NEURAL REGENERATION

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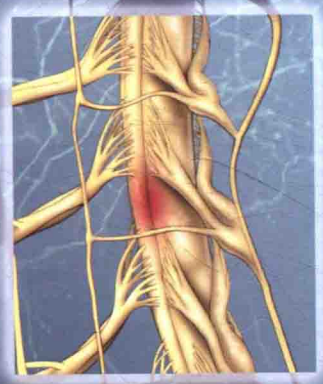
KWOK-FAI SO, XIAO-MING XU



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Edited by

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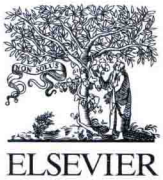

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TRANSPLANTATION-MEDIATED NEURAL REGENERATION

SECTION I

INTRODUCTION

CHAPTER

1

Advances and Challenges for Neural Regeneration Research

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1. NERVOUS SYSTEM, NERVE INJURY, AND NEURAL REGENERATION

The nervous system is divided into two parts: the central nervous system (CNS), which consists of the brain and spinal cord, and the peripheral nervous system (PNS), which consists of cranial and spinal nerves along with their associated ganglia. The function of the CNS and PNS is to relay information to and from all parts of the body. This communication is made possible through an extensive network of neurons and supporting cells called glia, including astrocytes, oligodendrocytes, and microglia.

Nerve injury, whether traumatic or degenerative, disrupts the normal flow of information and can, depending on the location and mechanism of injury, lead to deleterious effects. Injury or sudden trauma, such as from automobile accidents, falls, sports-related activities, etc., can cause nerve fibers or axons to be partially or completely severed, crushed, compressed, or stretched. When an axon is damaged, the distal segment undergoes Wallerian degeneration, losing its myelin sheath [1]. The axotomized neurons either die by necrosis or apoptosis or undergo a chromatolytic reaction, which is an attempt to repair. Injury to the nervous system also triggers the responses of glial cells, including oligodendrocytes, astrocytes, and microglia in the CNS; Schwann cells (SCs) in the PNS; and blood-derived macrophages that participate in both CNS and PNS injury processes. The responses of these cells to injury include cell death, proliferation, migration, and production of inflammatory mediators and growth factors, thus influencing processes of axonal degeneration and regeneration. Thus, nervous system injuries affect not only neurons and their processes but also glial cells.

Neural regeneration refers to the regrowth or repair of nervous tissues, cells, or cell products. Such mechanisms may include generation of new tissues, neurons, glia, axons, myelin, or synapses. Beyond the common knowledge of neurogenesis, a wider concept of neural regeneration may comprise endogenous neuroprotection leading to neuroplasticity and neurorestoration. Neural regeneration can also be promoted by implantation of viable tissues or cells. Neural regeneration differs between the PNS and the CNS owing to different neuronal and glia responses to injury as well as the different environments that the regenerative axons and cells encounter.

2. TECHNOLOGICAL ADVANCES IN NEURAL REGENERATION RESEARCH

2.1 Models

Preclinical animal models are critical for understanding regenerative neurobiology and for testing treatment strategies prior to implementation in clinical practice. For regeneration research, in vitro, ex vivo, or in vivo models, described below, have been used extensively and complementarily.

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2.1.1 *In vitro Model*

The flexibility and ease of control offered by the in vitro model make it a useful tool for the study of neural regeneration. Glass micropipettes can be used to sever processes from cultured neurons or tissue explants to study axonal and dendritic regrowth in vitro [2–4]. Although this method can cut many axon segments simultaneously, it cannot be used to isolate axon and dendritic segments. Fine knife cutting is another localized physical injury, which can precisely cut neurites [5,6]; this method, however, damages the coated substrate and sets up an artificial sulcus, which may prevent the truncated neurite from regrowing. Microdissection of a neurite with a laser beam offers more precise control [7,8] that provides a unique platform for regeneration research [9]. A nanocutting device with a cutting edge of less than 20 nm radius of curvature was developed that enables high-precision microdissection and subcellular isolation of neuronal structures [10]. With these devices, not only can a single-axon transection model be established, but also regeneration-related functional components of neurons, such as segments of axons, dendrites, dendritic spines, and nodes of Ranvier, can be isolated in culture.

2.1.2 *Ex vivo Model*

An ex vivo model is ethically advantageous, requires no postsurgical animal care, enables more reproducibility between lesions, and provides a tightly controlled artificial environment for regeneration studies. Published ex vivo spinal cord models include the culture of several hundred micrometers-thick transverse slices maintained for up to three weeks [11], unfixed longitudinal cryostat sections of spinal cord maintained for one week [12], and a novel ex vivo model that enables the culture of intact postnatal spinal cord segments for up to five days and the assessment of peripheral nerve grafting repair [13].

2.1.3 *In vivo Model*

Although invertebrates and lower vertebrates, such as *Caenorhabditis elegans* [14–16], lamprey [17–19], zebrafish [20–23], and lizard [24,25], have long been applied for neural regeneration research, the rat sciatic nerve, brain, and spinal cord injury models have been the most commonly used for studies of neural regeneration. Rodent models, such as rats and mice, are economical compared to large-animal models and primates, simple to handle and care for, very resistant to surgical infections, and can be investigated in large groups. Rodent models can be used for electrophysiology, functional recovery, muscle and nerve morphology, and other assessments of nerve regeneration [26,27]. The major value of the mouse model is the ability to answer mechanistic neural regeneration questions [28,29]. The rabbit, dog, and cat are large-animal species more frequently used for peripheral and central nervous system injury research. Large mammals such as sheep [30,31], pigs [32,33], and monkeys [34–37] have increasingly been employed to study neural regeneration. These large species are limited by extremely high costs related to animal care, the narrow range of assessments available, and the complexity of training for functional testing. Transgenic animals, particularly mice, that express fluorescent proteins in specific neuronal subsets provide potentially powerful tools for the study of neural regeneration. One strategy involves expressing fluorescent proteins under the control of neuron-type-specific promoters [38]. Another approach involves the use of bacterial artificial chromosome (BAC) mice [39,40]. Genetic labels can provide specificity in axonal labeling that is hypothetically independent of tracer transport [41]. Moreover, BAC mice bearing green fluorescent protein-tagged polyribosomes (BAC-TRAP mice) provide an exceptional opportunity to identify potential regeneration-associated transcriptional events in a cell-type-specific manner [40].

A book entitled *Animal Models of Acute Neurological Injuries* [42] has provided a wide array of animal models currently used for assessing acute neurological injuries, providing valuable resource for neural regeneration research.

2.2 Labeling and Imaging Technology

How to exquisitely label nerve fibers within the nerve system and their connections to their target continues to be an important concern for neural regeneration research. Transgenic animals that express fluorescent proteins in specific neuronal subsets provide potentially useful tools for the regeneration study of these neurons [38].

Axonal tract tracing technologies are also powerful tools for identifying axonal connections. With appropriate injury models and tracing techniques, the status of axons—sparing, die-back, sprouting, regeneration, or synaptogenesis—can be readily identified [43]. Based on axonal transport, a long series of tracers has been developed as anterograde tracing or retrograde tracing according to the preferential direction of their transport in the axon.

Viruses have been developed for tract-tracing studies. Compared to conventional tracers, viruses have the ability to traverse multisynaptic pathways and replicate to amplify signals at each step in the process [44]. Depending on the species and strain of the virus, viruses can travel preferentially in the anterograde or the retrograde direction or both [45,46]. For example, Wang et al. found that a recombinant adenovirus carrying a green fluorescent protein reporter gene (Adv-GFP) can preferentially, intensely, and bidirectionally label the rat rubrospinal

tract [46]. More recently, genetic modifications have allowed for many improvements. These include reduced pathogenicity, control of synaptic spread, addition of marker genes, pseudotyping for infection of selected cells, and addition of ancillary genetic elements for combining circuit tracing with manipulation of activity or functional assays.

Imaging plays an essential role in the diagnosis, treatment, and rehabilitation of nerve injury patients. Traditionally, imaging modalities have consisted of plain radiography, computed tomography, and magnetic resonance imaging (MRI). Despite their critical importance, these modalities offer comparatively less information regarding the microstructural changes after injury or regeneration. This has led to the development of novel imaging techniques that are principally focused on the microstructural and/or biochemical function of the nerve. These novel techniques include diffusion tensor imaging [47,48], MR spectroscopy [49,50], positron emission tomography [51,52], single-photon emission computed tomography [53,54], two-photon imaging [55,56], and functional MRI [57,58]. These techniques are currently in various development stages, including some whose applications are primarily limited to laboratory investigation, whereas others are being actively utilized in clinical practice. In 2011, a longitudinal coherent anti-Stokes Raman scattering imaging technique was reported to clearly monitor demyelination and remyelination of axons in live rats after spinal cord injury (SCI) [59]. A year later, a tetrahydrofuran-based clearing procedure that renders fixed and unsectioned adult CNS tissue transparent and fully penetrable for optical three-dimensional imaging was reported [60]. This procedure can be readily used to study neural regeneration.

2.3 Nanotechnology

The rapid expansion of nanotechnology during the past decade has led to new perspectives and advances in the neural regeneration field. As nanotechnology is defined by the size of a material or manipulation on the molecular level, it involves a broad range of nanoscaled materials used in various fields of regenerative medicine, including diagnosis, drug and gene delivery, tissue engineering (TE), and cell therapy. For example, to allow cells to be detected in vivo, superparamagnetic iron oxide nanoparticles have been successfully used to label transplanted cells for in vivo noninvasive MRI monitoring [61]. The basic strategy of TE is the construction of a biocompatible scaffold that, in combination with living cells and/or bioactive molecules, replaces or repairs damaged cells or tissue [62]. The large surface of nanostructured materials, such as two-dimensional (2D) electrospun nanofibers [63], 3D electrospun nanofibers [64,65], and self-assembling nanofibers [66,67], enhances the adsorption of adhesive proteins, such as fibronectin, which mediate cell-surface interactions through integrin cell-surface receptors [68]. For example, the self-assembling peptide RADA16-I supported the growth of PC12 cells and the formation of functional synapses of rat primary hippocampal neurons [69]. Modification of the RADA16 peptide by the immobilization of bone marrow homing protein motifs significantly enhanced the survival of mouse neural cells [70]. In vivo, RADA16-I repairs the disrupted optic tract [71], bridges the injured spinal cord of rats after transplantation [72], and helps to reconstruct lost tissue in the acutely injured brain [73]. Injured spinal tissue incubated with self-assembled monomethoxy poly(ethylene glycol)-poly(D,L-lactic acid) diblock copolymer micelles (60 nm diameter) showed rapid restoration of compound action potential and reduced calcium influx into axons for micelle concentrations much lower than the concentrations of polyethylene glycol, a known sealing agent for early-stage SCI. Intravenously injected micelles effectively recovered locomotor function and reduced the volume and inflammatory response of the lesion in injured rats [74]. Trends in TE include scaffold functionalization that is tailored to each specific application and cell response. Improving the cellular response and the loading and delivery of drugs or bioactive molecules as well as enhancing the scaffolds' bioactivity can lead to the optimization of nanofibrous materials for transplantation and clinical application.

3. PNS REGENERATION

The PNS comprises axons of motor neurons, which stem from the brain/spinal cord and convey information from the CNS to muscle cells, and sensory neurons, whose cell bodies reside in ganglia and transmit information to the CNS. After peripheral nerve injury, axons can readily regenerate. PNS regeneration is remarkably efficient in mammals and closely mimics neurodevelopment. When a peripheral axon is severed, the tip of the proximal segment develops a growth cone, which then samples its environment for growth signals emitted by its target cells and extends toward them. PNS axons can regrow several centimeters in this fashion [75].

Neuronal intrinsic pathways are critical for PNS regeneration. Dorsal root ganglia (DRG) neurons show a strong regenerative capability when their peripheral branches, but not their central ones, are damaged [76,77]. Interestingly, the limited regenerative capacity of the central branches can be enhanced when their peripheral axons are damaged prior to, at the time of, or following the injury of their central axon, a phenomenon defined as the conditioning effect [76–78]. The first molecule to be implicated in this phenomenon was cyclic adenosine monophosphate (cAMP) [79–81]. These studies strongly

suggest that PNS neurons have an intrinsic regenerative capacity, and the DRG model can be used to investigate the molecular and genetic mechanisms driving PNS axonal regeneration. Costigan et al. compared gene expression profiles of DRGs after axotomy of the sciatic nerve to naive conditions, identifying 240 genes involved in immunity, inflammation, and neurotransmission that were associated with DRG axonal regeneration [82]. In the past few years, a number of regeneration-associated genes, such as growth-associated protein-43 [83], small proline-repeat protein 1A [84], KLF4, p53, signal transducer and activator of transcription 3 (STAT3), NFAT, RARβ, c-Jun [85], activating transcription factor-3 [86], and Sox11, have been identified as critical factors associated with PNS axonal regeneration [87,88]. Furthermore, a number of neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin 3 and 4, have been shown to initiate and contribute to the prosurvival and progrowth response of axotomized PNS neurons [89,90]. Delivery of these neurotrophic factors alone or combined with other strategies [91–94] promotes PNS regeneration.

An important extrinsic player in PNS regeneration is a type of PNS-supporting cell named the Schwann cell. SCs execute the combined functions of astrocytes and oligodendrocytes, myelinating axons and encasing synapses in the PNS [95]. After injury, the SCs become activated, assume a more primitive phenotype, and stimulate axonal growth, with upregulation of growth-related genes, including those that encode intrinsic neurotrophic factors and key transcription factors [96,97]. Activated SCs produce collagen and laminin, creating a tunnel of extracellular matrix, and express cell adhesion molecules and receptors, including interleukin-1, N-cadherin, γ-integrins, and the neural cell adhesion molecule [98]. The resulting supportive environment yields SC proliferation, formation of bands of Büngner, and, finally, supporting axonal growth. This growth occurs at a rate of 1–4 mm per day, with progressive myelination of the fibers by the neighboring SCs. The final repaired nerve usually presents thinner myelin sheets with shorter nodal lengths, less functional than the original nerve [99]. Based on their potential benefits, SCs have been extensively applied in peripheral nerve regeneration research [100–102].

PNS axonal regeneration requires a complex interaction of a scaffold for axonal elongation, supportive cells such as the Schwann cells, growth factors, and extracellular matrix [97,103]. When end-to-end suture of the nerve is not possible, the interposition of a nerve conduit becomes necessary. Autologous nerve grafts are considered the gold standard for repairing peripheral nerve gaps [104]. Autologous grafts are often harvested from the sural, or sensory, nerves [105]; however, sometimes there are limitations such as tissue availability, size incompatibilities, and deformities. Less frequently, allografts can be used, with the disadvantages of requiring immunosuppression and of producing worse outcomes than autologous nerve grafts [106]. More recently, TE has provided nerve conduits, which function as guides for axonal regrowth. Depending on the materials used for their construction, nerve conduits can be classified as natural, based on laminin, collagen, or even vessels and decellularized nerves [107], or as artificial, usually made of polymers [108]. Although artificial, non-biodegradable scaffolds help nerve growth and provide beneficial results, they may lead to chronic inflammation and tissue compression and, therefore, must be surgically removed once the neural connection has been concluded. To avoid the hurdles of a second surgery on the injury site, biodegradable scaffolds are preferred [109]. The major limitation for use of nerve conduits is the low rate of axonal growth, which may not yield meaningful repair within the available time. Studies have shown that the conduits are effective in promoting repair of peripheral nerve gaps measuring up to 3 cm [108], whereas nerve autografts are required for bridging nerve gaps of larger distances.

4. CNS REGENERATION

In contrast to the PNS, in which severed axons often will regenerate, injured CNS neurons exhibit a burst of stymied growth but ultimately fail, with their axons stalling out and forming distinctive large endings dubbed "retraction bulbs" that fail to transverse the injury site. Numerous comparative studies have indicated phylogenetic differences in the regeneration capacity of various species. Whereas axons in the CNS of warm-blooded vertebrates (mammals and birds) do not regenerate, those in many lower vertebrates such as newts [110] can regenerate after injury. Young mammals are also capable of substantial CNS neural regeneration [111]. These studies demonstrate that the lack of CNS regeneration in warm-blooded vertebrates may be the result of evolutionary changes, although it is still unclear whether these varied responses are caused by differences in the expression of genes that are conserved across these organisms or by the presence of proteins that are specific to warm-blooded vertebrates. In the field's effort to define the failure of axonal regeneration after CNS injury in mammals, the neuron's intrinsic growth state, the glial scar, myelin inhibitors, and invading cells from the periphery have all been investigated as likely suspects involved in inhibiting CNS regeneration. Accordingly, numerous experimental research efforts aiming at these theories have been conducted and some exciting and promising interventions have been summarized below.

4.1 Intrinsic Growth Capability of CNS Neurons

As mentioned above, cAMP has been identified as the first molecule to be implicated in the conditioning effect [77,81]. Manipulation of signaling pathways by elevating the level of cAMP can similarly change a neuron's propensity to regenerate [77]. CNS neural regeneration can be enhanced in vivo by delivering a cAMP analog or by administering rolipram, which inhibits an enzyme that blocks the breakdown of cAMP [112,113]. Studies have also indicated that conditional knockout of PTEN (phosphatase and tensin homolog) or tuberous sclerosis complex 1, both negative regulators of the mammalian target of rapamycin (mTOR) pathway in adult retinal ganglion cells, promotes robust axon regeneration after optic nerve injury [114] and adult corticospinal tract [29], demonstrating that modulating neuronal intrinsic PTEN/mTOR activity represents a potential therapeutic strategy for promoting axon regeneration and functional repair after adult spinal cord injury [115]. More recently, the suppressor of cytokine signaling 3, a negative regulator of the Janus kinase/STAT pathway, was identified as another independent pathway that can act synergistically with PTEN/mTOR to promote enhanced axon regeneration [28,116].

4.2 The Glial Scar

Glial reaction is a hallmark of CNS injury. After CNS injury, astrocytes hyperproliferate and become "reactive," releasing extracellular matrix molecules, such as laminin, heparan, and especially chondroitin sulfate proteoglycans (CSPGs), which are considered to be major candidates for mediating the inhibitory activity of the scar [117]. Consistent with this possibility, therapeutic dissolution of the CSPG-rich matrix with chondroitinase ABC, an enzyme that selectively degrades CSPGs, has proven to be beneficial to axonal regeneration and functional recovery after SCI in preclinical studies in rodents [117–121]. In recent years this line of research has been further advanced with promising results [122–125]. Although it is well characterized that astrocytes produce several different CSPG family members that are differentially expressed after SCI [126–128], the molecular mechanisms through which CSPGs activate growth cone collapse are not fully understood. A transmembrane tyrosine phosphatase receptor, PTPσ, has been identified as one specific and high-binding-affinity receptor for CSPGs [129]. Subsequently, another member of the PTPR subfamily, LAR, was shown to bind to CSPGs with high affinity. Intervention with a LAR-targeting peptide improved axonal regeneration and motor functional recovery after SCI in rodents [130]. In addition, provocative reports have demonstrated that stromal cells derived from pericytes, which control the vasculature in the CNS, also constitute a substantial portion of the cells found at the glial scar. Genetically modified animals with severely reduced populations of pericytes failed to insulate spinal cord lesions with glial scar tissue [131].

4.3 Myelin-Associated Inhibitors

The clearance of myelin debris is extremely slow within the adult mammalian CNS. As these remnants stay for weeks and months after lesion, the possibility was raised that residual myelin may contain factors that can actively prevent injured neurons from regenerating. In vitro, cultured neurons are prevented from extending axons when plated on purified myelin extracts [132,133]. In vivo, animals that received irradiation to impair the formation of myelin-producing oligodendrocytes, or were immunized with myelin extracts, showed some regeneration [133]. Since 2000, three prominent myelin-associated inhibitors (MAIs) have been identified: Nogo-A [134–137], myelin-associated glycoprotein (MAG) [138,139], and oligodendrocyte myelin glycoprotein (OMgp) [140–142]. The inhibitory properties of Nogo, MAG, and OMgp have been tested in vitro and in vivo in different CNS injury models [138,141,143–146], indicating Nogo-A as the major actor in myelin-dependent CNS repair failure. An anti-Nogo-A antibody has advanced to clinical trials for SCI (http://www.research-projects.uzh.ch/p9471.htm). The potential synergistic inhibitory effect of these three proteins on axonal regeneration in injured adult CNS has been tested. Triple-knockout (TKO) mice for Nogo, MAG, and OMgp were independently generated in two laboratories, and the axonal regenerative capacity of the corticospinal tract (CST) and 5-hydroxytryptamine (5-HT), as well as the motor functional recovery of the TKO mice, compared to wild-type and single-mutant mice, was evaluated after SCI. The Strittmatter lab found that loss of Nogo-A allows corticospinal and raphe-spinal axon growth above and below the injury, as well as greater behavioral recovery than in wild-type or heterozygous mutant mice. In contrast, deletion of MAG and OMgp stimulates neither axonal growth nor enhanced locomotion. The triple-mutant mice exhibit greater axonal growth and improved locomotion, consistent with a principal role for Nogo-A and synergistic actions for MAG and OMgp, presumably through shared receptors, which provide the optimal chance for overcoming myelin inhibition and improving neurological function [142]. The Zheng lab, however, found that, whereas deleting any one inhibitor in mice enhanced sprouting of corticospinal or raphe-spinal serotonergic axons, there was neither associated behavioral improvement nor a synergistic effect of deleting all three inhibitors. Furthermore, they found that triple-mutant mice failed to exhibit enhanced regeneration of either axonal tract after