Sensory Axon Regeneration: A Review from an in vivo Imaging Perspective

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Injured primary sensory axons fail to regenerate into the spinal cord, leading to chronic pain and permanent sensory loss. Re-entry is prevented at the dorsal root entry zone (DREZ), the CNS-PNS interface. Why axons stop or turn around at the DREZ has generally been attributed to growth-repellent molecules associated with astrocytes and oligodendrocytes/myelin. The available evidence challenges the contention that these inhibitory molecules are the critical determinant of regeneration failure. Recent imaging studies that directly monitored axons arriving at the DREZ in living animals raise the intriguing possibility that axons stop primarily because they are stabilized by forming presynaptic terminals on non-neuronal cells that are neither astrocytes nor oligodendrocytes. These observations revitalized the idea raised many years ago but virtually forgotten, that axons stop by forming synapses at the DREZ.

Key words: dorsal root entry zone, sensory nerve regeneration, NG2 glia, oligodendrocyte precursor cells, in vivo imaging, astrocytes

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regeneration by all or most DRG neuron subtypes. A glial cell
line-derived neurotrophic factor (GDNF), artemin, induces
topographically specific regeneration of most DR axons in rodents
[24, 25] but, like other neurotrophic factors, severe side effects
preclude its use in clinic trials. The need to develop new treatments
is urgent.

DORSAL ROOT INJURY

Dorsal root injuries, which include brachial plexus, lumbosacral
plexus and cauda equina injuries, result in permanent loss of
primary afferent terminals in the spinal cord. These injuries
have profound effects on the spinal cord and evoke chronic,
often agonizing, pain and permanent loss of sensation. Brachial
plexus injury (BPI), the most common form of DR injury, results
from high-energy traction injuries in which the head and neck
are forced away from the shoulder. Obstetrical BPI is the most
common etiology of a plegic arm in infants, occurring in ~3 per
1,000 live births. In adults, BPI occurs most commonly in motor
vehicle accidents, particularly motorcycles, contact sports and
falls. Overall, DR injuries are 10–20 times more common than
spinal cord injury (SCI) [13, 26–28]. There is an urgent unmet
clinical need for effective therapies that can reduce the extent
of the initial injury or, at a later stage, enhance repair. The need
for effective treatment is increasing due to higher survival rates
following severe traumatic injuries and the increasing number of
elderly individuals susceptible to these injuries because of falls.

Clinical treatment of brachial plexus injury is often surgical.
In children, damaged peripheral components of the plexus may
be repaired with donor nerve (usually the patient’s own sural
nerve) or other graft material, including processed cadaver nerve
or tubes containing extracellular matrix [29, 30]. In adults, the
distance that regenerating nerves must grow is often too lengthy
for effective grafting. Nerve anastomosis to a nearby denervated
nerve and muscle may be useful in both children and adults [31,
32]. However, it must be emphasized that recovery after peripheral
injury is generally incomplete even with these treatments and that
there is no effective therapy for dorsal root avulsions.

CELLULAR CHANGES AT THE DREZ AFTER DORSAL ROOT
INJURY

At the DREZ, the glial ensheathment of the axons changes
abruptly from Schwann cells in the dorsal root to astrocytes
and oligodendrocytes in the spinal cord. Following DR injury,
on the PNS side, macrophages rapidly phagocytose myelin and
degenerating axons [33], while Schwann cells become activated/
dedifferentiated and occupy axon-free endoneurial tubes. By
contrast, on the CNS side, astrocytes rapidly undergo reactive
changes, which include proliferation and extension of their
processes further distally into DR, between the endoneurial tubes
(i.e., astrogliosis) [34, 35]. Following DR crush, injured axons
regenerate past the crush site, but turn around or stop at the DREZ.
DR injury evokes changes similar to those induced by direct spinal
cord injury (SCI), without the formation of a dense astrocytic glial
scar. Nevertheless, the axotomized DREZ prevents regeneration
surprisingly efficiently: Whereas peripheral conditioning lesions
promote intraspinal regeneration of their central axons in the
dorsal columns [36, 37], the same axons fail to regenerate through
the DREZ, as confirmed in our in vivo imaging study [5, 6, 38, 39].

ARE ASTROCYTES OR CSPGs DECISIVE FACTORS INHIBITING
REGENERATION AT THE DREZ?

What prevents axons from regenerating across the DREZ? The
prevailing view in the field is that regeneration is prevented at the
DREZ primarily by growth-inhibiting activities associated with
reactive astrocytes and/or degenerating oligodendrocytes. Because
axons contact astrocytes when they have stopped regeneration
at the DREZ [40–42], reactive astrocytes are thought to form the
primary regenerative barrier. Consistent with this notion, axons
grow through the DREZ that has been depleted of astrocytes by
X-irradiation [43], and, in general, (despite exceptions [6, 44]),
reactive astrocytes inhibit neurite outgrowth [8, 45] by producing
chondroitin sulfate proteoglycans (CSPGs) that collapse or repel
neurite outgrowth [46, 47]. Members of the CSPG family of extra-
cellular matrix molecules include neuroglycan 2 (NG2), aggrecan,
brevican, neurocan, vesican and phosphacan [45]. These CSPGs
are expressed at the DREZ both during development and after
dorsal root injury [48].

The differential expression and contribution of individual

Fig. 1. Cajal’s drawing illustrating DR axons growing away from (arrow)
or arrested (arrowheads) at the entrance into adult spinal cord.
members of the CSPG family have also been studied. NG2, the most important component, is a major inhibitory proteoglycan for sensory axons [49]. NG2 is expressed by oligodendrocyte progenitor cells, which react rapidly following CNS injury, and by some reactive astrocytes. Virus-mediated knockdown or antibody blocking of NG2 promotes intraspinal sensory axon regeneration [50]. Recently, a transmembrane protein tyrosine phosphatase, PTPσ, was identified as a high affinity receptor of CSPG that mediates its inhibitory effect [51, 52]. Disruption of the PTPσ gene reduced inhibition by CSPG.

Several lines of evidence argue that astrocytes and CSPGs are not the critical determinant of regeneration failure. For example, the same CSPGs are expressed abundantly in tissue that supports regeneration (dorsal roots) and in tissue in which regeneration fails (DREZ and spinal cord) [53]. The inhibitory properties of CSPGs are primarily due to glycosaminoglycan (GAG) side chains; enzymatic removal of GAG chains by chondroitinase ABC (ChABC) promotes intraspinal axon regeneration [46, 54]. However, pharmacological degradation of CSPGs by chondroitinase ABC or Phosphatidylinositol-specific phospholipase C (Pi-PLC), which enhances regeneration in the damaged spinal cord [55], does not promote regeneration across the DREZ [23; but see Cafferty et al., 2007]. How astrocytes prevent regeneration, if they indeed play a crucial role in the regeneration failure, remains uncertain.

ARE OLIGODENDROCYTES OR MYELIN-ASSOCIATED INHIBITORS DECISIVE FACTORS INHIBITING REGENERATION AT THE DREZ?

Oligodendrocyte/myelin-derived inhibitors, such as Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (Omgp), may also contribute to regeneration failure at the DREZ [7]. These molecules, except MAG, are distributed exclusively in CNS myelin synthesized by oligodendrocytes and are not found in PNS myelin synthesized by Schwann cells. All three of these myelin inhibitors bind to the glycosylphosphatidylinositol-anchored Nogo-66 receptor (NgR1), which is expressed by many CNS neurons [56, 57]. Other receptors, including NgR2 and the paired immunoglobulin-like receptor B (PirbB), have also been implicated in mediating the inhibitory growth signaling [58, 59].

Intrathecal application of the soluble Nogo-receptor peptide fragment of the NgR (sNgR) after DR crush elicited regeneration of myelinated, but not unmyelinated, sensory axons [18]. This result supports the idea that myelin-associated molecules contribute to regeneration failure. It is important to note, however, that myelin-associated molecules are eventually, although slowly, cleared, together with degenerating oligodendrocytes [35, 38, 40]. Thus, their actions are exerted only transiently during the initial phase of inhibition. In addition, it is noteworthy that axons can regenerate along degenerating white matter [60, 61], and that simultaneous elimination of multiple inhibitory molecules does not promote intraspinal regeneration [62, but see, 63]. Whether regeneration is promoted at the DREZ in these triple knockout mice has yet to be determined.

Lastly, but perhaps most importantly, myelin-derived inhibitors and CSPGs act as repellent cues that cause only brief growth cone collapse or retraction [10, 64, 65]. Furthermore, DRG axons can grow despite growth cone collapse [66-68]. Moreover, unlike in vitro, regenerating DR axons in vivo are accompanied by growth-promoting Schwann cells, which could provide attractive alternative growth pathways for axons with ‘transiently’ collapsed growth cones to turn around and grow back toward DRG. The myelin-associated growth inhibitory molecules and CSPGs could therefore explain why some axons turn around at the DREZ, but not why others become immobilized. These considerations highlight our current lack of understanding of the molecular and cellular mechanisms that account for regeneration failure at the DREZ. They also call into question whether astrocyte- or oligodendrocyte-associated growth inhibitory molecules are paramount in preventing sensory axon regeneration after dorsal root injury.

‘SYNAPTOID’: A FORGOTTEN IDEA

One provocative idea raised many years ago that has received little subsequent attention is that astrocytes induce DR axons to form synapses that immobilize them at DREZ. Carlstedt first made this proposal based on his observation of structures at DREZ that he termed synaptoids [40], because of their interesting resemblance to nerve terminals. Other investigators have observed similar nerve terminal-like structures [38,69], and Liuzzi and Laske also speculated that astrocytes provide a physiological ‘stop signal’ that prevents regeneration of DR axons at the DREZ [69]. As shown in Fig. 2, these axonal profiles resemble nerve terminals because mitochondria and vesicles are copious whereas microtubules and neurofilaments are sparse or absent. These ‘synaptoid’ profiles lack some characteristic features of pre- and postsynaptic differentiation, however, and mitochondria and vesicles are also abundant even in non-synaptic dystrophic endings [70]. In addition, none of the early studies provided direct evidence that these synapse-like profiles belong to sensory axon growth tips, rather than to other neuronal elements such as the
chronically remodeled dendrites of dorsal horn neurons. These studies also did not determine whether axon endings became ‘nerve terminal-like’ as they entered the DREZ or whether the nerve terminal-like appearance developed only after chronic remodeling.

Nonetheless, it is notable that these nerve endings clearly differ from the retraction bulbs, originally described by Cajal (Fig. 1), formed by severed dorsal column axons and from the dystrophic endings formed in an in vitro model of the glial scar [70], in which fragments of disorganized microtubules or vacuoles are abundant. Moreover, the idea that astrocytes act as a stop signal also receives support from recent studies demonstrating the ability of astrocytes to promote synapse formation and maintenance [71, 72]. Although supported by anecdotal evidence, these intriguing speculations await rigorous, and challenging, investigations with advanced techniques, such as the combined application of in vivo imaging, targeted electron microscopy (EM) and fluorescent mouse transgenics.

**IMAGING AXONS IN LIVING ANIMALS**

Until recently biologic processes could only be analyzed using static images obtained after death from animals euthanized at multiple time points. The temporal and spatial resolution of these analyses was limited because dynamic changes had to be deduced from comparisons of static images. Accordingly, prior studies based on conventional static analyses provided evidence that is often conflicting or inconclusive. Dynamic cellular processes, including axon regeneration and the interactions between axons and their environment that determine the success or failure of regeneration, are best studied with techniques that capture real-time events with multiple observations of each living animal.

Our ability to monitor neurons serially in vivo has increased dramatically owing to revolutionary innovations in optics and mouse transgenics. Several lines of thyl-XFP transgenic mice raised by Drs. Josh Sanes and Guoping Feng, in which subsets of neurons are genetically labeled in distinct fluorescent colors, have permitted individual neurons to be imaged in vivo, serving as a 21st century upgrading of Golgi staining. These mice have been used extensively for in vivo imaging of muscles [73-75] and brain [76-78], in both physiological and pathological conditions, and have provided novel insights into physiological mechanisms that static analyses could not have resolved. Surprisingly, however, imaging studies of living spinal cord have been limited. Lichtman and his colleagues first demonstrated their feasibility by tracking injured dorsal column (DC) axons with wide-field microscopy [60, 79]. Two-photon imaging of DC axons, microglia and blood vessels was also accomplished [80]. Recently, we provided the first study of dorsal root (DR) regeneration in living mice [5]. We used wide-field microscopy and a line of thyl-YFP mice (thyl-YFPH), in which a subset of large-diameter DRG neurons and their axons are brightly labeled by green fluorescence. This approach was more challenging to develop than we had anticipated but it generated unexpected insights in support of the ‘synaptoid’ hypothesis postulated many years ago but virtually forgotten.

**FEASIBILITY AND UTILITY OF IN VIVO IMAGING OF DR REGENERATION**

Axon sparing has been responsible for conflicting or inclusive observations in studies of spinal cord or root injuries [81]. We consider the power to distinguish between spared and regenerating axons an important asset of in vivo imaging studies of DR regeneration. This distinction is possible because in vivo imaging allows observation of the spatiotemporal responses of both regenerating proximal stump axons and of the same axons...
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Rapid immobilization of axons at the DREZ

If growth-inhibitory molecules prevent regeneration of DR axons at DREZ, in agreement with the predominant view in the field, then one would predict dynamic responses by DR axons at DREZ. After encountering CSPGs and myelin-associated inhibitors that are produced by reactive astrocytes and degenerating oligodendrocytes in the spinal cord and cause growth cone collapse, axons would be expected to regenerate back into the PNS, where growth-promoting Schwann cells are present, or to continue to try to grow within the DREZ and then to degenerate. Surprisingly, our in vivo imaging studies showed that > 95% of YFP-labeled axons rapidly stopped and became completely and permanently immobilized at the DREZ, even after conditioning lesions that enhanced their growth potential [5]. Notably, in vivo imaging also revealed that very few axons detoured back into PNS, and that they did so immediately after encountering the border between CNS and PNS ([5, 82, 83] and unpublished data).

Presynaptic differentiation at the DREZ and the resurgence of the forgotten idea

Light microscopic and ultrastructural analyses targeted specifically to axon tips monitored in vivo subsequently demonstrated that almost all axons stopped at the CNS territory of the DREZ [5], and that axon tips and adjacent shafts were intensely immunolabeled with synapse markers such as SV2 and synaptophysin [5]. Numerous axonal profiles demonstrated characteristic features of pre- but not postsynaptic endings [5]; these axonal profiles were filled with mitochondria and ~40-nm vesicles but lacked the vacuoles and disorganized microtubules that are typical of dystrophic endings. Postsynaptically, no indications of differentiation such as postsynaptic densities were observed (Fig. 4; red-pseudocolored).

Our findings conflict with the prevailing view that axons stop at the DREZ by forming swollen dystrophic endings [84]. We have found that surprisingly few stalled axons terminate in large swollen tips and that most end as slender tips that persist [5]. Notably, almost all axons, including those with large swollen endings, display small varicosities along their shafts that are intensely immunolabeled with synapse markers [5]. Our recent EM study confirmed that the large swollen endings are indeed dystrophic and lack synaptic characteristics. Importantly, however, these axon tips are completely surrounded by acellular collagenous fibers (Skuba et al., unpublished data), rather than by non-neuronal cellular elements, and they are located superficially where collagenous scars have formed adjacent to dura. Together,

Degenerating distal to the crush. As illustrated in the cartoon (Fig. 3), several features of crushed axons in vivo were very useful in differentiating regenerating axons from axons that had not been crushed or had recovered from crush. Within a few hours after crushing: 1) An ~30 μm wide non-fluorescent portion of the YFP+ axon crushed at the injury site becomes widened rather than narrowed (i.e., narrowed if axons survive the crush due to fluorescent cytoplasm refilling the squeezed/crushed axon site). 2) The distal portions of the same axons beyond the crush begin to degenerate and become fragmented. In addition, within a few days after crushing: 3) Fragments of fluorescent distal axons beyond the crush progressively shorten due to degeneration at both ends of each fragment. Moreover, 4) Regenerating axons or neurites are much thinner, less brightly fluorescent and more undulating than the intact axons (or recovered YFP labeled large diameter axons), and 5) The thin, dimly fluorescent regenerating neurites extend through the degenerating fragments of the original, brightly fluorescent axons. 6) Unlike surviving or spared axons, regenerating axons stop at the DREZ. 7) Spared axons exhibit nodes of Ranvier, whereas regenerating axons do not.

![Fig. 3. Schematic illustration of spared, degenerating and regenerating axons observed during in vivo imaging of YFP-labeled DR axons following crush injury (1). A non-fluorescent gap is obvious at the site of crush (2), and then in the distal axons undergoing fragmentation (3). Non-fluorescent gaps widen and become more numerous as distal axons degenerate (4). A thin regenerating neurite extends from the proximal stump of the same axon (3), which elongates through the much thicker and brighter fluorescent fragments of a degenerating axon (4, 5). A spared axon (blue arrow) and a crushed axon (green arrow) showing degeneration of an old axon and regeneration of a new neurite from its proximal stump.](http://dx.doi.org/10.5607/en.2012.21.3.83)
findings show that most, if not all, DR axons become arrested as they enter the CNS territory of the DREZ by forming presynaptic terminals on non-neuronal cellular elements. These observations are also in line with the provocative idea that astrocytes induce DR axons to form ‘synaptoids’ that cause their arrest at the DREZ [38, 40, 69].

ARE NG2 CELL OR SYNAPTOGENIC MOLECULES DECISIVE FACTORS INHIBITING REGENERATION AT THE DREZ?

The identity of the postsynaptic cells has yet to be determined. The absence of postsynaptic densities excluded the possibility that these profiles are axon-axon synapses [85], and the cells lack the intermediate filaments that are abundant in astrocytes [5]. Some evidence suggests that they may be NG2 cells forming synapses with neurons. NG2 cells are proteoglycan NG2 (nerve/glial antigen 2)-expressing oligodendrocyte progenitors which constitute a fourth major glial cell in the mammalian CNS, distinct from astrocytes, oligodendrocytes and microglia [86-93]. They are widely distributed in both gray and white matter, and have a complex stellate morphology with highly branched fine processes [86, 94-97]. They have also been reported to form a cellular net in the CNS territory of the DREZ [98, 99], proximal to the astrocyte-Schwann cell border, where our in vivo imaging showed that axons stop and become immobilized (Kim et al., unpublished observation). Many investigators have confirmed that NG2 cells form functional glutamatergic and GABAergic synapses with CNS neurons [100, 101], expressing a complex set of voltage-gated channels with AMPA/kainate and/or GABA receptors [86-93]. Interestingly, the presynaptic active zones observed in DR axons stopped at the DREZ are thinner than at neuron-neuron synapses and resemble those reported at neuron-NG2 cell synapses [100, 101]. Indeed, we have found that NG2 immunolabels are almost always colocalized with the synaptic label SV2 in DR axons stopped at the DREZ (Kim et al., unpublished data). An intriguing possibility therefore is that DR axons fail to regenerate into spinal cord primarily because they form synapses with NG2 glia, which transforms the axons from growth to differentiation mode (Fig. 5).

CAVEATS

Several caveats concerning the imaging studies, however, make our interpretation tentative and justify a more thorough investigation. A particular concern is that the limited spatiotemporal resolution of our axon growth tip imaging might have caused us to overlook subtle local motility. In addition, because we imaged only a few large-diameter axons in a small number of mice, we have little information about possible

Fig. 4. An electron micrograph of the DREZ 13 days after crush injury showing a presynaptic axonal profile. Vesicles are highly clustered and docked at an electron-dense membrane that resembles an active zone (white arrows). No postsynaptic densities are present on the non-neuronal, postsynaptic cell process, which also lacks the abundant intermediate filaments that are characteristic of astrocytes. Scale bar, 250 nm.

Fig. 5. Schematic illustration of the proposed model of regeneration failure at the DREZ. In addition to various growth inhibitory molecules, the CNS territory of the DREZ contains synaptogenic molecules associated with non-neuronal glial cells (possibly NG2 glia). Growth inhibitors fail to repel most axons at the interface. Most axons therefore invade the CNS territory of the DREZ and then cease their growth by forming presynaptic nerve terminals (red-colored swellings on axon tips and shafts). The few axons that are stopped and exhibit dystrophic endings (e.g., axon with grey-colored tip) may also be immobilized by forming presynaptic terminals on their axon shaft.
variability in the responses of the heterogeneous populations of DRG neurons. Imaging more DR axons of all different types at higher spatiotemporal resolution will therefore be necessary. Direct demonstration \textit{in vivo} of the consequences of selectively ablating NG2 glia and astrocytes and eliminating individual or combinations of growth-inhibiting molecules will also be extremely useful to identify the molecular and cellular factor(s) that play a definitive role in preventing regeneration at the DREZ.

CONCLUSION

Both intrinsic and extrinsic factors, particularly growth-inhibitory molecules associated with astrocytes and oligodendrocytes, are thought to contribute to regeneration failure at the DREZ. Several observations previously reported in the literature, however, make it unlikely that these factors are the critical determinant(s) of the regeneration failure: 1) Even after myelin-associated inhibitory cues have disappeared due to oligodendrocyte degeneration, the DREZ continues to prevent regeneration; 2) CSPGs associated with astrocytes and myelin-associated inhibitory cues collapse growth cones transiently, but do not immobilize or chronically stabilize growth cones; 3) Although central processes of DRG neurons in the DC and DR derive from the same neurons and encounter the same inhibitory cues, conditioning lesions enhance regeneration of DC axons [36, 102] but not DR axon regeneration across DREZ [38]; 4) CSPGs are expressed abundantly in the regenerating DR [53] and pharmacological degradation of CSPGs by ChABC does not promote regeneration across DREZ [23]. The first application of \textit{in vivo} imaging to the DREZ provides further support. These studies showed that DR axons rarely turn around or retract at DREZ but quickly become immobile within its CNS territory, and then remain in place for long periods of time, perhaps permanently, even without target innervation. These observations are better explained by mechanisms that stabilize rather than continuously inhibit axon growth and, intriguingly, axons immobilized at the DREZ show numerous features of presynaptic terminals. What causes them to form presynaptic nerve endings and whether they form functional synapses remain to be determined. Future in-depth investigations will determine whether this novel idea, raised many years ago, applies to all the different subtypes of DRG neurons and whether it provides a decisive explanation for failed regeneration at the DREZ and perhaps also within the spinal cord.

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