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Sensory Axons Inhibit Motor Axon Regeneration *in vitro*

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Abstract

During mammalian embryonic development sensory and motor axons interact as an integral part of the pathfinding process. During regeneration, however, little is known of their interactions with one another. It is thus possible that sensory axons might influence motor axon regeneration in ways not currently appreciated. To explore this possibility we have developed an organotypic model of post-natal nerve regeneration in which sensory and motor axons are color-coded by modality. Motor axons that express yellow fluorescent protein (YFP) and sensory axons that express red fluorescent protein (RFP) are blended within a three-dimensional segment of peripheral nerve. This nerve is then transected, allowing axons to interact with one another as they grow out on a collagen/laminin gel that is initially devoid of directional cues. Within hours it is apparent that sensory axons extend more rapidly than motor axons and precede them during the early stages of regeneration, the opposite of their developmental order. Motor axons thus enter an environment already populated with sensory axons, and they adhere to these axons throughout most of their course. As a result, motor axon growth is reduced dramatically. Physical delay of sensory regeneration, allowing motor axons to grow ahead, restores normal motor growth; direct axonal interactions on the gel, rather than some other aspect of the model, are thus responsible for motor inhibition. Potential mechanisms for this inhibition are explored by electroporating siRNA to the neural cell adhesion molecule (NCAM) and the L1 adhesion molecule (L1CAM) into dorsal root ganglia (DRGs) to block expression of these molecules by regenerating sensory axons. Although neither maneuver improved motor regeneration, the results were consistent with early receptor-mediated signaling among axons rather than physical adhesion as the mechanism of motor inhibition in this model.

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Introduction

The interactions of regenerating axons with the glia and substrates they contact have been explored for over a century. Similarly, sensory and motor axons have been shown to interact with one another during developmental pathfinding (Landmesser and Honig, 1986; Wang and Marquardt, 2013). The interactions of post-natal sensory and motor axons during regeneration, in contrast, have received little attention. It is thus possible that sensory axons might influence motor axon regeneration in ways not currently appreciated. Soon after nerve transection motor axons generate multiple sprouts that may each reinnervate a distinct Schwann cell tube in the distal nerve stump (Redett et al., 2005; Witzel et al., 2005). Individual tubes contain multiple axons from a variety of sources (the Regenerating Bundle: discussed in Brushart, 2011), so there is ample opportunity for sensory and motor axon collaterals to contact and influence one another after transection of any nerve containing both axon types. Although this possibility is obvious in mixed nerves, even so-called “motor nerves” contain more than twice as many afferent as efferent axons (Boyd and Davey, 1968; Brushart, 1988). As a result, there is no physiologic *in vivo* model that determines how well motor axons would perform if regenerating alone, and thus no comparative standard that can be used to determine whether sensory-motor interactions routinely influence motor axon regeneration.

We have overcome the limitations inherent in normal anatomy by developing an *in vitro* model of nerve repair and regeneration in which axons are color-coded by modality. The model is based on our original organotypic preparation in which motor axons that express yellow fluorescent protein (YFP) are led from a spinal cord slice into a three-dimensional segment of peripheral nerve (Siddique et al., 2014; Vyas et al., 2010). Although nominally yellow, YFP appears green with our filter system; henceforth we will describe motor axons as green. We now report modifying the model by co-culturing spinal cord with a DRG in which neurons express red fluorescent protein (RFP). A reversed segment of branched nerve is used to capture motor axons in one branch and sensory in the other, combining them to generate a mixed nerve. When this nerve is transected, green fluorescent motor and red fluorescent sensory axons grow out on an unstructured collagen/laminin hydrogel. Under these conditions, sensory axons extend more rapidly than motor axons and precede them during the early stages of regeneration. Furthermore, motor axons adhere to sensory axons throughout most of their course. As a result of this previously unappreciated interaction, sensory axons inhibit motor axon growth. Potential mechanisms for this inhibition are explored by delaying sensory axon regeneration and by injecting siRNA to adhesion molecules NCAM and L1CAM into DRGs to block expression of these molecules by regenerating sensory axons.

Materials and Methods

Organotypic Co-culture of Spinal Cord, DRG, and Peripheral Nerve

Organotypic cultures were prepared using spinal cord from mice that express YFP in motoneurons (B6.Cg-Tg[Thy1-YFP]16Jrs/J: (Feng et al., 2000), DRGs from mice that express RFP in all neurons (ROSA mT/mG), and peripheral nerve from non-expressing C57Bl6 mice. All procedures were approved by the Animal Care and Use Committee of the

Johns Hopkins Medical Institutions. Two basic configurations of the model were prepared: **Motor Only**, in which a spinal cord slice innervates a peripheral nerve segment with green fluorescent motor axons (Figure 1), and **Dual**, in which spinal cord and DRG contribute both green fluorescent motor axons and red fluorescent sensory axons to generate a mixed nerve in which axons are color-coded by modality (Figure 2). The **Dual** model was first used without modification to examine the interaction of regenerating sensory and motor axons, and was then modified to explore the effects of delaying sensory regeneration or of knocking down expression of the adhesion molecules NCAM and L1CAM. Throughout the following discussion each axon population is referred to first by the basic culture configuration, Motor Only or Dual, to which is appended specific descriptors when needed (e.g. Dual, Sensory Delay). This group descriptor is then followed by the axon type being considered, sensory axons or motor axons (e.g. Dual, Sensory Delay: motor axons).

Motor Only cultures (Figure 1; n=11) were prepared from neonatal (P4-P5) mice using a modification of our earlier technique (Siddique et al., 2014; Vyas et al., 2010). Mouse pups were chilled until all movement ceased, then decapitated. Their spinal cords were removed through a ventral approach and suspended in dissection medium (Hanks Balanced salt solution [HBSS, Gibco, Grand Island, NY], 4.3 mM sodium bicarbonate, 10mM HEPES [4–2-Hydroxyethyl, piperazine-1-ethanesulfonic acid], 33.3 mM D-glucose, 5.8 mM magnesium sulfate, 0.03% BSA and penicillin/streptomycin [Gibco]) to facilitate debridement of dura and root fragments. Lumbar spinal cords were then cut into 350 μ m transverse sections with a McIlwain tissue chopper and placed on Transwell[®] collagen-coated membrane inserts (Corning, Acton, MA.). The Transwell[®] system was modified by removing the walls of the insert, leaving only a ring of plastic to support the membrane, and by suspending it in a low-profile poly(dimethylsiloxane) (PDMS) base (Figure 1). These modifications facilitated imaging cultures directly from above, rather than from below through plastic and fluid, and improved accessibility for microsurgery. The culture was completed by opposing a segment of wild-type P4-P5 peripheral nerve to the ventral root exit zone to capture and channel regenerating motor axons. For successful reinnervation to occur, the cut face of the nerve must be in direct contact with transected motor axons as they exit the ventral surface of the spinal cord slice; if axons escape onto the surface of the membrane, they cannot be recaptured.

Cultures were maintained at 37°C in a humidified atmosphere with 5% CO₂, and were nourished by culture medium that contained 50% minimal essential medium (Gibco), 25% HBSS, 25% heat-inactivated horse serum (Hyclone, Logan UT), 25 mM HEPES, 35mM D-glucose, 2 mM glutamine, and penicillin/streptomycin. The reservoir in the PDMS base holds 1 cc of medium, which was changed every third day. Glial cell line derived neurotrophic factor (GDNF, 70 ng/mL; R&D systems Inc, Minneapolis MN) and insulin like growth factor 1 (IGF-1, 50 ng/mL; Peprotech Inc, Rocky hill, NJ) were added to the medium during culture preparation to protect newly axotomized neurons from apoptosis (Vyas et al., 2010), but were not included in subsequent medium changes.

Cultures were supported for one week during which motor axons fully reinnervated the peripheral nerve segment. The portion of the nerve farthest from the spinal cord section was then raised from the Transwell[®] membrane with microforceps, and cells that had migrated

from the nerve onto the membrane during the week of incubation were carefully removed to insure that the regeneration environment was not influenced by Schwann cells. The nerve was then transected with microscissors 3–5 mm from the spinal cord, and the freshly-cut end placed on an unstructured collagen/laminin hydrogel (Figure 1). We found that, once the nerve had been incubated for one week, no additional cells migrated from the nerve when it was transected. The hydrogel was made by mixing 400 μ l collagen type I solution (3 mg/ml, ThermoFisher Scientific), 50 μ l 10 $\times$ PBS, 50 μ l 7.5% sodium bicarbonate and 5 μ l laminin (1 mg/ml, ThermoFisher Scientific) on ice to prevent polymerization. This solution was then placed in 10mm $\times$ 4 mm PDMS frames and polymerized in a 37° incubator for 20 minutes. When hydrogels had gained sufficient tensile strength they were grasped with microforceps, lifted from the PDMS frame, and transferred to the surface of the Transwell® membrane. Cultures were then imaged daily for five days or once after 5 days of regeneration (see Data Collection and Analysis for breakdown).

Preparation of Dual cultures (Figure 2; n=26) was similar to that of motor only cultures, but also involved harvest and placement of DRGs. Two- to four-week-old ROSA mT/mG mice were anesthetized by intraperitoneal injection of a mixture of xylazine (16 mg/Kg) and ketamine (100 mg/Kg) before decapitation and exsanguination. Lumbar DRGs were excised through a ventral approach by sequentially removing lumbar intervertebral discs and vertebral bodies. DRGs were then dissected free, taking great care to preserve several millimeters of both dorsal root and peripheral nerve to lessen the stress imposed upon the neuron by proximal axotomy. Femoral nerves were excised from P4-P5 wild-type mice and placed on the Transwell® membrane with the muscle branch opposed to the ventral root exit zone of the spinal cord slice and the cutaneous branch opposed end-to-end to the peripheral nerve emanating from the DRG. During the following week sensory and motor axons reinnervated the femoral nerve segment in a retrograde direction, joining in the former femoral nerve trunk to produce a mixed nerve with intermingled green fluorescent motor axons and red fluorescent sensory axons. The remainder of the procedure followed the protocol already described for pure motor cultures.

Experimental Manipulations

The Dual culture model was used as described to study the interaction of regenerating sensory and motor axons (n=8), and was modified to delay sensory axon regeneration relative to motor regeneration (Dual, Sensory Delay; n=7), and to evaluate the contributions of NCAM (Dual, NCAM siRNA; n=5) and L1CAM (Dual, L1 siRNA; n=6) to heterotypic adhesion of sensory and motor axons (total 26 Dual cultures).

Sensory axon regeneration was delayed by crushing the peripheral nerve extension of the DRG at the time of nerve transection and hydrogel placement (Figure 3). As a result, motor axons could grow out on the hydrogel immediately, but sensory axons had to regenerate 2–3mm within the nerve before reaching its cut end, and their appearance on the hydrogel was thus delayed by 2–3 days. The role of adhesion molecules in the interaction of sensory and motor axons was evaluated by injecting and electroporating the DRGs with siRNA to NCAM or L1CAM (Dharmacon) to disrupt their expression (Figure 4: Saijilafu et al., 2011).

The siRNAs were dissolved in the buffer provided by the manufacturer. Four-week-old ROSA mT/mG mice were anesthetized by intraperitoneal injection of a mixture of xylazine (16 mg/Kg) and ketamine (100 mg/Kg), and their L3 and L4 DRGs were surgically exposed. One μl of siRNA solution ($50 \text{ pMol } \mu\text{l}^{-1}$) was injected into each DRG using a Picospritzer III (pressure: 30 psi; duration: 8 ms). Immediately thereafter electroporation was performed using tweezer electrodes powered by a BTX ECM830 Electro Square Porator (five 15 ms pulses at 35 V with 950 ms interval). After electroporation, DRG's were placed on the culture membrane and the dual culture was constructed as described previously. Additionally, Western blots were performed on DRGs one week after injection and electroporation to confirm the deletion efficacy for both siRNAs (Figure 5).

Data Collection and Analysis

Cultures were imaged at 10 $\times$ with a Nikon Eclipse E600 fluorescent microscope equipped with Nikon GFP and TRITC filter cubes, a motorized stage, and a Spot CCD camera. Eight cultures in which motor axons regenerated alone (Motor Only), six in which sensory and motor axons regenerated together (Dual) and an additional seven dual cultures in which sensory axon regeneration was delayed (Dual: Sensory Delay) were imaged daily for five days. The remaining 16 cultures were imaged once after five days of regeneration. Cultures were maintained throughout the imaging process in a Takai-Hit INU on-stage incubator that controlled temperature, humidity, and CO₂ level. NIS-Elements Microscope Imaging Software was used to generate and individually flatten z-stacks of the 6–18 microscope fields needed to encompass an entire culture. These images were then opened in Photoshop to adjust their contrast and brightness and to stitch the images together to form a single composite of the entire culture. A Photoshop layer containing a protractor with concentric lines at 0.25 mm intervals from the cut end of the peripheral nerve was then superimposed upon the composite image. Regeneration was evaluated by separately counting the green and red axons that crossed the lines delimiting each 0.25 mm of growth.

To evaluate the significance of our findings, we examined sensory and motor axon counts in two ways. First, to assess the synchrony of the onset of regeneration, we compared the number of axons crossing the 0.25 mm grid line at successive time intervals in three axon populations: sensory axons (Dual: sensory), motor axons growing alone (Motor Only), and motor axons growing with sensory axons (Dual: motor). Second, we evaluated the extent of regeneration in all experimental groups by plotting the percentage of axons counted at the 0.25 mm grid line that extended to each of the more distant grid lines after 5 days of regeneration. The number of axons at 0.25 mm was taken to represent 100%, and the percentage of these axons that reached each subsequent distance was calculated as the ratio of the number of surviving axons at that distance to the number present at 0.25 mm.

In the process of evaluating the synchrony of the onset of regeneration we found that it was not appropriate to assume a normal distribution (Shapiro Wilk test, $p < .05$), so we present descriptive data evaluated for this analysis as median and interquartile range (25th and 75th percentile, maximum and minimum). Comparisons in the number of axons crossing the 0.25 mm grid line between cultures were made using Poisson regression. Main effect variables were axon type (Motor Only, Dual: Sensory, and Dual: Motor) and time in hours (24, 48, 72,

96, 120). The interaction between axon type and time allowed for the test of a differential relationship between axon regeneration and time among axon types.

Comparisons in the proportion of axons present at 0.25 mm that crossed each subsequent protractor grid line (interval= 0.25 mm) were made using a non-parametric test of equality of the medians with a chi-squared continuity correction applied. This model was expanded to test for the effect of knocking down expression of L1CAM and NCAM on axon regeneration. Type I error threshold was set at 0.05. All analyses were conducted in Stata IC, version 14 (Stata Corp, College Station, TX).

Dual cultures were further analyzed to determine the percentage of motor axon length that was directly opposed to sensory axons. Composite images were imported into Wolfram Mathematica and cropped accordingly. To improve the contrast between green and red axons, images were adjusted using a pre-set function, Image Adjust. The “point-and-click” feature in the Graphics Drawing Tools was used to define endpoint coordinates of short line segments that defined portions of each green motor axon that were 1) contacting sensory axons, or 2) extending without sensory axon contact. These sets of coordinates were then compiled into a list. A “loop” was used to add together the total distances between the successive sets of coordinates. The totals from each category were then used to determine the percentage of motor axon length that was adherent to sensory axons within each dual culture.

Results and Discussion

Regeneration of Mixed Nerve with Axons Color-Coded by Modality

In our original organotypic model of nerve repair, motor axons expressing YFP were directed from spinal cord slices into three-dimensional segments of peripheral nerve (Vyas et al., 2010). Once reinnervated, the nerve could be repaired or grafted under a variety of conditions, and regeneration imaged in real time for several weeks. Presence of axons within nerve, rather than between nerve and the membrane surface, was confirmed by locating them at multiple levels throughout a 200–400 μ m-thick z-stack. The model closely approximates conditions *in vivo* in that neurons are post-natal, are supported within their normal tissue environment rather than on a flat surface, and regenerate through a cellular, three-dimensional segment of peripheral nerve. This model was the basis for development of a two-chamber model in which the environments of neuron and growth cone could be manipulated separately (Siddique et al., 2014), and presaged development of a rat model in which regenerating motor axons were demonstrated secondarily using immunohistochemistry (Gerardo-Nava et al., 2014).

To generate a color-coded mixed nerve as the source of axons, we have modified the model by co-culturing spinal cord with a DRG in which neurons express RFP (Brushart et al., 2016). A reversed segment of P5-P6 femoral nerve is used to capture motor axons in one branch and sensory in the other, combining them to generate a mixed nerve (Figure 2). Sensory and motor axons normally intermingle in the proximal femoral trunk (Brushart, 1993), so green fluorescent motor and red fluorescent sensory axons will be directly adjacent to one another at the level of subsequent nerve transection. In this iteration of the

model axons are not directed across a repair to another segment of peripheral nerve, but are allowed to grow out onto an unstructured collagen/laminin hydrogel. This environment is supportive of regeneration, does not provide physical guidance cues, and is devoid of cellular elements; Schwann cells and fibroblasts grow out of the femoral nerve soon after it is first placed on the membrane, but not when it is transected a second time and placed on the hydrogel. The model thus provides the opportunity for sensory and motor axons to contact one another in an environment in which interactions can be observed, quantified, and manipulated to study their underlying mechanism.

Sensory Axons Inhibit Motor Axon Regeneration

Comparison of motor axons growing alone (Motor Only: motor axons) with those growing in the company of sensory axons (Dual: motor axons) revealed a dramatic inhibition of motor axon regeneration when sensory axons were present (Figures 6, 7). The percentage of regenerating motor axons that extended 0.5 mm, 0.75 mm, 1.0 mm, and 1.25 mm from the cut nerve end was significantly lower ($p < .001$) in the Dual: motor axons population than in the Motor Only: motor axons group. To help understand this phenomenon we analyzed the two variables that determine the progress of regeneration for each axon: the delay between injury and the onset of regeneration, and the speed of regeneration itself. Many points on the left side of the growth curve (Figure 7) can potentially be reached either by slowly regenerating axons that get an early start, or by more rapidly regenerating axons that get a delayed start.

The percentage of available motoneurons that begin to regenerate soon after injury is expected to be the same in both Motor Only: motor axons and Dual: motor axons populations, as there are no differences in either the source of motoneurons or in the pathway they follow. To evaluate the rate at which additional neurons are added to the regenerating pool over time, we determined the number of axons crossing the 0.25 mm distance line in Motor Only: motor axons, Dual: sensory axons, and Dual: motor axon populations at 24, 48, 72, 96, and 120 hours (Figure 8). We found that, at each time period, the number of axons was significantly greater ($p < .001$) in the Motor Only: motor axons group than in the Dual: motor axons group, and in the Dual: sensory axons group than in both of the motor groups. These numeric differences are consistent with the structural details of the model: two motoneuron pools contributed axons in the Motor Only: motor axons group (Figure 2) while only one contributed in the Dual: motor axons group (Figure 3), and there are many more sensory neurons in the DRG than motoneurons in a spinal cord slice.

In all three groups, the number of axons crossing the 0.25 mm line increased significantly with time ($p < .001$; Figure 8). Furthermore, the rate of change in axon number with time did not differ significantly between the two motor groups ($p > .05$); the rate of adding sensory neurons, however, was greater than both motor groups at 72 hours ($p = .001$). Delay in the onset and/or progress of regeneration was thus similar in both motor groups. The staggered nature of regeneration was first observed by Forman and Berenberg using axonally transported radiotracers (Forman and Berenberg, 1978). They found that motor axons regenerated as a single front after nerve crush, but were widely dispersed after nerve transection and repair. Regeneration stagger following nerve transection was later quantified

with sequential double labeling after rat femoral nerve repair (Al-Majed et al., 2000; Brushart, 1993).

The relative speeds of sensory and motor axon regeneration can be evaluated in two ways: by identifying the location of each axon population at two time periods and calculating the regeneration speed of each, or by observing the relative locations of the two populations at a single time after identical lesions. The first approach was taken by Moldovan et al., (2006), who studied cat tibial nerve regeneration with implanted electrodes and found that the fastest sensory and motor axons regenerated at similar speeds. This comparison involved only the most rapidly elongating fibers (in one case only a single fiber), and measurements were taken a minimum of 8mm from the transection site. As a result, these landmark experiments address neither the onset of regeneration nor the speed at which the bulk of sensory and motor axons advance. We have taken the second approach by identifying the respective distances traveled by the longest 10% of each axon population (Figure 7). A potential weakness of this approach is the assumption that axons are added to both populations at the same rate. However, we have already demonstrated that both populations grow at similar rates (Figure 8). To eliminate the effect of the other potentially confounding factor, staggered regeneration, we have compared only the longest 10% axons in each group, automatically excluding axons that get a delayed start. This comparison revealed that Motor Only: motor axons and Dual: sensory axons regenerated significantly farther than Dual: motor axons ($p < .001$). Under these conditions, the longer distance traveled by Motor Only: motor axons and Dual: sensory axons must therefore represent a faster speed of axon regeneration. Motor axon regeneration is thus slowed significantly in Dual cultures, potentially by interaction with more rapidly-growing sensory axons.

Comparison of sensory and motor axon regeneration based on a single observation in other regeneration environments has produced results consistent with our findings. In organotypic culture, primary sensory neurons extended neurites that were consistently longer than those extended from spinal cord slices during the same time period (Allodi et al., 2011). After rat sciatic nerve repair, sensory regeneration dominated for the first two weeks as determined by retrograde labeling (Suzuki et al., 1998). Sensory regeneration was also dominant when regeneration was challenged by an unstructured gap (Madorsky et al., 1998) or a regenerative electrode (Madorsky et al., 1998; Negredo et al., 2004).

Inhibition involves a direct interaction between sensory and motor axons

A potential mechanism for the inhibition of motor regeneration by sensory axons is suggested by observation of early regeneration (Figure 9). Images taken 24 hours after nerve transection reveal that sensory axons have already grown substantially farther than motor axons. Furthermore, motor axons do not regenerate independently; throughout their length they adhere to the sensory axons that have preceded them. We extended these observations by examining each motor axon in the Dual: Motor group individually after 120 hours of regeneration and found that over 80% of motor axon length was directly adherent to sensory axons. This represents a complete reversal of the developmental relationship between sensory and motor axons; motor axons precede sensory axons into the limb bud (Landmesser, 1978; Wang et al., 2011) and are adept at choosing the correct pathways to

their muscle targets (Westerfield, 1988). Sensory axons, in contrast, rely on cues provided by the motor axons that precede them to reach appropriate destinations (Honig et al., 1986; Wang et al., 2011).

The experiments described above show that some aspect of the dual culture system inhibits motor axon regeneration. This is likely to involve heterotopic adhesion of sensory and motor axons, but could also be an unwanted consequence of adding a DRG to the culture. To confirm a pivotal role for inhibition of motor regeneration through direct contact with sensory axons on the hydrogel surface, we modified the dual culture model to delay the onset of sensory axon regeneration (Figure 3). During the second stage of culture preparation, transection of the femoral trunk and placing it on the collagen-laminin hydrogel, we also crushed the peripheral branch of the DRG. As a result, sensory axon growth onto the hydrogel was delayed for 2–3 days while the crushed sensory axons retraced their former pathway from the DRG through the femoral sensory branch and femoral trunk to the site of nerve transection. By the end of the 120 hour regeneration period, approximately half of the sensory axons had re-populated the femoral nerve and were at some stage of crossing the hydrogel (Figure 10). As a result of this delay, motor axons were able to regenerate on the hydrogel for at least 2–3 days without interference from sensory axons. The results of this maneuver were striking, in that regeneration of motor axons in the Dual, Sensory Delay: motor axons group closely resembled that in the Motor Only: motor axons group (Figure 11). In comparison with the Dual: motor axon group, significantly more motor axons reached the 0.5 mm, 0.75 mm, and 1.00 mm distances by 120 hours ($p < .05$). Motor inhibition in the dual cultures is thus the direct result of contact with sensory axons rather than the consequence of some other feature of the culture system.

The contributions of L1CAM and NCAM to sensory-motor adhesion

As shown above, motor axon regeneration after transection of mixed nerve is significantly improved by eliminating contact with sensory axons. Although delay of sensory regeneration is an effective means of accomplishing this *in vitro*, it is difficult to achieve *in vivo*. As an alternative, we have attempted to manipulate sensory-motor adhesion by down-regulating expression of the adhesion molecules L1CAM and NCAM by sensory axons.

L1CAM, a member of the immunoglobulin superfamily of cell adhesion molecules, is a likely contributor to sensory-motor adhesion. During peripheral nerve regeneration, axonal expression of L1CAM is limited to areas of contact with other axons (Martini, 1994; Martini and Schachner, 1988). It has been shown to promote fasciculation of regenerating peripheral axons (Martini, 1994), hippocampal neurons *in vitro* (Barry et al., 2010), sensory axons during development of the chick hindlimb (Honig et al., 2002), and thalamocortical and corticothalamic fibers in the developing mouse (Ohyama et al., 2004). L1CAM was initially shown to facilitate homophilic *trans*-binding of one axon to another (Lemmon et al., 1989). More recently, it has been found to stimulate regeneration through activation of the MAP kinase pathway in conjunction with the fibroblast growth factor receptor (FGFR; Huang et al., 2013; Schmid et al., 2000).

To explore L1CAM function the preparation of Dual cultures was modified by electroporating DRG neurons with siRNA to L1CAM at the time of initial DRG harvest

(Figure 4). Western blots performed one week after electroporation, at the time of nerve transection and hydrogel placement, confirmed elimination of L1 from the samples (Figure 5). Five days later, at the completion of the experiment, sensory axon regeneration was significantly reduced at distances from 0.5 mm to 1.25 mm (Figure 12). In spite of this reduction in sensory axon growth, however, motor axon regeneration was not improved. Impairment of sensory regeneration in this setting is unlikely to result from reduced axonal fasciculation per se. It could, however, be the consequence of reduced adhesion to the substrate or reduction in the MAPK signaling that results from homophilic interactions. Given that L1CAM has a minimal role in axon-substrate adhesion (Martini and Schachner, 1988), the latter seems most likely. Previous *in vivo* experiments have shown the consequences of L1CAM signaling to be highly context-dependent, as both knock-out of L1CAM expression (Guseva et al., 2009) and addition of function-triggering L1CAM antibodies to the regeneration environment (Guseva et al., 2014) have promoted mouse femoral nerve regeneration.

The failure to improve motor regeneration by impairing sensory regeneration suggests that the initial sensory-motor contacts in the first 0.25 mm of growth are sufficient to impair motor axon growth, suggesting signaling between sensory and motor axons rather than physical impairment through an adhesive mechanism. This hypothesis is consistent with the reduction in the number of motor axons regenerating to 0.25 mm in Dual cultures as compared with Motor Only cultures (Figure 8).

NCAM is also a member of the immunoglobulin superfamily of adhesion molecules. During peripheral axon regeneration it is expressed where axons contact fibroblasts and Schwann cells and where smaller axons contact each other or larger axons, but not where large axons contact one another (Martini and Schachner, 1988). NCAM was initially described as an adhesion molecule, and was found to promote side-to-side adhesion of post-natal DRG neurites to form fascicles (Rutishauser and Edelman, 1980). Subsequently, it was also found to signal through multiple context-dependent mechanisms involving kinases within lipid rafts and the FGF receptor to influence cell-cell and cell-substratum interactions (Maness and Schachner, 2007).

Assessment of the contributions of NCAM to axon regeneration is complicated by its role as the most common source of polysialic acid (PSA) in the regeneration environment. PSA bears a negative charge and swells when hydrated, increasing intercellular space and thus reducing cell-cell interactions such as the adhesion mediated by NCAM itself (Rutishauser et al., 1988). This role in attenuating axon fasciculation facilitates developmental pathfinding at plexus regions (Tang et al., 1994). During peripheral regeneration, PSA can have either positive or negative effects depending upon both its cellular location and level of expression (Jungnickel et al., 2009). Additionally, there is strong evidence that PSA expression is necessary to facilitate motor axon pathfinding during the process of preferential motor reinnervation (Franz et al., 2005, 2008).

In the current experiments, blocking expression of NCAM by sensory axons had no appreciable effect on the regeneration of either sensory or motor axons (Figure 13). Since the influence of sensory axons on motor regeneration is unchanged, the effect of removing

PSA displayed by NCAM (promoting fasciculation) must outweigh any reduction in axon-axon adhesion resulting from the removal of NCAM itself.

Conclusions

Only 10% of adults will regain normal or near-normal function after transection and repair of mixed nerves in the upper extremity (Brushart, 2011). Multiple factors contribute to this failure; since they interact with one another to differing degrees over time, the contributions of each are difficult to isolate and quantify. Given the complex interactions of sensory and motor axons during development, (Wang et al., 2011, 2013, 2014), we asked if similar interactions might influence the outcome of regeneration after nerve injury in post-natal animals.

The current experiments reveal that, at least under *in vitro* conditions, sensory axons interfere substantially with motor axon regeneration. Motor axons regenerate more slowly than sensory axons, and adhere to these axons throughout most of their length, providing ample opportunity for inhibitory interactions. Evidence obtained by excising rat dorsal root ganglia before nerve repair suggests that similar interference may occur *in vivo* (Redett et al., 2005), potentially leading motor axons into inappropriate afferent pathways. Development of minimally-invasive techniques to selectively inhibiting sensory axon regeneration *in vivo* should provide further insight into this possibility.

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Abbreviations:

DRG	Dorsal root ganglion
NCAM	Neural cell adhesion molecule
L1CAM	L1 cell adhesion molecule
YFP	Yellow fluorescent protein
PDMS	Poly(dimethylsiloxane)
RFP	Red fluorescent protein

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Highlights:

1. The interaction of regenerating, color-coded sensory and motor axons is studied in a new *in vitro* model of post-natal mammalian mixed nerve regeneration.
2. Sensory axons regenerate more rapidly than motor axons *in vitro*
3. Regenerating motor axons adhere to sensory axons throughout most of their course
4. Interaction with sensory axons inhibits motor axon regeneration
5. Delay of sensory axon regeneration restores normal motor regeneration
6. Knockout of NCAM and L1CAM in sensory neurons to reduce their adhesiveness fails to restore normal motor regeneration

Figure 1.

Steps in the construction of a Motor Only culture. Upper left: a custom PDMS frame is fabricated and bonded to a glass culture dish. Upper right: the Transwell[®] insert is modified by removing the plastic well, leaving only a ring of plastic to support the membrane. Lower left: the device is assembled by seating the plastic ring into the PDMS frame, which supports the membrane 1mm above the floor of the culture dish. The space within the PDMS enclosure beneath the membrane holds 1 cc of culture medium. Lower right: a spinal cord slice is placed on the membrane in contact with a segment of peripheral nerve that serves as a new ventral root. One week after the culture is set up, when green motor axons have re-populated the peripheral nerve, it is transected and the freshly cut end is placed on a collagen-laminin hydrogel.

Figure 2.

The configuration of Dual cultures. A reversed segment of P4-P5 wild-type femoral nerve is used to blend sensory and motor axons into a color-coded mixed nerve. The former muscle branch of the femoral nerve is opposed to the ventral exit zone of the spinal cord slice, and the former cutaneous branch is joined end-to-end with the peripheral nerve emanating from the DRG. The nerve is then reinnervated in a retrograde fashion so that green motor and red sensory axons intermingle in the former nerve trunk as shown on the right. When this nerve is transected, sensory and motor axons grow out on an unstructured surface in direct contact with one another.

Figure 3.

Modification of the dual culture model to delay sensory axon regeneration. One week after the culture is set up the femoral nerve trunk has been reinnervated by both sensory and motor axons and is transected and placed on the collagen/laminin hydrogel. At the same time, the femoral branch attached to the DRG is crushed to selectively degenerate sensory axons throughout the femoral graft. Motor axons are thus able to grow out from the transected nerve onto the hydrogel without being influenced by sensory axons for the first 2–3 days.

Figure 4. Modification of adhesion molecule expression with siRNA. At the time of initial culture set-up DRGs were injected with siRNA to either NCAM or L1, then electroporated to internalize the siRNA construct.

Figure 5.

Western blot of DRGs 1 week after injection of siRNA to NCAM (left) or L1 (right). Expression of NCAM is reduced substantially, and expression of L1 is eliminated.

Figure 6.

Representative cultures imaged after 120 hours of growth. In each instance 14 Z-stacks taken at 10× were combined in Photoshop®, composite images were manipulated to enhance brightness and contrast, and a scaled protractor was superimposed for measurements of axon growth. Top, Motor Only; middle, Dual: Motor; bottom, Dual: Sensory. The middle and bottom images are of the same culture during a single imaging session. Growth is vigorous in both Motor Only and Dual: Sensory cultures; there are more axons in the Dual: Sensory culture because there are many more sensory neurons in the DRG than there are motoneurons in a spinal cord slice. Motor axon growth is reduced substantially when sensory axons are present (Dual: Motor).

Figure 7.

Growth of motor and sensory axons in Motor Only and Dual cultures 120 hours after nerve transection (Figure 6). There are more parent DRG neurons than parent motoneurons in the culture system, so sensory axon counts will always be higher than motor axon counts. Axon counts at each distance from the nerve end are thus graphed as a percentage of the total number of axons regenerating, approximated by the axon count at 0.25 mm. It is impossible to count axons closer to the nerve end, as they are too closely packed to be resolved. Growth of motor axons in the Motor Only group is strikingly similar to the growth of sensory axons in the Dual group. However, the percentage of regenerating motor axons that reached 0.5 mm, 0.75 mm, 1.0 mm, and 1.25 mm is significantly lower ($p < .001$) when sensory and motor axons are regenerating together (Dual: Motor). Some feature of the dual culture system is thus inhibiting motor axon growth relative to growth in Motor Only cultures. The intersection of each curve with the horizontal lines defines the distance traveled by 50% (upper line) and the longest 10% (lower line) of regenerating axons in that population. Over a 120 hour period both 50% and the longest 10% of axons traveled significantly farther in the Motor Only group than in the Dual: Motor group $p < .0001$.

Figure 8.

Box plot of the absolute number of axons that reach the 0.25 mm protractor line after 24, 48, 72, 96, and 120 hours of regeneration. Horizontal lines represent the mean of each group, the boxes delimit 75% and 25%, and the ends of the vertical lines identify the maximum and minimum. There were significant differences among the three groups at each time period ($p < .001$). Among all groups, as time increased, so did the number of axons crossing the 0.25 mm line ($p < .001$). The onset of regeneration was thus staggered, with new axons joining the regenerating population at each time period.

Figure 9.

The relationship of red sensory and green motor axons in a dual culture imaged 24 hours after nerve transection. The cut nerve end is at the left margin, so regeneration is from left to right. Sensory axons have already outdistanced motor axons. Motor axons are uniformly adherent to the sensory axons that have preceded them onto the hydrogel surface.

Figure 10.

The absolute numbers of axons that cross each distance line 120 hours after transection of the donor nerve in Dual: Sensory and Dual: Sensory Delay groups. Even though the peripheral branch of the DRG was crushed at the time of nerve transection and hydrogel placement in the Dual: Sensory Delay group, substantial numbers of axons have regenerated from the crush site, through the femoral nerve, and onto the hydrogel in the ensuing 120 hours. Sensory regeneration has thus been delayed, but not prevented.

Figure 11.

Growth of motoneurons in Motor Only, Dual: Motor, and Dual, Sensory Delay: Motor groups. In the Dual, Sensory Delay: Motor group, sensory axon regeneration has been delayed by crushing the sensory branch of the DRG at the time of nerve transection and placement of the hydrogel. Motor axons have the opportunity to grow out on the hydrogel before, instead of after, sensory axons, and their growth is improved to a level that closely resembles the Motor Only group. At 120 hours, significantly more motor axons reached the 0.5 mm, 0.75 mm, and 1.00 mm distances ($p < .05$). Motor inhibition in the dual cultures is thus the direct result of contact with sensory axons rather than some other feature of the culture system.

Figure 12.

Regeneration of sensory and motor axons in Dual cultures after reduction of L1 expression by sensory axons. Motor axon regeneration is unchanged, but sensory axon regeneration is reduced significantly at all distances from 0.5 mm to 1.25 mm.

Figure 13.

Regeneration of sensory and motor axons in Dual cultures after reduction of NCAM expression by sensory axons. Neither sensory nor motor axon regeneration are changed significantly.