experiments were done with oligodendrocyte progenitor cells derived from human embryonic stem cells (hESC-OPCs). However, injection of these cells into the damaged spinal cord elicited nerve remyelination and locomotor recovery 1 week after injury, but not 10 weeks after injury [127], hinting that such approach might not help the many patients living with SCI. Nevertheless, the successes in these preclinical studies led to the inception of a clinical trial by a company, Geron, where hESC-OPCs were injected in SCI patients who had motor impairment and impaired control of their bowel and bladder. The trial was approved by the Food and Drug Administration and $25 million were awarded to Geron to conduct the tests [128]. Yet, Geron abandoned the project a few months later alleging capital scarcity [129]. The few patients who received one injection of these cells showed neither beneficial nor adverse effects. Hence, the true potential of cell therapies for SCI in humans still awaits demonstration.

Molecular Therapies: Targeting Intrinsic and Extrinsic Factors

Other successful preclinical studies in rodents have targeted neuronal and axonal responses (intrinsic factors) using molecules such as growth factors and blocking antibodies. The spinal cord encompasses several ascending and descending tracts, which are responsible for the control of sensory and motor functions and, importantly, possess distinct regrowing capacities after damage in physiological contexts. The corticospinal tract (CST) has very limited regrowing ability after trauma in physiological contexts in rodents, and it is responsible for control of fine motor movements of the limbs, such as grasping [130], rather than locomotion [131]. In contrast, locomotion is predominantly controlled by the CST in humans [132], making CST regeneration and functional recovery a relevant target for clinical translation. Injection of neurotrophin-3 (NT-3), but not of BDNF, at the lesion site of SCI rats has been shown to enhance CST collateral sprouting, but not regrowth caudal from the lesion [133]. Nevertheless, later experiments showed that continuous release of NT-3 in the lesion site from a graft of genetically modified fibroblasts not only improved CST collateral sprouting but also increased axonal regrowth up to 8 mm caudal to the injury. This was paralleled by improved recovery of motor function and coordination. Transplant of fibroblasts genetically modified to produce NGF did not show effects on recovery of motor function nor CST axonal regrowth [134]. Strikingly, BDNF, but not NT-3, delivery promoted functional recovery and regeneration of the rubrospinal tract, another motor-spinal descending tract [135, 136]. Altogether, regeneration of different spinal tracts appears to be modulated by distinct factors, likely in part due to the differential expression of the Trk neurotrophin receptor subtypes and to the heterogeneous specific experimental setup.

Other strategies aim to counteract the inhibitory environment for axonal regeneration imposed by CNS myelin

proteins or by the glial scar (extrinsic factors), and some have already produced some promising results in clinical trials[109, 110]. Treatment with an antibody against Nogo-A, IN-1, or with a Nogo receptor peptide antagonist, NEP1-30, ameliorated the sprouting of the CST and serotoninergic fibers and functional recovery in rodents [90, 103, 137]. Similar results for IN-1 administration were observed in primates with cervical spinal cord lesions [138–140]. Most importantly, IN-1 treatment is now in clinical trials with SCI patients. Phase I of this study was successfully completed in Europe and North America, where good tolerance to the treatment without relevant side effects was observed; the subsequent phase II clinical trial is underway [141]. This is the first treatment for SCI that has reached clinical trials to date.

In addition to anti-Nogo antibodies, anti-scarring treatments have been successfully undertaken in animal models. Following SCI, enzymatic digestion of CSPGs with Chondroitinase ABC intrathecally administered at the lesion site resulted in upregulation of regenerative associated proteins, such as GAP-43, increased sprouting of ascending and descending fibers, restoration of post-synaptic activity of corticospinal neurons below the injury site upon electrical stimulation, and recovery of locomotor and proprioceptive functions [118].

Finally, it is worth mentioning that a chemical biology screening for axonal regeneration inducers performed early this decade identified four hit compounds capable of augmenting neurite elongation in neurons plated on myelin, CSPGs and co-cultured with an in vitro glial scar model. Notably, one of the hits also proved to promote regeneration of the dorsal column after SCI [142]. We hope that similar high-throughput studies will identify novel pro-neuroregenerative drugs in a near future.

Combinatorial Approaches

The concomitance of many different events at both the glial and neuronal levels during CNS trauma has motivated the development of combinatorial therapies, where different cell types and growth factors or blocking antibodies are combined to achieve a synergistic effect.

Promising results in rats were obtained by modulating both the intrinsic and extrinsic neuronal responses to SCI. Six weeks after inflicting spinal cord dorsal column injury, peripheral nerves adjacent to the damage site were crushed, eliciting regeneration in neurons [26], and bone marrow stem cells were injected into the resulting cavity along with NT-3. Furthermore, a gradient of this neural growth factor was provided by infecting dorsal column cells surrounding the wound site. The three procedures were sufficient to induce axon regeneration across the wound and, importantly, provoked the same effect even if carried out more than 1 year after injury [143], providing hope to chronic SCI patients. Along the same lines, stimulation of the sensory neuron cell body with injection of cAMP analogs prior to injury, and NT-3 delivery past the lesion site in association with a bone marrow stromal cell graft, resulted in regeneration of the dorsal columns rostral to the lesion. However, single treatments did not show significant axonal regeneration beyond the lesion site [144].

Another strategy that has been revealed to be powerful both in acute and chronic SCI when used together with other approaches is the enzymatic digestion of the CSPG-rich matrix by chondroitinase ABC (ChABC) [145, 146]. For instance, combination of PNS grafts with ChABC following SCI in rodents results in longer and more numerous axons in the spinal cord regenerating beyond the lesion and peripheral graft than grafting of the peripheral nerve at the lesion site alone [25], improving motor functional recovery [147]. Recently, this approach was also successfully applied in functional recovery of respiratory muscles and breathing through the improvement of diaphragmatic function [148]. When combined with rehabilitation protocols, ChABC treatment improved functional neurological recovery and axonal sprouting even when given 4 weeks after SCI [145, 146]. Specifically, rats that received task-specific rehabilitation training of the forepaws and ChABC showed an enhanced recovery of motor functions compared to the group that

Table 1 Preclinically tested therapeutic approaches addressed in this review

Promoting neuronal intrinsic response:	**Counteracting glial inhibitory environment:**
cAMP analog injection [29, 30]	IN-1 antibody treatment [90, 103, 137–140]
NT-3 delivery [133]	ChABC treatment [118]
BDNF delivery [135, 136]	hCNS-SC injection [125]
	hESC-OPC injection [127]
Combinatorial:	
Conditioning lesion+NT-3 delivery+BMSCs graft [143]	
cAMP analog injection+NT-3 delivery+BMSCs graft [144]	
Rehabilitation+ChABC treatment [145, 146]	
ChABC treatment+NT-3 delivery+NR2D expression [149]	
PN graft+ChABC treatment [147, 148]	

cAMP cyclic adenosine monophosphate, *NT-3* neurotrophin-3, *BDNF* brain-derived neurotrophic factor, *ChABC* chondroitinase ABC, *BMSC* bone marrow stromal cells, *hCNS-SC* human central nervous system-derived stem cells, *hESC-OPC* human embryonic stem cell-derived oligodendrocyte progenitor cells, *PN* peripheral nerve

received task-specific training only [146]. Strikingly, however, such amelioration was restricted to the trained performance and accompanied by a negative impact in the recovery of other motor functions [145]. Indeed, rats treated with ChABC and trained for skilled reaching tasks, such as grasping, performed poorly in general locomotor tasks compared with ChABC-treated animals that did not undergo any rehabilitation. Conversely, animals rehabilitated for general locomotion performed worse than non-rehabilitated animals in skilled reaching tasks when ChABC was administered [145]. These observations suggest that ChABC is a valid procedure to enhance functional recovery following SCI and that rehabilitation influences what motor skills are regained. Of note, this strategy allows pharmacological treatment to be initiated at the time of rehabilitation, an optimal window for clinical translation [145, 146]. Interestingly, combination of ChABC with secreting NT-3 fibroblast grafts and viral expression of NRD2, a subunit of NMDA receptor, in the adult injured rat spinal cord resulted in partial improvement of hindlimb locomotor functions. More interestingly, the lesion performed was a lateral hemisection that left intact the axons on the controlateral side of the spinal cord. Electrical stimulation above the lesion induced a significant synaptic response in motor neurons below the injury site in the animals that received the combinatorial approach, indicating the formation of a detour pathway, corresponding to a better axonal sprouting and suggesting a synergistic effect targeting three different aspects influencing recovery after SCI : axonal plasticity, growth, and synaptic function [149]. Table 1 provides a summary of the different therapeutic approaches discussed in this section.

Conclusions

The reasons why the regenerative capacities differ so widely between phylogenetically close species remain to be elucidated. Regeneration shall not be considered as a mere recapitulation of development. Unique mechanisms had to evolve to respond to injury and other stresses that are not present in the embryo. Nevertheless, the study of development can inform us on how complex patterns are formed and restored in living systems.

Our expanding knowledge about neuroregeneration entices that dramatic improvements can be achieved as regards human repair and regeneration. It is possible to coax mature adult mammalian neurons to divide [150] and migrate [66] in response to artificial electric fields. Indeed, a phase I clinical trial has shown that implanting an oscillating field stimulator in the site of spinal cord injury for 15 weeks ameliorates the patients' motor and sensory reflexes without any deleterious side effects [151]. Recently, a stunning report showed that GFP-expressing embryonic hypothalamic progenitors and newborn hypothalamic neurons transplanted to the hypothalamus of adult mice were able to differentiate into various neuron and glial cell types and to become functionally integrated [152]. When put together with the aforementioned successful methodologies to ameliorate SCI, these results endorse that the adult mammalian CNS retains enough plasticity for functional recovery after trauma.

The nervous system has been revealed to be prolific in terms of provided insight into regeneration. In particular, much has been learned about the discrepancies between different animals regarding axonal regeneration. Such renaissance of the field may allow the promise of regenerative medicine, which aims to accelerate the pace at which our bodies heal themselves by enhancing tissue and organ regeneration, to be fulfilled in a not so distant future. Indeed, CNS injuries now considered incurable may be repaired by providing appropriate cells, substrates, or small molecules to the site of injury or in locations functionally connected to it, to efficiently drive axonal regeneration and functional recovery

Acknowledgments Thanks are due to Prof. John Dowling for insightful discussions. We would like to thank the Hertie Foundation, the DFG (DI 14931), and WFL research grants (awarded to SDG).

Conflict of interest The authors declare no conflicts of interest

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