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MOLECULAR MECHANISMS UNDERLYING FUNCTIONAL REGENERATION FOLLOWING NERVE INJURY

by

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Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
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To my parents,

I love you both very much.

I know I don't say so very often, but I am grateful for all you have done and sacrificed for me. When others were doubtful, you always believed in me, and this has given me the confidence to pursue my dreams.
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ABSTRACT

Peripheral nerves have a robust capacity to regenerate following injury. Nevertheless, recovery of motor function after nerve injury is often sub-optimal due to the mistargeting of regenerating motor axons to incorrect muscles or skin. Consequently, therapies aimed at improving the accuracy of regenerating motor axons are required. Since the limits of mechanical nerve repair were reached decades ago, the next generation of therapies must arise from exploiting the molecular mechanisms underlying nerve regeneration. Therefore, the major goal of this thesis was to explore the roles of Neural Cell Adhesion Molecule (NCAM) and its Polysialic Acid (PSA) moiety in neuromuscular recovery after nerve injury. Previous studies had shown that these molecules were required for the appropriate targeting of embryonic motor axons to muscle, as well as the normal development and function of the neuromuscular junction (NMJ). Furthermore, PSA and NCAM are known to be up-regulated after nerve injury. I more closely examined PSA up-regulation after nerve injury and found that it was an intrinsic property of neurons because distinct motor and sensory neurons expressed it differently. To assess the functional consequence of this, I applied an established model of nerve regeneration where motor axons preferentially regenerate to muscle over skin despite having equal access to both. I discovered that the ability to preferentially regenerate to muscle requires PSA since its genetic or enzymatic ablation abolished this ability. Furthermore, I found that PSA levels and regeneration accuracy were both enhanced by brief electrical stimulation applied to cut motor axons. The benefit of electrical stimulation was directly related to increased PSA levels because it was completely blocked by PSA removal. Finally, I studied the role of NCAM in NMJ formation, maintenance and function following nerve injury. In the absence of NCAM, reinnervated muscle initially recovered normal function, but then experienced a dramatic loss of muscle strength. Immunohistochemical analysis of the muscle revealed a severe reduction in muscle area that was due to a selective loss of fast muscle fibers. In summary, PSA and NCAM are crucial for neuromuscular recovery after nerve injury and present promising targets for future molecular based nerve repair strategies.
**LIST OF ABBREVIATIONS USED**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>CAM</td>
<td>Cell adhesion molecule</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin-β</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyography</td>
</tr>
<tr>
<td>Endo-N</td>
<td>Endoneuraminidase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>Fas II</td>
<td>Fasciclin II</td>
</tr>
<tr>
<td>FN III</td>
<td>Fibronectin type III</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial derived neurotrophic factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycophosphatidylinositol</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>MAPK</td>
<td>MEK/mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural cell adhesion molecule</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal cutting temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PMR</td>
<td>Preferential motor reinnervation</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PSA</td>
<td>Polysialic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>PST</td>
<td>Polysialyltransferase ST8SiaIV</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFN</td>
<td>Society for neuroscience</td>
</tr>
<tr>
<td>STX</td>
<td>Polysialyltransferase ST8SiaII</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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</table>
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CHAPTER 1: Introduction

Neuromuscular Recovery Following Peripheral Nerve Injury

Overview

Nerve injuries are common and occur in a wide range of diseases, traumatic injuries, and mass lesions. In the central nervous system (CNS) injured axons have little capacity for spontaneous regeneration, but in the peripheral nervous system (PNS) injured axons are capable of robust regeneration. Despite this, recovery of function after peripheral nerve injury is often incomplete because regenerating neurons frequently fail to re-establish specific connections with their appropriate end organs (Sunderland, 1978b; Mackinnon and Dellon, 1988). Re-establishment of specific connections to appropriate end organs is essential for normal processing of sensory information as well as for effective performance of motor tasks (Duff, 2005). Therefore, it is widely accepted that improving axon regeneration accuracy will lead to better clinical outcomes.

Unfortunately, very little is known about the molecular mechanisms that promote axon guidance during regeneration. The main focus of this thesis is to improve our understanding of the molecular mechanisms that guide regenerating motor axons back to their appropriate muscles. A second, but related focus is to better understand how cell adhesion molecules promote the formation of stable synapses at the neuromuscular junction (NMJ). Ultimately, we hope that this information will contribute to the design of superior treatment strategies for managing peripheral nerve injuries.
Restoring Muscle Function

In order to achieve complete recovery of muscle function after peripheral nerve injury several conditions must be met: (1) axons must be able to regenerate all the way back to the denervated muscle fibers; (2) axons must selectively grow down the appropriate pathway leading back to its original muscle; (3) upon reaching its original muscle, axons must reestablish appropriately matched nerve-muscle connections; and (4) the muscle must have its pre-denervation physiological and structural properties restored (Kernell, 2006). Unfortunately, at least one of these conditions usually fails to occur after peripheral nerve injury. Consequently, full recovery of muscle function is rarely achieved.

Based on the strong propensity for PNS axons to regenerate (Ramon y Cajal, 1928), the first step to the recovery of muscle function should be achievable. However, in circumstances where motor axons have to regenerate over long distances to reach their muscle, such as in the case of a brachial plexus injury in humans, regenerating all the way back to distal muscles can be difficult (Gordon et al., 2003). This appears in large part due to the progressive decline in the ability for peripheral tissues to support axon regeneration after prolonged periods (i.e. > 8 weeks) of denervation (Fu and Gordon, 1995b; Sulaiman and Gordon, 2000). In contrast, the intrinsic capacity for a neuron to regenerate its axon after chronic axotomy appears to be relatively less compromised (Fu and Gordon, 1995a). In more severe nerve injuries there may be an accumulation of scar tissue in the nerve that can “trap” axons in their basal lamina sheaths and prevent them from regenerating (Mackinnon and Dellon, 1988). Associated with scarring are specific
molecules known to inhibit axon growth during regeneration such as myelin associated glycoprotein (Schafer et al., 1996; Mears et al., 2003) and chondroitin sulfate proteoglycans (Zuo et al., 1998b; Zuo et al., 1998a; Zuo et al., 2002; English, 2005). However, the expression of these growth inhibiting molecules can be successfully counteracted by the abundance of growth supporting molecules expressed after injury including ciliary neurotrophic factor (Sendtner et al., 1990; Sendtner et al., 1992), nerve growth factor (Heumann et al., 1987), brain derived neurotrophic factor (BDNF; Meyer et al., 1992; Kobayashi et al., 1996; Al-Majed et al., 2000a), glial cell line-derived neurotrophic factor (GDNF; Naveilhan et al., 1997; Burazin and Gundlach, 1998), Insulin-like growth factors (Hammarberg et al., 1998), NCAM (Daniloff et al., 1986; Martini and Schachner, 1988; Thornton et al., 2005), L1/NILE (Martini and Schachner, 1988), N-Cadherin (Shibuya et al., 1995), J1/tenascin (Martini et al., 1990; Fruttiger et al., 1995), and laminin (Hammarberg et al., 2000; Wallquist et al., 2002). Therefore, in circumstances where the distance to regenerate is not far, and the injury is managed in a timely fashion, virtually all axons can return to their muscle target (Lundborg, 2000; Hoke, 2006).

The ability for motor axons to attain the second and third conditions of functional muscle recovery can be a major challenge, especially when the continuity of the basal lamina surrounding individual axons is disrupted by the lesion (Sunderland, 1978b; Mackinnon and Dellon, 1988). If the basal lamina is not interrupted regenerating axons can be mechanically guided with remarkable precision not just to their original muscle, but specifically back to their original synaptic sites on individual muscle fibers (Nguyen et al., 2002). Following transection injury, the basal lamina is no longer continuous and
the extent of axon mistargeting becomes progressively worse as you increase the number of axons and/or target choices involved (Brushart, 1988; Madison et al., 1996). Also, depending on the type of targeting choice to be made motor axons can display varying degrees of specificity. For example, motor axons preferentially reinnervate muscle fibers even when they have equal access to cutaneous targets (Brushart, 1993; Madison et al., 2000). They also show a bias for returning to their original intramuscular region (Laskowski and Sanes, 1988; Wang et al., 2002; Wang and Kernell, 2002) and synapsing with their appropriate physiological class of muscle fiber (Soileau et al., 1987; Thompson et al., 1987; Nguyen et al., 2002). However, motor axons appear to be non-selective when choosing between their original muscle versus other muscles supplied by the same nerve (Brushart and Mesulam, 1980; Gillespie et al., 1986). It should be made clear that even in situations when selectivity is demonstrated by regenerating motor axons it is not completely free of mistargeting (Soileau et al., 1987; Brushart, 1993; Wang and Kernell, 2002). Overall, identifying molecular mechanisms of axon guidance during regeneration, which are at present poorly understood (Fu and Gordon, 1997; Madison et al., 2007), has tremendous implications for the development of new treatments for peripheral nerve injuries (Lundborg, 2000; Gordon et al., 2003).

The muscle is usually amenable to having its pre-denervation physiological and structural properties restored after reinnervation if the first 3 conditions are sufficiently accomplished (Kernell, 2006). Typically, 2-6 months after reinnervation most physiological (Gordon and Stein, 1982a, b; Badke et al., 1989; Desypris and Parry, 1990; Leterme and Tyc, 2004) and structural (Desypris and Parry, 1990; Fu and Gordon, 1995a; Bishop and Milton, 1997) properties of the muscle are restored. However, the ability of
the muscle to be completely restored is dramatically diminished with prolonged periods of muscle denervation (Sunderland, 1978b; Irintchev et al., 1990; Fu and Gordon, 1995b; Kobayashi et al., 1997).

**Motor Axon Targeting: Development versus Regeneration**

Unlike regenerating motor axons, developing motor neurons always innervate their appropriate muscles. In order to do so, embryonic motor neurons use a combination of instructive signals that guide them to their correct post-synaptic targets (Landmesser, 1994; Krull and Koblar, 2000). Developing axons grow towards gradients of chemoattractant molecules (Fig. 1.1A, Guide cell 1), and avoid gradients of chemorepulsive molecules (Fig. 1.1A, Guide cell 2), that diffuse from "guidance" cells along the growth trajectory. The surfaces of guide cells and target tissues also express attractive and repulsive molecules that either permit, or restrict, growth over their surfaces. Developing motor neurons forming a common nerve express cell surface molecules that promote adhesion, which in turn, cause their axons to selectively fasciculate together (Fig. 1.1B, right insert). Finally, growth cones temporally express de-adhesive molecules, such as polysialylated NCAM (see following sections), when they need to branch away from the main nerve trunk to form individual muscle nerves (Fig. 1.1B, green molecules in left insert; Rutishauser and Landmesser, 1996). Identified and/or putative long-range, diffusible guidance molecules used by developing motor neurons include BDNF (Song et al., 1997; Ming et al., 2002), GDNF (Haase et al., 2002), acetylcholine (Zheng et al., 1994), netrin (Colamarino and Tessier-Lavigne, 1995), and neuropilin-2 (Giger et al., 2000). Characterized cell surface guidance molecules used by
**Figure 1.1. Mechanisms of axon targeting.** A, Motor axons locate their correct postsynaptic targets by using chemoattractants (Guide cell 1) and chemorepellants (Guide cell 2) that diffuse from "guidance" cells along the growth trajectory. Surfaces of guide cells and target tissues also display attractant and repellant guidance molecules. B, Developing motor neurons forming a common nerve express cell surface molecules that promote adhesion, which in turn, causes their axons to selectively fasciculate together (right insert). De-adhesive molecules, such as polysialylated NCAM, are expressed on axons when they need to branch away from the main nerve trunk to form individual muscle nerves. Artwork by Monique Guilderson (Maritime Medical Design).
developing motor neurons include EphA4 (Helmbacher et al., 2000; Eberhart et al., 2002; Eberhart et al., 2004), BEN (Weiner et al., 2004), and polysialylated NCAM (Landmesser et al., 1990; Tang et al., 1992; Tang et al., 1994; Rafuse and Landmesser, 2000).

Many of the guidance molecules expressed by developing motor neurons are re-expressed by regenerating motor neurons after peripheral nerve injury (Daniloff et al., 1986; Martini and Schachner, 1988; Meyer et al., 1992; Kobayashi et al., 1996; Moscoso et al., 1998; Madison et al., 2000; Scarlato et al., 2003). The temporal and spatial re-expression of these guidance molecules suggests that they are, at the very least, strategically situated to influence motor axon guidance during regeneration. However, despite this correlative data, very few studies have attempted to directly test whether regenerating motor neurons use developmental guidance cues to selectively reinnervate their appropriate target. One explanation for this apparent oversight is the general misconception that regenerating motor axons reinnervate post-synaptic targets in a purely random fashion (Sunderland, 1978b; Mackinnon and Dellon, 1988). This strong conviction arises from studies showing that regenerating motor neurons often reinnervate inappropriate muscles when the nerve is transected proximal to its formation of individual muscle nerves (Brushart and Mesulam, 1980; Gillespie et al., 1986; Evans et al., 1991; English, 2005). Furthermore, muscle fiber-type grouping is a prominent feature of many nerve pathologies that result in reinnervation of denervated muscle fibers (Sunderland, 1978b). Fiber-type grouping occurs when fast and slow motor neurons do not reinnervate their appropriate class of fast and slow twitch muscle fibers (Edstrom and Kugelberg, 1968). However, despite these clear examples of axon mistargeting, it would be incorrect to conclude that motor neurons cannot preferentially reinnervate their
appropriate postsynaptic targets. In fact, there are several examples where selective motor axon regeneration likely occurs. Transected motor neurons often reinnervate complex, compartmentalized muscles in the same topographically correct manner as occurs during development (Laskowski and Sanes, 1988; Wang et al., 2002; Wang and Kernell, 2002). The absence of fiber-type grouping (Nemeth et al., 1993; Unguez et al., 1993; Rafuse and Gordon, 1996b, a) in reinnervated cat muscles strongly suggests that feline fast and slow motor neurons preferentially reinnervate their appropriate class of muscle fibers. Finally, regenerating femoral motor axons preferentially grow down distal motor pathways even when they have equal access to sensory pathways (Brushart, 1988, 1993; Al-Majed et al., 2000b, Krarup et al., 2002; Robinson and Madison, 2003). Taken together, these later studies suggest that guidance cues are expressed after nerve injury and that regenerating motor axons can use them to selectively reinnervate their correct post-synaptic targets. Therefore, identification of specific guidance molecules involved in the aforementioned examples of selective regeneration will be an important advancement towards developing strategies to manage peripheral nerve injuries.

**Preferential Motor Reinnervation: A Model for Selective Motor Axon Regeneration**

Developmental biologists have often applied simple, but extremely useful model systems to identify and study specific axon guidance molecules. To this end, Brushart and colleagues developed a femoral nerve regeneration model that reproducibly demonstrates that regenerating motor neurons selectively grow down distal motor pathways even though they have equal access to cutaneous pathways (Brushart, 1988; 1993; Al-Majed, 2000). In this injury model (Fig. 1.2), the femoral nerve is sectioned
Figure 1.2. Model of preferential motor reinnervation. A, Motor axons in the femoral nerve normally project to the quadriceps femoris muscle. B, One of 2 fluorescent dyes (fluororuby and fluoroemerald) was applied to the reinnervated muscle and cutaneous nerve branches after nerve injury in order to quantify the number of motor neurons extending axons into one or both branches. C, Motor neuron cell bodies retrogradely labelled with fluororuby (red cells) can easily be distinguished from those labelled with fluoroemerald (green cells). A cell backlabelled with both dyes appear yellow (arrow) in this overlay.
and repaired before it bifurcates into a separate cutaneous pathway that innervates skin and a muscle pathway that innervates the quadriceps femoris muscle group (Greene, 1935). The cutaneous and muscle branches are cut distal to the bifurcation three to six weeks later so that the motor neurons innervating each branch can be backlabelled with two different fluorescent dyes. The number of motor neurons reinnervating each branch is quantified by counting the backlabelled cell bodies within the spinal cord.

Permutations of this injury model have been used in rats (Brushart, 1988, 1993; Madison et al., 1996; Al-Majed et al., 2000b), mice (Mears et al., 2003; Robinson and Madison, 2003) and primates (Madison et al., 1999). In all cases, motor axons preferentially grow into the muscle pathway. Treatments such as conditioning lesions (Brushart et al., 1998) and brief electrical stimulation (Al-Majed et al., 2000b) have been shown to increase motor axons preference for the muscle pathway, whereas factors such as old age (Le et al., 2001) and a more proximal site of nerve transection (Madison et al., 1996) have been shown to reduce it. This model of selective motor neuron regeneration is commonly known as preferential motor reinnervation (PMR).

The Forms and Functions of the Neural Cell Adhesion Molecule

General Structure and Localization in the Nervous System

Cell adhesion molecules (CAMs) are abundantly expressed throughout the CNS and PNS on neurons, glial cells, as well as the surrounding extracellular matrix. It is widely accepted that CAMs play an essential role in both the assembly and maintenance of neural circuitry, and they include members of the cadherin, immunoglobulin, integrin and semaphorin superfamilies (for reviews see: Clegg et al., 2003; Rougon and Hobert, 2003;
Kruger et al., 2005; Takeichi, 2007). As their name suggests, CAMs have the ability to influence the extent to which adjacent cell membranes either adhere or de-adhere from each other. The first discovered and one of the most abundantly expressed CAMs in the nervous system is neural cell adhesion molecule (NCAM; Rutishauser et al., 1976; Thiery et al., 1977; Hoffinan et al., 1982).

NCAM is a member of the immunoglobulin superfamily and primarily exists in three isoforms that are generated through alternative splicing of a single gene (Goridis and Brunet, 1992). These three NCAM isoforms are named according to their molecular weight: NCAM 120, 140 and 180 kilodaltons (kDa). They share identical extracellular portions consisting of five Ig-like (Ig1-5) and two fibronectin type III (FN1-2) domains (Fig. 1.3A). The Ig domains are generally associated with homophilic binding to other NCAM molecules (Zhou et al., 1993), whereas the FN III domains can bind to fibroblast growth factor (FGF) receptor and is associated with activation of intracellular signaling pathways (Anderson et al., 2005; Kiselyov et al., 2005). Of the three major isoforms NCAM 140 and 180 kDa have cytoplasmic components, while NCAM 120 kDa is linked to membranes via glycophostidylinositol (GPI) (Fig. 1.3B; Panicker et al., 2003). The 140 kDa isoform has a shorter cytoplasmic component and is predominately found on growing axons and growth cones, whereas the 180 kDa isoform has a longer cytoplasmic component and tends to be found at synapses and other sites of close cell to cell contact (Dityatev et al., 2000). The GPI linked NCAM 120 kDa has been reported to be primarily found on glial cells (Noble et al., 1985) and developing muscle fibers (Rafuse and Landmesser, 1996).
Figure 1.3. Schematic representation of the structure and function of NCAM and polysialylated NCAM. A, NCAM protein has a N-terminal extracellular part with five immunoglobulin-like domains (Ig1-5) and two fibronectin type III repeats (FN1-2), a transmembrane domain and a C-terminal intracellular portion. The polysialic acid (PSA) chain is synthesized on an N-linked core carbohydrate attached to asparaginyl residues (Asn) located on the Ig5 domain (inset; glm, glucosamine; man, mannose; glc, galactose). B, Three main isoforms of NCAM characterized by different cytoplasmic domains (NCAM 180 and NCAM 140) or attachment to the membrane (NCAM 120) through a glycosylphosphatidylinositol anchor (GPI). C, PSA, a linear homopolymer of α 2-8-linked N-acetylneuraminic acid containing from about 8 to over 100 monomers, is synthesized by polysialyltransferases (PST and STX). Sialic acid is synthesized in the cytosol in four consecutive reactions (arrows) from UDP-N-acetylgalactosamine, the key enzyme being the UDP-N-acetylgalactosamine 2-epimerase/N-acetylmannosamine-kinase (GNE). Then monomers are polymerized to PSA in the Golgi by PST and/or STX. The PSA chains form a large molecule with steric properties due to a high density of negative charges (represented as grey ovals in D). D, The steric properties of PSA macromolecule hinder the apposition of cell membranes (middle) regulated both by NCAM and by different receptors (Rec). This effect can be exerted by trans interactions, when molecules belong to other cells (left), or cis interactions if they are placed on the same cell membrane (right). Adapted from Rutishauser (1998) and Bonfanti (2006).

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Post-Translational Addition of Polysialic Acid

It is becoming increasingly recognized that glycoproteins, which are a type of organic molecule whose composition includes both a carbohydrate and protein, play many critical roles in the development and function of the nervous system (Sampathkumar et al., 2006). Polysialylated NCAM is one of the most studied glycoproteins and is formed from the post-translational addition of polysialic acid (PSA) to the Ig5 extracellular domain of any of the three major NCAM isoforms (Fig. 1.3C; Crossin et al., 1984; Nelson et al., 1995). PSA is a linear homopolymer of alpha 2,8-linked neuraminic acid (commonly referred to as sialic acid) that possesses a slightly negative charge, and when added to NCAM, it exists with chain lengths that range from a few to more than 100 repeats (Livingston et al., 1988; Ashwell et al., 1994). This process is often referred to as polysialylation and is an essentially exclusive feature of NCAM as evidenced by the finding that the genetic deletion of NCAM results in an approximate 85% decrease in total PSA (Cremer et al., 1994). The remaining PSA in the NCAM null mice has primarily been attributed to the autopolsialylation of the sialyltransferases responsible for addition of PSA to NCAM (Muhlenhoff et al., 1996; Close and Colley, 1998), although there is evidence that PSA can be expressed in the absence of NCAM on the alpha subunit of sodium channels in rat olfactory bulbs (Zuber et al., 1992).

The polysialylation of NCAM is mediated by the activity of one of two sialyltransferases known as alpha-2,8-sialyltransferase II (STX) and IV (PST). STX is expressed highest during embryonic development but then diminishes postnatally, whereas PST levels remain relatively similar in the embryonic and adult brains (Kurosawa et al., 1997; Ong et al., 1998). Polysialylated NCAM is abundantly expressed
on neurons and glial cells during development. PSA is dramatically down-regulated in the adult nervous system post-natally, but remains high in specific regions of the brain associated with neural plasticity such as the dentate gyrus of the hippocampus, the hypothalamo-neurohypophysial and olfactory bulb systems (for review see: Durbec and Cremer, 2001). PSA is also up-regulated after nerve injury (Zhang et al., 1995). Therefore, the temporal and spatial pattern of PSA expression corresponds well with events related to the assembly, maintenance and repair of the nervous system.

**General Molecular Mechanisms**

NCAM is traditionally viewed as a regulator of both positive and negative adhesions between cells and their environment. It can act as a positive mediator of cellular adhesion through homophilic (i.e. NCAM to NCAM) and heterophilic binding with other immunoglobulin adhesion molecules such as L1 (Horstkorte et al., 1993) and Tag-1/Axonin-1 (Milev et al., 1996). When NCAM is polysialylated it acts as a negative mediator of cellular adhesion due to a combination of steric and electrostatic hindrances that interfere with its binding and consequently cause the intracellular distance between adjacent membranes to increase (Rutishauser et al., 1988; Yang et al., 1992). The relative abundance and widespread distribution of NCAM allows it to play a major role in regulating membrane biophysics of the nervous system. However, it has become evident that NCAM is involved in more than just regulating adhesiveness in the brain.

Beyond its capacity for trans interactions with other adhesion molecules on adjacent membranes (Fig. 1.3D), NCAM can also form linkages with neighbouring molecules on the same membrane in a cis manner (Fig. 1.3D) and is involved in the
activation of several intracellular signaling cascades (reviewed by Panicker et al., 2003). For instance, NCAM has been shown to dimerize with isoforms of the fibroblast growth factor receptor and promote neurite extension via the phospholipase-C gamma/diacylglycerol lipase/Ca^{2+}/protein kinase C pathway (Williams et al., 1994; Doherty and Walsh, 1996; Crossin and Krushel, 2000; Kiselyov et al., 2003). Homophilic cis and trans interactions involving the NCAM 140 isoform can lead to neurite outgrowth through the Ras/MAP kinase pathways (Beggs et al., 1997; Schmid et al., 1999). Also, it appears that NCAM 140 kDa dimerizes with GDNF Receptor Alpha 1 to form an alternative receptor for GDNF that promotes neurite extension, activation of Fyn and FAK, and reduction of cell adhesion (Paratcha et al., 2003). There are many other documented heterophilic binding partners for NCAM such as chondroitin/heparan sulfate proteoglycans including phosphacan (Grumet et al., 1993; Milev et al., 1994), neurocan (Friedlander et al., 1994; Retzler et al., 1996) and agrin (Cole et al., 1986; Lubec, 1986; Storms and Rutishauser, 1998), prion protein (Schmitt-Ulms et al., 2001), rabies virus (Thoulouze et al., 1998), and adenosine triphosphate (Dzhandzhugazyan and Bock, 1993, 1997; Skladchikova et al., 1999).

Roles in Neuromuscular Development and Regeneration

NCAM and PSA have critical roles in neuromuscular development and regeneration (Walsh and Doherty, 1996; Kiss et al., 2001). Classic experiments by Landmesser and colleagues showed that developing avian motor axons must express PSA in order to appropriately innervate their correct muscle targets (Landmesser et al., 1990; Tang et al., 1992; Tang et al., 1994; Rafuse and Landmesser, 2000; Hanson and
Landmesser, 2004). In these studies, Landmesser and colleagues took advantage of the fact that endoneuraminidase-N (Endo-N) selectively ablates PSA, but spares other sialic acid containing structures, because it only cleaves α2,8-linked sialic acid polymers with a minimum chain length of eight (Vimr et al., 1984). Consequently, when they injected Endo-N into the developing avian limb to enzymatically remove PSA from NCAM they found that motor axons formed overly fasciculated trajectories as they exited the spinal cord (Tang et al., 1994). These abnormal trajectories resulted in mistargeting of motor axons to inappropriate muscles (Tang et al., 1992). In a more recent study Landmesser and colleagues depressed PSA levels in motor axons by pharmacologically blocking normal patterns of spontaneous motor neuronal activity, which is initiated prior to muscle contact (Milner and Landmesser, 1999). Decreased PSA levels in these pharmacologically treated embryos also caused the over-fasciculation of motor axons and targeting errors (Hanson and Landmesser, 2004).

Whether PSA plays a similar role in regeneration is not known, however there are several lines of evidence to suggest that it does. First, PSA and NCAM were known to be highly expressed on both developing (Tosney et al., 1986; Martini and Schachner, 1988) and regenerating motor axons (Daniloff et al., 1986; Martini and Schachner, 1988; Zhang et al., 1995). Second, the formation of many motor axon sprouts over a wide field of arborization is important for axon targeting during regeneration (Brushart, 1993; Madison et al., 1999; Witzel et al., 2005), and PSA is known to regulate axon defasciculation (Tang et al., 1994; Hanson and Landmesser, 2004), arborization (El Maarouf and Rutishauser, 2003) and branching (Landmesser et al., 1990; Rafuse and Landmesser, 2000) during development. Finally, electrical stimulation of regenerating
motor axons enhances PMR (Al-Majed et al., 2000b) and motor recovery (Eberhardt et al., 2006). The expression of PSA on motor neurons during development requires motor neuron activity (Hanson and Landmesser, 2004).

Mice lacking NCAM (i.e. NCAM -/-; Cremer et al., 1994) have been invaluable for studying NCAM and/or PSA function during neuromuscular development and regeneration. For example, several aspects of the postnatal development of neuromuscular junctions (NMJs) in NCAM -/- mice are delayed including the assembly of post-synaptic junctional folds (Moscoso et al., 1998), the withdrawal of polyneuronal innervation (Rafuse et al., 2000), and the localization of synaptic vesicles to the synaptic terminals (Rafuse et al., 2000). In addition, NCAM -/- mice have permanently impaired neuromuscular transmission at high frequencies (Rafuse et al., 2000; Polo-Parada et al., 2001), which appears to be a consequence of their inability to down-regulate L-type calcium channels after development causing them to maintain an immature vesicle release mechanism (Polo-Parada et al., 2001). The genetic ablation, or small peptide interference, of the NCAM 180 isoform results in even more severe transmission deficits suggesting that NCAM 180 has a non-redundant role in sustaining transmitter release at high frequencies (Polo-Parada et al., 2004; Polo-Parada et al., 2005). Interestingly, while the established roles of NCAM at the mouse NMJ have been attributed to presynaptic deficiencies (Rafuse et al., 2000; Polo-Parada et al., 2001; Polo-Parada et al., 2005) its expression is abundant and highly localized to both the pre- and postsynaptic components of the NMJ (Covault and Sanes, 1986).

Prior to the generation of NCAM -/- mice, the role of NCAM in neuromuscular regeneration examined in studies using NCAM antibodies to perturb function. These
experiments provided functional (Remsen et al., 1990) and anatomical (Langenfeld-Oster et al., 1994) evidence of delayed endplate reinnervation in mice, incomplete myelination of axons, absence of many terminal Schwann cells capping the NMJ and ectopically located NMJs in frog (Rieger et al., 1988). However, the complete functional recovery of muscle requires at least 2-6 months after nerve injury (Gordon and Stein, 1982a, b; Badke et al., 1989; Desypris and Parry, 1990), which makes the use of NCAM antibodies impractical for studying long-term reinnervation processes (Remsen et al., 1990; Langenfeld-Oster et al., 1994). Therefore, NCAM −/− mice offer a better option for this issue, but the only study to examine neuromuscular reinnervation in NCAM −/− mice focused on recovery 1 week after a nerve crush injury. In contrast to the previous studies that used NCAM antibodies, Moscoso et al. (1988) did not did find any evidence of delayed endplate reinnervation or the absence of terminal Schwann cells capping reinnervated NMJs. However, this study was far from conclusive because many basic parameters of neuromuscular recovery weren’t assessed such as the number of motor neurons that reinnervated the muscle, gross morphology of muscle, re-establishment of muscle fiber contractile properties and muscle fiber type composition. Therefore, we decided to perform a detailed investigation into the role of NCAM in synapse formation, function and maintenance during neuromuscular recovery from peripheral nerve injury.

Summary of Objectives

The overall objective of this thesis is to better understand what role NCAM and/or PSA plays a role in motor axon targeting after a peripheral nerve injury. In addition, we
also felt that muscle reinnervation was a good model to study the role of NCAM in synapse formation, function and maintenance because the highly localized pre- and postsynaptic NCAM expression (Covault and Sanes, 1986) has yet to be completely explained (Rafuse et al., 2000; Polo-Parada et al., 2001; Polo-Parada et al., 2005). Thus, the specific objectives of this thesis were:

1. To characterize the re-expression of PSA after transection of muscle and cutaneous nerve branches of the femoral nerve.

2. To determine if PSA was necessary for the selective targeting of regenerating motor neurons after femoral nerve transection.

3. To assess the influence of PSA on motor axon sprouting after femoral nerve transection.

4. To determine whether intrinsic differences exist between different motor neuron pools after nerve injury.

5. To determine whether intrinsic differences between different motor neuron pools regulate selective axon targeting.

6. To determine if all regenerating motor axons must express PSA in order to preferentially reinnervate muscle.

7. To determine whether brief electrical stimulation improves regeneration accuracy because it increases PSA expression on regenerating axons.

8. To assess the influence of electrical stimulation on motor axon sprouting.

9. To determine the role of NCAM over the full time course of functional recovery of muscle after nerve crush.
10. To determine the role of NCAM in the recovery of normal muscle structural properties after nerve crush.
References


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CHAPTER 2: Polysialylated Neural Cell Adhesion Molecule is Necessary for the Selective Targeting of Regenerating Motor Neurons

Preface and Significance to the Thesis

It is well established that peripheral nerves regenerate following injury. Therefore, incomplete functional recovery usually results from misguided axons rather than a lack of regeneration per se. Despite this knowledge very little is known about the molecular mechanisms regulating axon guidance during regeneration. In the developing neuromuscular system, neural cell adhesion molecule (NCAM), and its polysialic acid (PSA) moiety, are essential for proper motor axon guidance. In this study we used a well-established model of nerve transection and repair to examine whether NCAM, and/or PSA, promote selective regeneration of femoral motor nerves in wild-type and NCAM null mice.

Preliminary results of this work have been presented at the Society for Neuroscience (SFN) Annual Meeting in New Orleans, Louisianna, USA, October 2003, and were published in Abstract form as Franz CF & Rafuse VF (2003, . SFN Annual Meeting, New Orleans, LA, 357.18.). This entire chapter was published in the “Journal of Neuroscience” as Franz CF, Rutishauser U, & Rafuse VF (2005, Polysialylated neural cell adhesion molecule (PSA-NCAM) is necessary for the selective targeting of regenerating motor neurons. Journal of Neuroscience, 25: 2081-2091). I would like to acknowledge substantial intellectual contributions from my supervisor Dr. Victor Rafuse
in the preparation of this manuscript. Dr. Urs Rutishauser provided many helpful comments on the manuscript and provided the Endo-N. I would like to thank Mr. David Fillmore for his technical assistance with cryostat sectioning and Mr. Stephen Whitefield for teaching me confocal microscopy. I would also like to recognize Monique Guilderson (Martime Medical Design) for her artwork.

Introduction

The degree of functional recovery attained after a peripheral nerve injury is, to a large extent, dependent on the number of lesioned axons that reinnervated their appropriate post-synaptic target(s) (Fu and Gordon, 1997). During development, it is well established that growing axons respond to a combination of both long- and short-range guidance cues that specifically direct them to their correct target(s) (Tessier-Lavigne and Goodman, 1996). Moreover, many of the guidance molecules and cellular mechanisms responsible for precise targeting of axons during development have been well characterized (Chisholm and Tessier-Lavigne, 1999). It is therefore rather surprising that very little is known about the molecular mechanisms regulating selective targeting of regenerating peripheral axons. One explanation for this apparent oversight is the general conception that regenerating axons do not selectively reinnervate their original targets after peripheral nerve injury (Sunderland, 1978a). However, there are several examples where selective axon regeneration likely occurs. For example, physiologically distinct classes of fast and slow motor neurons have a propensity to preferentially reinnervate their appropriate class of muscle fibers (Soileau et al., 1987;
Brown and Everett, 1990, 1991; Nemeth et al., 1993; Unguez et al., 1993; Rafuse and Gordon, 1996a; Wang et al., 2002). Similarly, transected motor neurons reinnervate the diaphragm in the same topographically correct manner as occurs during development (Laskowski and Sanes, 1988) suggesting that many of the same guidance cues expressed during embryogenesis are re-expressed after peripheral nerve injury.

Developmental biologists have often applied simple, but extremely useful model systems to identify specific axon guidance molecules. To this end, Brushart and colleagues developed a femoral nerve regeneration model that reproducibly demonstrates that regenerating motor neurons selectively grow down distal motor pathways even though they have equal access to sensory pathways (Brushart, 1988, 1993; Al-Majed et al., 2000b). This example of selective motor neuron regeneration is known as Preferential Motor Reinnervation (PMR).

The molecular mechanisms regulating PMR are not well understood. However, there are several lines of evidence to suggest that neural cell adhesion molecule (NCAM) and its polysialic acid (PSA) moiety are involved. PSA and NCAM are both re-expressed by regenerating motor axons after peripheral nerve injury (Zhang et al., 1995; Rutishauser and Landmesser, 1996). During development motor axons must express PSA in order to selectively innervate their appropriate muscle target(s) (Tang et al., 1992; Tang et al., 1994). Finally, the speed and accuracy of PMR is increased with brief electrical stimulation (Al-Majed et al., 2000b; Brushart et al., 2002), which suggests that the guidance molecules responsible for PMR can be regulated by neuronal activity. Neuronal expression of PSA is increased with cell activity (Kiss et al., 1994).
To determine whether PSA and NCAM regulate PMR we compared motor axon regeneration in wild-type (WT) and NCAM null (-/-) mice. We found that PMR was present 3 weeks after nerve transection in WT, but not NCAM -/- mice. PMR was also absent in WT mice when PSA was enzymatically removed from the regenerating nerve. Finally, transgenic mice were generated to show that axonal sprouting was restricted, and withdrawal of misprojecting axons more inhibited, in mice lacking PSA compared to WT mice. Taken together, these results indicate that PSA-dependent growth of motor axons is required for selective targeting of regenerating motor axons.

Methods

Mice

Five different strains of mice were used in this study. Wild-type (WT) C57BL/6 mice were obtained from Charles River Inc. (Wilmington, MA). In the majority of the experiments, the same strain of NCAM -/- mice, on a C57/B6 background, generated by Cremer et al. (1994) were used. To control for possible strain differences a second strain of NCAM -/- mice was obtained from The Jackson Laboratory (Bar Harbor, MA). This latter strain has been extensively backcrossed with C57BL/6 mice for at least 9 generations (Delling et al., 2002). A transgenic mouse line (mHb9-Gfp1b), in which enhanced green fluorescent protein (eGFP) is expressed under the control of the mouse Hb9 promoter (Wichterle et al., 2002: a kind gift from Dr. T.M. Jessell, Columbia University), was used to specifically visualize motor axons in WT mice. To visualize motor axons in NCAM -/- mice, mHb9-Gfp1b mice were bred with NCAM -/- mice to
generate NCAM-/- mice that express the \textit{mHb9-Gfp1b} gene. We designated these latter mice \textit{NCAM-/- Hb9-Gfp}. All strains of mice were housed and bred locally in the animal facilities at Dalhousie University.

Mouse genotyping was performed by polymerase chain reaction (PCR). The NCAM mutant allele was detected as a 336 base pair DNA fragment generated by PCR using a 5' primer that anneals to the NCAM sequence (5'-GCT CAT GTT CAA GAA TGC ACC) and a 3' primer in the \textit{neo} sequence (5'-ATC CAT CTT GTT CAA TGG CCG). The NCAM WT allele was detected as a 403 base pair DNA fragment using the 5' primer described above and a 3' primer in the NCAM sequence (5'-CCT CAG GTA TTA TGG TGT TGG). Amplification was: 95°C 1 min, 55°C 30 s, 72°C 1 min, 30 cycles of 94°C 30 s, 58°C 30 s, 72°C 30 s, and finished with 72°C 5 min. The eGFP allele was detected as a 650 base pair DNA fragment generated by PCR using a 5' primer (5'-GCT GTT CAC CGG GGT GGT GC) and a 3' primer (5'-TCC AGC AGG ACC ATG TGA TC). Amplification was: 94°C 5 min, 30 cycles of 94°C 30 s, 60°C 60 s, 72°C 60 s, and finished with 72°C 5 min.

\textbf{Nerve Transection and Repair}

All surgeries were performed on young adult (8-12 weeks) mice under aseptic operating conditions. Mice were anesthetized with isoflurane (Baxter International, Toronto, ON), a small incision was made in the skin to expose the femoral nerve, and 1 of 3 nerve transection and repair surgeries was performed (Fig. 2.1). For immunohistochemistry, the muscle and cutaneous pathways of the femoral nerve of C57BL/6 mice were sharply cut at points 3 mm distal to where they bifurcate from the
common nerve trunk (Fig. 2.1A). The proximal stumps were surgically joined to the distal stumps with 11-0 nylon sutures (Fine Science Tools, North Vancouver, BC). 2) For analysis of PMR, the femoral nerve was transected and repaired 2 mm proximal to where it bifurcates into separate muscle and cutaneous pathways. This transection site is distal to where the iliacus nerve separates from the main femoral nerve, but proximal to the divergence of the pectineus nerve (Fig. 2.1B). The femoral nerve was sharply cut and the proximal and distal nerve stumps were secured together with a single 11-0 nylon suture. Care was taken to ensure that the suture held the proximal and distal femoral nerve stumps tightly together so that their cut ends were closely apposed. Due to the very small diameter of the mouse femoral nerve (~300 μm), it was not possible to convincingly align the proximal and distal femoral nerve stumps with the same precision as described previously in the rat (Brushart, 1988, 1993; Al-Majed et al., 2000b). Thus, no attempt was made to do so. 3) To selectively remove PSA from the regenerating nerve in WT mice, 0.5 μl (8.7U/μl) of endoneuraminidase N (Endo-N) or 0.9% saline was injected into the femoral nerve 2 mm proximal to where it bifurcates into separate muscle and cutaneous pathways 30 minutes before the nerve was cut and sutured as described above. As shown previous, Endo-N injected into the chick hindlimb effectively removes PSA from developing nerve and muscle fibers for several days in vivo (Landmesser et al., 1990; Rafuse and Landmesser, 2000). For pain relief, all of the operated mice were administered ketoprofen (5mg/kg) after the surgeries.
**Immunohistochemistry**

To study the expression of PSA in regenerating muscle and cutaneous axons the muscle and cutaneous pathways of the femoral nerve were harvested from WT C57BL/6 mice 3, 8, and 15 days following transection and repair (surgery #1, described above). To study the re-expression of PSA on distal muscle targets in Endo-N and saline injected mice the quadriceps femoris muscle were dissected out of WT C57BL/6 mice 7 days after transection, injection, and repair (surgery #3, described above). Mice were deeply anesthetized with 300 μl of Somnotol (240 mg/ml) and perfused with 4% paraformaldehyde (PFA). Three millimeter segments of the muscle and cutaneous branches of the femoral nerve were dissected free from the surrounding tissue, post-fixed in 4% PFA overnight at 4°C, and cryoprotected in 30% sucrose-PBS overnight at 4°C. The harvested nerve segments contained axons located immediately proximal to the original site of transection (Fig. 2.1A). Muscles and nerves were frozen in OCT (Miles, Elkhart, IN) and 14 μm coronal sections were cut using a cryostat. Muscle and nerve sections were immunolabelled with 5A5 (a monoclonal IgM antibody that exclusively recognizes PSA; Dodd et al., 1988) (Developmental Studies Hybridoma Bank, Iowa City, IA). The muscle and nerve sections were incubated overnight at 4°C in the 5A5 primary antibody, washed several times in PBS, incubated for 90 minutes at room temperature in a rhodamine-conjugated IgM secondary antibody (1:500; Sigma), washed several times in PBS and then mounted in 50% glycerin-PBS containing 0.03 mg/ml p-phenylenediamine. Sections for comparison were photographed at the same time, and with identical exposures, using a Nikon CoolPix 4500 digital camera attached to an upright Leica DM/LFS fluorescent microscope.
The cross-sectional area of muscle fibers in WT and NCAM-/- mice was measured from quadriceps femoris muscles harvested from non-perfused animals. The muscles were quickly mounted on cork with OCT and frozen in isopentane that was cooled with dry ice. Fourteen μm sections were cut using a cryostat, photographed with a Nikon CoolPix digital camera and cross sectional areas measured using Scion Image software.

**Retrograde Labelling of Motor Neurons**

At the end of the regeneration period (i.e. 1.5, 3 and 6 weeks), the muscle and cutaneous branches of the femoral nerve in mice that received surgery #2 or #3 (see above) were isolated, cut and backlabelled with neurotracers to quantify the number of motor neurons innervating each branch. Under aseptic conditions, the mice were anesthetized with isoflurane and the femoral nerve exposed. A 10% solution of fluororuby (Molecular Probes, Eugene, OR), or fluoroemerald (Molecular Probes), was randomly applied to the muscle or cutaneous branch of the femoral nerve 5mm distal to the point of bifurcation (Fig. 2.1B). In each mouse, one branch was labelled with fluororuby while the other was labelled with fluoroemerald. Application of the neurotracers was performed by pre-soaking the dyes in a piece of Gelfoam (Upjohn, Don Mills, ON) that was subsequently placed at the end of a freshly cut nerve stump for 1 hr. To avoid cross contamination of dyes, temporary wells were created around the sensory and motor nerve branches with petroleum jelly and cotton. Before closing the wound, the gel foam and wells were removed and sterile saline was used to thoroughly wash out any dye not taken up by the axons. The mice were permitted to recover for 3 days to allow
sufficient time for the retrograde neurotracers to be transported back to the cell bodies.

The mice were then deeply anesthetized with Somnotol and perfused with 4% PFA. The lumbar region of the spinal cords were dissected free from the surrounding vertebrae, post-fixed in 4% PFA overnight at 4°C, and finally immersed in 30% sucrose-PBS overnight at 4°C for cryoprotection. Spinal cords were then frozen in OCT and sectioned at 40 μm with a cryostat.

**Motor Neuron Counting**

The total pool of labelled motor neurons was counted by a person who was unaware of which dye had been applied to the muscle and cutaneous pathways. Cells were only counted if their nucleus was present in the section and raw cell counts were corrected via the method described by Abercrombie (1946).

**Analysis of Regenerating Motor Axons**

Surgery #2 or #3 (see above) was performed on mHb9-Gfp1b and NCAM-/- Hb9-Gfp mice (see above). Three weeks after nerve transection the mice were deeply anesthetized with Somnotol and perfused with 4% PFA. A large segment of the femoral nerve, extending 3mm proximal to the repair site to 3 mm distal to the bifurcation point, was dissected out for analysis. The femoral nerve was post-fixed in 4% PFA overnight at 4°C, cryoprotected overnight in 30% sucrose-PBS at 4°C, and then frozen in OCT. Coronal sections (14 μm) were cut using a cryostat, mounted in 50% glycerin-PBS containing 0.03 mg/ml p-phenylenediamine and imaged via a laser-scanning confocal
microscope (Zeiss LSM 510; Zeiss, Thornwood, NY). Representative sections from a
region of the femoral nerve that was distal to the lesion but proximal to the pectineus
branch (Fig. 2.1B) were selected for quantitative analysis using Stereo Investigator
software (MicroBrightfield Inc., Williston, VT). To quantify the amount of collateral
sprouting the total number of eGFP positive axons in the representative sections was
counted. To quantitatively characterize the distribution of motor axons within the nerve a
50 μm² box was randomly applied to 30 sites in a single nerve cross-section. The number
of eGFP positive fibers within each of the 30 boxes was counted and used to calculate the
coefficient of variation (CV; standard deviation/mean X 100%). The CV allows the
comparison of variability between populations with different means. A low CV indicates
that the axons were distributed evenly across the cross-section while a high CV indicates
more segregation.

**Statistical Analysis**

Unpaired student t-tests were used to make comparisons between groups. Means
values are given with ± the standard error of the mean (SEM).
Figure 2.1. Diagrammatic representations of surgeries performed and examples of retrogradely labelled motor neurons. A, The femoral nerve bifurcates into a cutaneous nerve branch that innervates the skin of the lower leg and a muscle nerve branch that supplies the quadriceps femoris muscle. The expression of PSA on regenerating axons in each nerve was determined by cutting the branches 3 mm distal to the bifurcation point and suturing together the proximal and distal stumps. B, PMR was assessed by cutting and repairing the femoral nerve 2 mm proximal to the bifurcation point. Application of neurotracers fluororuby and fluoroemerald was used to quantify motor neurons that regenerated their axons 5 mm into the muscle and cutaneous branches of the cut and repaired femoral nerve. The cell bodies of motor neurons retrogradely labelled with fluororuby (C) can readily be distinguished from those labelled with fluoroemerald (D). E, Overlay of C and D.
Results

PSA Is Differentially Up-Regulated On Nerves Reinnervating Muscle and Cutaneous Pathways

Although it is well established that NCAM is up-regulated on axons and muscle fibers after peripheral nerve injuries (Covault and Sanes, 1985, 1986; Rieger et al., 1988), the extent to which PSA is expressed by regenerating peripheral axons is still, surprisingly, poorly understood. Furthermore, no study to date has distinguished between regenerating muscle and cutaneous neurons and thus it is not known whether these two distinct neuronal phenotypes differentially express PSA after injury. To address these questions we took advantage of the unique anatomical characteristics of the murine femoral nerve. Like the majority of peripheral nerves, the femoral nerve is composed of both motor and sensory axons. However, unlike most peripheral nerves, the femoral nerve ultimately segregates into distinct muscle and cutaneous pathways that are approximately the same size (Brushart and Seiler, 1987; Brushart, 1988; Figs. 2.1 & 2.4). The cutaneous branch only contains sensory neurons that innervate the skin while the muscle branch contains both motor neurons and muscle afferents that innervate the quadriceps femoris muscle. This distinct characteristic of the femoral nerve enabled us to first determine whether PSA is differentially expressed by regenerating muscle and cutaneous neurons and, second, to determine whether PSA regulates PMR.

To determine to what extent PSA is expressed by regenerating muscle and cutaneous neurons the muscle and cutaneous branches of the femoral nerve were transected and repaired 3 mm distal to their bifurcation (Fig. 2.1A). The animals were
Figure 2.2  Regenerating muscle and cutaneous branches of the femoral nerve differentially express PSA. A, Immunolabelling shows that the cutaneous pathway normally expresses PSA while the muscle pathway does in unoperated adult WT mice. B, PSA is up-regulated by both the muscle and cutaneous pathways 3 days (R3) after nerve transection and repair. However, PSA levels are noticeably higher in the muscle pathway at R3. C, The muscle pathway, but not the cutaneous pathway, continues to express high levels of PSA 8 days (R8) after nerve transection and repair. D, The intensity of PSA immunohistochemistry returns to control levels 15 days (R15) after surgery in both the motor and cutaneous pathways.
sacrificed 3 to 15 days post-transection and the two 3 mm nerve segments immediately proximal to the transection sites were harvested and processed for PSA immunohistochemistry. For comparison, the same regions of the muscle and cutaneous branches of the femoral nerve were also harvested from un-operated, control, adult mice. Figure 2.2A shows that PSA is expressed in the cutaneous, but not the muscle, pathway of the un-operated femoral nerve in adult WT mice. These results are in agreement with previous studies showing that, while PSA is down-regulated in motor neurons and muscle afferents after birth, it is continually expressed by cutaneous neurons that project to laminae I and II of the dorsal horn in adult rats (Boisseau et al., 1991; Bonfanti et al., 1992; Seki and Arai, 1993).

As expected, the expression of PSA in both the muscle and cutaneous pathways increased significantly from control levels 3 days after complete nerve transection and repair (Fig. 2.2B). Although it is impossible to determine the source of the PSA using light microscopy, previous studies using electron microscopy have shown that the vast majority of PSA re-expressed in regenerating nerves is neural in origin (Zhang et al., 1995). Consequently, the dramatic up-regulation of PSA in the muscle and cutaneous pathways is most likely due to the re-expression of PSA by regenerating axons and not to the synthesis of PSA by neighboring Schwann cells. Interestingly, the axons in the muscle pathway increased their expression of PSA to a much greater extent compared to axons in the cutaneous pathway. The PSA levels remained high on regenerating axons in the muscle pathway 8 days after nerve transection while it returned to near control levels on axons in the cutaneous pathway (Fig. 2.2C). Intriguingly, the level of PSA near the transection site returned to preoperative levels 15 days post-transection (Fig. 2.2D).
Because withdrawal of polyneuronal innervation in reinnervated muscles takes 1-2 weeks (Thompson, 1978; Gorio et al., 1983) after nerve-muscle contact these results indicate that the temporal expression of PSA along the axon corresponds better with the time it takes regenerating axons to cross a surgical repair site (Guttmann et al., 1942; Brushart et al., 2002) than the time it takes to restore mature synaptic endplates. Taken together, these results indicate that PSA is differentially up- and down-regulated by regenerating muscle and cutaneous neurons and that its down-regulation along the axon occurs subsequent to crossing the surgical repair site, but prior to the cessation of the reinnervation process.

**PMR Occurs in WT, But Not in NCAM -/- Mice**

The temporal and spatial expression of PSA suggests that it could play a role in regulating selective axon targeting. To directly test this possibility we adapted the well-established rat model of PMR (Brushart, 1988, 1993; Al-Majed et al., 2000b) to WT C57BL/6 mice and mice that lack both NCAM and PSA (i.e. NCAM -/-; Cremer et al., 1994). A schematic of the mouse model used is shown in Figure 2.1B. The mouse femoral nerve was transected 2 mm proximal to the bifurcation point where the nerve segregates into a muscle and cutaneous branch. The proximal end of the nerve was sutured directly to the distal stump with a single 11-0 monofilament suture. No attempts were made to correctly reorient the proximal and distal stumps during the suturing process. The animals were allowed to recover for 1.5, 3 or 6 weeks. Motor neurons correctly innervating the muscle pathway, or incorrectly innervating the cutaneous
Figure 2.3  PMR occurs in WT, but not NCAM -/- mice.  A, The mean number (± SEM) of retrogradely labelled motor neurons that regenerated into the appropriate muscle branch (filled bars), inappropriate cutaneous branch (gray bars), and both branches (open bars) 1.5, 3 and 6 weeks after femoral nerve transection and repair in WT and NCAM -/- mice (**, p<.01, Mu compared with corresponding Cu).  B and C, The same data as shown in A, except that the total number (T) of regenerating neurons is included for both WT (B) and NCAM -/- mice (C).  Mu, labelled axons in muscle branch; Cu, labelled axons in cutaneous branch; B, labelled axons innervating both branches.
pathway, were identified by backlabelling the axons with two fluorescent neurotracers 5 mm from the point of bifurcation (Fig. 2.1B; see Methods for details). To determine the number of femoral motor neurons in control C57BL/6, and NCAM -/- mice, preliminary experiments were performed whereby the femoral muscle branch in un-operated mice was cut and backlabelled with a single fluorescent tracer. Using this technique we determined that adult C57BL/6 mice have 173 ±10.3 (n=5) quadriceps femoris motor neurons, while adult NCAM -/- mice have 163 ± 4.7 (n=4). The numbers of quadriceps femoris motor neurons in the C57BL/6 mice and NCAM -/- mice were not significantly different from each other (p<0.05) and both agree well with previously published data (McHanwell and Biscoe, 1981; Mears et al., 2003; Robinson and Madison, 2003). To rule out the possibility that NCAM -/- mice have gross muscle abnormalities that could complicate the interpretation of our data we measured the cross sectional area of muscle fibers in quadriceps femoris muscles in WT and NCAM -/- mice. The mean (±SEM) muscle fiber cross sectional area in WT mice (1962 ±81.2 μm²) was not significantly different from NCAM -/- mice (1845 ±83.5 μm²) indicating that muscle fiber development was not notably impaired in mice lacking NCAM (Moscoso et al., 1998; Rafuse et al., 2000).

As shown in Figure 2.3, less than 40 cut femoral motor neurons regenerated to a point 7 mm from the site of surgical repair in both WT (Fig. 2.3B), and NCAM -/- (Fig. 2.3C) mice 1.5 weeks after nerve transection. Those that did regenerate 7 mm showed the same propensity to grow down the inappropriate cutaneous pathway as down the appropriate muscle pathway (i.e. no PMR evident) (Figs. 2.3A-C). A few motor neurons (<10) were double-labelled with both neurotracers indicating that they had extended
collateral axons 7 mm down both the muscle and cutaneous pathways (Figs. 2.3A-C). The number of motor neurons that regenerated 7 mm from the transection and repair site increased dramatically from 1.5 to 3 weeks post transection in both the WT (Fig. 2.3B) and NCAM -/- mice (Fig. 2.3C). As previously described in rats (Al-Majed et al., 2000b, Brushart et al., 2002), this progressive increase in number of backlabelled motor neurons over time indicates that mouse motor axons regenerate at different speeds. However, while staggered reinnervation in the WT mice was associated with progressively more motor neurons growing down the correct muscle pathway (Fig. 2.3A,B) there was no evidence of PMR in NCAM -/- mice (Fig. 2.3A,C). PMR was also not observed at 3 weeks in a different strain of NCAM -/- mice that was extensively backcrossed with C57/B6 mice (n=3, data not shown). Thus, PMR occurs in WT mice between 1.5 and 3 weeks post transection and its emergence appears to require NCAM and/or PSA because it is completely absent in NCAM -/- mice even after 6 weeks. Interestingly, there was only a small decline in the number of double-labelled motor neurons in WT mice between 1.5 to 6 weeks. Consequently, like the emergence of PMR in the rat, PMR in the mouse primarily occurs because motor axons either specifically grow into the appropriate muscle branch from the onset of regeneration or incorrect collaterals are withdrawn proximal to the distal site of retrograde tracer application.

Previous PMR studies in mice indicate that there is a tremendous amount of variability in the number of motor axons that regenerate beyond the lesion site. For example, Robinson and Madison (2003) showed that between 22 to 161% of the total number of the transected motor neurons regenerated to the dye application site in the
Figure 2.4  The distribution of quadriceps femoris motor axons in the femoral nerve is similar in WT and NCAM -/- mice. Left figure, Cartoon illustrating the basic anatomy of the murine femoral nerve and the site of the coronal sections illustrated to the right. A, Coronal sections through the muscle (arrow) and cutaneous branches (arrowhead) of the femoral nerve in WT mHb9-Gfp1b and NCAM -/-/Hb9-Gfp mice indicates that eGFP expressing axons are only located in the muscle branch. B, Coronal sections taken proximal to the divergence of the pectineus nerve shows that the quadriceps femoris motor axons are clustered together (circled by yellow dotted line) in both WT mHb9-Gfp1b and NCAM -/-/Hb9-Gfp mice. C, Quadriceps femoris motor axons are predominantly segregated in the ventral lateral (bottom right) quadrant of the femoral nerve in coronal sections taken between the divergence of the iliacus and obturator nerves in both WT mHb9-Gfp1b and NCAM -/-/mHb9-Gfp mice. D, The discrete grouping of quadriceps femoris motor axons is less distinct in coronal sections taken between the obturator nerve and the point where the spinal nerves join to form the femoral nerve. In all sections dorsal is up and medial is left.
distal nerve stump. Despite this variable degree of innervation, there was no correlation between the number of motor neurons that regenerated and the extent of PMR (calculated by dividing the number of motor neurons in the muscle branch by those in the sensory branch; Robinson and Madison, 2003). In the present study, the number of motor neurons that regenerated beyond the lesion site varied between 33 to 85% in WT mice. As observed previously, there was no correlation between the number of motor neurons that regenerated beyond the lesion site and the extent of PMR ($r^2=0.22$). The reason for the wide range in regenerated motor neurons is not known. However, it is likely due to variability in the number of axons that regenerated through the site of surgical repair and not because neurons died because the number of motor axons proximal to the transection site was very similar to that observed in control femoral nerves (data not shown).

The Absence of PMR in NCAM +/- Mice Is Not Due to an Abnormal Distribution of Femoral Motor Axons in Mice Lacking NCAM

Motor axons normally preferentially clustered together in major limb nerves before they exit as a coherent group at the level of their appropriate muscle in birds (Lance-Jones and Landmesser, 1981; Milner et al., 1998), amphibians (Wilson et al., 1988; Brown et al., 1989) and mammals (Browne, 1950; Brown and Booth, 1983). Whether the same degree of motor axon compartmentalization occurs in NCAM +/- mice is not known. It is possible that motor axons are not compartmentalized in NCAM +/- mice and that this initial lack of motor axon organization hinders the process of PMR. In order to examine the topographical organization of femoral motor axons in WT mice we used a transgenic mouse line ($mHb9-Gfp1b$) in which eGFP is expressed under the
control of the mouse Hb9 promoter (Wichterle et al., 2002). Hb9 is a homeodomain protein that is expressed by somatic motor neurons (Arber et al., 1999; Thaler et al., 1999) and thus eGFP expression can be used to identify motor axons in nerve coronal sections. To visualize motor axons in NCAM -/- mice we bred mHb9-Gfp1b mice with NCAM -/- mice to create eGFP expressing motor neurons in NCAM -/- mice (see Methods for details). We designated these latter mice NCAM -/- Hb9-Gfp. As shown in the coronal sections in Figure 2.4, eGFP expressing motor neurons are located, as expected, in the muscle (Fig. 2.4A, arrow), but not the cutaneous pathway (Fig. 2.4A; arrowhead) of the femoral motor nerve in both WT and NCAM -/- mice. The motor axons in the motor pathway innervate the quadriceps femoris muscle group. Coronal sections taken proximal to the bifurcation, but distal to the divergence of the iliacus nerve, indicate that the quadriceps femoris motor axons are clustered together within the femoral nerve as a segregated, coherent group (Fig. 2.4B; encircled by dotted lines) in both WT and NCAM -/- mice. The eGFP expressing axons in the small branch located outside the dotted lines presumably belong to motor neurons innervating the pectineus muscle. Quadriceps femoris motor axons remain clustered in the nerve proximal to the divergence of the iliacus as the majority of the fluorescent axons are still located in the ventro-lateral quadrant in both WT and NCAM -/- mice (Fig. 2.4C). The distinct clustering of quadriceps femoris motor axons only becomes inconspicuous immediately distal to where the spinal nerves join to form the femoral nerve (Fig. 2.4D). Taken together, these results indicate that quadriceps femoris motor axons preferentially clustered together in the femoral nerve long before they bifurcate to form a distinct motor pathway. Furthermore, quadriceps femoris motor axons in WT and NCAM -/- mice are
similarly compartmentalized and topographically located indicating that an initial lack of motor axon organization does not account for the absence of PMR in NCAM deficient mice.

**PMR Is Abolished After Removal of PSA from Regenerating WT Femoral Nerves**

NCAM -/- mice lack both NCAM and PSA (Cremer et al., 1994). Thus, the absence of PMR in NCAM -/- mice could be due to the loss of NCAM, PSA or both. To distinguish between these possibilities, we enzymatically removed PSA from regenerating nerve in WT mice by injecting 0.5 μl of Endo-N (8.7U/μl) into the lesion site of the femoral nerve 30 minutes prior to transection and surgical repair. To ascertain that PSA was completely removed from the regenerating femoral nerve during the onset of the regeneration process we first performed preliminary experiments whereby Endo-N injected femoral nerves were removed 2 (data not shown) and 7 days (Fig. 2.5B) post transection, and processed for PSA immunohistochemistry. Furthermore, to rule out the possibility that some Endo-N defused from the injection site and caused a systemic loss of PSA we dissected out the distal muscle target (i.e. the quadriceps femoris muscle) of the femoral nerve 7 days after the nerve was transected and injected with Endo-N or saline. As shown in Figure 2.5, the level of PSA was substantially less on a regenerating, Endo-N injected, femoral nerve (Fig. 2.5B) compared to a non-injected regenerating femoral nerve (Fig. 2.5A) 7 days after nerve transection and repair. In contrast, PSA was up-regulated in denervated muscle fibers in both saline (Fig. 2.5C; see also Olsen et al., 1995) and Endo-N (Fig. 2.5D) injected mice. In addition, the spatial distribution of PSA
Figure 2.5  PMR does not occur in WT mice when PSA is enzymatically removed during the onset of regeneration. A, PSA is expressed by regenerating axons in the femoral nerve of saline injected WT mice 7 days post-transection. B, In contrast, no PSA is expressed by regenerating axons injected with Endo-N at the time of the surgery 7 days previously. C and D, PSA expression in quadriceps muscle is similar 7 days following transection and repair of saline (C) and Endo-N (D) injected femoral nerves. E, The mean number (± SEM) of retrogradely labelled motor neurons that reinnervated the appropriate muscle branch (Mu; open bars), inappropriate cutaneous branch (Cu; gray bars), and both branches (B, cross-hatched bars) 3 weeks after femoral nerve transection and repair in WT mice injected with saline or Endo-N.
expression was very similar to that reported previously for NCAM in denervated rat muscles (Covault and Sanes, 1985). Thus, PSA can be selectively removed from the regenerating femoral nerve, at least during the first week post surgery.

As shown in Figure 2.5E, like non-injected WT mice (Fig. 2.3A), saline injected WT mice displayed PMR 3 weeks after transection and repair (p<0.05). However, like the NCAM -/- mice (Fig. 2.3A), regenerating motor neurons in the Endo-N injected mice did not show any preference to selectively reinnervate the distal muscle pathway. Consequently, the absence of PMR in the NCAM -/- mice is most likely attributable to the loss of PSA from the regenerating nerve and not to the absence of NCAM. Furthermore, these results also support the conclusion that PMR does not occur in NCAM -/- mice because they lack PSA and not because there are possible underlying strain differences.

**Impaired Sprouting of Axon Collaterals in NCAM -/- and Endo-N Treated Mice**

Lesioned peripheral axons generally extend several collateral sprouts that explore, and then grow along, Schwann cell basal lamina tubes in the distal nerve stump (Cajal, 1928). This generation of supernumerary collateral sprouts likely enhances regeneration accuracy because it increases the chances that at least one collateral will enter the appropriate distal basal lamina tube that will ultimately direct it to its correct target (Brushart, 1988; Madison et al., 1999). Enzymatic removal of PSA from developing motor neurons reduces axonal branching (sprouting) by inappropriately enhancing axon-axon adhesion (Landmesser et al., 1990; Tang et al., 1992; Tang et al., 1994; Rafuse and Landmesser, 2000). Based on these developmental studies, it seemed reasonable to
Figure 2.6  Motor axon sprouting is impaired in NCAM -/- mice and WT mice following enzymatic removal of PSA.  A, Coronal section through WT mHb9-Gfp1b femoral nerve between the surgical repair site and pectineus muscle nerve shows a large number of collateral sprouts that are evenly distributed throughout the nerve 3 weeks post-transection and repair.  B and C, The collateral sprouts appear fewer in number and less evenly distributed in cut femoral nerves from NCAM -/-/Hb9-Gfp mice (B) and WT mHb9-Gfp1b mice (C) treated with Endo-N.  D, The mean number (±SEM) of eGFP fibers in coronal sections of femoral nerve between the surgical repair site and the pectineus nerve branch in uncut mHb9-Gfp1b (WT; gray cross-hatched bars), uncut NCAM -/-/Hb9-Gfp (NCAM; cross-hatched bars), 3 weeks after transection and repair in WT mHb9-Gfp1b (WT 3wk; gray bar), NCAM -/-/Hb9-Gfp (NCAM 3wk; white bar), and WT mHb9-Gfp1b mice injected with Endo-N (EndoN 3wk; black bar).  E, The mean CV (±SEM) (see text for details) calculated from coronal sections of femoral nerves between the surgical repair site and the pectineus branch of uncut WT mHb9-Gfp1b (WT; gray cross-hatched bars), uncut NCAM -/-/Hb9-Gfp (NCAM; cross-hatched bars), 3 weeks after transection and repair in WT mHb9-Gfp1b (WT 3wk; gray bar), NCAM -/-/Hb9-Gfp (NCAM 3wk; white bar), and WT mHb9-Gfp1b mice injected with Endo-N (EndoN 3wk; black bar).  *, p<.05 (compared with WT 3wk).  **, p<.01 (compared with WT 3wk).
determine whether collateral sprouting is attenuated in mice lacking PSA. Consequently, the femoral nerves in WT mHb9-Gfp1b, NCAM -/-/mHb9-Gfp and Endo-N injected mHb9-Gfp1b mice, were transected and repaired as shown in Figure 2.1B. Three weeks later, segments between the transection site and bifurcation point were harvested and cut coronally. As expected from Cajal (1928), the number of motor fibers in the femoral nerve of WT mice increased dramatically after nerve transection and repair (Fig. 2.6D; compare Figs. 2.6A and 2.4B) indicating that extensive collateral sprouting had occurred. The numbers of motor fibers in the femoral nerves of NCAM -/- (compare Figs 2.6B and 2.4B), and Endo-N injected mice (compare Figs. 2.6C and 2.4B) were also higher than normal after nerve transection (Fig. 2.6D). However, compared to transected WT mice there were significantly fewer axons in mice lacking PSA (p<0.05).

The distributions of collateral sprouts in the transected nerves were also visibly different in WT mHb9-Gfp1b, NCAM -/-/mHb9-Gfp and Endo-N injected mHb9-Gfp1b mice (compare Figs. 2.6A-C). In WT mice, motor fiber collaterals were evenly distributed across the entire cross-section (Fig. 2.6A) while collateral sprouts in transected nerves lacking PSA appeared more segregated such that there were obvious areas without eGFP fibers (Figs. 2.6B,C). To quantitatively characterize the distribution of motor axons in an unbiased manner a 50 μm² box was randomly applied to 30 sites in a single nerve cross-section. The number of eGFP positive fibers within each box was counted. These numbers were then used to calculate a coefficient of variation (CV; standard deviation/mean X 100%), which allowed comparison of variability between populations with different means. The CVs were then used to quantitatively assess clustering of motor fibers. As shown in Figure 2.6E, the CVs were high (~120%) in both
WT and NCAM +/- mice. These high CV values reflect the highly clustered distribution of motor axons in the uncut femoral nerves (Fig. 2.4B). The mean CV in WT mice 3 weeks after transection and repair was substantially lower (~60%) than uncut WT and NCAM +/- mice. This low CV reflects the even distribution of collateral sprouts in WT animals (Fig. 2.6A). The CVs were significantly greater (p<0.01) in transected nerves from NCAM +/- and Endo-N injected mice (~90%) as compared to transected nerves in WT mice (Fig. 2.6E). However, these CVs were also significantly less (p<0.01) than uncut nerves in WT and NCAM +/- mice (Fig. 2.6E). Taken together, these results indicate that collaterals of regenerating motor axons do not become evenly distributed throughout the entire nerve in the absence of PSA, although they do become significantly less segregated than uncut axons.

Abnormal Persistence of Motor Axon Collaterals Distal to the Bifurcation Occurs in Mice Lacking PSA

In order for individual motor axons to respond to distant guidance cues, and branch from the main nerve trunk during development, they must decrease axon-axon adhesion by expressing PSA (Landmesser et al., 1990; Tang et al., 1992; Tang et al., 1994; Rutishauser and Landmesser, 1996). Studies in juvenile rats (Brushart, 1988, 1993) and non-human primates (Madison et al., 1999) suggest that PMR occurs, in part, because misguided regenerating femoral motor axons are "pruned" from inappropriate cutaneous pathways. Whether PSA is required for creating a level of de-adhesion necessary for axonal pruning is not known. Interestingly, our neurotracer studies showed only a small decline in the number of double-labelled motor neurons over time.
Figure 2.7 Abnormal persistence of motor axon collaterals distal to the bifurcation in NCAM -/- and Endo-N treated mice. A and B, Coronal section through WT mHb9-Gfp1b (A) and NCAM -/- Hb9-Gfp (B) femoral nerves approximately 3 mm distal to their bifurcation. The number of motor axon collaterals found in the muscle pathway (arrow) is not visibly greater than in the cutaneous pathway (arrowhead) in either A or B. C, Mean percentage (±SEM) of motor axon collaterals found distal to the bifurcation compared to the number of collaterals found immediately distal to surgical repair in WT mHb9-Gfp1b (WT 3wk; gray bar), NCAM -/-Hb9-Gfp (NCAM 3wk; white bar), and mHb9-Gfp1b mice injected with Endo-N (EndoN 3wk; black bar). *, p<.05 (compared to WT 3wk).
suggesting that selective pruning does not occur in WT mice (Fig. 2.3). However, it is possible that misguided mouse collaterals were selectively withdrawn prior to the distal site where the fluorescent tracers were injected (Fig. 2.1B). To examine this possibility we sectioned the muscle and cutaneous branches 3 mm distal to the bifurcation (i.e. 5 mm from the lesion site) in WT mHb9-Gfp1b, NCAM /-/-mHb9-Gfp and Endo-N injected mHb9-Gfp1b mice and counted the number of eGFP nerve fibers in single cross-sections. Quite surprisingly, the number of eGFP fibers in the muscle branch was not visibly greater than those in the cutaneous branch 3 weeks post-transection in both WT (Fig. 2.7A) and NCAM /-/- mice (Fig. 2.7B). However, when the number of eGFP fibers in the distal branches was expressed as a percentage of those counted in the nerve immediately distal to the transection site it became clear that the distal branches contained fewer (~60%) motor axons in WT mice (Fig. 2.7C). In contrast, the distal branches had about the same number (~100%) of eGFP fibers as the nerve immediately distal to the lesion site in NCAM /-/- and Endo-N injected mice (Fig. 2.7C). Combined with our backlabelling experiments (Fig. 2.3), these results suggest that PMR occurs in WT mice because misguided motor axons do not regenerate very far down, and/or they are selectively withdrawn from, inappropriate distal pathways. PMR does not occur in mice lacking PSA because misguided motor axons are not "pruned" and/or they continue to reinnervate inappropriate sensory pathways.

**Discussion**

Motor axons must express PSA in order to selectively innervate their correct muscle targets during chick neuromuscular development (Landmesser et al., 1990; Tang
et al., 1992; Tang et al., 1994; Rafuse and Landmesser, 2000). Although PSA is not essential for regeneration per se (Moscoso et al., 1998) our results indicate that PSA is required for selective targeting of motor neurons during peripheral nerve regeneration in the adult mouse. Furthermore, using transgenic mice to visualize motor axons, we serendipitously showed that motor axons destined to innervate specific muscle groups are highly clustered together in the main nerve truck well before they diverge to form individual muscle nerves. These results have important clinical implications in the development of surgical strategies designed to improve functional recovery after peripheral nerve injury.

PMR: Rat versus Mouse

Since it was originally described in juvenile (Brushart, 1988, 1993) and adult rats (Al-Majed et al., 2000b), studies have shown that cut femoral motor neurons preferentially reinnervate muscle targets in nonhuman primates (Madison et al., 1999; Krarup et al., 2002) indicating that selective regeneration of femoral motor axons is not a phenomena restricted to lower vertebrates. However, a recent study by Mears et al. (2003) indicated that PMR does not occur in C57BL/6 WT mice unless transection of the femoral nerve is followed by daily injections of antibodies to either myelin associated glycoprotein or HNK-1. These results clearly disagree with the present study where unambiguous PMR was reported in the same strain of C57BL/6 WT mice (Fig. 2.3). The reasons for this discrepancy are not obvious. However, there are differences between the two studies. Mears et al. (2003) transected the femoral nerve between the divergence of the iliacus and obturator nerves (TM Brushart; personal communication) while we cut the
nerve more distally between the divergence of the iliacus and pectineus nerves (see Fig. 2.4 for anatomy). Although gross anatomical differences cannot account for the discrepancy because the organization of motor axons at the two levels is very similar (Fig. 2.4), it has been reported that PMR in the rat is better when the transection and repair occurs at a site closer to the bifurcation (Madison et al., 1996). Consequently, it is possible that subtle molecular differences exist at these two sites that account for the conflicting mice results.

**PMR and PSA**

It is well established that, during the initial stages of peripheral nerve regeneration, each cut axon produces "dozens" of collaterals at the injury site (Ranson, 1912; Cajal, 1928). Studies in rodents indicate that cut femoral axons typically extend 5-10 collateral sprouts with fields of arborization spreading 50-100 \( \mu \)m ten days after nerve transection and repair (Brushart et al., 1998; Brushart TM, 2003). Although most sprouts presumably degenerate within a few days, 2-4 collaterals can persist in the distal nerve stump for 1-2 years (Shawe, 1955; Mackinnon et al., 1991; Nishimura et al., 1991).

The generation of supernumerary collateral sprouts is believed to enhance regeneration accuracy because it increases the chances that at least one sprout will reinnervate an appropriate basal lamina tube (Brushart, 1988, 1993; Madison et al., 1999) that will direct it back to its original muscle target (Sanes et al., 1978, Nguyen et al., 2002). Although it is not known whether PSA regulates sprouting of single motor axons, several lines of evidence suggest that it does. The numbers of collateral sprouts diverging from motor nerves are higher when PSA expression is experimentally
increased during neuromuscular development (Landmesser et al., 1990). Branching of individual retinal ganglion cells is reduced during development when PSA is enzymatically removed (El Maarouf and Rutishauser, 2003). Finally, we found that the distal stump near the lesion site contained 3 times more collateral sprouts than the proximal stump 3 weeks post transection and repair in WT mice. Mice lacking PSA had only 2 times as many axons (Fig. 2.6D). These results suggest that PSA promotes better PMR because it enhances the formation of supernumerary collateral sprouts.

The probability of contacting an appropriate basal lamina tube is presumably greater when the field of arborization is large because different sprouts from a single axon can sample many tubes distributed in various regions across the nerve cross-section. Using detailed morphometric analysis, El Maarouf and Rutishauser (2003) recently showed that the volume of arborization of individual developing retinal ganglion cells is reduced when PSA is enzymatically removed. Our CV data (Fig. 2.6E) suggests that collateral sprouts from cut motor axons extend farther and form larger fields of arborization if they express PSA. Thus, PSA may also promote PMR by enhancing the volume of arborization that, in turn, enables individual axons to sample many basal lamina tubes located at different sites throughout the nerve cross-section.

Indiscriminately sampling, and then reinnervating, multiple basal lamina tubes cannot in itself promote PMR unless misprojected collateral sprouts are selectively withdrawn from inappropriate pathways (Brushart, 1988, 1993). Indeed we found that there were significantly fewer eGFP motor fibers in the distal pathways compared to immediately after the lesion site in WT mice 3 weeks post-transection (Fig. 2.7C).
Figure 2.8  **Regeneration differs between WT mice and mice lacking PSA.** A single motor axon can form numerous sprouts between the proximal and distal stumps in WT (A) and mice lacking PSA (B). However, both the number and volume of arborization is less when PSA is absent. Furthermore, while pruning of motor axon collaterals occurs in WT mice, it is impeded in mice lacking PSA. Green fascicles indicate pre-existing muscle pathways while red fascicles indicate pre-existing cutaneous pathways. Artwork by Monique Guilderson (Maritime Medical Design).
indicates that collateral sprouts were either being withdrawn or they did not have sufficient time to reinnervate the distal branches in the first place. The latter seems unlikely because axons typically regenerate at a rate of 3 mm/d (Guttmann et al., 1942; Seddon et al., 1943; Brushart et al., 2002) and the number of backlabelled motor neurons in transected WT mice did not change between 3 and 6 weeks post-transection (Fig. 2.3). The visibly high number of collateral sprouts remaining in the cutaneous branch was initially quite puzzling. However, electrophysiological studies have shown that transected motor neurons remain viable for at least 2 years when they exclusively innervate skin (Nishimura et al., 1991). Thus, it seems likely that misprojected collaterals only withdraw if they belong to motor neurons that also extended at least one collateral down an appropriate muscle pathway. The number of motor fibers in the distal pathways was the same as the number of fibers immediately distal to the lesion site in mice lacking PSA (Fig. 2.7C). This indicates that withdrawal of misprojecting motor axons is impeded if PSA is not present. The small number of double labelled motor neurons in mice lacking PSA is presumably due to the fact that sprouting is restricted and thus the likelihood of collaterals reinnervating both muscle and cutaneous pathways is low (Fig. 2.8).

What impedes motor axons from withdrawing from inappropriate pathways when they lack PSA? At present it is not known. However, numerous cell adhesion molecules, such as laminins in the basal lamina and L1 in Schwann cells, are expressed in the distal nerve stump after peripheral nerve injury (Fu and Gordon, 1997). PSA is known to decrease these types of adhesive interactions (Tang et al., 1994; Fujimoto et al., 2001). Consequently, withdrawal of misprojected axons may be impeded in mice lacking PSA.
because the forward directed growth, mediated by cell adhesion molecules, outweighs the signals to withdraw.

**PMR: Permissive versus Instructive Guidance Molecules**

When PSA is removed during development, nerves become more fasciculated (Landmesser et al., 1990; Tang et al., 1992; Rafuse and Landmesser, 2000) and many motor axons misproject to inappropriate muscles (Landmesser et al., 1990; Tang et al., 1992). Because PSA attenuates cell-cell interactions (Acheson et al., 1991; Rutishauser and Landmesser, 1996) these results indicate that PSA regulates axon targeting by allowing discrete groups of developing motor axons to respond to specific, instructive guidance molecules.

Based on developmental studies it is logical to hypothesize that PSA also promotes PMR because it provides the necessary level of axon-axon de-adhesion that permits regenerating motor axons to respond to instructive guidance molecules expressed in the distal pathway and/or muscle fibers. The carbohydrate epitope HNK-1, is preferentially expressed by Schwann cells associated with the motor pathway (Martini et al., 1992) and thus it could act as a short-range axon guidance molecule instructing regenerating motor axons to innervate the correct basal lamina tubes at the lesion site (Mears et al., 2003). However, several lines of evidence suggest that the distal targets are also important in regulating PMR. First, the number of motor neurons that correctly reinnervate the muscle pathway is reduced when all end organ (i.e. muscle and skin) contact is prevented (Brushart, 1993). Second, femoral motor neurons preferentially reinnervate cutaneous pathways under conditions where skin contact is maintained, but
muscle contact is prevented (Robinson and Madison, 2004). Although it is not known why this occurs the authors suggest that motor neurons withdraw collaterals from distal targets containing low levels of trophic support while they simultaneously extend axons into pathways with relatively higher concentrations of trophic factors (e.g. Campernot, 1982). In this way the distal target providing the highest level of trophic support is preferentially reinnervated overtime. When the end organs are present, as in the current study, the muscle provides more trophic support than the cutaneous pathway and, as a result, the motor pathway is preferentially reinnervated. However, when muscle reinnervation is prevented, the cutaneous pathway provides relatively more trophic support and thus becomes preferentially reinnervated (Robinson and Madison, 2004).

Regardless of the instructive mechanisms underlying PMR, it is clear from the present study that regenerating motor neurons must express PSA in order to sprout supernumerary collaterals and mediate the withdrawal of inappropriate axons that project down cutaneous pathways. Thus, as occurs during development, instructive signals from distal targets cannot selectively guide regenerating motor axons unless PSA is present.
References


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CHAPTER 3: Intrinsic Neuronal Properties Control Selective
Targeting of Regenerating Motor Neurons: An Essential Role for
Polysialylated Neural Cell Adhesion Molecule

Preface and Significance to the Thesis

Despite advances in microsurgical techniques, recovery of motor function after peripheral nerve injury is often poor because regenerating axons reinnervate inappropriate targets. Consequently, surgical repair must evolve treatment strategies that improve motor axon targeting. The development of new treatments requires a better understanding of the molecular mechanisms governing selective motor axon targeting. In this study we used a well established model of nerve transection and repair to examine (1) whether intrinsic differences exist between different pools of motor neurons after peripheral nerve injury, (2) if such differences regulate selective axon targeting, (3) if regenerating motor axons must express polysialic acid (PSA) in order to preferentially reinnervate muscle, and (4) whether brief electrical stimulation improves regeneration accuracy because it increases PSA expression on regenerating axons.

Preliminary results of this work have been presented at the Society for Neuroscience (SFN) Annual Meeting in Washington, District of Columbia, USA, November 2005, and were published in Abstract form as Franz CF & Rafuse VF (2005, Re-expression of polysialylated neural cell adhesion molecule is necessary but not sufficient for the selective targeting of regenerating motor neurons. SFN Annual Meeting, Washington, DC, 29.12.). I would like to acknowledge substantial intellectual contributions from my supervisor Dr. Victor Rafuse in the preparation of this manuscript.
Dr. Urs Rutishauser provided the Endo-N, and Dr. Melitta Schachner the NCAM-floxed mice. I would like to thank Lindsay Fisher and Alexandra Nelson for their technical assistance with cryostat sectioning and genotyping mice. I would also like to recognize Monique Guilderson (Martime Medical Design) for her artwork.

**Introduction**

Unlike axons in the central nervous system, peripheral nerves are capable of robust regeneration following injury. Unfortunately, functional recovery is often poor because many regenerating axons are misdirected to inappropriate end-organs. In the case of motor axons, mistargeting contributes to varying degrees of muscle weakness, movement impairments, and physical deformity (Sunderland, 1978b). Even when peripheral nerves are repaired in a timely fashion using advanced microsurgery techniques, normal function is almost never restored (Mackinnon and Dellon, 1988). Consequently, surgical repair must eventually include treatment strategies that promote better motor axon targeting. In order to develop these new treatments the molecular mechanisms governing selective motor axon targeting must be determined.

The rodent femoral nerve is an invaluable model to explore the mechanisms of axon targeting during regeneration. Originally described in the rat (Brushart, 1988), transected femoral motor neurons preferentially reinnervate the muscle branch of the femoral nerve even though they have equal access to the cutaneous branch. This example of selective motor neuron regeneration was termed preferential motor reinnervation (PMR). Subsequent studies examining the mechanisms underlying PMR have primarily focused on guidance factors expressed by the nerve branches and/or end organs.
(Brushart, 1993; Martini et al., 1994; Robinson and Madison, 2004, 2005; Hoke et al., 2006; Madison et al., 2007). However, intrinsic properties unique to distinct groups of motor neurons cannot be overlooked as they will likely determine if the regenerating axons can respond to the appropriate guidance cues.

One intrinsic property unique to distinct subtypes of developing motor neurons is the expression of polysialic acid (PSA; Landmesser et al., 1988; Allan and Greer, 1998; Soundararajan et al., 2006). PSA is expressed by limb innervating motor neurons in the developing chick (Landmesser et al., 1988; Tang et al., 1992) and its expression is essential for proper motor axon guidance during limb innervation (Tang et al., 1992; Tang et al., 1994). PSA is down-regulated on developing motor neurons after muscle innervation, but is dramatically up-regulated on many motor axons after nerve injury including those in the femoral nerve (Franz et al., 2005). The functional importance of this re-expression was recently demonstrated when it was shown that regenerating mouse femoral motor axons must up-regulate PSA in order to exhibit PMR (Franz et al., 2005). Motor neurons not expressing PSA during embryogenesis may not re-express it after nerve injury because many intrinsic neuronal properties expressed in the adult are established during development (Fawcett, 1992; Goldberg, 2004). If true, these results would influence our understanding of selective motor axon regeneration and consequently the development of treatment strategies designed to promote better functional recovery after peripheral nerve injury.

In the present study we show that distinct pools of motor neurons differentially express PSA during development and after nerve injury. Furthermore, we show that motor neurons expressing high PSA levels exhibit PMR while PSA negative motor
neurons do not. Finally, brief electrical stimulation, a process known to enhance PMR of femoral motor neurons (Al-Majed et al., 2000b), increases regeneration accuracy because it up-regulates PSA on regenerating axons. Taken together, these results indicate that phenotypes established during development can determine whether regenerating motor neurons selectively reinnervate their appropriate target after injury.

Methods

Animals

Embryonic day 10 (E10) wild-type (WT) C57BL/6 mice (Charles River, Wilmington, MA) and Stage 25 (Hamburger and Hamilton, 1951) chick embryos were used to study PSA expression patterns during embryonic development. The mice were bred and housed at Dalhousie University and White Leghorn chicken eggs (Cox Bros, Truro, NS) were maintained at 38°C in a humidified incubator. Nerve regeneration experiments were performed on 4 different strains of mice. WT C57BL/6 mice (Charles River) and NCAM null (-/-) mice (Cremer et al., 1994) were bred locally and housed at Dalhousie University. A transgenic mouse line (mHb9-Gfp1b), that expresses enhanced green fluorescent protein (eGFP) under the control of an Hb9 promoter (a kind gift from Dr. T. M. Jessell, Columbia University, New York; Wichterle et al., 2002), was used to visualize and quantify regenerating motor axons in WT and enzymatically treated adult mice (Franz et al., 2005). A fourth strain, which lacks NCAM and PSA expression in motor neurons, was generated by first crossing NCAM-floxed (NCAM^{lox/lox}) mice (Bukalo et al., 2004) with transgenic mice expressing cre-recombinase under the control of the Hb9 promoter (Yang et al., 2001). The progeny of this cross were all heterozygous for the NCAM-floxed allele, while 50% expressed the Hb9 Cre transgene.
(NCAM^{lox^+/Hb9^{cre^+}}). The NCAM^{lox^+/Hb9^{cre^+}} mice were subsequently bred with NCAM^{lox/lox} mice in order to generate NCAM^{lox/lox/Hb9^{cre^+}} mice that selectively lack NCAM and PSA expression in motor neurons.

The NCAM^{+/} and mHb9-Gfp1b mice were genotyped as previously described (Franz et al., 2005). A 5' primer (5'-GTT ATT CGT ATC ATC AGC TAC ACC) and 3' primer (5'-GTC CAA TTT ACT GAC CGT ACA CC) were used to genotype the Hb9 cre-recombinase transgene. Amplification was: 94°C 1 min, 35 cycles of 94°C 0.5 min, 60°C 1 min, 72°C 1 min, and finished with 72°C 10 min. The NCAM^{lox/lox} mutant allele was genotyped as described previously (Bukalo et al., 2004).

All procedures were conducted in accordance to the guidelines of the Canadian Council on Animal Care and the policies of Dalhousie University.

**Nerve Transection and Repair**

Surgeries were performed on young adult mice (8-12 weeks) under aseptic conditions. Animals were anesthetized with isoflurane (Baxter, Toronto, ON, Canada), a small incision was made in the skin overlying the ventral thigh in order to expose the femoral, obturator and/or genitofemoral nerves after which 1 of 3 surgeries was performed. (1) The femoral nerve was cut midway between the divergence points of the iliacus and pectineus nerve branches (Fig. 3.1A). The proximal end of the cut femoral nerve was then sutured back together with its distal stump using a single 11-0 nylon suture (Fine Science Tools, North Vancouver, BC, Canada). As previously described, the very small size of the mouse femoral nerve (~300 µm) precluded any attempt to anatomically re-align the nerve fascicles (Franz et al., 2005). The obturator nerve was
also cut and ligated as a control for the cross-reinnervation surgeries (see below). (2) The obturator nerve, which is located immediately adjacent to the femoral nerve (Fig. 3.1A), was cut proximal to its division into anterior and posterior branches. The nerve was subsequently sutured to the distal stump of the femoral nerve, which was cut midway between the divergence of the iliacus and pectineus nerve branches (Fig. 3.1B). A tight ligature was tied around the proximal end of the cut femoral nerve to prevent regeneration of femoral axons (Fig. 3.1B). (3) The genitofemoral nerve was cut proximal to its lumbo-inguinal and external spermatic nerve branch points, and then sutured to the distal stump of the femoral nerve that was cut as described above. When transected at this level the proximal end of the genitofemoral can be deflected to the femoral nerve without causing axonal stress. A tight ligature was tied around the proximal end of the cut femoral nerve to prevent regeneration of femoral axons.

**Electrical Stimulation of Nerves**

In some noted experiments, the surgeries were modified such that the proximal end of the cut nerve (femoral, obturator, or genitofemoral) was stimulated at 20 Hz for 1 hr (100 μs pulse duration; 20-60 μA stimulus current) using a fine-tipped polyethylene suction electrode (PE-190; Clay Adams, Sparks, MD) that was manually pulled with a small flame (Fig. 3.1E). A S88 stimulator (Grass Technologies, West Warwick, RI) was used to generate balanced biphasic stimuli, which were isolated from ground via two photoelectric stimulus isolation units (PSIU6; Grass Technologies), according to the manufacturer's directions. The amplitude of the current was set at 2 times the threshold required to elicit a muscle twitch upon stimulation of the distal nerve stump immediately
after nerve transection. The stimulated nerve was subsequently connected to the distal femoral nerve stump with a single 11-0 suture as described above. Non-stimulated controls in this experimental group were prepared in the same manner except the stimuli were not applied.

**Endoneuraminidase-N (Endo-N) Treatment**

The femoral nerve was cut and electrically stimulated and repaired as described above. In addition, immediately after suturing, 0.5 μl of Endo-N (8.7U/μl) or 0.9% saline was injected into the lesion site. Endo-N specifically cleaves α2,8-linked sialic acid polymers with a minimum chain length of eight (Vimr et al., 1984). Endo-N completely removes PSA from regenerating axons during the period of peak expression which last for 1 week after injury (Franz et al., 2005).

**Immunohistochemistry and Quantitative Optical Analysis**

E10 mice and Stage 25 chick embryos were harvested and fixed with 4% paraformaldehyde in phosphate buffered saline (PFA-PBS) solution overnight at 4°C. Embryos were cryoprotected with 30% sucrose/PBS, frozen in OCT compound, sectioned coronally at 40 μm with a cryostat, and dried overnight at 37°C. Slides with sectioned embryos were incubated overnight at 4°C with an anti-PSA monoclonal IgM (1:10 000; 5A5 ascites fluid; Dodd et al., 1988) and an anti-neuronal class III β-Tubulin (1:1000; MMS-435P; Covance, Berkeley, CA) antibodies, washed several times in PBS, incubated for 60 minutes with rhodamine-conjugated Goat anti-Mouse IgM (1:500,
M7019, Sigma, Oakville, ON, Canada) and Alexa Fluor 488 Goat anti-Mouse IgG
(1:500; A11001; Invitrogen, Burlington, Ontario, Canada) secondary antibodies, washed
several times in PBS, and finally mounted in 50% glycerol/PBS mixture containing 0.03
mg/ml ρ-phenylenediamine to prevent fading.

To examine PSA on regenerating axons we perfused the deeply anesthetized
animals with 4% PFA-PBS and removed a 3 mm nerve segment immediately distal to the
lesion site. Nerves were fixed further in 4% PFA-PBS overnight, cryoprotected in 30%
sucrose/PBS, quickly frozen in OCT compound, sectioned coronally at 14 μm with a
cryostat, and dried overnight at 37°C on a slide warmer. Care was taken to ensure that
the nerve was oriented in the same direction and that each section was the same
thickness. The slides were then incubated for 12 hours at 4°C with an anti-PSA
monoclonal IgM antibody (1:50 000; 5A5 ascites fluid; Dodd et al., 1988), washed
several times in PBS, incubated for 60 minutes with rhodamine-conjugated Goat anti-
Mouse IgM secondary antibody (1:500; M7019; Sigma), washed several times in PBS,
and finally mounted in 50% glycerol/PBS mixture containing 0.03 mg/ml ρ-
phenylenediamine. This concentration PSA antibody did not saturate its binding sites
and thus could be used to quantitatively compare PSA expression levels in different
animals. The sections for quantitative comparison were immunolabelled at the same time
and photographed with a digital camera (C4742; Hamamatsu Photonics, Hamamatsu,
Japan) using IPLab acquisition software (Version 4.0; BD Biosciences, Rockville, MD).
All exposure times and adjustments for the captured images were identical for each nerve
analyzed. Pixel intensity was recorded within a linear region of interest that extended
across the entire length of each representative nerve section using IPLab software. The raw data was exported to a spreadsheet for numerical analysis.

Retrograde Labelling of Motor Neurons

At the end of the 6 week regeneration period, a second operation was performed to expose the muscle and cutaneous branches of the femoral nerve. The two branches were cut and backlabelled with neurotracers to quantify the number of motor neurons innervating each branch (Fig. 3.2D) according to the methods described by Franz et al. (2005). Briefly, solutions of 10% fluororuby (D-1817, Invitrogen) and fluoroemerald (D-1820, Invitrogen) were randomly applied to the cut ends of the muscle or cutaneous branches of the femoral nerve for 1 hr. To prevent cross-contamination of the dyes temporary wells were created around each branch using sterile cotton. Sterile saline was generously applied to the cut nerves to thoroughly wash out any residual dye not taken up by the severed axons. The mice were allowed to recover for 5 days following application of the dyes to ensure optimal backlabelling of the motor neurons. The animals were then deeply anesthetized and perfused with 4% PFA-PBS. The lumbar region of the spinal cord was postfixed in 4% PFA-PBS overnight at 4°C, and then immersed in 30% sucrose/PBS for cryoprotection. Spinal cords were isolated from the surrounding vertebrae, frozen in OCT compound, and sectioned longitudinally at 60 µm with a cryostat.
Motor Neuron Counting

The entire pool of retrogradely labelled motor neurons was counted by a person unaware of which dye had been applied to the muscle or cutaneous nerve branch. Fluorescent cells were counted only if their nuclei and a primary dendrite was clearly resolved. Raw cell counts were corrected by the Abercrombie method (Abercrombie, 1946).

Anatomical Analysis of Regenerating Motor Axons

Regenerating motor axon dynamics were quantitatively compared in mHb9-Gfp1b mice that were electrically stimulated and injected with Endo-N or saline (Modified Surgery #1; see above). A control group of mHb9-Gfp1b mice received a saline injection after standard nerve repair without electrical stimulation. After 3 weeks the mice were deeply anesthetized and perfused with 4% PFA-PBS. The femoral nerve was freed from surrounding tissue, postfixed overnight in 4% PFA-PBS, and cryoprotected in 30% sucrose/PBS, frozen in OCT compound, cut coronally at 14 μm with a cryostat and mounted on slides that were dried overnight at 37°C. In order to enhance the eGFP signal the nerve sections were incubated overnight at 4°C with rabbit GFP antibody (1:1000; AB3080P; Chemicon, Temecula, CA), washed in PBS, incubated for 60 minutes with FITC-conjugated goat anti-rabbit IgG secondary antibody (1:500; F-0382, Sigma), washed several times in PBS, and coverslipped in 50% glycerol/PBS containing 0.03 mg/ml ρ-phenylenediamine. Nerve sections were photographed with digital camera (Hamamatsu) using IPLab acquisition software. Collateral sprouting was quantified by counting the number of eGFP positive axons distal to the lesion site using
ImageJ software (Version 1.36b, National Institute of Health, USA; http://rsb.info.nih.gov/ij). The lengths of lateral axonal deflections made by motor axons crossing the injury site were measured using Axiovision LE software (V4.3.0.101; Carl Zeiss; http://www.zeiss.de/en).

Statistical Analysis

Mean values are shown ± Standard Error of the Mean (SEM) throughout. A one-way ANOVA was used to make comparisons between the average peak pixel intensities of PSA immunostaining of motor nerves, number of eGFP positive fibers distal to the lesion and the length of lateral axonal deflections at the lesion site. Tukey’s Honestly Significant Difference (HSD) Post Hoc test was used to determine where the significant differences occurred if the F-value exceeded F-critical. Unpaired student’s t-tests were used to make comparisons between motor neuron numbers.
Figure 3.1  Diagrammatic representation of the femoral, obturator and genitofemoral nerves, cross-reinnervation surgery and an example of the retrogradely labelled motor neurons.  A, Lumbar spinal nerves L2-4 supply the femoral, obturator and genitofemoral nerves. The femoral nerve eventually bifurcates into a muscle branch that innervates the quadriceps femoris muscle and a cutaneous (saphenous) branch that innervates skin. The obturator and genitofemoral nerves are located medial to the femoral nerve and both contain neurons innervating skin and muscle.  B, The obturator (depicted) or genitofemoral nerve (not shown) was cut and cross sutured onto the distal stump of the femoral nerve that was cut midway between the iliacus and pectineus nerve branches. The proximal end of the cut femoral nerve was ligated to prevent possible regeneration.  C, Motor axons in the femoral nerve normally project to the quadriceps femoris muscle.  D, One of 2 fluorescent dyes (fluororuby and fluoroemerald) was applied to the reinnervated muscle and cutaneous nerve branches 6 weeks after nerve injury in order to quantify the number of motor neurons extending axons into one or both branches.  E, A suction electrode was used in some experiments to electrically stimulate the cut motor neurons immediately after nerve transection.  F, Motor neuron cell bodies retrogradely labelled with fluororuby (red cells) can easily be distinguished from those labelled with fluoroemerald (green cells). A cell backlabelled with both dyes appear yellow (arrow) in this overlay.
Results

PSA Is Differentially Expressed by Sub-Groups of Developing Motor Neurons

It is well established that PSA is expressed by motor neurons during embryonic development (Landmesser et al. 1988). Its expression reduces axon-axon adhesion and thus allows discrete groups of developing motor axons to respond to specific, instructive guidance molecules that ultimately direct them to their appropriate target (Tang et al., 1992; 1994; Rutishauser and Landmesser, 1996). PSA is down-regulated postnatally, but is re-expressed on regenerating axons and denervated muscle fibers after peripheral nerve injury (Covault and Sanes, 1985; Daniloff et al., 1986; Martini and Schachner, 1988; Zhang et al., 1995). The functional consequence of this re-expression was recently demonstrated when it was shown that regenerating mouse femoral motor axons must up-regulate PSA in order to preferentially reinnervate their appropriate muscle pathway after injury; a process known as PMR (Franz et al., 2005).

Interestingly, distinct pools of chick motor neurons express varying levels of PSA during embryonic development. Whether different groups of mouse motor neurons differentially express PSA during early neuromuscular development is not known. To test this possibility, we sectioned E10 mouse hindlimbs at the level of the lumbosacral plexus and double immunolabelled the sections with antibodies directed against neurofilament (Fig. 3.2A; NF) and PSA (Fig. 3.2B). Figure 3.2A shows a cross-section of the developing hindlimb containing three major nerve branches as they exit the developing spinal cord (Fig. 3.2A; asterisk). The dorsal (Fig. 3.2A; D) and ventral nerve trunks (Fig. 3.2A; V) innervate limb muscles while motor axons in the ventral flank nerve
Figure 3.2 Sub-groups of motor neurons differentially express PSA during neuromuscular development. A, Double immunolabelling for neurofilament and PSA shows that the dorsal and ventral nerve trunks innervating the mouse hindlimb bud at E10 express PSA (arrowheads) while axons in the ventral nerve flank do not (arrow). B, Double immunolabelling for neurofilament and PSA shows that the dorsal nerve trunk innervating the hindlimb bud of a St. 26 chick embryo expresses more PSA (arrowhead) than the ventral nerve trunk (small arrow) and ventral flank nerve (large arrow). NF, neurofilament; D, dorsal nerve trunk; V, ventral nerve trunk; VF, ventral nerve flank.
(Fig. 3.2A; VF) innervate muscles in the groin. Interesting, while PSA was clearly expressed on the limb innervating nerve trunks (Fig. 3.2B; arrowheads), no PSA was detectable on the ventral flank nerve (Fig. 3.2B; arrow). This differential pattern of PSA expression is slightly different in the developing chick embryo where axons in the dorsal nerve trunk (fig. 3.2B, arrowhead), which includes femoral motor neurons (Jones, 1979), express significantly more PSA compared to both the ventral nerve trunk (Fig. 3.2B, small arrow) and ventral flank nerve (Fig. 3.2B; large arrow) of the lumbosacral plexus (see also Tang et al., 1992). Taken together, while the pattern of PSA expression differs somewhat in the chick and mouse, it is clear that different pools of developing motor neurons differentially express PSA in the two species.

**PSA Is Differentially Up-Regulated on Different Motor Neuron Pools after Peripheral Nerve Injury**

Peripheral neurons up-regulate many developmentally expressed growth associated molecules after injury (Goldberg, 2004; Bosse et al., 2006; Rossi et al., 2007). Consequently, our present results raised the possibility that PSA may be differentially expressed by different pools of regenerating motor neurons. To determine to what extent PSA is expressed by different pools of regenerating motor neurons, we transected 3 distinct motor nerves that innervate ventral limb, dorsal limb or groin muscles during embryonic development (Jones, 1979). These nerves include the muscle branch of the femoral nerve (derived from the dorsal nerve trunk), the obturator nerve (derived from the ventral nerve trunk), and the genitofemoral nerve (derived from the ventral flank
Figure 3.3  PSA expression levels vary between different motor nerves after transection. A, Cartoon and accompanying representative femoral nerve cross-sections immunolabelled for PSA (i and ii) show that its expression is higher on the nerve near the growth cones (ii) compared to more proximal sites (i). B-J, Immunolabelling shows that control femoral (B), obturator (E) and genitofemoral nerves (H) express very low levels of PSA. PSA is up-regulated on regenerating femoral axons (C), but not regenerating obturator (F) or genitofemoral axons (I), 5 days after injury. PSA expression is further increased on regenerating femoral axons (D), and it is up-regulated on obturator neurons (G), 5 days after nerve transection and brief electrical stimulation. Genitofemoral neurons do not alter PSA expression levels after nerve injury and electrical stimulation (J).
nerve). The mice were killed 5 days later because previous studies have shown that PSA levels peak near the lesion site at this time point (Franz et al., 2005). A 3 mm segment from each nerve, immediately proximal to the lesion, was isolated, cut in cross-section, and processed for PSA immunohistochemistry (see Methods for details). Immediately upon inspection of the immunolabelled sections we noticed that there was a gradation of PSA expression along the length of the transected nerves. This gradient is shown in Figure 3.3A where the cartoon illustrates the site of femoral nerve transection and suture, as well as the location of the two representative nerve sections (i & ii, where i is closer to the spinal cord) that were immunolabelled for PSA. As observed during development (e.g. Soundararajan et al., 2006), we found that PSA expression was higher on the nerve near the growth cones (Fig. 3.3Aii) compared to more proximal sites (Fig. 3.3Ai). These results indicate that PSA is required at the level of the distal axon and/or growth cone, but not along the proximal axonal shaft. Furthermore, it highlights that necessity to compare PSA expression in sections taken from the same region of the nerve. Consequently, the location of each section along the length of the nerve segments (with respect to the injury site) was recorded and great care was taken to ensure that sections from the same level were used for comparison between conditions.

To establish baseline levels of PSA expression, a comparable segment from each nerve was dissected from un-operated, age matched control mice and immunolabelled for PSA. Figure 3.3 shows that PSA was expressed at similarly low levels on uncut control femoral (Fig. 3.3B), obturator (Fig. 3.3E) and genitofemoral nerves (Fig. 3.3H). Interestingly, while PSA was dramatically up-regulated on the femoral nerve 5 days after nerve transection (Fig. 3.3D; see also Franz et al., 2005), its expression was not
noticeably different from control levels on transected obturator (Fig. 3.3F) and
genitofemoral nerves (Fig. 3.3L). These results demonstrate that up-regulation of PSA
after axotomy is not an inherent property of all regenerating motor nerves. Instead, its re-
expression is intrinsic to a subset of motor neurons including those forming the femoral
nerve.

Brief Electrical Stimulation Increases PSA Expression in a Subset of Regenerating
Motor Neurons after Injury

Previous studies have shown that PMR is enhanced in rats if the transected
femoral nerve was electrically stimulated for 1 hour prior to surgical repair (Al-Majed et
al., 2000b). The molecular mechanisms underlying this improvement in PMR likely
involves several factors (Maehlen and Nja, 1982; Manivannan and Terakawa, 1994; Al-
Majed et al., 2000a; English et al., 2006; see Discussion). However, because PSA
expression is increased with activity in excitable cells (Kiss et al., 1994; Rafuse and
Landmesser, 1996) we hypothesized that PSA is a mediator of this effect. To determine
whether brief electrical stimulation modulates PSA expression on regenerating motor
axons we stimulated the proximal end of the femoral, obturator and genitofemoral nerves
immediately after transection and repair (1 hr stimulation at 20 Hz). The mice were
killed 5 days later and the proximal ends of the transected nerves were processed for PSA
immunohistochemistry. Nerves from each group were stained simultaneously to ensure
that variability in incubation times and antibody concentrations were minimized. Images
used for analysis were captured using identical exposure settings that were standardized
to ensure that the highest PSA levels observed were not saturated. Figure 3 shows that
Figure 3.4  Pixel intensity profiles indicate that PSA expression varies between different motor nerves after transection. A-I, The pattern of the PSA staining profiles consists of many sharp peaks and valleys that correspond well with the fact the PSA is predominately expressed on the cell membrane. Pixel intensity profiles show that control femoral (A), obturator (D) and genitofemoral nerves (G) express very low levels of PSA. PSA is clearly up-regulated on regenerating femoral axons (B), but not regenerating obturator (E) or genitofemoral axons (H), 5 days after injury. The amplitude of the peaks is further increased on regenerating femoral axons (C), and it is up-regulated on obturator neurons (F), 5 days after nerve transection and electrical stimulation. Pixel intensity is modestly increased on the genitofemoral neurons when they were transected and stimulated (I). J-L, The average peak pixel intensities of the electrically stimulated femoral nerve was significantly higher than all other conditions studied (J; white bar; ## p<0.01). The level of PSA expression on the transected femoral nerves (J; grey bar) was not significantly different from the electrically stimulated obturator nerves (H; white bar; # p<0.01), however both were statistically higher than all remaining groups. While there was a trend for the average pixel intensities to be higher on the regenerating genitofemoral nerves with (L; open bar), or without (L; shaded bar), stimulation the average increase was not significantly higher than unoperated nerves (J-L; filled bars) or regenerating obturator axons (K; shaded bar).
previously stimulated regenerating femoral nerves expressed substantially higher levels of PSA (Fig. 3.3D) compared to unstimulated cut femoral axons (Fig. 3.3C). PSA expression was also up-regulated on cut obturator nerves after electrical stimulation (Fig. 3.3G) even though PSA levels did not increase following transection alone (Fig. 3.3F). Interestingly, the level of PSA on the stimulated cut obturator nerve (Fig. 3.3G) was very similar to the level expressed by the regenerating femoral nerves that were not stimulated (Fig. 3.3C). In contrast to the stimulation-induced increase in PSA levels on femoral and obturator nerves, brief electrical stimulation did not affect the levels of PSA expressed by the regenerating genitofemoral nerves (Fig. 3.3J). In fact, the expression level of PSA was the same regardless as to whether the nerve was uncut, transected or stimulated after transection (Fig. 3.3H-J).

Pixel intensity profiles were used to quantify and compare PSA expression levels on the three distinct nerves, under the aforementioned experimental conditions (Fig. 3.4). Profiles were produced by plotting the values of PSA staining in arbitrary units of relative pixel intensity across the diameter of the nerve cross section. As expected, the pattern of the PSA staining profile consisted of many sharp peaks and valleys (eg. Fig. 3.4C) that correspond well with the fact the PSA is predominately expressed on the cell membrane and not in the cytoplasm (Cremer et al., 1994). Mean pixel intensities from multiple representative nerve sections from 3 separate mice per condition were analyzed and statistically compared (Fig. 3.4J-L).

Figure 4 shows that the PSA pixel intensity profiles for the control femoral (Fig. 3.4A), obturator (Fig. 3.4E), and genitofemoral (Fig. 3.4I) nerves were very similar and support our previous conclusion that all three nerves express the same low level of PSA
prior to transection (see above). Pixel intensity profiles also showed that PSA levels were up-regulated on the femoral nerve 5 days after transection and that the expression levels were even higher when the cut femoral nerve was electrically stimulated (compare Figs. 3.4A-C). The profiles further illustrate that PSA levels only increased on the obturator nerve after transection when the nerves were electrically stimulated (compare Figs. 3.4E-G). Finally, the genitofemoral nerve expressed relatively low levels of PSA in all conditions (Figs. 3.4 I-K). When the peak pixel intensities were quantified we found the average intensity of the electrically stimulated femoral nerve was significantly higher than all other conditions studied (Fig. 3.4D; open bar, p<0.01). Interestingly, the level of PSA expression on the transected femoral nerves (Fig. 3.4D, grey bar) was not significantly different from the electrically stimulated obturator nerves (Fig. 3.4H, white bars), however both were statistically higher than the remaining groups including the electrically stimulated genitofemoral nerve (Fig. 3.4L; open bars, p<0.01). Taken together, these results indicate that up-regulation of PSA after a nerve injury, or after a nerve injury with brief electrical stimulation, is an intrinsic property that is not shared by all motor neuron subtypes.

**PMR Does Not Occur When Foreign Motor Neurons Cross-Reinnervate the Femoral Nerve**

The rodent femoral nerve model is ideally suited for studying motor axon regeneration specificity because the growing axons only have to make a binary decision between innervating a muscle or cutaneous pathway (Fig. 3.1C). Brumfield and colleagues used the simplicity of this system to show that regenerating femoral motor neurons
preferentially grow down the distal muscle pathway even though they have equal access to the cutaneous pathway when transected proximal to their bifurcation (Brushart, 1988, 1993). The accuracy of motor axon regeneration is also easily assessed by counting backlabelled motor neurons after applying 1 of 2 different fluorescent dyes to the reinnervated muscle and cutaneous nerve branches (Fig. 3.1D and F).

Mouse femoral motor axons must express PSA is order to preferentially reinnervate muscle fibers (i.e. exhibit PMR) after nerve injury (Franz et al., 2005). Consequently, it seems reasonable to hypothesize that motor neurons incapable of up-regulating PSA do not exhibit PMR after nerve injury. To directly test this hypothesis the obturator or genitofemoral nerves were cut and sutured onto the distal femoral nerve stump (Fig. 3.1B) and motor axon regeneration accuracy was compared to when the femoral nerve was cut and simply repaired onto itself. The femoral to femoral nerve surgeries were performed as described in Franz et al. (2005) except that the obturator nerve was cut and ligated as a control. In the case of the obturator and genitofemoral nerve cross-reinnervation surgeries the proximal femoral nerve was ligated in order to prevent self-reinnervation of its distal stump. The mice were allowed to recover for 6 weeks before a second operation was performed to apply fluorescent dyes (fluororuby and fluoroemerald) to the muscle and cutaneous nerve branches to quantify the extent of PMR (Fig. 3.1D,F). As shown previously (Robinson and Madison, 2003; Franz et al., 2005; Robinson and Madison, 2005), PMR occurred when PSA+ femoral motor neurons reinnervated their own distal nerve stump (Fig. 3.5A; p=0.021). In striking contrast, regenerating PSA- obturator (p=0.118) and genitofemoral (p=0.253) motor neurons did not exhibit PMR, and reinnervated the muscle and cutaneous pathways with equal
Figure 3.5  Different pools of motor neurons display varying degrees of PMR after nerve injury with, or without, stimulation.  A, The mean ± SEM number of retrogradely labelled motor neurons that regenerated into the appropriate muscle branch (black bars), inappropriate cutaneous branch (gray bars), and both branches (open bars) 6 weeks after the femoral, obturator or genitofemoral nerve was transected and sutured onto the distal femoral nerve stump without (Standard Repair) or with electrical stimulation (* p<0.01 and ** p<0.01; Mu compared to corresponding Cu).  B, The number of motor neurons that reinnervated the appropriate muscle branch after electrical stimulation was normalized as a percentage of the mean number of motor neurons that reinnervated the muscle branch after standard repair. The number of femoral motor neurons reinnervating the muscle pathway after electrical stimulation was significantly greater than after standard repair (p<0.05).  C, The number of motor neurons that reinnervated the cutaneous branch after electrical stimulation was normalized as a percentage of the mean number of motor neurons that reinnervated the cutaneous branch after standard repair. There was a significant decrease in the number of femoral and obturator motor neurons that incorrectly reinnervated the cutaneous branch after electrical stimulation (p<0.05). FEM, femoral-femoral; OBT, obturator-femoral; GNF, genitofemoral-femoral nerve surgeries; Mu, labelled axons in muscle branch; Cu, labelled axons in cutaneous branch; B, labelled axons innervating both branches.
preference (Fig. 3.5A; Standard Repair). These results support the hypothesis that selective growth of regenerating motor axons requires the expression of PSA and PMR is not an intrinsic property exhibited by all motor neuron pools.

**Electrical Stimulation Improves Regeneration Accuracy in Specific Subsets of Motor Neurons Capable of Up-Regulating PSA**

Brief electrical stimulation increases PSA expression on regenerating femoral and obturator nerves (Figs. 3.3 & 3.4) and improves PMR of transected rat femoral motor neurons (Al-Majed et al., 2000b). Whether electrical stimulation improves PMR because it increases PSA levels on regenerating motor axons is not known. To address this issue we cut 1 of the 3 nerves (femoral, obturator, or genitofemoral) and applied brief electrical stimulation to its proximal end using a suction electrode (Fig. 3.1E). Immediately after the stimulation was completed the proximal end of the nerve was sutured to the distal stump of the femoral nerve. Mice were allowed to recover for 6 weeks when a second surgery was performed to apply the two neurotracers as described previously (Fig. 3.1D,F). Figure 3.5A shows that electrical stimulation dramatically improved femoral motor neuron regeneration accuracy over non-stimulated femoral nerves (p<0.001;). There was no difference in the total number of regenerated motor neurons (p=0.931), so this stimulation-induced improvement was attributed to significantly more motor neurons reinnervating the muscle pathway (Fig. 3.5A,B; p=0.036;) and significantly fewer motor neurons reinnervating the cutaneous pathway (Fig. 3.5A,C; p=0.013). Because stimulated femoral motor axons express more PSA compared to non-stimulated femoral axons (Fig. 3.3,3.4), these results suggest that the degree of PMR is correlated to the level of PSA expressed by the regenerating nerve.
Obturator motor neurons elicited PMR when electrically stimulated (p<0.001) even though no PMR was evident using standard repair conditions (Fig. 3.5A). This improvement was primarily due to significantly fewer motor neurons reinnervating the cutaneous pathway after stimulation (p=0.032; Fig. 3.5C). The overall number of regenerating motor neurons was not significantly different from non-stimulated obturator nerves (p=0.816) because there was a small increase in the number of motor neurons reinnervating the motor pathway (Fig. 3.5A,B). These results indicate that foreign axons are capable of responding to guidance cues that preferentially guide them down the motor pathway when cross-reinnervated onto the distal femoral nerve stump provided they express PSA (Figs. 3.3,3.4).

Genitofemoral motor neurons do not exhibit PMR even when electrically stimulated (Fig. 3.5; p=0.147). Once again, stimulation did not effect the number of regenerating motor neurons (Fig. 3.5, p=0.812). Overall, these findings indicate that the extent of PMR is directly correlated with the amount of PSA expressed by the regenerating motor neurons, irrespective of their origin.

**Electrical Stimulation Does Not Improve Regeneration Accuracy if PSA Is Removed from the Reinnervating Motor Neurons**

The results above strongly suggest that there is a direct correlation between PSA levels and regeneration accuracy. In addition, electrical stimulation appears to enhance PMR because it increases the expression of PSA over non-stimulated levels. However, brief electrical stimulation also accelerates and enhances the expression of other regeneration-associated genes in motor neurons (Al-Majed et al., 2000a; Al-Majed et al., 2004). Consequently, to dissociate the functional roles of PSA from other regeneration-
Figure 3.6  Electrical stimulation only improves femoral motor neuron regeneration accuracy when PSA is up-regulated on regenerating motor axons. A-D, PSA immunolabelling shows that regenerating femoral neurons within the muscle branch express PSA 5 days after injury and repair in saline injected WT mice (A). In contrast, regenerating femoral neurons within the muscle branch in NCAM -/- (B), Endo-N injected WT (C), and NCAMloxP/+Hb9cre+ (D) mice express little to no PSA. E, The mean number ± SEM of retrogradely labelled motor neurons that reinnervated the appropriate muscle branch (black bars), the inappropriate cutaneous branch (gray bars), or both branches (white bars) was determined 6 weeks after femoral nerve transection, electrical stimulation, and repair in saline injected WT (saline), NCAM -/- mice, Endo-N injected WT mice, and NCAMloxP/+Hb9cre+ mice (**p<0.01, Mu compared with corresponding Cu). Mu, Labelled axons in muscle branch; Cu, labelled axons in cutaneous branch; B, labelled axons innervating both branches.
associated genes after brief electrical stimulation we transected and stimulated the femoral nerve of NCAM -/- mice, that also lack PSA, and WT mice whose femoral nerve was injected with Endo-N to remove PSA at the time of nerve transection. Previous studies have shown that injecting 0.5 μl of Endo-N (8.7 U/μl) immediately prior to the nerve repair prevents up-regulation of PSA on regenerating axons throughout the period of peak expression (Franz et al., 2005). For comparison, the femoral nerve was cut and stimulated in WT mice that were injected with 0.5 μl of saline as a control. PMR was assessed 6 weeks later as described earlier.

Figure 3.6A-D show PSA immunolabelled cross sections, from the femoral nerve muscle pathway, that were harvested from saline injected WT, NCAM -/-, Endo-N injected, and NCAM$^{lox/lo}x/Hb9^{cre+}$ mice (see below) 5 days after nerve transection/stimulation. Not surprisingly, PSA was only expressed by the stimulated/saline injected femoral nerves. Also, as expected, stimulated/saline injected femoral nerves in WT mice displayed very robust PMR Fig. (3.6E; p<0.001). In striking contrast, PMR was completely absent when the femoral motor neurons were stimulated in NCAM -/- mice (Fig. 3.6E; p=0.221) and WT mice injected with Endo-N (Fig. 3.6E; p=0.602). The lack of PMR was likely due to the absence of PSA, rather than NCAM, because regeneration accuracy was equally impaired in the NCAM -/- and Endo-N treated mice.

Gordon and colleagues (Al-Majed et al., 2000b) applied TTX proximal to the transection site while electrically stimulating the cut rat femoral nerve to prevent action potentials from reaching the motor neuron cell body. PMR enhancement was dramatically attenuated in these rats indicating that stimulation mediates its effect via the
motor neurons and not the surrounding glia. To confirm that PSA is up-regulated on femoral motor neurons and not the surrounding glia after electrical stimulation, we conditionally ablated NCAM and PSA in motor neurons by breeding NCAM-floxed mice (Bukalo et al., 2004) with mice in which cre-recombinase was expressed under the control of the Hb9 promoter (Yang et al., 2001; Hess et al., 2007). Hb9 is a homeobox gene that is expressed by motor neurons and a few interneurons (Arber et al., 1999; Thaler et al., 1999; Wilson et al., 2005). The very low level of PSA expression in the muscle pathway of the femoral nerve 5 days after nerve transection in NCAM\textsuperscript{lox/lox}/Hb9\textsuperscript{cre/+} mice indicates that the vast majority of PSA expressed in the cut femoral nerve is associated with regenerating motor neurons. Interestingly, the low levels of PSA expression (Fig. 3.6D) also indicates that muscle afferents, which do not express the Hb9 gene, do not up-regulate PSA after injury. To conclusively determine whether PSA expression by motor neurons regulates enhanced PMR we cut, electrically stimulated and repaired the femoral nerve in NCAM\textsuperscript{lox/lox}/Hb9\textsuperscript{cre/+} mice as described above and assessed PMR 6 weeks later. As anticipated, PMR was absent in the reinnervated NCAM\textsuperscript{lox/lox}/Hb9\textsuperscript{cre/+} mice (Fig. 3.6E; p=0.169) indicating that increased PSA expression by motor neurons, and not glia, is necessary to enhance PMR after brief electrical stimulation.

**Motor Axon Sprouting Is Enhanced by Electrical Stimulation and Requires the Expression of PSA**

It is well established that cut motor axons form multiple collateral sprouts as they extend distally across the injury site (Ramon y Cajal, 1928). The formation of these supernumerary collaterals is believed to increase the probability that at least one sprout
will innervate an appropriate basal lamina tube in the distal nerve stump (Brushart, 1988, 1993; Madison et al., 1999). Once the appropriate tube is innervated, the basal lamina guides each regenerating axon to its correct distal target (Sanes et al., 1978; Nguyen et al., 2002). Collateral sprouts extending into inappropriate basal lamina tubes in the sensory branch withdraw provided at least one sprout correctly contacts a basal lamina tube in the muscle branch. The probability of contacting an appropriate basal lamina tube is likely improved if the collateral sprouts arborize over a large area of the distal stump (Franz et al., 2005). These assumptions are supported by the fact that the number and distribution of collateral sprouts are significantly reduced, and regeneration accuracy severely compromised, when PSA is removed from regenerating femoral motor neurons (Franz et al., 2005).

If regeneration accuracy is compromised with reduced sprouts and fields of arborization it seems reasonable to assume that PMR will be improved if they increase. Unfortunately, this hypothesis is not easily testable because it is experimentally difficult to simply increase the number of collaterals sprouts and/or their fields of arborization. However, previous studies have shown regenerating sympathetic ganglion cells in vivo (Maehlen and Nja, 1982; Manivannan and Terakawa, 1994) and dorsal root ganglion cells in vitro (Itoh et al., 1995) form supernumerary collateral sprouts when electrically stimulated. Consequently, we used brief electrical stimulation to determine whether regeneration accuracy is improved because it increases both the number of sprouts and fields of arborization. Second, if stimulation increases motor axon sprouting and/or arborization size, we wished to determine whether PSA is involved in these two processes.
The number of collateral sprouts and changes in arborization size were quantified in mHb9-Gfp1b mice. These transgenic mice express eGFP in motor neurons and a few interneurons (Wichterle et al., 2002; Franz et al., 2005; Wilson et al., 2005). As described earlier, the femoral nerve was transected, injected with either saline or Endo-N, and in some cases electrically stimulated at 20 Hz for 1 hour immediately before it was sutured to its distal stump. The nerves were allowed to regenerate for 3 weeks after which time the femoral nerve was removed and sectioned from the surgical repair site to another site 1 mm distally. For comparison, cross-sections were taken from the same level of the femoral nerve in un-operated mHb9-Gfp1b mice. The number of eGFP+ axonal sprouts and their length of lateral deflections were quantified from several representative cross-sections from each mouse (see Methods for details). Figure 3.7A shows that the femoral nerve in control mHb9-Gfp1b mice contains two groups of motor axons that are distributed in a very stereotypic manner at the level of the nerve injury (also see; Franz et al., 2005). Motor axons innervating the quadriceps femoris muscle fasciculate together in a relatively large cluster of eGFP+ axons near the ventral surface of the nerve (Fig. 3.7A; bottom is ventral). These axons eventually form the muscle branch of the femoral nerve. The second small cluster of motor axons at the dorsal surface of the nerve eventually branches off to form the pectineus nerve (Fig. 3.1A). The unlabelled area separating the two clusters of motor axons contains sensory axons that form the cutaneous nerve branch. As reported previously (Franz et al., 2005), there was a significant increase in the number of eGFP+ motor axons distal to the lesion site after nerve transection and standard repair (Fig. 3.7B, I; Saline). Furthermore, rather than re-establishing their stereotypical organization, the reinnervated eGFP+ axons were now
evenly distributed throughout the nerve cross-section (Fig. 3.7B). As hypothesized, brief electrical stimulation further increased the number of eGFP⁺ collateral sprouts in the distal nerve stump (Fig. 3.7C, I; Saline + Stim). This increase in sprouting was dramatically attenuated when Endo-N was injected into the transected nerve immediately prior to the onset of stimulation (Fig. 3.7D, I; Endo-N + Stim).

The sagittal section in Figure 3.7E shows an example of the complex axonal trajectories eGFP⁺ femoral motor axons take as they traverse across the surgical repair site (delineated by the two opaque lines). These elaborate paths establish the field of arborization and appear as lateral axon excursions in cross-sections taken from the site of nerve injury (Fig. 3.7F, arrow; see also Witzel et al., 2005). Nerves containing long excursions reflect the presence of motor axons with large fields of arborization (Witzel et al., 2005). Lateral excursion lengths in transected and electrically stimulated femoral nerves in mHb9-Gfp1b mice were quantified (see Methods for details) and compared with mHb9-Gfp1b mice that had undergone standard repair without electrical stimulation or received electrical stimulation/repair followed by enzymatic removal of PSA. The cross-section in Figure 3.7G shows multiple long lateral excursions traversing the repair site 3 weeks after brief electrical stimulation and nerve repair. The mean length of the excursions was significantly greater in stimulated animals (Fig. 3.7J; Saline + Stim) compared to animals receiving standard nerve repair (Fig. 3.7J; Saline). In contrast, motor axon collaterals had noticeably shorter lateral excursions when PSA was removed immediately after electrical stimulation (Fig. 3.7H; Endo-N + Stim). In fact, when quantified, the mean length of the lateral excursions was significantly shorter in the stimulated/Endo-N injected mice (Fig. 3.7J; Endo-N + Stim) than the unstimulated/saline
Figure 3.7  Electrical stimulation increases the number of sprouts and length of lateral deflections at the repair site. Cross-section of the femoral nerve in mHb9-Gfp1b mice shows that femoral motor axons are normally distributed in two highly stereotyped clusters (A). Three weeks after nerve transection and repair, femoral motor axon numbers increase (B, I), and become evenly distributed throughout the nerve 1 mm distal to the repair site (B). The number of sprouts is enhanced further when the cut nerve is electrically stimulated (C, I) and nearly abolished if Endo-N is injected immediately after stimulation (D, I). A sagittal section through the lesion site shows multiple lateral deflections (E) that appear as lateral excursions in cross-sections (F, arrow). The lengths of the lateral deflections significantly increase when the cut nerve is electrically stimulated (G, J), an effect that is completely abolished if Endo-N was injected immediately after stimulation (H, J). The mean ± SEM number (I) and length of lateral deflections (J) of eGFP positive femoral motor axons from uncut control, standard repair with saline injection (Saline), electrical stimulation repair with saline injection (i.e. Saline + Stim), and electrical stimulation repair with Endo-N injection (Endo-N + Stim). # p<0.05, as compared to Saline; ** p<0.01, as compared to Endo-N + Stim; * p<0.05, as compared to Endo-N + Stim; α p<0.01, as compared to all groups; α p<0.01, as compared to all groups. K-M, Proposed model showing that a single PSA+ femoral motor neuron forms multiple collateral sprouts that explore both the muscle (green fascicles) and cutaneous (red fascicles) pathways in the distal stump after standard repair (K). Enhancing PSA expression increases both the number of sprouts and field of arborization (L) while decreasing PSA expression has the opposite effect (M). Artwork by Monique Guilderson (Maritime Medical Design).
injected animals (Fig. 3.7J; Saline). Taken together, these results strongly suggest that PMR is enhanced with brief electrical stimulation because it increases the number of collateral sprouts and arborization size and that these increases require up-regulation of PSA over normal expression levels.

**Discussion**

Regenerating femoral motor neurons preferentially reinnervate the quadriceps femoris muscle after nerve injury even though they have equal access to the skin (Brushart, 1988, 1993). Although the molecular mechanisms underlying this example of PMR are complex, it is generally accepted that selective motor axon regeneration is promoted by guidance factors expressed by the regenerating axons and distal targets (Madison et al., 2007). Interestingly, an underlying assumption in most PMR studies is that the guidance factors are generic for all types of regenerating motor neurons, irregardless of origin. The results in the present study challenge this presumption by showing that not all motor neurons are capable of PMR. In addition, we show that selective reinnervation of the muscle target requires up-regulation of PSA; an intrinsic neuronal property that is likely established during neurogenesis. Finally, brief electrical stimulation was found to enhance PMR because it increases PSA expression on the regenerating axons, which in turn promotes more collateral sprouting and larger fields of arborization.
Molecular Differences between Motor Pools during Development and Regeneration

One of the most striking findings in the present study was the observation that obturator and genitofemoral motor neurons do not preferentially reinnervate the quadriceps femoris muscle when cross-reinnervated with the femoral nerve. This lack of selectively was not simply due to cross-reinnervation per se because cross-reinnervated obturator motor neurons exhibited PMR when electrically stimulated. Because it seems likely that the expression of guidance molecules in the distal targets do not vary between experimental conditions (femoral nerve reinnervation versus cross-reinnervation), these results indicate that neuronal properties that are intrinsic to distinct motor pools dictate whether they elicit PMR.

Molecular distinctions in individual motor pools arise during early spinal cord development (Landmesser, 2001). Differential expression of ETS and LIM homeodomain transcription factors (Livet et al., 2002), as well as Hox genes (Dasen et al., 2005), are responsible for instructing motor pool development. These genes, in combination with epigenetic factors such as neuronal activity (Hanson and Landmesser, 2004, 2006), regulate the expression of axonal guidance factors that ultimately guide their axons to their appropriate targets. Guidance molecules differentially expressed by distinct groups of developing motor neurons include type II cadherins (Price et al., 2002), EphA4 (Helmbacher et al., 2000) and PSA (Tang et al., 1992; Allen and Greer, 1998). In the developing mouse, PSA is expressed by dorsal (including femoral neurons) and ventral (including obturator neurons) projecting hindlimb motor neurons, but is nearly absent on ventral flank neurons (including genitofemoral neurons). Whether individual pools of motor neurons retain and/or re-express intrinsic molecular differences during
regeneration is poorly understood. Motor neurons innervating the extensor digitorum longus and soleus muscles differentially express tyrosine kinase C (trkC) after axotomy (Simon et al., 2002). However, it is not known whether these two motor pools differentially express trkC during development. Thus, our finding that PSA is differentially re-expressed by regenerating femoral, obturator, and genitofemoral motor neurons represents the first example where distinct molecular differences, which were likely established during development, are recapitulated after injury. In other words, the capacity to up-regulate PSA after injury appears to be an intrinsic neuronal property that is determined during embryogenesis. Furthermore, because PSA expression is essential for PMR, these results strongly suggest that neuronal properties that are established during development have a profound influence on the neuron's capacity to respond to instructive guidance cues after peripheral nerve injury.

**Potential Mechanisms Underlying PMR**

Potential regulators of PMR include the 1) distal nerve pathways, 2) end organs, and 3) motor neurons (Reviewed by Madison et al., 2007). Transected femoral motor neurons preferentially reinnervate the muscle pathway in the absence of end organs (muscle and skin) in young rats (Brushart, 1993) indicating that molecular differences must exist between the two pathways at this stage in development. Indeed, HNK-1 is at least one axon guidance molecule that is expressed to a greater extent in the muscle pathway compared to the cutaneous pathway in rats (Martini et al., 1992) and mice (Eberhardt et al., 2006). HNK-1 enhances neurite outgrowth from motor, but not sensory neurons *in vitro* (Martini et al., 1992) and thus it is a potential guidance molecule.
regulating PMR. This assumption is supported by the fact that BDNF/trkB dependent up-regulation of HNK-1 appears to be required for enhanced PMR after brief electrical stimulation (Eberhardt et al., 2006). Pleiotrophin and glial cell line-derived neurotrophic factor are up-regulated to a greater extent by Schwann cells in denervated ventral roots compared to dorsal roots (Hoke et al., 2006). Consequently, these neurotrophins could be guidance molecules differentially expressed in the denervated femoral nerve muscle pathway.

Selective recognition of the muscle pathway by regenerating femoral motor axons has recently been challenged as a possible mechanism promoting PMR (reviewed by Madison et al., 2007). In an elegant series of experiments, Madison and colleagues (Robinson and Madison; 2004, 2005, Uschold et al., 2007) showed that regenerating femoral motor neurons in adult rats preferentially reinnervate the cutaneous pathway provided the muscle target is removed. Furthermore, they propose that the length of the distal pathway is a greater determinant of PMR than the actual identity of the pathway. Taken together, the authors propose a hierarchy of axon guidance cues with muscle contact being the highest, followed by the length of the nerve pathway and/or contact with the skin (Madison et al., 2007).

Regardless of the source, the capacity to respond to distal guidance cues ultimately rests with the reinnervating motor neurons. This fact is definitively shown in the present study where regenerating obturator and genitofemoral motor neurons failed to preferentially reinnervate the muscle pathway even though they had access to the same guidance molecules as the regenerating femoral neurons. Furthermore, the capacity to respond to the guidance molecules was directly related to the motor neuron's capacity to
up-regulate PSA because PMR was absent in WT mice treated with Endo-N, NCAM-/-
mice, and NCAM^{lox/lox}/Hb9^{cre/+} mice. The latter mice were used to definitively show that
PSA must be up-regulated on the regenerating motor axons and not cells in the distal
pathways or end organs.

PSA is required for proper motor axon targeting in the developing chick (Tang et
al., 1992; 1994) because it enables individual axons to respond to distant guidance cues
by decreasing axon-axon adhesion (Landmesser, 2001). A similar phenomenon may also
occur during regeneration whereby PSA permits individual motor axons to respond to
distant guidance cues emanating from the distal pathways and/or muscle. In addition,
PSA expression promotes the formation of collateral sprouts. The presence of
supernumerary collaterals increases the probability that at least one sprout will contact
the appropriate muscle pathway (Brushart, 1993; Brushart et al., 1998; Franz et al., 2005;
Redett et al., 2005). Once contacted, the basal lamina guides each regenerating axon to its
correct distal target (Sanes et al., 1978; Nguyen et al., 2002). Collaterals extending into
the cutaneous pathway are selectively pruned provided a single sprout correctly
innervated the muscle (Brushart, 1993; Brushart et al., 1998; Franz et al., 2005; Redett et
al., 2005).

The exact mechanism by which PSA promotes sprouting is not known. Sprouting
is initiated through the stabilization of individual filopodia that normally form and
collapse within the growth cone. By attenuating cell-cell adhesion, PSA may allow
filopodia to explore the environment for positive signals which, when encountered,
results in the formation of a stabilized sprout (El Maarouf and Rutishauser, 2003). PSA
expression may also attenuate neuronal contact with growth inhibiting molecules such as
myelin associated glycoprotein (MAG). Mice lacking MAG have significantly more axonal sprouts distal to the injury site (Schafer et al., 1996), and daily treatment of regenerating femoral motor axons with antibodies to MAG enhances PMR (Mears et al., 2003). Finally, PSA expression modulates BDNF signaling in neurons (Vutskits et al., 2001; Vutskits et al., 2003; Gascon et al., 2007). This modulation may explain the correlation between BDNF/trkB signaling and enhanced motor axon regeneration/PMR after electrical stimulation (Eberhardt et al., 2006).

Electrical Stimulation, PMR & PSA

Brief electrical stimulation of the proximal femoral nerve stump immediately after nerve transection hastens the onset of PMR and recovery of motor function (Al-Majed et al., 2000b; Eberhardt et al., 2006). Electrical stimulation itself promotes axon collateral sprouting (Maehlen and Nja, 1982; Manivannan and Terakawa, 1994), increases axon defasciculation (Itoh et al., 1995), and accelerates axonal growth (Nix and Hopf, 1983; Pockett and Gavin, 1985; Al-Majed et al., 2000b; Brushart et al., 2002; English, 2005; English et al., 2006). The effects of electrical stimulation on PMR have been linked to axon regeneration associated molecules including brain-derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT-4/5), neurotrophin receptor trkB, and the HNK-1 carbohydrate (Al-Majed et al., 2000a; Eberhardt et al., 2006; English et al., 2006; Geremia et al., 2007). The present results extend these findings to show that brief electrical stimulation only enhances PMR in motor neuron pools capable of increasing PSA expression. Furthermore, the capacity to up-regulate PSA appears to be limited to motor pools that normally express PSA during neuromuscular development. Finally,
improved reinnervation accuracy requires a PSA-dependent increase in the number of collateral sprouts and arborization size.

Taken together, these results indicate that therapeutics aimed at increasing PSA levels on regenerating motor axons could lead to superior functional outcomes following nerve injury. However, clinical treatments must also consider that motor pools have different intrinsic neuronal properties that may limit the use of a global treatment strategy.
References


CHAPTER 4: Neural Cell Adhesion Molecule is Indispensable for the Maintenance of Reinnervated Neuromuscular Junctions.

Preface and Significance to the Thesis

It is likely that many of the molecules involved with the development of the neuromuscular junction (NMJ) are re-expressed and have similar functions during reinnervation after peripheral nerve injury. However, the molecular basis for synapse reformation, maintenance and function after nerve injury is inadequately understood. Neural cell adhesion molecule (NCAM) is likely involved in all of these processes based on 1) its persistent pre- and postsynaptic expression at the NMJ throughout adulthood, 2) its up-regulation on growing axons and uninnervated muscle fibers during both development and regeneration, and 3) the well known functional deficiencies that persist at the NMJs of NCAM-/- mice. In order to evaluate the functional role of NCAM in synapse formation, function and maintenance we chose to examine synaptogenesis at the NMJ after peripheral nerve injury.

Preliminary results of this work have been presented at the Society for Neuroscience (SFN) Annual Meeting in Atlanta, Georgia, USA, October 2006, and were published in Abstract form as Franz CF, Dawson S & Rafuse VF (2003, Neural cell adhesion molecule is required for the maintenance of reinnervated neuromuscular junctions after peripheral nerve injury. SFN Annual Meeting, Atlanta, GA, 716.5).
would like to acknowledge substantial intellectual contributions from my supervisor Dr. Victor Rafuse in the preparation of this chapter. I am grateful for the contributions of Sonia Dawson, an undergraduate student in the lab, who performed many of the tibial crush surgeries, as well as provided assistance with some of the cryostat sectioning and muscle measurements. The work of another undergraduate student, Alexandra Nelson, was also invaluable as she performed all the H & E stains, cut some of the muscle and nerve sections on the cryostat, and assisted with muscle measurements.

**Introduction**

The recovery of motor function after peripheral nerve injury is not achieved simply by the initial reformation of the neuromuscular junction (NMJ). In fact, the recovery of motor function progresses for several months after axons first make contact with the muscle (Gordon and Stein, 1982a, b; Badke et al., 1989; Desypris and Parry, 1990; Leterme and Tyc, 2004). Unfortunately, very little is known about the molecular mechanisms regulating NMJ reformation and maintenance after nerve injury. This is particularly surprising when you consider that the molecular mechanisms controlling NMJ development have been extensively studied (Sanes and Lichtman, 2001). This oversight is likely attributable to the presumption that the mechanisms of synaptogenesis during reinnervation and development are essentially the same because reinnervated NMJs can reestablish normal structural and physiological properties (Sanes and Lichtman, 1999). Conversely, this line of thinking suggests that identifying novel molecular mechanisms of NMJ synaptogenesis and maintenance after nerve injury should also offer insight into NMJ development.
Neural cell adhesion molecule (NCAM) is abundantly expressed on both axons and muscle fibers after nerve injury (Covault and Sanes, 1985; Rieger et al., 1985; Daniloff et al., 1986; Martini and Schachner, 1988) and during development (Nieke and Schachner, 1985; Covault and Sanes, 1986; Martini and Schachner, 1986; Tosney et al., 1986). Following nerve-muscle contact, NCAM becomes widely down-regulated on nerve and muscle with the exception of its persistent pre- and postsynaptically expression at the NMJ (Rieger et al., 1985; Covault and Sanes, 1986). Based on this pattern of expression NCAM has been proposed to play roles in neuromuscular regeneration, development and maintenance (Rutishauser and Landmesser, 1996; Walsh and Doherty, 1996).

Support for this assumption comes from studies using antibodies to NCAM during neuromuscular regeneration. In vivo injections of NCAM antibodies after nerve injury delayed both endplate reinnervation (Langenfeld-Oster et al., 1994) and recovery of function (Remsen et al., 1990) in mice. Similar studies in the frog lead to incomplete myelination of axons, absence of many terminal Schwann cells capping the NMJ, and ectopically located synapses (Rieger et al., 1988). Interestingly, the one study examining neuromuscular reinnervation in NCAM (-/-) mice found no evidence of delayed endplate reinnervation or absence of terminal Schwann cells capping the NMJ 1 week after nerve injury (Moscoso et al., 1998). Taken together, these studies indicate that, while NCAM is involved in the regeneration process, its function is not indispensable.

NCAM -/- mice have several deficits in neuromuscular function including an impaired capacity to release neurotransmitter at during high rates of neural activity (Rafuse et al., 2000; Polo-Parada et al., 2001). This abnormal capacity to release
neurotransmitter likely underlies the increased neuromuscular fatigue exhibited by these mice (Polo-Parada et al., 2001). Taken together with the persistent expression of NCAM at the adult NMJ these studies suggest that NCAM plays a role in the formation and/or maintenance of the synapse during development and possibly after nerve injury. To examine its function during and after synaptogenesis we conducted anatomical and functional studies on soleus muscles in wild-type (WT) and NCAM -/- mice 1-6 months after the tibial nerve was crushed. Our results showed that 3 and 6 months after nerve injury, the maximum force output of NCAM -/- muscles was dramatically less than reinnervated soleus muscles from WT mice as well as their unoperated controls. These deficits in strength existed despite the fact that reinnervated NCAM -/- and WT mice recovered to normal force levels as early as 1 month after nerve injury. Morphological analysis of muscles revealed a dramatic reduction in the size of NCAM -/- muscle 3-6 months after nerve injury. The reduction in NCAM -/- muscle size occurred primarily because of a selective loss of physiologically fast muscle fibers from 1 to 6 after nerve injury. Overall, these findings indicate that NCAM plays an indispensable role in the maintenance of reinnervated NMJs following nerve injury.

**Materials and Methods**

**Mice**

Experiments were performed on 2 different stains of mice in this study. WT C57BL/6 mice were obtained from Charles River (Wilmington, MA). NCAM -/- mice were generated on a C57BL/6 background (Cremer et al., 1994), and locally bred and housed. Mouse genotyping of NCAM -/- mice was performed by polymerase chain
reaction (PCR) as described previously (Franz et al., 2005). All procedures were conducted in accordance to the guidelines of the Canadian Council on Animal Care and the policies of Dalhousie University.

**Surgery**

All surgeries were performed on 12 week old adult mice unless otherwise noted. Animals were anesthetized with isoflurane (Baxter, Toronto, ON, Canada) and 1 of 2 surgeries was performed under aseptic conditions. (1) A small incision was made in the skin above the knee in order to expose the tibial nerve. The tibial nerve was crushed 2 times consecutively, using fine forceps, 10 mm distal to its divergence from the sciatic. Denervation was visually confirmed by noting muscle contraction and subsequent transparency of the nerve at the crush site. (2) A small incision was made in the skin overlaying the dorsal shank muscles. The soleus muscle was exposed by separating the lateral gastrocnemius muscle from the medial gastrocnemius and extensor digitorium longus muscles with forceps. A 0.5 μl volume of 1% Cholera toxin subunit b (CTB) conjugated to Alexa Fluor 594 (C22842; Invitrogen, Burlington, ON, Canada) was injected into the soleus muscle near the nerve entry point with the purpose of retrogradely labelling its motor neuron pool.

**In Vitro Isometric Tension and EMG Measurements**

Mice were killed with isoflurane (Baxter), their right hindlimb was quickly dissected free of its skin and then immediately placed into ice cold, bubbling (95% O₂, 5% CO₂) Tyrode’s solution (125 mM NaCl, 24 mM NaHCO₃, 5.37 mM KCl, 1 mM
MgCl₂, 1.8 mM CaCl₂, and 5% dextrose). The soleus muscle and its nerve supply was rapidly isolated from surrounding tissues by cutting it free at its insertion points on the femur and calcaneous bones. A small piece of bone attached to each tendon was removed along with the muscle and great care was taken to ensure that the soleus muscle nerve supply was not damaged during the dissection. The proximal muscle tendon was securely pinned down on a Sylgard (Dow Corning, Midland, MI) coated recording chamber that was perfused with well oxygenated Tyrode's solution maintained at 20-22°C. Suture (1-0) was tied to the distal tendon so as to hold the soleus muscle at its approximate physiological length while connecting it to a force transducer (FT 03; Grass Technologies, West Warwick, RI). A fine-tipped polyethylene stimulating suction electrode, pulled from polyethylene tubing (PE-190; Clay Adams, Sparks, MD), was used to deliver electrical current to the soleus nerve. The stimulus was produced by a S88 stimulator (Grass Technologies), and it was isolated from ground via photoelectric stimulus isolation unit (PSIU6; Grass Technologies). Soleus muscle electromyography (EMG) was recorded from the belly of the soleus muscle just proximal to its nerve insertion point with a second polyethylene suction electrode and amplified with a bandwidth between 3 Hz and 10 kHz (EX4-400; Dagan Corporation, Minneapolis, MN). Short (0.1-1 msec), monophasic electrical stimuli were used to elicit maximal isometric contractions and EMG responses that were acquired at 10 kHz using a Digidata 1322A A/D board and Axoscope 9.2 software (Axon Instruments, Union City, CA). The muscle was left in the perfusion medium for at least 1 hour before force and EMG measurements were made.
Fatigue Test

At the conclusion of isometric tension and EMG measurements a “Burke-type” fatigue test was performed (Burke et al., 1973). The test consisted of 13 pulses delivered at 40 Hz every second for 2 minutes. The fatigue index was calculated by dividing the peak isometric tension produced at the 1 and 2 minutes time points by the peak isometric tension of the first contraction.

Motor Neuron Counting

The motor neurons that were retrogradely labelled 3 days after CTB injection into the soleus muscle (described previously) were only counted if their nuclei and at least a primary dendrite could be clearly resolved. Raw cell counts were corrected by the Abercrombie method (Abercrombie, 1946).

Immunohistochemistry and Histology

For NCAM immunohistochemistry, mice were deeply anesthetized with Somnotol (240 mg/ml) and perfused with 4% paraformaldehyde in phosphate buffer saline (PFA-PBS) solution. The tibial nerve or soleus muscle was dissected out, postfixed for 1 hour in 4% PFA-PBS at 4°C, immersed in 1 part 20% sucrose in PBS and 2 part OCT compound mixture and then rapidly frozen in 2-methylbutane (i.e. isopentane) cooled by dry ice. The tibial nerves and soleus muscles were sectioned with a cryostat at 14 and 30 μm respectively. Slides with tissue sections were dried overnight at 37°C on a slide warmer, then incubated with anti-NCAM IgG primary antibody (1:200; MAB310;
Chemicon, Temecula, CA) overnight at 4°C, washed several times in PBS, incubated with Alexa Fluor 488 Goat anti-Mouse IgG (1:500; A11001; Invitrogen) for 1 hour at room temperature, washed several times in PBS, and finally mounted in 50% glycerol/PBS mixture containing 0.03 mg/ml ρ-phenylenediamine to prevent fading. In order to label acetylcholine receptors some muscle sections were also incubated with rhodamine-conjugated α-bungarotoxin (1:100; Invitrogen), which was applied simultaneously with the secondary antibody.

Fast and slow myosin immunohistochemistry was performed on soleus muscles that were fresh frozen. Unfixed muscles were pinned out on cork at their physiological length, immersed in 1 part 20% sucrose in PBS and 2 part OCT compound mixture and then rapidly frozen in isopentane cooled with dry ice. Muscles were sectioned at 30 μm on a cryostat, dried overnight at 37°C on a slide warmer, incubated with anti-slow myosin IgA primary antibody (1:10; S58; Developmental Studies Hybridoma Bank, Iowa City, IA) overnight at 4°C, and washed several times in PBS. Slides with muscle sections were then postfixed for 15 minutes in 4% PFA-PBS, washed several times in PBS, incubated with anti-fast myosin IgG primary antibody (1:500; M4276; Sigma Aldrich, Oakville, ON, Canada) at room temperature for 1 hour, washed several times in PBS, incubated with Alexa Fluor 546 Goat anti-Mouse IgG (1:500; A11003; Invitrogen) and Fluorescein-conjugated Goat anti-Mouse IgA (1:500; 55492; Cappel, Aurora, OH) secondary antibodies for 1 hour at room temperature, washed several times in PBS, and finally mounted in 50% glycerol/PBS mixture containing 0.03 mg/ml ρ-phenylenediamine to prevent fading. All fluorescently labelled tissues were photographed with a Hamamatsu
camera (C4742; Hamamatsu Photonics, Hamamatsu, Japan) using IPLab acquisition software (Version 4.0; BD Biosciences, Rockville, MD).

Hematoxylin and Eosin (H & E) staining was performed on fresh frozen soleus muscles cross sections cut on a cryostat as described for fast and slow myosin immunohistochemistry.

**Statistical Analysis**

For quantified data the mean values were reported ± standard error of the mean (SEM) throughout. One-way ANOVAs were used to make comparisons of WT or NCAM -/- results across multiple time points. If the F-value exceeded F-critical then Bonferroni’s post hoc test was used to determine where the reinnervated muscles differed significantly relative to their unoperated controls (α = 0.05/n; Holm, 1979). Unpaired student’s t-tests were used to make comparisons between time matched WT and NCAM -/- data points (α = 0.05).
Results

Alterations in NCAM Expression on Muscle and Nerve Following Nerve Crush

It is well established that NCAM is highly expressed along the entire length of developing muscle fibers and motor nerves prior to synapse formation (Covault and Sanes, 1985; Nieke and Schachner, 1985; Covault and Sanes, 1986; Martini and Schachner, 1986; Sanes et al., 1986; Tosney et al., 1986). NCAM is later down-regulated on both after synaptogenesis, except at the NMJ where it remains highly expressed pre- and postsynaptically throughout adult life (Rieger et al., 1985; Covault and Sanes, 1986). After nerve injury, NCAM is dramatically up-regulated along denervated muscle fibers in the diaphragm, gastrocnemius, soleus and extensor digitorum longus muscles (Covault and Sanes, 1985; Rieger et al., 1985; Daniloff et al., 1986; Sanes et al., 1986) and regeneration motor axons in the sciatic nerve (Daniloff et al., 1986; Martini and Schachner, 1988) until the synapses are reestablished when it is again down-regulated extra-synaptically (Rieger et al., 1985; Covault and Sanes, 1986; Daniloff et al., 1986). Interestingly, while the temporal and spatial expression patterns of NCAM have been known for over two decades, its function during neuromuscular development, regeneration and synaptogenesis remains largely unknown.

Before examining NCAM function during synaptogenesis we first characterized its expression in soleus muscles and nerves, before and after peripheral nerve injury. Acetylcholine receptors at the NMJs were identified as pretzel shaped structures in longitudinal sections of unoperated soleus muscle fibers using rhodamine conjugated α-bungarotoxin (Fig. 4.1A). As expected, the same sections immunolabelled for NCAM showed that it was highly expressed at the NMJ. Furthermore, its pattern of expression
Figure 4.1  NCAM expression at the neuromuscular junction, on denervated muscle fibers, and on regenerating nerve.  A, Control motor endplates from soleus muscle visualized with rhodamine-conjugated α- bungarotoxin.  B, NCAM immunostaining of the motor endplates from A demonstrate its highly localized expression at the neuromuscular junction in control muscle.  C-D, NCAM immunostaining of muscle cut in cross section show that in control soleus muscle (C) there is low levels of expression, but 1 week after tibial nerve crush (D) NCAM is widely up-regulated on the surface of denervated muscle fibers.  E-F, NCAM immunostaining of nerve cut in cross section shows that in control tibial nerve (E) there is only small amount of NCAM, but 1 week after tibial nerve crush (G) there is an up-regulation of NCAM around the injured axons.
closely resembled the distribution of acetylcholine receptors (compare Figs. 4.1A and 4.1B). NCAM was absent, or weakly expressed, at extra-synaptic sites in unoperated soleus muscles (Fig. 4.1B).

NCAM immunolabelling was dramatically up-regulated along the entire length of denervated soleus muscle fibers 1 week after the tibial nerve was crushed. This up-regulation is clearly demonstrated in soleus muscle cross-sections where NCAM is undetectable in unoperated soleus muscles (Fig. 4.1C), but is highly expressed at, or near the cell membrane in denervated soleus muscle fibers (Fig. 4.1D). Similarly, the vast majority of axons in the unoperated tibial nerve do not express NCAM (Fig. 4.1E). Those axons expressing NCAM are likely cutaneous axons because previous studies have shown that cutaneous axons in the femoral nerve do not down-regulate NCAM expression after birth (Saito et al., 2005). While the number of intensely stained axons does not noticeably increase 1 week after the tibial nerve was crushed, the overall expression of NCAM is up-regulated on all axons distal to the injury site (Fig. 4.1G). As observed previously (Daniloff et al., 1986; Martini and Schachner, 1988), the highest level of NCAM expression appears to be located on the surface of the regenerating axons. Taken together, these results are in agreement with several previously published studies (Rieger et al., 1985; Covault and Sanes, 1986; Daniloff et al., 1986; Sanes et al., 1986; Martini and Schachner, 1988) showing that NCAM is expressed at the NMJ in unoperated soleus muscles and is re-expressed by denervated muscles fibers and regenerating axons after peripheral nerve injury.
Figure 4.2  **Isometric force of the reinnervated soleus muscle.** Maximum isometric force was measured in response to a 50 Hz, 0.5 s train of stimuli applied to the soleus muscle nerve. A, Traces show no difference in the ability of control WT and NCAM -/- soleus muscles to generate force. B, Traces indicate that 1 month after tibial nerve injury the WT and NCAM -/- soleus muscles have recovered to control force levels. C & D, Three and 6 months after tibial nerve injury traces illustrate that the reinnervated soleus muscle of NCAM -/- mice have a reduced force generating capacity while reinnervated WT mice remain normal. E, The mean ± SEM muscle tension (mN) that was generated by WT (gray bars) and NCAM -/- (white bars) soleus muscles at 1, 3, and 6 months after tibial nerve crush as compared to unoperated controls. **p< 0.01, WT compared with corresponding NCAM -/-; #p< 0.017, as compared to its control.
Functional Assessment of Muscle Reinnervation

The persistent expression of NCAM at the NMJ and its extensive up-regulation after nerve injury suggests that it plays some role in synapse formation/function and neuromuscular reinnervation (Fig. 4.1; Covault and Sanes, 1985; Rieger et al., 1985; Covault and Sanes, 1986; Daniloff et al., 1986; Sanes et al., 1986; Martini and Schachner, 1988). However, NCAM is clearly not indispensable for neuromuscular development or regeneration because NCAM -/- mice are viable (Cremer et al., 1994) and regeneration of motor axons occurs in mice lacking NCAM (Moscoso et al., 1998; Franz et al., 2005). Nevertheless, Landmesser and colleagues have clearly shown that neurotransmitter release at the NMJ is abnormal in NCAM -/- mice (Rafuse et al., 2000; Polo-Parada et al., 2001), which results in atypical locomotor behavior (Polo-Parada et al., 2001).

Consequently, while the loss of NCAM can be compensated for during neuromuscular development and regeneration, it clearly has a function in the establishment, function and/or maintenance of the NMJ.

In order to study the functional role of NCAM in synapse formation, function and maintenance we chose to examine synaptogenesis at the NMJ after peripheral nerve injury. The rationale for studying synaptogenesis in reinnervated rather than developing muscles was based on the hypothesis that compensation by differentially expressed molecules is less pronounced in the adult after injury. In addition, parameters of synaptic function are more easily recorded in adult muscles. The regeneration model we chose was the reinnervation of the mouse soleus muscle (WT and NCAM -/-) after a tibial nerve crush injury. Nerve crush was chosen over a cut injury because crushed axons rapidly regenerate across the lesion site, which decreases the amount of time the muscle
fibers are denervated (Brushart et al., 2002), crushed motor axons reinnervate their original NMJs (Nguyen et al., 2002), and a previous regeneration study using NCAM -/- mice used a crush injury (Moscoso et al., 1998). The soleus muscle was chosen because normal anatomical and physiological reinnervation of this muscle has been extensively characterized in previous studies (Badke et al., 1989; Desypris and Parry, 1990; Bishop and Milton, 1997), it has a relatively small number of motor units (Lewis et al., 1982), and unlike the vast majority of mouse muscles, it has a substantial population of both fast and slow muscle fibers (Dribin and Simpson, 1977; Lewis et al., 1982; Wigston and English, 1992). The latter makes the soleus muscle amendable for evaluating fiber type grouping; a muscle pathology characterized by the abnormal grouping of fibers of the same physiological class (Kugelberg et al., 1970).

Synaptic function was initially screened by comparing recovery of isometric tetanic tension 1-6 months after tibial nerve crush in WT and NCAM -/- mice. The contraction profiles (Fig. 4.2A) and mean ± SEM isometric tension, elicited by a 50 Hz, 0.5 s train of pulses, from unoperated WT (79.2 ± 3.6 mN) and NCAM -/- (84.3 ± 16.5 mN) soleus muscles were not different (Fig. 4-2A,E) indicating that neuromuscular transmission and excitation-contraction (E-C) coupling is not severely compromised in NCAM -/- mice at this stimulation rate (see also Rafuse et al., 2000). Tetanic contractions (Fig. 4.2B) and isometric tensions (Fig. 4.2B) from WT (76.6 ± 11.0 mN) and NCAM -/- (64.5 ± 6.4 mN) muscles were not significantly different from each other or from their respective controls (Fig. 4.2A,B,E) 1 month after tibial nerve crush indicating that the motor axons had regenerated from the injury site and successfully reestablished normal synaptic function at the NMJ. Surprisingly, 3 months after tibial
Figure 4.3  Soleus muscle EMG amplitude following tibial nerve crush. A & B, Examples of EMG recorded from the soleus muscle during a 50Hz, 0.5 sec train of stimuli applied to its nerve. The comparison of traces from 3 month reinnervated WT (A) and NCAM -/- (B) soleus muscles reveal that 3 month reinnervated NCAM -/- mice elicit greatly diminished EMG amplitudes. C, The mean ± SEM peak to peak EMG (mV) that was recorded from the soleus muscle of control, 1, 3, and 6 month WT (gray bars) and NCAM -/- (white bars) during a maximal twitch. **p< 0.01, WT compared with corresponding NCAM -/-; *p< 0.05, WT compared with corresponding NCAM -/-.
nerve crush the NCAM -/- soleus muscle exhibited reduced isometric tension (36.1 ± 7.8 mN) compared to both 3 month reinnervated WT (91.2 ± 9.4 mN) (p<0.001) and NCAM -/- control (p=0.006) muscles (Fig. 4.2A,C,E). The reduction in strength of the NCAM -/- mice was progressive, because 6 months after injury the reinnervated NCAM -/- muscle produced even less isometric tension (16.2 ± 3.6 mN) than its 3 month reinnervated (p=0.028) and control (p<0.001) time points (Fig. 4.2A,C-E). The 6 month reinnervated WT (96.5 ± 12.2 mN) also produced significantly more tension than the 6 month reinnervated NCAM -/- muscle (p<0.001), but it did not differ from any of the other WT conditions. Overall, these findings show that axon regeneration and reformation of the NMJ is functionally normal in mice lacking NCAM (Fig. 4.2A,B,E; Moscoso et al., 1998; Franz et al., 2005). However, the capacity to maintain normal contractile force is severely compromised in NCAM -/- mice (Fig. 4.2C,D,E).

EMGs were recorded concurrently with isometric tension measurements to assess whether decreased force in the NCAM -/- mice was due to a neuropathic or myopathic condition. Surface EMGs are comprised of the sum of motor unit action potentials trains from all active muscle fibers within the electrode recording range. Thus, while the shape of the EMG wave is due to many factors, its peak to peak amplitude is proportional to the number of active muscle fibers (Henneman and Olson, 1965; Kosarov, 1974; Milner-Brown and Stein, 1975) and it varies as the square root of force in both normal and reinnervated muscles (Totosy de Zepetnek et al., 1991). Small EMG amplitudes generally indicate the presence of fewer contracting muscle fibers. Decreased force in the absence of small, or attenuating, EMG signals indicates contraction coupling defects or a diminished metabolic capacity of the muscle fibers to generate force. Finally,
fluctuations in EMG amplitudes during repetitive stimulation indicate possible neurotransmission defects (Polo-Parada et al., 2001; Polo-Parada et al., 2004).

Figure 4.3 shows two representative EMG recordings from WT (Fig. 4.3A) and NCAM -/- (Fig. 4.3B) soleus muscles during a 0.5 second stimulus train at 50 Hz, 3 months after nerve injury. The amplitude of the EMG was markedly diminished throughout the stimulus train in the NCAM -/- mice compared to those recorded from WT muscles. Interestingly, the amplitude of the EMG did not decrease or fluctuate during the train indicating that neurotransmission, contraction coupling and metabolic activity in the NCAM -/- muscles are not severely compromised at this rate of activity. The mean ± SEM peak to peak EMG amplitudes were plotted for unoperated and reinnervated WT and NCAM -/- soleus muscles 1, 3 and 6 months after nerve transection (Fig. 4.3C). In agreement with the isometric tension results (Fig. 4.2E), NCAM -/- values were significantly reduced at 3 (p=0.005) and 6 (p=0.037) months reinnervation as compared to their corresponding WT time point.

NCAM -/- mice are unable to maintain locomotion when placed on a rotor rod rotating at 20 revolutions per minute (Polo-Parada et al., 2001). Such locomotor deficits are compatible with the inability of NCAM -/- mice to maintain neuromuscular transmission at high stimulus rates or an inability to generate force due to neuromuscular fatigue. To assess neuromuscular fatigue we conducted fatigue test on unoperated and reinnervated WT and NCAM -/- muscles (see Methods for details; Burke et al., 1973). The mouse soleus muscle is composed of slow and fast fatigue resistant muscle fibers and is thus resistant to fatigue (Clarke et al., 1995; Brotto et al., 2002). Isometric force recordings from reinnervated WT and NCAM -/- soleus muscle, 3 months after nerve
Figure 4.4  Fatigability of the reinnervated soleus muscle. A, The force of repetitive maximal isometric contractions were recorded during fatiguing stimulation (13 pulses at 40Hz every second for 2 min). The top trace is from a 3 month reinnervated WT soleus muscle and the bottom trace is from a 3 month reinnervated NCAM +/- soleus muscle. B-C, Despite the discrepancy in absolute force values, the relative extent of fatigue did not differ for WT and NCAM +/- soleus muscle as determined by normalizing contraction force to the first contraction of the fatigue test (i.e. Fatigue Index = F_i/F_0).

The mean ± SEM of the fatigue indices at 1 (B) or 2 (C) min for WT (gray bars) and NCAM +/- (white bars) soleus muscles 1, 3, and 6 months after tibial nerve crush as well as for their unoperated controls.
injury (Fig. 4.4A), showed that the relative decline in muscle force during the fatigue test was similar in both WT and NCAM -/- muscles. The mean ± SEM fatigue index (see Methods) at 1 and 2 minutes were compared for unoperated WT and NCAM -/- soleus muscles and reinnervated soleus muscles, 1, 3 and 6 months after nerve injury (Fig. 4.4B,C). No significant differences were observed between any of these conditions. Taken together, these results indicate that the decreased force generating capacity of reinnervated soleus NCAM -/- muscle, 3 months after nerve injury, is not due to abnormal contraction coupling, metabolic capacity or transmission failure, but it is likely due to a significant reduction in functional muscle fibers.

The Number of Motor Neurons That Reinnervated the Soleus Muscle

The dramatic reduction in isometric force 3 months after nerve injury (Fig. 4.2) suggests that NCAM plays a critical role in maintaining reinnervated NMJs. The withdrawal of synapses over time could result in fewer reinnervated motor units (i.e. all reinnervated synapses from single motor neurons are withdrawn) and/or smaller motor units (i.e. reinnervated synapses are randomly withdrawn). To distinguish between these two possibilities we injected rhodamine conjugated CTB into unoperated (control) and reinnervated soleus muscles to backlabel all of its innervating motor neurons. Figure 4.5 shows that there was no difference between the total number of soleus motor neurons (alpha and gamma) in unoperated (control) WT (38.5 ± 2.0) and NCAM -/- (39.3 ± 3.4) mice). These results agree with previous findings showing that NCAM -/- mice do not have fewer motor neurons than aged matched WT mice (Franz et al., 2005). The number of motor units in unoperated (control) and reinnervated soleus muscles, 3 months after
Figure 4.5  The number of motor neurons that reinnervated the soleus muscle.

The mean ± SEM number of motor neurons that were backlabelled and counted after injection of CTB into the soleus muscle of WT (gray bars) and NCAM +/- (white bars) mice 3 months after tibial nerve crush and in unoperated controls.
nerve injury, were not significantly different in WT (36.7 ± 2.5) and NCAM-/- (42.1 ± 1.6) mice (Fig. 4.5). These results indicate that the decrease in force over time was not due to the loss of motor units, but rather resulted from the gradual decrease in size of the reinnervated motor units.

**Morphological Analysis of the Reinnervated Soleus Muscle**

The two major determinants of muscle force are muscle fiber number and cross-sectional area (Kernell, 2006). Consequently, the decrease in force over time in reinnervated NCAM -/- mice could be due to the loss of muscle fibers and/or muscle fiber atrophy. To distinguish between these two possibilities we cut the soleus muscle immunolabelled the sections with antibodies that selectively recognize fast myosin heavy chain. Figure 4.6A shows fast myosin immunostaining of representative sections through the midbelly of the soleus muscle from unoperated (control) and reinnervated WT mice 1, 3, and 6 months after nerve injury. The gross morphology of the muscle (Fig. 4.6A; Control) was altered after tibial nerve crush and the total cross-sectional area appeared diminished 1 month after injury (Fig. 4.6A; 1 Month). However, the size of the reinnervated muscle largely recovered 3 and 6 months after nerve injury (Fig. 4.6A; 3 and 6 Month). The overall proportion and distribution of fast (stained) and slow (unstained) fibers were similar across all WT conditions, with the fast type being noticeably more prevalent than the slow (Fig. 4.6A). As expected from a nerve crush injury, there was no evidence of fiber-type grouping. Figure 4.6B shows fast myosin immunostaining of representative sections through the midbelly of the soleus muscle from unoperated (Control), 1, 3 and 6 month reinnervated NCAM -/- mice. The size of the soleus muscle
Figure 4.6  Anatomical analysis of the reinnervated soleus muscle. Muscle cross sections were taken through the largest region of the soleus muscle and immunolabelled for fast (shown) and slow myosin in order to characterize muscle morphology during the recovery from tibial nerve crush. A-B, Representative cross sections taken from a control, 1, 3, and 6 month reinnervated WT (A) and NCAM -/- (B) soleus muscle stained for fast myosin. C, The mean ± SEM total muscle area of control, 1, 3, and 6 month reinnervated WT (gray bars) and NCAM -/- (white bars) soleus muscles. D, The mean ± SEM fiber area of control, 1, 3, and 6 month reinnervated WT and NCAM -/- soleus muscles. E, The mean ± SEM number of slow muscle fibers in control, 1, 3, and 6 month reinnervated WT and NCAM -/- soleus muscles. F, The mean ± SEM number of fast muscle fibers in control, 1, 3, and 6 month reinnervated WT and NCAM -/- soleus muscles. **p< 0.01, WT compared to corresponding NCAM -/-; #p< 0.017, As compared to corresponding control.
in NCAM -/- and WT mice (Figs. 46A,B; Control), and the distribution of fiber types, were very similar in unoperated (control). Differences were also not apparent between reinnervated WT and NCAM -/- soleus muscles 1 month after injury (Fig. 4.6A,B; 1 Month). However, the reinnervated NCAM -/- muscle appeared smaller than its control (Fig. 4.6B; Control and 3 Month) and reinnervated WT muscle (Fig. 4.6A,B; 3 Month) 3 months after nerve injury. The reinnervated NCAM -/- muscle also had an apparent reduction in the number of fast muscle fibers to the point where the majority of its fibers were no longer immunopositive for fast myosin (Fig. 4.6B; 3 Month). A further reduction in size and number of fast muscle fibers occurred in the NCAM -/- muscles 6 months after nerve injury (Fig. 4.6B). Overall, the decreased NCAM -/- muscle size 3 and 6 months after nerve injury correlates well with the dramatic reductions in isometric force and EMG observed at these time points (Fig. 5.2C-E).

In order to objectively compare differences between the unoperated and reinnervated WT and NCAM -/- soleus muscles we quantified total muscle cross-sectional area (Fig. 6.C), mean muscle fiber cross-sectional area (Fig. 6.D), and numbers of slow (Fig. 6.E) and fast muscle fibers (Fig. 6.F). ANOVA analysis indicated that there were no statistical differences in the total muscle cross-sectional areas between unoperated (control) WT soleus muscles (mean ± SEM; 1.04 ± 0.09 mm²) and reinnervated WT muscles 1 (0.74 ± 0.06 mm²), 3 (0.86 ± 0.13 mm²) and 6 months (0.85 ± 0.08 mm²) after nerve injury. This was congruent with our finding that soleus muscle isometric tension was not significantly less than its unoperated control 1-6 months after nerve injury (Fig. 6.2), and agreed with previously reported adult mouse soleus muscle area (Brooks and Faulkner, 1988; Wang and Kernell, 2001). In contrast, ANOVA
analysis indicated significant differences (p<0.001) in total muscle cross-sectional areas existed between the NCAM -/- unoperated control (mean ± SEM; 0.93 ± 0.07 mm²) and reinnervated muscles 1 month (0.72 ± 0.06 mm²), 3 month (0.60 ± 0.07 mm²) and 6 month (0.36 ± 0.05 mm²) after nerve injury. Post hoc analysis (α=0.017; see Methods for details) revealed significant decreases from control muscle cross-sectional area 3 (p=0.009; #) and 6 months (p=0.001; #) after nerve injury. The 6 month reinnervated NCAM -/- muscle was also significantly smaller than its corresponding WT time point (p=0.008; **). Therefore, decreased total muscle cross-sectional area is a major contributor to the progressive loss of force after neuromuscular reinnervation in NCAM -/- mice (Fig. 6.2).

The average muscle fiber cross-sectional area was estimated based by dividing the total muscle cross-sectional area by the total number of muscle fibers in the cross-section (see below). ANOVA analysis indicated that there was no significant differences in muscle fiber cross-sectional areas between unoperated control WT soleus muscles (mean ± SEM; 1276.7 ± 120.4 μm²) and reinnervated WT muscles 1 (909.4 ± 76.6 μm²), 3 (1002.6 ± 134.1 μm²) and 6 months (1039.7 ± 104.3 μm²) after nerve injury. In contrast, ANOVA analysis indicated that there were significant differences (p=0.047) in muscle fiber cross-sectional areas between unoperated control NCAM -/- soleus muscles (1172.0 ± 100.5 μm²) and reinnervated muscles 1 (955.2 ± 107.0 μm²), 3 (881.6 ± 133.7 μm²) and 6 months (786.8 ± 50.8 μm²) after nerve injury. Post hoc analysis (α=0.017) revealed a significant decrease from control muscle fiber cross-sectional area for 6 month reinnervated (p=0.016; #), but not muscles reinnervated 1 or 3 months after the tibial nerve was crushed. However, comparisons made between WT and NCAM -/- muscles at
corresponding time points yielded no significant differences, which suggests that changes in muscle fiber cross-sectional area are not responsible for the large differences in isometric tension (Fig. 4.2; 3 and 6 Month) or total muscle area (Fig. 4.6; 6 Month).

The total number of muscle fibers in WT mice did not vary significantly between unoperated control (mean ± SEM; 823 ± 61) soleus muscles and reinnervated muscles 1 (819 ± 78), 3 (903 ± 71) and 6 months (825 ± 88) after nerve injury. These values corresponded well with previously published findings (Desypris and Parry, 1990; Irintchev et al., 2002). However, as expected, the number of soleus muscle fibers in the NCAM -/- mice were significantly different between unoperated control (793 ± 35) and reinnervated muscles 1 (757 ± 18), 3 (651 ± 50) and 6 months (459 ± 39) after peripheral nerve injury (ANOVA; p<0.001). Post hoc analysis (α=0.017) of the total number of muscle fibers in NCAM -/- mice found no significant change from control values 1 month after nerve injury, but there were significantly fewer fibers after 3 (p=0.048) and 6 months (p<0.001).

In contrast to the distribution and number of fast and slow fibers in reinnervated WT muscles (Fig. 4.6A), the number of fast muscle fibers appears to gradually decrease 3 months after nerve injury in NCAM -/- mice (Fig. 4.6B). Indeed, quantification showed that there were no significant changes in the number of slow and fast fibers (Fig. 4.6E,F) between unoperated WT control muscles (mean ± SEM; 278 ± 18 and 544 ± 54; slow and fast, respectively) and reinnervated WT muscles1 (315 ± 14 and 475 ± 68), 3 (326 ± 33 and 576 ± 37) and 6 months (307 ± 33 and 516 ± 69) after nerve injury. Interestingly, while the number of slow fibers (Fig. 4.6E) in NCAM -/- mice varied significantly as a group (ANOVA; p=0.014), post hoc analysis (α=0.017) revealed no specific differences
Figure 4.7  H & E staining of reinnervated soleus muscle. A-B, Control muscle cross sections from WT and NCAM -/- mice. C-D, 1 month after reinnervation the appearance of WT and NCAM -/- muscle fibers was very similar. E-F, 3 months after reinnervation WT muscle fibers were similar to their control (A), but NCAM -/- muscle had signs of partial denervation including necrotic fibers filled with nuclei (black arrows) intermixed with fibers that extremely hypertrophied (asterisk).
from unoperated control values (290 ± 14) 1 (263 ± 7), 3 (399 ± 54) and 6 months (307 ± 33) after tibial nerve crush (p=0.119-0.566). In contrast, the number of fast muscle fibers (Fig. 4.6F) were in NCAM -/-. control (504 ± 22), 1 month (471 ± 10), 3 month (237 ± 36) and 6 month (186 ± 43) reinnervated muscles were highly significant (ANOVA; p<0.001). Post hoc analysis (α=0.017) showed that the number of fast muscle fibers in control and 1 month reinnervated did not differ, but that relative to control there were significant fewer fast muscle fibers 3 (p=0.002) and 6 months (p=0.004) after nerve injury in NCAM -/- muscles. Furthermore, direct comparisons between WT and NCAM -/- muscles after 3 month (p=0.001) and 6 month (p=0.016) reinnervation showed 50-60% fewer fast muscle fibers in NCAM -/- mice.

The loss of such a large proportion of fast fibers without a corresponding increase in the number of slow fibers suggests muscle fiber death. To determine if this was the case H & E staining was performed on muscle cross sections in order to further characterize reinnervated WT and NCAM -/- muscle fibers (Fig. 4.7). H & E staining was comparable between WT and NCAM -/- control muscle fibers (Fig. 4.7A,B). WT and NCAM -/- muscle fibers 1 month after tibial nerve crush appeared smaller (Fig. 4.7C,D) but otherwise similar to their unoperated controls (Fig. 4.7A). However, there were clear signs of muscle necrosis 3 months after nerve injury in NCAM -/- soleus muscles (Swash and Schwartz, 1984). Some muscle fibers were filled with nuclei indicating macrophage invasion (black arrows; Fig. 4.7F), while others were abnormally hypertrophied (asterisks; Fig. 4.7F). In contrast, 3 month reinnervated WT fibers (Fig. 4.7E) showed no evidence of necrosis (Fig. 4.7A). Overall, these results strongly indicate that the selective loss of fast muscle fibers 3 months after reinnervation in NCAM -/-
muscle (Figs. 4.6F) is likely due to necrosis (Fig. 4.7F) and appears to account for most, if not all of the corresponding reduction in muscle strength (Fig. 4.2).

The selective loss of fast muscle fibers (Fig. 4.6F) occurs despite the fact that the total number of motor neurons reinnervating the soleus muscle remains the same in WT and NCAM -/- mice (Fig. 4.5). It is unknown whether this strictly represented a reduction in the size of some or all fast motor units, or if there was any inter-conversion of muscle fiber types after neuromuscular reinnervation (Desypris and Parry, 1990). To gain insight into the possibility that muscle fiber types might be converted after reinnervation we quantified the incidence of fast and slow myosin double immunolabelled fibers. In adult mice, less than 1% of muscle fibers express both myosin types (Wigston and English, 1992). In contrast, during the first week of postnatal development approximately 15% of mouse soleus muscle fibers stain positive for both fast and slow myosin, but this decreases to less than 5% by the second week (Wigston and English, 1992) and at least in part seems to reflect the withdrawal of polyneuronal innervation known to occur around this time (Brown et al., 1976). In agreement with that study we found that 12 week WT and NCAM -/- control muscles rarely expressed fibers double immunolabelled for fast and slow myosin (Fig. 4.8A-D; Control). However, 1 month after neuromuscular reinnervation fast and slow myosin double labelled fibers were found more frequently in WT and NCAM -/- muscle (Fig. 4.8A-D; 1 Month). The incidence of double labelled fibers was similar to control for 3 and 6 month reinnervated WT muscle (Fig. 4.8A,B; 3 and 6 month), but in 3 month reinnervated NCAM -/- muscle the occurrence of double labelled fibers was still relatively high and wasn’t reduced to control levels until 6 month reinnervation (Fig. 4.8C,D; 3 and 6 month). Figure 4.8E
Figure 4.8  Slow and fast myosin immunohistochemistry of the reinnervated soleus muscle.  A & C, Slow myosin immunohistochemistry on muscle cross sections from control, 1, 3 and 6 month reinnervated WT (A) and NCAM /-/- (C) soleus muscle.  B & D, Fast myosin immunohistochemistry on muscle cross sections from control, 1, 3 and 6 month reinnervated WT (B) and NCAM /-/- (D) soleus muscle.  Muscle fibers that were positive for fast and slow myosin are denoted by asterisks.  E, The mean ± SEM number of fast and slow double labelled muscle fibers in control, 1, 3, and 6 month reinnervated WT (gray bars) and NCAM /-/- (white bars) soleus muscles.  \( \Psi p<0.05 \) WT compared to corresponding NCAM /-/-;  \#p< 0.017, As compared to corresponding control.
shows quantification of the mean ± SEM number of fast and slow double immunolabelled fibers. For WT mice, the number of double labelled fibers is significantly greater than its control (1.8 ± 1.2) at 1 month (28.3 ± 3.3; p=0.001, #), but not 3 (1.3 ± 0.4) or 6 (2.0 ± 2.4) months, after muscle reinnervation. For NCAM -/- mice, the number of doubled labelled fibers is significantly greater than control (2.0 ± 1.2) for 1 (22.7 ±4.7; p=0.017, #) and 3 (14.8 ± 2.7; p=0.009, #) months muscle reinnervation, but not for 6 months (1.7 ± 1.5) reinnervation. Also, there were significantly more double labelled fibers in NCAM -/- as compared to WT muscle at 3 month reinnervation (p=0.012, ‡). The persistence of double labelled muscle fibers at 3 months for NCAM -/- mice corresponded with a small, but not significant, increase in the absolute number of slow fibers seen at this time point (Fig. 4.6E), and might have represented an initial attempt to compensate for the loss of fast fibers (Fig. 4.6F). These results demonstrate that fiber type inter-conversion occurs, albeit to a relatively small proportion of fibers at a given time (i.e. <3%), after neuromuscular reinnervation in WT and NCAM -/- muscle; however, in NCAM -/- mice it occurs over an extended duration.

**The Influence of Age on the Recovery of Isometric Tension**

The most severe deficits in neuromuscular reinnervation in NCAM -/- mice aren't apparent until 3 and 6 months after tibial nerve crush (i.e. ~6-9 months in age; Figs. 4.2, 4.6). Interestingly, the expression of NCAM is known to be increased on muscle and nerve during the normal aging process (Kobayashi et al., 1992; Andersson et al., 1993). Thus, to what extent an impaired aging process can account for the inability of NCAM -/- mice to maintain reinnervated neuromuscular connections is not known. To determine
this we analyzed the recovery of isometric tension after 1 month neuromuscular reinnervation in aged (i.e. 9-12 months in age) WT and NCAM -/- soleus muscles (Fig. 4.9). If aging alone could account for the deficits at 3 and 6 month reinnervation in young adult NCAM -/- mice (Fig. 4.2 C-E) we would have expected to see comparable deficits present at 1 month reinnervation in aged NCAM -/- mice. The mean ± SEM isometric tension for aged control WT (85.6 ± 24.6 mN) and NCAM -/- (75.6 ± 10.7 mN) mice were not statistically different. Following 1 month recovery from tibial nerve crush the mean ± SEM isometric tension for aged WT (77.27 ± 5.6 mN) and NCAM -/- (75.44 ± 18.1 mN) also did not significantly differ. Therefore, the loss of force generating capacity at 3 and 6 months after neuromuscular reinnervation in NCAM -/- mice is not directly related to the aging process.
Figure 4.9  The recovery of isometric force 1 month after tibial crush in aged mice. The mean ± SEM muscle tension (mN) that was generated by WT (gray bars) and NCAM -/- (white bars) control and 1 month reinnervated soleus muscles.
Discussion

Based on its abundant expression on regenerating nerves (Fig. 4.1E,F; Daniloff et al., 1986; Martini and Schachner, 1988) and denervated muscle fibers (Fig. IC,D; Covault and Sanes, 1985; Sanes et al., 1986; Rieger et al., 1988), NCAM has long been assumed to play an important role in neuromuscular reinnervation (Walsh and Doherty, 1996). However, previous studies have provided evidence to both support (Rieger et al., 1988; Remsen et al., 1990; Langenfeld-Oster et al., 1994) and refute this assumption (Moscoso et al., 1998). In the present study, we uncovered striking deficits following neuromuscular reinnervation in the absence of NCAM. Although there were no apparent discrepancies between WT and NCAM -/- soleus muscle 1 month after nerve crush, by 3 and 6 months the size and strength of NCAM -/- muscle was dramatically reduced (Figs. 4.6,4.2). This reduction in muscle size and strength was primarily attributable to a loss of fast muscle fibers (Fig. 4.6B,F). These findings demonstrate for the first time that vertebrate NCAM has a critical role in the maintenance of NMJs.

Role of NCAM: Axon Regeneration versus Synaptic Maintenance

The discrepancy between the loss of function studies that either supported or refuted an essential role for NCAM in motor axon regeneration can largely be accounted for by their method of perturbation. Studies that used NCAM antibodies to interfere with motor axon regeneration found functional (Remsen et al., 1990) and anatomical (Langenfeld-Oster et al., 1994) evidence of delayed endplate reinnervation in mice, as well as incomplete myelination of axons, absence of many terminal Schwann cells
capping the NMJ and ectopically located synapses in frog (Rieger et al., 1988). In contrast, the one study that specifically examined neuromuscular reinnervation in mice where NCAM was genetically ablated (i.e. NCAM -/-) found no evidence of delayed endplate reinnervation or absence of terminal Schwann cells capping the NMJ (Moscoso et al., 1998). Recently, we examined the ability of cut motor axons in NCAM -/- mice to preferentially reinnervate a muscle versus cutaneous nerve pathway given equal access to both and noted that even though the pathway selection was impaired, the rate of regeneration did not differ between WT and NCAM -/- motor axons (see Fig. 2.3; Franz et al., 2005). In agreement, the present study also found no differences between WT and NCAM -/- mice in the rate of initial recovery of isometric force (Fig. 4.2B,E) and EMG (Fig. 4.3C) 1 month after tibial nerve crush. Taken together, these findings indicate that function-blocking antibodies are likely more effective than genetic ablation at disturbing neuromuscular reinnervation. This is probably due to the rapid action of function-blocking antibodies, which may not provide sufficient opportunity for compensatory changes in gene expression that might obscure NCAM's function following genetic ablation (Moscoso et al., 1998). However, the fact that regenerating NCAM -/- motor axons can re-grow at a normal rate and initially reform functional NMJs demonstrates that axon regeneration per se can occur in the absence of NCAM (Moscoso et al., 1998; Franz et al., 2005).

Surprisingly, despite the fact that the development of NMJs are clearly delayed (Moscoso et al., 1998) in NCAM -/- mice, no previous studies have examined the function of NCAM at reinnervated synapses. Since it has been found that the maturation of murine muscle neurotransmission after tibial nerve transection requires at least 2
months (Badke et al., 1989; Desypris and Parry, 1990; Remsen et al., 1990), the striking
decline in NCAM -/- muscle tension (Fig. 4.2) and EMG (Fig. 4.3) that emerged 1 to 6
months after nerve injury pointed to a deficiency in maintenance, rather than reformation,
of NMJs. Furthermore, the loss of muscle function in reinnervated NCAM -/- muscle
occurred in a progressive fashion with an approximately 50% decrease in force (Fig.
4.2C,E) and EMG (Fig. 4.3B,C) at 3 months, which increased to a greater than 80%
decline in force (Fig. 4.2D,E) and EMG (Fig. 4.3C) by 6 months. In stark contrast, there
were no physiological differences detected between reinnervated WT mice and their
unoperated controls at this time points. Therefore, the long-term deficits in muscle
function after nerve crush in NCAM -/- mice appear to be due to their inability to
maintain reinnervated NMJs.

To increase our insight into the possible mechanisms underlying the inability of
reinnervated NCAM -/- NMJ to be maintained, we continued on with a more in depth
comparison of physiology and anatomy following muscle reinnervation in WT and
NCAM -/- mice. For example, fatigue tests and motor neuron backlabelling experiments
were performed because it is known that disorders involving the ongoing death of motor
neurons (e.g. postpolio syndrome, ALS) lead to muscle weakness and fatigability
(Thomas and Zijdewind, 2006). The results of these experiments gave no indications that
the significantly weakened NCAM -/- muscles at 3 and 6 months reinnervation were any
less fatigue resistant (Fig. 4.4) or had fewer motor neurons supplying them (Fig. 4.5) than
controls. However, morphometric analysis of cross sections taken from reinnervated WT
and NCAM -/- muscles revealed a progressive decrease in muscle area by approximately
35% at 3 months and 60% at 6 months (Fig. 4.6A-C). These decreases in muscle area
agreed well with the corresponding decreases in NCAM−/− muscle isometric tension (Fig. 4.2C-E) and EMG (Fig. 4.3) at these time points; in particular when you consider that the area of the non-contractile elements of the muscle probably remained constant (Thomason et al., 1987).

The reduction in NCAM−/− muscle area at 3 and 6 months reinnervation were due to a loss of muscle fibers rather than muscle atrophy. The average fiber area of WT and NCAM−/− muscles tended to be slightly decreased at all time points post-nerve crush (Fig. 4.6D), but only at 6 months reinnervation in NCAM−/− mice was one of these decreases significantly less than its control. It should also be noted that at no time point did the average muscle fiber area differ significantly between WT and NCAM−/− mice. On the other hand, the number of muscle fibers did decrease, but interestingly the number of slow fibers remained relatively constant throughout all conditions (Fig. 4.6E) and it was a selective loss of more than 60% of the fast fibers by 6 months (Fig. 4.6F) that appeared to be the major reason for the diminished muscle area (Fig. 4.6) and strength (Fig. 4.2) after neuromuscular reinnervation in NCAM−/− mice. H & E staining revealed that the reduction in the number of fast fibers in NCAM−/− was most likely due to necrosis (Fig. 4.7), although there may have been some inter-conversion from fast to slow fiber type (Fig. 4.8). Furthermore, the observation that aged WT and NCAM−/− mice were both able to recover normal levels of muscle force 1 month after nerve crush indicates the inability of adult NCAM−/− muscles to maintain reinnervated NMJs is not simply a consequence of a defective aging process (Fig. 4.9), but rather a specific consequence of muscle reinnervation.
The specificity of the fiber loss for fast versus slow fibers at 3 and 6 months reinnervation suggested that fast motor units were either exclusively affected or relatively more compromised in NCAM -/- muscle. We favour the latter possibility based on several lines of evidence. First, other studies of peripheral nerve injuries and motor neuron disease support the hypothesis that slow motor units are more resilient to trauma than fast motor units (Lowrie and Vrbova, 1984; Desypris and Parry, 1990; Pun et al., 2006). Second, the population of slow fibers in unoperated adult WT and NCAM -/- soleus muscle makes up about 35% of the total population (Fig. 4.6; Wigston and English, 1992), and although the same absolute number of slow fibers is present 6 months after tibial nerve crush, along with about one third of the original number of fast fibers, the reinnervated NCAM -/- muscle produces less than 20% of its original isometric tension. This suggests that at least some of the slow motor units present 6 months after reinnervation in NCAM -/- mice were also weakened. Finally, following partially denervation or extensive peripheral nerve injury, as few as 20% of the original number of motor units can sprout and innervate all of the surrounding denervated muscle fibers (Rafuse et al., 1992; Gordon et al., 1993; Rafuse and Gordon, 1996b). In the present study, NCAM -/- slow motor neurons appear unable to form enlarged motor units (Fig. 4.6E), despite that some collateral sprouting likely occurred (Fig. 4.8). However another interpretation of the absence of slow motor unit enlargement is that reduction in fast motor unit size is secondary to the necrotic death of fast muscle fibers. That is, sprouting does not occur because the fast muscle fibers died.
Role of NCAM in Synaptic Maintenance: Motor Neuron or Muscle?

NCAM is localized pre- and postsynaptically at mature NMJs (Fig. 4.1A,B; Covault and Sanes, 1986; Sanes et al., 1986). Consequently, the inability of reinnervated NCAM -/- muscle to maintain isometric force and EMG could be a result of the absence of NCAM pre- and/or postsynaptically. Insights into the mechanism of this phenotype can be drawn from elegant work done in Drosophila where the NCAM homolog, Fasciclin II (Fas II; Schuster et al., 1996). Similar to the highly conserved developmental patterns of vertebrate NCAM expression (Sanes et al., 1986; Tosney et al., 1986; Rieger et al., 1988), Fas II is initially expressed extensively on developing muscle fibers and growing motor axons but then becomes highly localized to the NMJ after synaptogenesis (Vactor et al., 1993; Schuster et al., 1996). Like in the present study, genetic ablation of Fas II does not interrupt the initial formation of the synapse (Baines et al., 2002) rather it results in the regression of newly formed NMJs (Schuster et al., 1996). As a consequence, Fas II -/- larvae exhibit uncoordinated and sluggish motor behavior that rapidly deteriorates and ultimately results in death within hours of hatching (Schuster et al., 1996). Attempts to rescue the Fas II -/- phenotype through crosses with “enhancer detector” lines (O'Kane and Gehring, 1987) that specifically drive transgene expression in neurons, muscles, or both showed that Fas II must be expressed by neurons and muscle fibers in order to rescued the larvae from lethality (Schuster et al., 1996). Overall, these results strongly suggest that NCAM must be expressed by both muscle and nerve in order for NMJs to be maintained.

Based on the dramatic deficits found in Fas II mutants it is surprising that genetic ablation of NCAM in mice did not cause more severe deficits in neuromuscular
development (Cremer et al., 1994; Moscoso et al., 1998; Rafuse et al., 2000). Moscoso et al. (1998) speculated that this discrepancy between Fas II/NCAM loss of function experiments in Drosophila and mouse could be attributed to the greater redundancy of synaptic cell adhesion molecules (CAMs) known to be involved in vertebrate versus invertebrate synaptogenesis and maturation (Akins and Biederer, 2006). Many clear examples of gene redundancy and compensation in the murine neuromuscular system have been documented (Moscoso et al., 1998), for instance the loss of dystrophin from muscle leads to compensatory up-regulation of its homolog, utrophin, which is sufficient to prevent muscular dystrophy from occurring (Deconinck et al., 1997; Grady et al., 1997). So why is there greater compensation in the NCAM -/- mice during developmental compared to after nerve injury? The answer to this question is unclear, but one reasonable explanation would be that there is incomplete recapitulation of developmental gene expression during nerve regeneration (Vogelaar et al., 2003; Vogelaar et al., 2004). This decreased gene expression may reduce redundancy and therefore decreases the potential for other synaptic CAMs to compensate in the absence of NCAM. In support of this explanation, our previous work has shown that the projection patterns of NCAM -/- femoral motor axons develop normally (see Fig. 2.4; Franz et al., 2005) despite the well established role of its polysialylated form in motor axon guidance and patterning in embryonic chick (Landmesser et al., 1988; Tang et al., 1992; Tang et al., 1994; Rafuse et al., 1996). However, following nerve transection, NCAM -/- femoral motor axons are more likely to project to the inappropriate cutaneous pathway compared to femoral neurons in WT mice (see Fig. 2.3; Franz et al., 2005).
Whatever the case may be, the fact that the elimination of NCAM in mice is not lethal has permitted examination its role during axon regeneration (Moscoso et al., 1998; Franz et al., 2005), cell migration (Ono et al., 1994; Chazal et al., 2000), and learning (Cremer et al., 1994; Schellinck et al., 2004). The most pronounced developmental alterations at the NCAM-/- NMJ appear to be presynaptic in origin and include delayed localization of synaptic vesicles to the presynaptic terminal, an absence of paired pulse facilitation, periodic transmission failures during high frequency stimulation, and an impaired vesicle cycling mechanism apparently related to the persistence of immature L-type calcium channels (Rafuse et al., 2000; Polo-Parada et al., 2001). Interestingly, a specific deletion of the NCAM 180 isoform (Tomasiewicz et al., 1993) caused even more transmission failures during high frequency stimulation than those in the total NCAM -/- mice (Polo-Parada et al., 2004). The application of peptides which interfere with NCAM 180 function reproduced the deficits found in the NCAM 180 -/- mice. Interestingly, there were no detrimental effects when the peptide was introduced postsynaptically, indicating that NCAM 180 is required presynaptically (Polo-Parada et al., 2005). In contrast, in vitro studies on hippocampal neurons found deficiencies in NCAM -/- synaptogenesis that appeared to be postsynaptic in origin (Dityatev et al., 2000; Dityatev et al., 2004). When WT neurons were postsynaptic they received more synapses and had larger excitatory postsynaptic currents as compared to NCAM -/- neurons, regardless if the presynaptic neurons were WT or NCAM -/- (Dityatev et al., 2000). The preferential formation of synapses on WT versus NCAM -/- neurons could be abolished by enzymatic removal of polysialic acid (PSA) from NCAM, which indicates that postsynaptic expression of Polysialylated NCAM is responsible for this phenomenon (Dityatev et al.,
2004). In line with this finding, transfection of NCAM -/- neurons with any of the three major NCAM isoforms (i.e. 120, 140 and 180), all of which can be post-translationally modified to express PSA, rescued the phenotype (Dityatev et al., 2004). Overall, future experiments that take advantage of conditional genetic ablation technologies (Sternberg and Hamilton, 1981; Sauer and Henderson, 1988), will offer a more systematic approach to identify the function of pre- and postsynaptic NCAM during the development and maintenance of the vertebrate NMJ.
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CHAPTER 5: Conclusion

In comparison to axons in the CNS, axons in mammalian peripheral nerves have a strong propensity to regenerate following nerve injury (Ramon y Cajal, 1928). However, even under the most ideal circumstances, where peripheral nerves are repaired in a timely fashion with the use of advanced microsurgery techniques to re-align individual nerve fascicles, the recovery of normal function is usually incomplete (Sunderland, 1978b; Mackinnon and Dellon, 1988). This highlights two critical issues in nerve regeneration: (1) achieving robust axon regeneration in itself is not sufficient for functional recovery to occur; and (2) with the limits modern microsurgical strategies to mechanically repair nerves having been reached, novel therapeutic approaches are required to improve functional outcomes (Lundborg, 2000; Gordon et al., 2003). Based on our rapidly expanding understanding of the molecular mechanisms underlying nerve regeneration in animal models (Fu and Gordon, 1997; Makwana and Raivich, 2005), molecular based treatments will likely yield the next generation of therapeutic breakthroughs in human nerve regeneration. Therefore the purpose of the present set of experiments was twofold: (1) to increase our knowledge of the molecular mechanisms of nerve regeneration; and (2) to identify and test strategies aimed at exploiting these molecular mechanisms to improve peripheral nerve injury functional outcomes.

Since motor axons in the PNS can regenerate, the failure to achieve functional recovery must involve deficiencies in at least one of the following steps of neuromuscular reinnervation: (1) selection of an appropriate pathway in order to return to their original
muscle target; (2) reestablishment of appropriately matched nerve-muscle connections in muscle; and (3) restoration of the pre-denervation structure and physiology of the muscle (Kernell, 2006). Chapters 2 and 3 of this thesis were primarily focused on the first step of functional motor recovery, and Chapter 4 focused on the latter two steps. Considered first will be the findings of Chapter 2 and 3 where we applied a well established rodent model of nerve regeneration (Brushart, 1988, 1993), which has been shown to elicit reproducible results in several different laboratories (Madison et al., 1996; Al-Majed et al., 2000b; Franz et al., 2005; Eberhardt et al., 2006; Geremia et al., 2007), to investigate the molecular mechanisms of motor axon targeting to muscle. Our findings showed that: (i) PSA, a known developmental guidance molecule, must be re-expressed for motor axons to be selectively targeted to muscle; (ii) PSA re-expression was an intrinsic neuronal property established during development; (iii) motor axons lacking PSA, either because of intrinsically low expression or due to its removal, were incapable of being selectively targeted to muscle; (iv) electrical stimulation increases PSA levels and promotes better regeneration accuracy, but only in motor neurons capable of PSA expression; (v) removal of PSA completely abolished the improvements in regeneration accuracy after electrical stimulation; and (vi) PSA expression appears to permit accurate motor neuron regeneration by increasing the number and distribution of motor axon collateral sprouts. However, whether future treatments designed to elevate PSA levels in all regenerating motor neuron sub-types would be generally effective at promoting better regeneration accuracy is not known.

The present findings suggest that high levels of PSA expression on regenerating motor axons would act universally to improve the targeting of all types of motor neuron...
because it enhances the number and distribution of its axon collateral sprouts (see Fig. 3.7). However, enhanced sprouting alone would not improve the selectivity of axon regeneration, rather there is strong evidence to suggest that these sprouts are specifically pruned and/or guided to the muscle branch by attending to the relatively superior levels of generic trophic support emanating from the muscle pathway and/or its end organ as compared to the skin (Brushart, 1988, 1993; Robinson and Madison, 2005; Uschold et al., 2007). Furthermore, recent studies propose that the trophic support from these sources are generic for different types of neurons (i.e. motor and sensory) because surgical manipulations altering pathway length and/or availability of end organ contact can cause motor neurons to demonstrate preference for the skin as well as the muscle pathway (Robinson and Madison, 2004, 2005; Uschold et al., 2007). Therefore treatments known to enhance sprouting, such as conditioning lesions (Brushart et al., 1998) or electrical stimulation (see Fig. 3.7; Mehele and Nja, 1982; Itoh et al., 1995; Al-Majed et al., 2000b), applied in combination with surgical manipulations that appear to shift the relative balance of trophic support even more in favour of the muscle pathway, such as ligating the cutaneous pathway (Uschold et al., 2007) while leaving the muscle pathway intact, might act synergistically to yield the best possible regeneration accuracy.

In Chapter 4 we examined the role of NCAM after peripheral nerve injury in reestablishing the normal pattern of nerve-muscle connectivity within muscle, as well as in restoring the muscle structure and physiology to its pre-denervation state. NCAM was widely assumed to play a role in muscle reinnervation based on its abundant re-expression on regenerating nerve (Daniellof et al., 1986; Martini and Schachner, 1988) and denervated muscle fibers (Covault and Sanes, 1985; Sanes et al., 1986; Rieger et al.,
1988), so it was surprising that the only study to examine neuromuscular reinnervation in NCAM -/- mice did not find any deficiencies 1 week following sternomastoid nerve crush (Moscoso et al., 1998). In our study, we compared the recovery of the soleus muscle after tibial nerve crush in WT and NCAM -/- mice, but unlike the previous study (Moscoso et al., 1998) we assessed recovery at timepoints well beyond 1 week (i.e. 1-6 months) and used much more extensive outcome measures that included muscle physiology, retrograde labelling of motor neurons, and myosin specific immunohistochemistry. Our findings revealed that: (i) NCAM is dispensable for axon regeneration and the initial reformation of functional NMJs; (ii) NCAM is required for the maintenance of muscle strength 3 and 6 months after nerve crush; (iii) in the absence of NCAM muscle area is progressively lost from 1-6 months after nerve crush primarily due to the selective loss of fast muscle fibers; and (iv) the deficits in maintaining reinnervated NMJs after nerve crush are independent age. However, further studies are required to determine if it is the absence of pre- and/or postsynaptically NCAM which is at the root of this dramatic phenotype.

When the gene for Fas II, the homolog of NCAM in drosophila, was completely ablated it resulted in regression of NMJs and consequential death of these mutants early in their postnatal lives (Schuster et al., 1996). Taking advantage of the power of drosophila genetics, Schuster et al. (1996) then crossed their Fas II -/- line with “enhancer detector” lines (O’Kane and Gehring, 1987) that were used to specifically rescue Fas II expression in muscle, neurons, or both to show that Fas II must be expressed pre- and postsynaptically to maintain the drosophila NMJ. Until recently, equivalent experiments were not possible in mice. The emergence of mouse conditional genetic ablation
technologies, such as Cre-Lox recombination (Stemberg and Hamilton, 1981; Sauer and Henderson, 1988), should allow for questions like this to be addressed through specific deletion of NCAM (i.e. NCAM-floxed; Bukalo et al., 2004) in discrete cell types like motor neurons (e.g. Hb9-Cre; Yang et al., 2001) and striated muscle (e.g. Human Skeletal Actin-Cre; Miniou et al., 1999). Furthermore, since our experiments demonstrate that removal of NCAM impairs the maintenance of NMJs, it seems reasonable that over-expression of NCAM might augment it. Therefore, over-expression of NCAM should be considered for its therapeutic potential to enhance synaptic maintenance. If NCAM over-expression can help to maintain synapses then this could conceivably be part of combinatory treatments for conditions such as amyotrophic lateral sclerosis, where recent studies suggest the need for therapies aimed at both preventing motor neuron death along with maintaining NMJs (Ferri et al., 2003; Gould et al., 2006).

In conclusion, the vigorous capacity of PNS axons to regenerate combined with our growing knowledge of the molecular events that underlie it should translate to improved therapies for nerve injuries in the near future. In this thesis, NCAM and PSA have been shown to play an important part in each of the major steps to functional neuromuscular recovery after nerve injury (Kernell, 2006). Consequently, NCAM and PSA are promising molecular targets for the new wave of peripheral nerve injury treatments such as pharmaceuticals or gene therapy (Tepper and Mehrara, 2002). The lessons we are learning about functional recovery after peripheral nerve injury will certainly also have implications for recovery from CNS injury as researchers are taking major strides towards overcoming the barriers to axon regeneration in the brain and spinal cord (Thuret et al., 2006).
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