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ABSTRACT


Successful regeneration of a severed peripheral nerve is insufficient to restore the stretch reflex. This deficit occurs despite successful muscle reinnervation and functional restoration of the circuit components. For example, Ia afferents encode muscle stretch, Ia-motoneuron synapses reform to the extent of restoring low frequency transmission, and activation of motoneurons results in muscle force output. However, when recording intracellularly from reinnervated rat motoneurons during ramp hold and release muscle stretch, no excitatory synaptic potentials are detected in 2/3 motoneurons (Haftel et al., 2005), a clear sign that the synapses connecting Ia afferents with motoneurons are physiologically disrupted. This thesis examines extensively the cellular properties of the presynaptic Ia afferent, postsynaptic motoneuron, and the function of their central synapse to identify where along this circuit deficits are occurring. Using intraaxonal recordings from regenerated muscle afferents, we found that not only were regenerated afferents capable of encoding stretch information, but they were, by many parameters, indistinguishable from normal. In addition, intracellular recordings from regenerated motoneurons indicated that intrinsic motoneuron properties (rheobase, input resistance, and afterhyperpolarization potential) returned to control levels with reinnervation. To
study synaptic function between regenerated afferents and motoneurons, muscle stretch and electrical stimulation at group I strength were used to activate regenerated afferents. Whereas electrical stimulation at both low (1 pps) and physiologic frequencies was capable of producing synaptic responses, muscle stretch was entirely ineffective in some motoneurons. Examination of individual synapses using spike triggered averaging suggested that many regenerated afferents responding to muscle stretch in patterns typical of Ia do not make physiologic monosynaptic connections with motoneurons. In total, these data demonstrate a disconnect between peripheral reinnervation by regenerated afferents and central connectivity with motoneurons: many afferents regaining normal stretch responses are not centrally connected to motoneurons, however, many group I afferents that do not reinnervate muscle spindles maintain central synaptic connections with motoneurons. This finding may result from factors including nonspecific reinnervation, synaptic stripping of afferent input at proximal/distal sites, and electrotonic decay of afferent input at distal synapses and may explain why the stretch reflex is not restored even after successful muscle reinnervation.
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Abbreviations
Ia Group Ia primary afferent
AHP Afterhypolarization potential
EPSP Excitatory post synaptic potential
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<td>SSP</td>
<td>Stretch evoked synaptic potential</td>
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<tr>
<td>TTSSP</td>
<td>Tendon tap stretch evoked synaptic potential</td>
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<td>STA</td>
<td>Spike triggered averaged</td>
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CHAPTER 1: General Background

Peripheral nerve injury undeniably damages axons and interrupts neuronal circuits. Axon regeneration is an important step in restoring these circuits and is to a large degree successful. However, despite the ability of the axons to reinnervate peripheral targets, normal function is not restored. The aim of this proposal is to identify cellular factors that may not only limit recovery from peripheral nerve damage, but may also have significance in preventing recovery from central axon injury. Using the peripheral nervous system as our model, we are able to study the injured system after successful regeneration, a system currently unavailable for brain and spinal cord injury. This may lead us to conclusions that are of great significance once central nerve regeneration can be achieved. In other words, similar cellular problems may exist for all injured axons; therefore, the work proposed here may promote our understanding of the precise cellular changes that must be reversed to achieve full functional recovery from both central and peripheral nerve injury. The studies proposed here focus on the stretch reflex, a behavior necessary for normal control of movement and the body’s quickest response to compensate for unexpected postural disturbances. Central to this thesis is the surprising finding that peripheral target reinnervation does not restore the stretch reflex.

Behaviorally, animals exhibit a corresponding ataxic gait. The stretch reflex behavior is controlled neurally by a well-known two-neuron circuit consisting of primary sensory neurons (Ia afferent) synapsing directly onto alpha motoneurons. In studying this circuit
we have the unique ability to link behavioral deficits to a single connection between two cells. This clearly simplifies the task of identifying cellular mechanisms that limit recovery. Interestingly, our focus has been directed toward central cellular mechanisms given the unexpected knowledge that certain components of the circuit substantially recover following nerve injury and regeneration. Using current recording techniques, physiologic properties of Ia afferents, motoneurons and their synaptic connection can be measured in response to the very stimulus that typically elicits the reflex behavior: muscle stretch. Additionally, the circuit can be manipulated experimentally to test the reliability of each circuit component. The most remarkable and unique feature of the proposed experiments is the ability to study the circuit in vivo. Because tissue is not removed from the animal to obtain data, the results of this work are truly relevant to the living, breathing animal. In total, the proposed experiments provide a detailed, yet relevant, examination of the most likely cellular factors limiting functional recovery from injury.

The Stretch Reflex Circuit

The stretch reflex circuit is a feedback system that regulates muscle length. The reflex is the body’s first response to postural perturbations, and it may have a role in all movements and postures (Latash, 1993). In total, the Ia-motoneuron stretch reflex circuit functions in the following manner: muscle spindle afferents are activated by muscle stretch. The resulting action potentials propagate centrally to their axon terminals, where they make monosynaptic connections with homonymous motoneurons. Through the process of chemical transmission, monosynaptic excitatory post synaptic potentials
(EPSPs) are produced in these motoneurons. When a motoneuron is sufficiently depolarized, it initiates action potentials that propagate to the neuromuscular junction where chemical transmission again occurs. Muscle fibers contract as a result of excitation-contraction coupling, and the reflex is complete. The components of the stretch reflex circuit provide the body with useful information about limb position, regulate muscle stiffness, aide in locomotor activity, and allow the body to respond to unexpected changes in posture. Disruption of this circuit, as with injury, results in severe deficits to limb movement and coordination.

*Stretch Reflex Components*

The stretch reflex circuit consists of a two-neuron system connecting peripheral sensory receptors and muscle fibers through a single synapse in the spinal cord (see Figure 1.1). The specific components include muscle spindle receptors, muscle afferents, alpha motoneurons, and extrafusal muscle fibers. Input from descending and polysynaptic sources may influence the circuit but are not considered here as our experimental protocol minimizes their contribution.

Muscle Spindle Receptors

Initial studies by Ruffini (1898), Sherrington (1894), Matthews (1933) and Barker (1948) provided a thorough histological analysis of the sensory fibers innervating intrafusal muscle. In total, their work has led to a number of conclusions about the muscle spindle apparatus and the afferents that innervate it. Muscle spindles lie within the extrafusal muscle mass and consist of intrafusal fibers that are innervated by both afferent and
Figure 1.1. *Components of the stretch reflex.* The stretch reflex circuit consists of Ia afferents which innervate muscle spindle receptors and alpha motoneurons which innervate extrafusal muscle fibers. Ia afferents and motoneurons communicate through a monosynapse in the ventral spinal cord.
efferent nerve endings. The afferents supplying the muscle spindle apparatus terminate on small, encapsulated intrafusal fibers in two distinct arrangements: annulospiral rings at the equatorial position and flowerspray patterns at the juxtaequatorial position, corresponding to primary and secondary endings, respectively. While they both fire in response to muscle stretch, their differing locations likely account for the specific encoding properties associated with each.

Primary Muscle Afferents
Muscle proprioceptors innervating muscle spindles respond to changes in muscle length, velocity, and even acceleration. Their cell bodies are located in the dorsal root ganglia, and their axons project centrally into the spinal cord where branches provide segmental input to a variety of cells (interneurons, motoneurons, etc.) and ascend to higher centers. These afferents have the capacity to fire at a range of frequencies, and collectively, they encode very specific information about limb position and body movement. While all muscle proprioceptors discharge when the muscle is stretched, specific differences in their firing characteristics, sensitivity to different types of muscle stretch, and conduction velocity allow them to be classified into two groups: Ia and group II (Bradley and Eccles, 1953, Hunt, 1954, Matthews, 1933, Matthews, 1972). Group Ia afferents have greater dynamic responses to muscle stretch than group II afferents indicating a specific ability to detect rate of muscle stretch in addition to muscle length. Group Ia afferents are also exquisitely sensitive to high frequency (167-250 Hz, 80 um) muscle vibration. Group II afferents serve primarily as length sensors and are far less sensitive to vibration (responding to 25-50 Hz, 80 um) (De-Doncker et al., 2003). It is clear in the cat that
primary endings correspond to group Ia fibers and the secondary endings correspond to
group II fibers, however, in the adult rat this is still a point of discussion (De-Doncker et
al., 2003).

Alpha Motoneurons

Spinal motoneurons are among the largest neurons in the nervous system. The cell
bodies of alpha motoneurons are located in Lamina IX and are topographically arranged
in “pools” corresponding to their target muscle. Their massive dendritic trees extend
radially throughout the spinal cord allowing input from a variety of other neurons. In
total, motoneurons receive approximately 50,000 synaptic inputs from supraspinal and
segmental sources with an estimated 2-4 % of synaptic input coming directly from
monosynapses with Ia afferents (Fyffe, 2001). Motoneuron dendrites have both active
(voltage-dependent channels) and passive (electrotonic structure) properties that
collectively determine motoneuron firing characteristics. Functionally, motoneurons
connect with skeletal muscle and, thereby, are fundamental to voluntary and reflexive
movement (MacIntosh et al., 2006).

Skeletal Muscle

Skeletal muscle is designed to provide force generation, and serves as the final
component of voluntary and reflexive movement. The process of muscle contraction
begins when impulses initiated in motoneurons travel peripherally to synapses with
skeletal muscle at the neuromuscular junction. Near this junction, efferent axons lose
their myelin sheath and divide to supply multiple muscle fibers (MacIntosh et al., 2006).
Acetylcholine released by the motoneuron depolarizes skeletal muscle and through, excitation-contraction coupling, results in muscle contraction. Generation of muscle force is initiated by an influx of calcium and occurs through the interaction of key contractile proteins actin and myosin (Lieber and Lieber, 2002). Muscle fiber types and motor units are classified physiologically and histologically into three general types: fast glycolytic (fast fatigable), fast oxidative glycolytic (fast fatigue-resistant), and slow oxidative (slow) (Lieber and Lieber, 2002). Motor units are generally recruited in a systematic manner with small motoneurons being more easily activated, according to Henneman’s size principle (Henneman et al., 1965).

Special Emphasis on the Ia-motoneuron synapse: Ia-motoneuron organization
Muscle spindle afferents project into the ventral spinal cord where both Ia afferents and some group II afferents synapse on alpha motoneurons (Brown and Fyffe, 1981, Munson et al., 1982). Ia contacts on motoneurons are rarely found at the soma but may occur throughout the length of the dendritic tree (Brown and Fyffe, 1981, Burke et al., 1979, Burke and Glenn, 1996). Physiologic data are consistent with this notion based on the multiple shapes seen in individual EPSPs elicited by stimulation of single Ia afferents (Jack et al., 1971, Mendell and Henneman, 1971). Each Ia afferent may make as many as 32 contacts on a motoneuron (Burke and Glenn, 1996). Those contacts arising from a single Ia afferent collateral may be observed in clusters on the dendrite (Brown and Fyffe, 1981). Ia afferents project to nearly all homonymous motoneurons and to a smaller portion of the motoneurons from heteronymous synergists (Scott and Mendell, 1976). When contributions from motoneuron size and position are minimized, individual
EPSPs generated in homonymous motoneurons from single afferents are similar in size to those generated in heteronymous motoneurons (Webb and Cope, 1992). Electrophysiologic evidence using potent AMPA and kainate antagonists strongly suggests that this synapse is glutamatergic and acts through non-NMDA receptors (Finkel and Redman, 1983, Jahr and Yoshioka, 1986, Walmsley and Bolton, 1994).

Ia-motoneuron physiology
When whole peripheral nerves are stimulated at levels sufficient to activate multiple or all Ia afferents, composite, or aggregate, EPSPs can be recorded in the homonymous and synergistic alpha motoneurons (Eccles et al., 1957). The steady-state synaptic strength, or the peak EPSP amplitude during low frequency stimulation (~1 pps), is generally steady throughout continual stimulation (Curtis and Eccles, 1960), and is relatively constant from animal to animal. However, steady-state synaptic strength can be altered in a positive or negative direction under experimental conditions. Composite EPSPs are likely influenced largely by Ia contacts, but the possibility exists that monosynapses formed by group II afferents and polysynapses formed by group Ib afferents with similar electrical thresholds contribute to the EPSP (Cope et al., 2001). This is particularly true for the rat where the distribution of group I and group II afferent conduction velocities makes an overlap in electrical thresholds likely. Therefore, caution is required when interpreting the composite EPSPs. This shortcoming can be addressed by studying individual EPSPs arising from single Ia afferents synapsing on motoneurons which can be recorded using the technique of spike-triggered averaging. This technique averages
sweeps using a presynaptic action potential as the trigger to amplify postsynaptic activity time locked to the presynaptic spike (Mendell, 1971).

Under normal physiologic conditions, Ia afferents fire at high frequencies (up to 600 pps). When Ia afferents are electrically stimulated at physiologically relevant frequencies, amplitude modulation of postsynaptic potentials can occur in the direction of depression (decrease in EPSP amplitude throughout train) or, less often, facilitation (increase in EPSP amplitude throughout train) (Honig et al., 1983). Frequency sensitivity appears to be primarily determined by the postsynaptic cell, with slow-type motoneurons exhibiting more depression (Mendell et al., 1995). Muscle stretch activates Ia afferents at high frequencies and, in control animals, always produces stretch-evoked excitatory postsynaptic potentials (SSPs) in corresponding motoneurons. The amount of depolarization varies with the characteristics of muscle stretch (Westbury, 1972).

Study of the Ia motoneuron synapse

The aim of this thesis is to use the Ia afferent-motoneuron synapse to identify cellular factors that change as a result of peripheral nerve injury and regeneration. The Ia-motoneuron synapse provides us with a prime location for studying central synaptic transmission for a number of reasons. First, this system has been well-characterized in both adult rats and cats. The Ia-motoneuron synapse has been the subject of a variety of experiments dating back to the 1950’s and continues to provide us with novel and important information about synaptic function. Second, current recording techniques allow us to physiologically examine the actual connection between the pre and
postsynaptic cells, which can be studied at the level of individual afferent input. And finally, this synapse can be accessed using *in vivo* preparations, whereby one can manipulate the cells of interest without massive experimental disruption of other synapses. This is particularly important in studies of injury where synaptic transmission may be influenced by trophic factors, activity of the surrounding neurons, and the immune system’s response. The Ia-motoneuron synapse has already provided us with valuable insight into how the central nervous system responds to peripheral nerve injury. For example, several previous experiments have illustrated the interesting effects whereby synaptic strength both increases and decreases following axotomy.

**Axotomy**

The mere act of transecting a peripheral muscle nerve sets off a myriad of changes within the Ia-motoneuron circuit. Axotomy disrupts the connection between sensory and motor axons and their peripheral targets, causing their distal portions to undergo Wallerian degeneration. The remaining proximal components respond to this injury with a number of cellular and functional changes, most of which reverse upon muscle reinnervation.

**Afferent Response to Injury**

After axotomy, changes occur throughout the cell body and central and peripheral axons of sensory neurons. The cell body swells and the nucleus displays signs of chromatolysis (Degn et al., 1999). Some afferents eventually die as a result of peripheral injury; studies report cell loss as high as 50% in the dorsal root ganglion (Degn et al., 1999; Devor, 1995). Axon caliber is reduced (Kreutzberg, 1995) and, consequently, central and
peripheral conduction velocity of injured axons decreases (Czech et al., 1977). The activity of severed afferents is dramatically reduced (Michaelis et al., 2000).

Approximately 80% of myelinated axons are silent, while 20% fire spontaneously in irregular patterns at low frequencies. Active afferents fire spontaneously at mean rates of approximately 25 Hz (range 1.1-140 Hz), with the number of active afferents declining over time (Seburn et al., 1999). Centrally, levels of vesicular glutamate transporter 1 (Vglut1), a protein that transports glutamate into presynaptic vesicles (Bellocchio et al., 2000, Takamori et al., 2000), and a histologic marker for primary afferents (Alvarez et al., 2004, Landry et al., 2004, Li et al., 2003, Oliveira et al., 2003, Todd et al., 2003, Varoqui et al., 2002), decrease (Brumovsky et al., 2007, Hughes et al., 2004). For example, Hughes et al. (2004) found that luminescence values for Vglut1 staining in spinal cord lamina IX were decreased to 65% of the control value. Vglut1 staining decreased over time with the lowest recorded value at 8.5 weeks post-axotomy.

**Motoneuron Response to Injury**

When a peripheral nerve is cut, motoneuron axons are damaged and physically disconnected from their target muscle fibers. Motoneurons respond to this injury with metabolic and cellular changes that affect conduction velocity, intrinsic motoneuron properties, and the motoneuron’s synaptic capacity. Shortly after injury, conduction velocity decreases. In addition, motoneuron excitability, as measured by rheobase current, increases, input resistance increases and afterhyperpolarization potential (AHP) duration increases. These changes are well-accepted as they have been published for various species and motoneuron pools (Bichler et al., 2007, Foehring et al., 1986,
Gardiner and Seburn, 1997, Gustafsson and Pinter, 1984, Mendell et al., 1995, Nakanishi et al., 2005, Yamuy et al., 1992). In addition to the motoneuron electrical properties, the cell body swells, dendritic volume and dendritic membrane area are reduced (Brannstrom et al., 1992). Several reports have shown substantial decreases in postsynaptic glutamatergic (Alvarez et al., 2000, Croul et al., 1998, Popratiloff et al., 1996), GABAergic (Vassias et al., 2005), and glycinergic (Eleore et al., 2005) receptor subunits in the ventral horn and other motoneuron nuclei.

Retrograde changes following axotomy also include a profound stripping and reorganization of motoneuron synapses. 12-weeks post axotomy, the number of boutons contacting axotomized cat motoneurons is greatly reduced on the soma and proximal dendrites (Brannstrom and Kellerth, 1998). Synaptic loss differs in time course and severity according to synapse type with preferential elimination of glutamatergic S type boutons (Brannstrom and Kellerth, 1999, Linda et al., 2000). Ultrastructural analysis of stripped synapses indicates vesicle displacement, synaptic membrane detachment and degeneration of terminals (Linda et al., 2000, Tiraihi and Rezaie, 2004). Synaptic stripping appears to be controlled, at least in part, by expression of major histocompatibility compatibility (MHC) class I (Thams et al., 2008). Experiments performed on mice lacking crucial components of the MHC molecule indicate that MHC molecule is important for stabilizing and maintaining inhibitory synapses after injury (Oliveira et al., 2004, Thams et al., 2008). However, stripping of excitatory synapses may occur through an altogether separate mechanism. In general, tissue reinnervation
results in recovery of central synapses and is best for S and L type boutons (Brannstrom et al., 1999).

**Synaptic Response to Injury**

Peripheral nerve injury has enormous effects on synaptic transmission in the central nervous system. Following axotomy of a peripheral muscle nerve, a robust increase is seen in Ia-motoneuron steady-state synaptic strength despite all central projections and cell bodies being experimentally untouched. Miyada and Yasuda (1988) showed a significant increase in Ia-motoneuron EPSP amplitude peaking three days after medial gastrocnemius (MG) axotomy. This effect was seen in both axotomized MG homonymous and intact lateral gastrocnemius (LG) heteronymous motoneurons. In both cases, the synaptic enhancement observed lasted less than one week and was followed by a long period of synaptic depression. EPSPs recorded at synapses made between intact LG Ia afferents and both MG and LG motoneurons appeared normal following MG axotomy suggesting that Ia afferents, not motoneurons, must be cut to trigger the effect. Seburn and Cope (1998) confirmed this synaptic enhancement and added that greater negative modulation (synaptic depression) is seen in EPSPs throughout 167 Hz trains. Specifically, EPSPs occurring later in the train had reduced amplitude compared to those at the start of the train, and this reduction was more pronounced in axotomized circuits than in control circuits. This may be the result of greater susceptibility to transmitter depletion (caused by increased probability of neurotransmitter release) or receptor desensitization.
Weeks after axotomy, the Ia-motoneuron connection weakens. Physiological studies performed by Mendell and colleagues (1976) illustrate that individual Ia EPSPs recorded from motoneurons with axotomized peripheral nerves have reduced amplitudes, slower rise times, and longer half-widths. This physiologic slowing may reflect early elimination of synaptic boutons on the soma and proximal dendrites or postsynaptic changes (Vanden Noven and Pinter, 1989). Following these changes, connectivity, defined by the presence of an individual EPSP, decreased to levels as low as 50% (compared to 96% in controls). Instances in which no individual Ia EPSP was recorded may represent total disconnection of all boutons arising from the single Ia afferent being studied.

It is clear that injury induces a number of changes in the central elements of the Ia-motoneuron circuit. In addition to these changes, the synaptic connection between Ia afferents and motoneurons can actually be removed. In order for central function of the Ia-motoneuron circuit to be completely restored, a great degree of cellular recovery must take place to either bring the circuit back to its preinjury state or to compensate for any remaining deficits.

**Regeneration**

In the absence of substantial compensatory mechanisms, recovery from injury requires that damaged axons regenerate and the connections between neurons reform. Cellular changes promoting regeneration begin almost immediately after injury. Proximal to the injury site, axonal growth cones form within 3-48 hours of injury (Bisby, 1995). The cell
body begins to produce new mRNA, cytoskeletal proteins, microtubule associate proteins, and other molecules associated with cellular growth such as GAP-43 (Benowitz and Lewis, 1983, Seckel, 1990, Skene and Willard, 1981, Skene et al., 1986). Schwann cell and fibroblast production of constitutively expressed nerve growth factors and neurotrophic factors is increased. The injury site is infiltrated by Schwann cells and macrophages that phagocytose and degrade the distal axon and surrounding myelin. Schwann cells proliferate and form sheaths within the endoneurial tubes that guide regenerating axons to their target (Thomas and Jones, 1967). Axons are directed through the endoneurial tubes by chemotropic molecules, adhesion molecules, and glycoproteins within the basal lamina (Seckel, 1990). Axon elongation proceeds at rates of up to 5-6 mm per day, corresponding to the maximum rates of slow anterograde transport of actin, tubulin, and neurofilaments (Tetzlaff and Bisby, 1989).

Reinnervation of peripheral targets is crucial to functional recovery. Studies of self-reinnervation (muscle nerve is cut and surgically rejoined) indicate that axons have the capacity to reinnervate their original targets (Brown and Butler, 1976, Gregory et al., 1982). Interestingly, axons can also reinnervate inappropriate, or foreign, targets (Collins et al., 1986, Banks and Barker, 1989). When the proximal end of a cut medial gastrocnemius muscle nerve is surgically joined to the distal portion of a cut sural cutaneous nerve, muscle afferents not only survive and respond to cutaneous stimulation, but some even begin to take on characteristics resembling cutaneous afferents (Lewin and McMahon, 1991a). Some evidence exists for concurrent changes in central connectivity when gastrocnemius afferents reinnervate skin (Lewin and McMahon, 1991b). However,
when cutaneous afferents inappropriately reinnervate muscle, the afferents have properties similar to when they reinnervated their original targets and changes in central connectivity are not observed (Lewin and McMahon, 1991c).

Successful reinnervation depends on a number of variables. The age of the subject, type of lesion, site of injury, and surgical intervention all play a role in determining the outcome of recovery. Even when these variables are controlled to optimize reinnervation, the outcome will not be perfect. Some cells will likely die during the process, and other cells will reinnervate inappropriate targets. Myelination of reinnervated axons may be thinner and nodes may be closer (Bisby, 1995). Injury to surrounding tissue may also result in imperfect reinnervation.

It is clear, however, that both sensory and motor reinnervation is to a large degree successful after peripheral injury to the Ia-motoneuron circuit. Recordings from dorsal root axons show that regenerated sensory afferents respond to muscle stretch (Banks and Barker, 1989, Lewin and McMahon, 1991b) with the majority of response properties being similar to normal (Haftel et al. 2005). Whole cell electrical properties of motoneurons, e.g. AHP, rheobase, input resistance, return to pre-injury values (Bichler et al., 2007, Foehring et al., 1986, Mendell et al., 1995), muscle weights increase from their atrophied state (Cope and Clark, 1993), muscle tension recovers (Gordon and Stein, 1982), and muscle fibers contract (in response to electrical or reflex stimulation). Motoneuron synapses become functional as many reflexes (cutaneous, crossed extension, etc.) are regained (Cope and Clark, 1993) and Ia-EPSPs can be electrically evoked in
motoneurons at low stimulus frequencies (1pps) (Haftel et al., 2005). The observations listed above indicate that all components of the Ia-motoneuron circuit are structurally intact and functional. However, despite a great deal of recovery, reinnervation is not entirely successful. The stretch reflex is virtually absent (Cope and Clark, 1993, Cope et al., 1994): stretch of a reinnervated muscle does not elicit reflex contraction! This finding is entirely surprising given that motoneurons actively respond to other reflexes. Furthermore, muscle stretch is completely ineffective in eliciting a synaptic response from several motoneurons even when sensory afferents are firing. (This conundrum was first reported in a previous study from our lab using decerebrate rats and has been verified here using isoflurane anesthetized rats.) Together, these data lead us to the unexpected conclusions that factors other than poor peripheral reconnection account for stretch areflexia and that the signals produced by muscle afferents are suppressed within the spinal cord. The implications of these findings are paramount as they indicate that changes within the central nervous system limit full functional recovery and could explain movement deficits and proprioceptive abnormalities in patients suffering from neurologic trauma and disease. Consequently, isolation of the central factors responsible for stretch areflexia may lead to novel approaches for improved recovery from peripheral nerve injury or other types of neuronal injury.

There are undoubtedly a number of extrinsic factors (immunologic, neurotrophic, network) that have the potential to influence central processing in the regenerated Ia-motoneuron circuit. Factors intrinsic to the Ia-motoneuron circuit that are capable of influencing synaptic activity include presynaptic activity and synchronization of this
activity, postsynaptic anatomy (cell size, membrane area, and dendritic morphology) and intrinsic membrane properties (membrane resistivity and capacitance), and the characteristics of the synapse itself (neurotransmitter release, receptor sensitivity, synaptic reversal potential, and levels of connectivity) (Burke, 1987). For this dissertation, the function of the presynaptic cell, postsynaptic cell, and the actual synapse were studied to determine their role in synaptic dysfunction after peripheral nerve regeneration.

In studying the function of the Ia-motoneuron synapse, we have focused our attention on factors that may explain why the circuit fails at high physiological frequencies (ca. 20-600 Hz) such as that during stretch, but not at much lower frequencies (1 pps). Consideration has been further limited by giving less attention to factors that are known to reverse following regeneration. Two key mechanisms have led us to question the reliability of cellular components intrinsic to the Ia-motoneuron synapse and will be examined in the proposed studies. Conduction block within Ia afferents and synaptic depression at the Ia-motoneuron synapse could limit recovery by contributing to the loss of stretch reflex and the lack of stretch evoked synaptic excitation seen in regenerated circuits.

**Limitations on Recovery**

*Conduction Block*

Conduction block occurs when action potential propagation is prematurely terminated and the signal no longer reaches its target. Several factors including ion channel
function, firing frequency, and anatomic features all influence the success of action potential propagation. The classic action potential is mediated by both voltage-gated sodium and potassium channels, with the former initiating the depolarizing event and the latter opposing it. A reduced sodium gradient, sodium channel inactivation, or simply a lack of functional channels all may result in action potential blockade. Outward current produced by potassium channels is also a source of conduction block (Bostock et al., 1978). Whether changes in ion channel location or function occur centrally in regenerated primary afferents remains largely unknown. However, remodeling of membrane electrical properties, particularly involving sodium channels, may be the basis for abnormal excitability in injured afferents (Devor, 1995, Michaelis et al., 2000). Studies using combinations of microarray, RT-PCR, and in situ hybridization show increases in sodium and calcium channel and decreases in potassium channel subunit expression in the DRG and dorsal spinal cord following axotomy (Costigan et al., 2002, Takahashi et al., 2003, Yang et al., 2004). Additionally, pharmacologic studies on regenerating rat whole peripheral nerves suggest changes in the organization and function of voltage-gated channels (Mert et al., 2004).

Conduction block can occur following high frequency stimulation of normal axons. Human cutaneous afferents exhibit considerable hypoexcitability when stimulated at moderate to high frequencies (Vagg et al., 1998). Hypoexcitability, induced by repetitive stimulation of axons, can result from membrane hyperpolarization or sodium channel inactivation and can prohibit action potential conduction (Debanne, 2004).
Certain anatomic sites are more susceptible to frequency-dependent conduction block. Locations of low safety margin occur when axons change structurally and/or axon diameter changes. Vulnerable sites include axonal branch sites and boutons en passant (Debanne, 2004), which for Ia afferents occur centrally. The first electrophysiological experiments demonstrating conduction block at areas of low safety margin were performed on invertebrates due to the sheer size and accessibility of their axons, but similar results have since been confirmed in vertebrates (Debanne, 2004). Studies of the deep abdominal extensors in the spiny lobster illustrate conduction block at branch points (Grossman et al., 1979). When axons were stimulated at high frequency (30 Hz), action potential amplitude within the parent branch decreased by 10-15% and patch clamp recordings indicated a concurrent decrease in inward current just before the branch point. \textit{In vivo} extracellular recordings from daughter branches revealed conduction failure at this frequency.

Peripherally, regenerated Ia afferents do not appear to experience conduction block. Action potentials can be recorded intracellularly in dorsal roots upon low frequency stimulation of the peripheral nerve, high frequency activation of afferents produces a train of action potentials, and muscle stretch also produces a series of action potentials similar to control. However, centrally conduction block may be more probable for regenerated afferents responding to stretch as they form new synaptic boutons, create new branch points, and fire at high frequencies.
**Synaptic Depression**

Peripheral nerve axotomy and regeneration induces a number of changes at the synaptic level. As discussed previously, the steady-state EPSP increases dramatically within days of injury but begins to fall thereafter, eventually reaching levels decreased from normal (Miyata and Yasuda, 1988). When injured axons are stimulated at high frequencies, EPSP amplitude is not maintained throughout the stimulus train (Seburn and Cope, 1998). These observations are less surprising when the cellular impact of injury is considered. As motoneurons are stripped of synapses, synaptic machinery undergoes drastic changes. Hundreds of genes are up or down regulated within three days of nerve injury, including many that code for synapse-related proteins. One study using microarrays to detect changes in gene expression observed at least 1.5 fold changes for genes responsible for intracellular signaling, ion transport, ion channels, neurotransmission, and vesicle trafficking (Costigan et al., 2002). MRNA levels of SNAP-25, a contributor to the exocytotic vesicular fusion complex, are reduced beginning 3 days after injury and continue to decrease for at least 2 weeks when regeneration is prevented. Spinal cord injury results in significant changes in synpatotagmin, synaptobrevin, and synaptogyrin mRNA expression and increased expression of Rab 13, a protein that regulates synaptic vesicle movement to and from the plasma membrane (Di Giovanni et al., 2005). Levels of vesicular glutamate transporters also change after nerve injury (Brumovsky et al., 2007, Hughes et al., 2004). While data are not available to determine whether synaptic proteins return to their normal levels and original function upon regeneration, it is obvious that the potential for less than perfect recovery exists. Experimental studies using knockout mice provide insight to the...
problems that could exist after reinnervation if synaptic proteins were not restored to original level and function. Fremeau (2004) showed that cells from hippocampal slices lacking Vglut1 experience greater synaptic depression following stimulation at 10 Hz for 30 seconds than slices from wild type animals. In a study using embryonic chromaffin cells lacking SNAP-25, amperometric currents and capacitance values indicated nearly no synchronized secretory response to triggered increases in intracellular calcium (Sorenson et al., 2003). And, finally, pronounced synaptic depression is seen in mice deficient in synapsin II. Synaptic physiology experiments were performed using whole cell patch clamp recordings in hippocampal slices from synapsin I, synapsin II, and synapsin I/II double knockout mice. Electrical stimulation at 10 Hz for 30 seconds resulted in synaptic depression in both synasin II and synapsin I/II double knockout mice (Rosahl et al., 1995).

Changes in vesicle loading, docking, or release, may underlie the increased synaptic depression seen at the Ia-motoneuron synapse following injury. The ability of these to recover following regeneration is unknown but is enormously important as it determines the extent to which they could contribute to the loss of the stretch reflex.
CHAPTER 2: General Methods

Animals

All experiments were performed using adult female Wistar rats (250-425 g, Charles Rivers Laboratories, Wilmington, MA). This is largely due to published work from our lab detailing afferent firing patterns and stretch evoked synaptic potentials following reinnervation in this species (Haftel et al., 2005). All animals were housed in the Wright State University LAR facility. Food and water was available ad libitum, and rats were given wooden chew sticks to encourage activity. Animals were randomly placed in one of two treatment groups: control or regenerated. Those rats assigned to the regenerated group were subjected to chronic nerve treatment detailed below. All animals underwent a terminal experiment for data collection.

Chronic Nerve Treatment

To study the injured and regenerated Ia-motoneuron circuit, animals were subjected to a chronic nerve treatment in which the medial gastrocnemius muscle nerve was cut and immediately rejoined. The surgical procedures performed to obtain this treatment condition are described here. Anesthesia was induced by isoflurane (4-5% in 100% O2, inhalation in induction chamber) and maintained by isoflurane (1-3% in 100% O2, through nose cone). The incision site was shaved and surgically prepared. An incision was made through the skin overlying the popliteal fossa of the left hindlimb and blunt
dissection was used to visualize the medical gastrocnemius muscle nerve. Once the nerve was dissected free of the surrounding nerves, it was cut and surgically rejoined with 10-0 suture. After irrigation with 0.9% sterile saline, the disrupted fascial planes were sutured together and the wound was closed with subcuticular simple interrupted surgical knots. Upon completion of the surgery, the animal was removed from anesthesia and monitored until full recovery. Animals were given a subcutaneous injection of buprenorphine (0.1 mg/kg) every 12 hours for 48 hours to alleviate pain. During all nonterminal experiments, great care was taken to maintain aseptic conditions. Animals having undergone chronic nerve treatment were studied 6 to 21 months following nerve cut to allow ample time for nerve regeneration.

**Terminal Experiments**

*Anesthesia and Vitals*

Rats were studied in single terminal recording sessions lasting up to 18 hours. Therefore, it is necessary to ensure adequate health of the animal throughout the experiment. In doing so, a number of physiological measures were recorded and maintained at normal levels. Anesthesia was induced by isoflurane (4-5% in 100% O2, inhalation in induction chamber) and maintained by isoflurane (1-3% in 100% O2, through tracheal cannula). Ringer/dextrose solution was injected subcutaneously to maintain mean arterial pressure. Respiratory rate and end tidal pCO2 in addition to withdrawal reflexes (in the absence of paralytic drug) were used to ensure an adequate level of anesthesia throughout the experiment. Heart rate and oxygen saturation were also monitored to ensure the health of
the animal. Core body temperature was measured via rectal probe and maintained at 37 ± 1°C with both a hot water heating pad and radiant heat.

Dissection

Standard research procedures were used to dissect the spinal cord and hindlimb for the purposes of recording bioelectric signals from single motoneurons (Seburn and Cope, 1998) and afferent axons (Haftel et al., 2005). The left hind limb was exposed and the medial gastrocnemius muscle nerve was freed and isolated. The medial gastrocnemius, lateral gastrocnemius, and soleus muscles were freed from the surrounding tissue. Their distal tendon was detached from the calcanei and tied to a motor (Model 305B-LR, Aurora Scientific Inc.) designed to stretch the muscle within physiological range. The lumbar spinal cord (T10-S1) was exposed dorsally by laminectomy and the dorsal dura mater was removed. The rat was fixed in a rigid recording frame that stabilizes the animal and maintains knee and ankle joint angles at 90°. Skin flaps were used to construct mineral oil pools that bathe all exposed tissue to prevent drying.

Recording Preparation

The medial gastrocnemius muscle nerve was placed on a monopolar stimulating hook electrode that allowed antidromic identification of impaled motoneurons and orthodromic stimulation of muscle afferents. A rootlet dissected from the L5 or L6 dorsal root was placed on bipolar recording hook electrodes so the dorsal root volley could be measured. Peripheral nerve stimulation strength was set at 2.5 times the dorsal root volley threshold to minimize the activation of small afferents and maximize activation of alpha
motoneurons and group I afferents. Muscle resting length was set to the length where muscle force was approximately 10-15 g. The muscle was stretched in characteristic ramp hold and release patterns in order to stimulate mechanoreceptors within the MG muscle.

Data Collection

**Ia afferent** Using a micromanipulator system (Burleigh Microdrive, Transvertex Arc), afferents were impaled with borosilicate glass microelectrodes (1.2 mm OD, 25-35 MΩ resistance, World Precision Instruments). Microelectrodes were filled with either 2M potassium acetate for typical recordings or neurobiotin (10%, in 2M potassium acetate) for filling and visual identification. Group Ia afferents were suspected when an orthodromic action potential was present upon stimulation of the medial gastrocnemius muscle nerve. If impalement was adequate to discriminate spikes from noise, a series of ramp hold and release (3mm stretch amplitude, 20 mm/s ramp and release velocity) and vibratory (50, 100, 167, 250 500 Hz, 80 μm amplitude) stretches were performed using the motor. Classification as a Ia afferent (as opposed to group Ib or group II) ultimately occurred when the following criteria were met 1) had an initial burst at the onset of muscle stretch 2) responded to vibration at 100 Hz and 3) paused during medial gastrocnemius muscle contraction.

**Motoneuron** Motoneurons were impaled as borosilicate glass microelectrodes (1.2 mm OD, 7-10 MΩ resistance, World Precision Instruments) advanced through the spinal cord using a micromanipulator. Cells were identified as medial gastrocnemius motoneurons
when peripheral nerve stimulation resulted in antidromic action potentials. Upon identification, the antidromic action potential was recorded, and its amplitude was measured from the baseline noise to the peak voltage. Only those cells whose membrane potential was stable and action potential amplitude was maintained at at least +60 mV were deemed acceptable. In the cases where these conditions were met, the motoneuron’s intrinsic electrical properties: rheobase current, afterhyperpolarization (AHP) half decay time and peak amplitude, and input resistance were recorded. Additionally, synaptic efficacy at the Ia-motoneuron synapse was determined by recording low frequency EPSPs, high frequency synaptic responses, and stretch evoked synaptic responses in the impaled motoneuron.

Intrinsic Properties

Rheobase current

Rheobase current serves as a measure of motoneuron excitability and is quantified as the minimal amount of injected current necessary to evoke an action potential. To determine a cell’s rheobase current, depolarizing current pulses (50 ms) were delivered intracellularly in 1 nA steps until the cell fired (see Figure 2.1). In the rat, typical control values for rheobase range from 1 nA to 25 nA with averages between 7 and 12 nA (Seburn and Cope, 1998, Bichler et al., 2007).

After-hyperpolarization half decay time

After-hyperpolarization half decay time and amplitude were determined from action potentials occurring as the result of suprathreshold intracellular current injection (0.5 ms...
Figure 2.1. *Rheobase Current.* Top traces show membrane potential and bottom shows intracellular current steps. Rheobase current is measured as the amount of depolarizing intracellular current required to activate a cell.
AHP half decay time was measured as the time it takes for membrane potential to decay to half of the after-hyperpolarization peak amplitude (see Figure 2.2). While slow type medial gastrocnemius motoneurons have AHP half decay times > 20 ms, values for fast type motoneurons are never longer than 19 ms (Gardiner, 1993). Recent publications from our lab reported a mean value of 11.15 for control rat medial gastrocnemius motoneurons (Bichler et al., 2007).

Input resistance

To determine the cell’s resistivity, hyperpolarizing current pulses (50ms) of 1 nA and 3 nA were injected into the motoneuron (see Figure 2.3). The resulting change in membrane potential for a given amount of current was used to calculate the cell’s input resistance. Normal input resistance values for rat medial gastrocnemius motoneurons are 1.9 ± 1.1 MΩ (Button et al., 2008).

Synaptic Efficacy

The work done for this dissertation relies almost entirely on measurements of synaptic efficacy. As discussed earlier, normal Ia afferents make monosynaptic connections with homonymous motoneurons. When the circuit is functioning properly, Ia afferent action potentials propagating centrally result in excitatory postsynaptic potentials that can be experimentally recorded in homonymous motoneurons, providing us with a measure of synaptic efficacy. Because Ia afferents are sensitive to changes in muscle length, muscle stretch is one way of activating Ia afferents and eliciting Ia-motoneuron stretch evoked synaptic potentials. Two forms of muscle stretch were used here to elicit synaptic
Figure 2.2. *Afterhyperpolarization potential*. AHP half decay time and AHP peak amplitude were measured from action potentials produced by suprathreshold intracellular current injection.
Figure 2.3. *Input Resistance.* Hyperpolarizing current (top) was injected intracellularly at steps of -1 and -3 nA. The relationship between subthreshold current and the resulting membrane potential change (bottom) was used to calculate input resistance.
activity, ramp hold and release stretch and tendon tap stretch. During ramp hold and release stretches, the muscle was displaced 3mm with a 150 ms rise and release time (20 mm/s) and 500 ms hold time. Tendon taps displaced the muscle 1 mm from resting length over a period of 5 ms including 2.5 ms for the ramp and 2.5 ms for the release. Alternatively, intracellular or extracellular electrical stimulation can be used to activate Ia afferents at varied frequencies. Postsynaptic potentials were quantified using measurements of amplitude, half-width, and latency.

Nerve Stimulation
To determine the functional capacity of the circuit, low frequency Ia-motoneuron EPSPs were recorded. This was done by stimulating the entire peripheral nerve (composite EPSP) extracellularly (Iso-Flex Stimulus Isolation Unit, AMPI) using 50 μs pulses delivered at 0.5 Hz. When the whole peripheral nerve is stimulated, both afferent and efferent axons are activated. Monosynaptic EPSPs arriving in the motoneuron can be obscured by the antidromic action potential arriving shortly before. To deal with this problem, the stimulus strength was reduced to a level just below the motoneurons firing threshold. At these stimulus strengths, it is likely that some but not all medial gastrocnemius Ia afferents were activated. Varying levels of motoneuron excitability make it likely that different numbers of Ia afferents were activated in each motoneuron, therefore care is taken to account for this during data analysis.
High Frequency Pulse Train

To test whether transmission fails at rates that encode stretch, Ia afferents were stimulated using a pulse train that simulates stereotyped Ia afferent firing in response to ramp hold and release stretches (see Figure 2.4). This pulse train stimulus was created by averaging the interspike intervals of several control Ia afferents and then using the averages as the actual intervals between stimulus triggers. The pulse train, therefore, is entirely physiologically relevant. This stimulation paradigm was used to activate Ia afferents extracellularly and intracellularly at physiologic frequencies.

Spike Triggered Averaging

Synaptic efficacy between individual Ia afferents and motoneurons was measured using the spike triggered averaging technique. This technique uses the presynaptic cell’s action potentials as a trigger for postsynaptic activity, and allowed the collection of individual EPSPs from arising single regenerated Ia afferents synapsing on motoneurons. Dorsal roots were dissected into fine filaments that contained a single discriminable MG afferent with firing properties typical of group Ia. MG motoneurons were impaled using the procedure described above. Upon penetration of a motoneuron, the muscle was stretched to a length that caused sustained firing (usually ~25 Hz) in the discriminable afferent. Action potentials generated by the afferent were recorded by the extracellular electrodes at the dorsal root, and motoneuron membrane potential was simultaneously recorded by the intracellular recording electrode. Triggered by the occurrence of presynaptic action potentials, segments of motoneuron membrane potential were recorded, collected and averaged over many sweeps (300-2000). These averages were used to assess the
Figure 2.4. *High frequency pulse train used to stimulate Ia afferents for the recording of postsynaptic potentials in motoneurons.* Each stimulus is represented by a bar and the instantaneous firing rate is shown below.
presence/absence of an individual EPSP. When an individual EPSP was present, its amplitude, latency, rise time and half-width were measured.

**Euthanasia**

Animals were euthanized by intraperitoneal overdose of euthasol. However, in cases where immunohistochemistry was performed on the spinal cord tissue, animals were euthanized by exsanguination and fixation.

**Data acquisition and analysis**

Data were recorded and analyzed using Spike2 software (Cambridge Electronic Design). This software is ideal for these experiments as it allows experimenters to easily control the parameters of muscle stretch and create long continuous recordings. Using this software, waveforms can be averaged using a stimulus trigger. This feature was used when obtaining AHP, input resistance, and all synaptic efficacy measures. Several sweeps were averaged together to create a trace in which noise was reduced and the biological signal was enhanced.

**Statistics**

Regenerated and control afferent firing rates, motoneuron properties and synaptic efficacy were statistically compared across treatment groups with consideration for animal to animal variation using Nested Analysis of Variance (Nested ANOVA) (SYSTAT, Systat Software, Point Richmode, CA). Pearson correlations were used to analyze relationships between properties and were performed using Statistica software.
(Statsoft, Inc., Tulsa, OK). For all statistical tests, the level of significance was set at \( p<0.05 \). Statistical help was achieved through Wright State University Statistical Consulting Center.
CHAPTER 3: Regenerated muscle afferents respond normally to muscle stretch.

Introduction

Synaptic responses to muscle stretch are abnormal long after injury to a peripheral nerve even when the nerve is permitted to regrow. One factor influencing activity of a synapse is the information it receives from the presynaptic cell (Burke, 1987). In the case of the Ia-motoneuron circuit, input to the postsynaptic cell comes from activity within group Ia afferents, and it is, therefore, necessary that Ia afferents recover from injury for synaptic function to be restored. Peripheral nerve injury damages Ia afferent axons and disrupts connections between the sensory neurons and their peripheral receptors, muscle spindles. Recovery from this insult requires that damaged axons regenerate, reinnervate target receptors, encode information properly and communicate properly with the central nervous system. Group Ia muscle afferents have a specific anatomic configuration in which they wrap around intrafusal fibers, and functionally they encode information about muscle stretch in very precise patterns and at specific rates (Matthews, 1972).

Seemingly, a system like this could be difficult to reproduce. However, current evidence suggests that muscle afferents recover from injury quite successfully. Months after injury, muscle afferents do a remarkable job of reconnecting with muscle spindles, encoding muscle stretch information, and conducting information into the spinal cord (Banks and Barker, 1989, Barker et al., 1986, Lewin and McMahon, 1991a, Haftel et al.,
Studies of self-reinnervation (muscle nerve is cut and surgically rejoined) indicate that muscle afferents have the capacity to reinnervate their peripheral targets (Brown and Butler, 1976, Gregory et al., 1982). In the adult rat, when the medial gastrocnemius muscle nerve is cut and surgically rejoined, approximately 83% of muscle spindles are reinnervated and 75% of those have normal appearing annulospiral endings that are structurally associated with intrafusal muscle fibers (Haftel et al., 2005). The remaining 25% of muscle spindles do not have annulospiral endings structurally associated with intrafusal muscle fibers, but may receive some nerve supply from thin axons resembling free nerve endings (Haftel et al., 2005). In other words, the majority of muscle spindles are successfully reinnervated.

Peripheral reinnervation is also successful from a functional standpoint. Muscle afferents produce robust responses to muscle stretch and fire at normal rates. Many regenerated afferents even exhibit initial bursting activity. Despite nearly normal responses, a few differences have been reported between control and regenerated afferents. In the cat, some reinnervated afferents lack a tonic discharge and/or cannot sustain firing during the hold phase of ramp hold and release stretch (Banks and Barker, 1989). Likewise, an increased proportion of regenerated rat muscle afferents cannot sustain firing during sustained muscle stretch at constant muscle length (Lewin and McMahon, 1991a). Multiple reports show that regenerated afferents have significantly greater length thresholds than control (Banks and Barker, 1989, Haftel et al., 2005). While minor differences may exist between control and regenerated afferents, many regenerated
muscle afferents are capable of sending almost normal information to the central nervous system.

This thesis focuses on cellular deficits to the Ia-motoneuron circuit after peripheral nerve regeneration. The ability of regenerated afferents to fire in normal patterns and at normal rates is critical because it indicates that the deficits occurring within the circuit are not the result of abnormal spindle encoding or peripheral action potential conduction. Here the ability of regenerated afferents to encode muscle stretch information is tested by a number of different measures in a more stringent way than has ever been reported. In the studies described here, the physiology of “provisional” group Ia sensory neurons are examined following self-reinnervation of the medial gastrocnemius in the adult rat with specific attention given to action potential firing rates and patterns during ramp hold and release muscle stretch. We find that regenerated afferents produce firing responses nearly indistinguishable from controls.

**Methods**

*Animals*

Data were collected from adult female Wistar rats using procedures approved by Wright State University Laboratory Animal Care and Use Committee. All animals were housed individually or in pairs in cages, and were given food and water ad libitum.
Animal Treatment

Control data from 37 control Ia afferents were collected from animals as a control database for a separate study not reported in this dissertation. These animals were given i.p. injections of dextrose 1/week for 4 weeks prior to the terminal recording session. The remaining 13 control afferents were collected from animals untreated in any way.

Nerve Treatment

Animals in the reinnervated group underwent a survival surgery in which the nerves supplying their left medial and lateral gastrocnemius and soleus muscles were cut and immediately surgically rejoined. Animals were anesthetized with isoflurane (induced at 4-5% in 100% O₂, inhalation in induction chamber, and maintained at 1-3% in 100% O₂, on nose cone). An incision was made through the skin overlying the popliteal fossa. The left hindlimb MG and LGS muscle nerves were exposed, isolated, and cut using microscissors. Immediately after axotomy, the proximal nerve was surgically rejoined with its original distal portion using 10-0 suture. The wound was irrigated with 0.9% saline and the muscle and skin layers were closed. Animals were returned to their cages upon recovery from anesthesia.

Terminal Experiments

Animals were studied in single terminal recording sessions. Anesthesia was induced (4-5% in 100% O₂, inhalation in induction chamber) and maintained (1-3% in 100% O₂, through tracheal cannula) by isoflurane. Ringer/dextrose solution was injected subcutaneously (~1mL/hour) to maintain mean arterial pressure. End tidal pCO₂ and
withdrawal reflexes were used to ensure an adequate level of anesthesia throughout the experiment. Whole body temperature was monitored and maintained at approximately 37°C by radiant heat. Oxygen content and pulse rate were also monitored and maintained above 90% and 300 respectively.

Standard research procedures were used to dissect the spinal cord and one hind limb for the purposes of recording bioelectric signals from single afferent axons (Haftel et al., 2004). The lumbar spinal segments L3-L6 were exposed dorsally by laminectomy. The left hind limb was dissected such that the medial gastrocnemius muscle nerve was isolated and placed on bipolar hook electrodes. All other hindlimb nerves were crushed. The medial and lateral gastrocnemius and soleus muscles were freed from the surrounding tissue. Their tendon was detached from the calcaneous and tied to a servomotor. Resting length was determined by matching suture ties in the soleus muscle and the surrounding connective tissue that were lined up prior to the tendon being detached. The rats were fixed in a rigid recording frame with knee and ankle joints fixed at 90° angles. Skin flaps were used to construct mineral oil pools to bathe all exposed tissue. After data had been collected, animals were euthanized by isoflurane overdose (5% in 100% O₂).

Data Collection

The purpose of this experiment was to examine the effect of reinnervation on afferent encoding in response to muscle stretch. Physiologic data were collected from individual muscle afferents either intracellularly by penetration of sharp glass micropipettes (25-35
MΩ, 2M K-acetate) in the dorsal roots or extracellularly by recording from small dorsal root filaments using bipolar silver hook electrodes (see Figure 3.1). Afferents were identified by orthodromic stimulation as arising from the medial gastrocnemius muscle. Data collection proceeded when action potentials from a single afferent could be discriminated from action potentials produced by other afferents and electrical noise. We first stimulated the peripheral nerve to evoke orthodromic action potentials which were used (1) to provide action potential conduction delay and (2) to determine whether firing rate accelerated or decelerated during muscle contraction, a characteristic which was used in classifying afferents. Next, we recorded afferent firing in response to ramp-hold-release (20 mm/s, 3 mm), triangular (1 mm/s, 1 mm) and vibrational (50, 100 and 167 Hz, 80 um) muscle stretch. All stretches began at resting length. Records of membrane potential, muscle length, and muscle force were collected, digitized (20 kHz) and stored on a computer for later analysis using Spike2 software.

**Afferent Classification**

Strict criteria were used to group each afferent as a provisionary group Ia afferent. The descriptor “provisional” will be used to indicate that although these afferents fired as if they are Ia afferents, their original identity is unknown. For example, an afferent may have originally innervated the muscle spindle at the juxtaequatorial position or a golgi tendon organ but reinnervated the muscle spindle annulospiral ending. Those afferents exhibiting initial bursting activity during ramp-hold-release stretches, decelerating during muscle contraction, and firing 1:1 in response to vibration (100Hz) were classified as provisional group Ia muscle spindle afferents and were further analyzed. When no initial
Figure 3.1 *Experimental paradigm for measuring presynaptic activity.* Muscle stretch was used to activate muscle afferents. Afferent responses to stretch were recorded either intraaxonally or extracellularly at the dorsal roots.
bursting activity was seen, firing accelerated during muscle contraction or the cell did not respond 1:1 to vibration, the afferent was excluded from further analysis.

Statistics

Afferent firing properties were compared across control and regenerated afferents with consideration of animal to animal variability using nested ANOVA (SYSTAT, Systat Software, Point Richmode, CA). Data were pooled; values are reported as mean ± SD unless stated otherwise. For all statistical tests, significance was reached when p<0.05.

Results

In a total of 27 different rats, the firing responses of 50 control and 12 regenerated afferents were studied. These afferents were selected for study as their responses characterized them as firing in patterns typical of normal Ia afferents. As shown in Figure 3.2, regenerated afferents produced vigorous responses to muscle stretch. Not only did they respond to muscle stretch, their responses were qualitatively indistinguishable from normal (see Figure 3.3). They exhibited initial bursting activity in which the highest firing rates achieved are seen at the onset of muscle stretch, responded with increasing firing rates during the ramp when muscle length increases, and decelerated firing during the hold phases when muscle length is kept static. To look for any quantitative differences in the firing behaviors between control and regenerated afferents, several parameters were studied. Because muscle spindle afferents primarily encode muscle length and velocity of muscle stretch (Matthews, 1972), special attention
Figure 3.2. *Regenerated afferents are capable of producing robust responses to triangular, ramp hold and release, and vibrational (100, 167 Hz) stretch.* The upper trace shows the instantaneous firing rates (pps). Notice the initial bursting activity at the start of each stretch. The middle trace shows the activity recorded from a dorsal root filament, and the lower trace shows muscle length.
Figure 3.3. *Regenerated afferents respond to muscle stretch in patterns and at rates similar to control.* Shown here is the instantaneous firing rate (pps) for one control (top) and one regenerated (middle) afferent during ramp hold and release muscle stretch (length trace on bottom). As with control Ia afferents, regenerated provisional Ia afferents respond to ramp hold and release stretch with an initial burst, a dynamic response during the ramp, and then a static period of firing during the hold phase.
was given to measures that explore the ability of regenerated afferents to accurately encode dynamic and static muscle stretch.

**Conduction delay**

Conduction delay was measured to be certain that the afferents included in the study had delays falling in the range typical for group I afferents. Conduction delay was measured as the time between stimulation of the peripheral nerve and arrival of the orthodromic action potential at the dorsal roots. Mean conduction delay (± SD) for control and regenerated provisional Ia afferents was 1.62 ± 0.72 ms and 1.82 ± 0.37 ms, respectively. This measure was not statistically different between control and regenerated provisional Ia afferents (nested ANOVA, p = 0.157). Conduction distance (the distance from the site of peripheral nerve stimulation to the dorsal root recording site) contributes to conduction delay. This distance was not measured here and may be a source of variance.

**Response properties to dynamic muscle stretch**

Regenerated afferents responded to changing muscle length with responses quite similar to controls. Group Ia afferents often respond to muscle stretch initially by firing at high rates that can reach up to 600 pps. This bursting activity was one of the criteria for classifying regenerated afferents as provisional group Ia, therefore all afferents studied had exhibited an initial burst. Firing rates (reported in pulses per second (pps)) during the initial burst were compared for regenerated and control afferents and, as seen in Figure 3.4, were remarkably similar. Regenerated afferents on average reached maximum rates of 391.63 ± 83.22 pps during their initial burst. Similarly, control afferents had average
maximum firing rates of 396.32 ± 93.47 pps during the initial burst. Firing rates were also examined at the peak of the ramp during the last portion of muscle velocity increase. These values were also comparable between groups with regenerated afferents firing at 208 ± 38.92 pps and control afferents firing at 164.96 ± 52.34 pps (see Figure 3.4). The total number of spikes during dynamic muscle stretch did not differ between groups either. Consideration was given to how fast afferents accelerated their firing rates by measuring the slope of the change in firing rate over time during the ramp portion of muscle stretch after the initial burst. The rate in which control and regenerated afferents increased their firing rates over time, 692.71 ± 497.60 pps/s and 667.56 ± 652.64 pps/s respectively, was highly variable and not statistically different between groups (nested ANOVA, p = 0.958). Finally, the dynamic index calculated as the difference in firing rate at the peak of the ramp and halfway through the hold phase was not statistically significant, but trended in the direction of regenerated afferents having larger values (regenerated 157.54 ± 33.03 pps, control 110.59 ± 40.14 pps; nested ANOVA, p = 0.058).

Response properties to static muscle stretch

When the muscle was stretched from resting length by 3 mm and held constant during the hold phase of stretch, regenerated afferents also had similar responses to controls. No parameters measured during static muscle stretch differed statistically between control and regenerated afferents. In general, afferents were able to sustain firing throughout the entire hold phase. As with dynamic muscle stretch, control and regenerated afferents produced similar numbers of spikes during the static hold phase. Firing rates during
Figure 3.4. *Regenerated provisional group Ia afferents respond to dynamic muscle stretch at rates indistinguishable from control.* Shown are the mean firing rates of both control and regenerated afferents during the initial burst and at the ramp peak during ramp hold and release muscle stretch. Error bars represent S.D. No statistical differences were observed between the groups (nested ANOVA, initial burst: p = 0.332, ramp peak: p = 0.218).
static muscle stretch were not significantly different between regenerated afferents and controls (see Figure 3.5). Finally, the rate of deceleration in firing over time during the hold phase of muscle stretch was similar in regenerated afferents and control afferents. Firing decelerated at a rate of -47.23 ± 13.73 pps/s in regenerated afferents compared to -32.06 ± 19.02 pps/s in controls (nested ANOVA, p = 0.125).

History Dependence

Normal rat Ia afferents exhibit a behavior, history dependence, in which successive muscle stretch causes a reduction in the afferents’ dynamic responses. For example, when three identical triangular muscle stretches are performed consecutively, the number of spikes fired in response to the final stretch is often reduced from the number of spikes fired during the initial stretch and the firing rates are also reduced in the final stretch compared to the initial stretch (Haftel et al., 2004). To see if this behavior was preserved in regenerated afferents, the difference in the number of spikes fired during the initial and final stretch in a series of three triangular stretches was measured. History dependent behavior was seen in both regenerated and control afferents, but this behavior was more pronounced in control afferents (see Figure 3.6, nested ANOVA, p = 0.011). Control afferents experienced an average reduction of 15.92 ± 6.84 spikes from the first triangular stretch to the third whereas the regenerated afferents only experienced a reduction of 10.08 ± 4.66 spikes.
Figure 3.5. *Regenerated afferents fire at similar rates during static muscle stretch as control afferents.* The data are represented as Mean ±SD. There were no significant differences in the firing rates between control and regenerated afferents (nested ANOVA; halfway through hold, $p = 0.118$, end of hold, $p = 0.456$).
Figure 3.6. *Control afferents exhibited a larger degree of history dependence than* regenerated afferents. Means ± SD are shown for control and regenerated afferents (nested ANOVA, p = 0.011). History dependence was measured as the reduction in the number of spikes fired during the final triangle compared to the initial triangle in a series of three consecutive triangular stretches (see insert).
Evaluation of afferents on an individual basis

The regenerated afferents studied here were used in experiments in which individual axons were filled for study of their central projections or in separate studies testing whether physiologic connections existed between the selected afferent and homonymous motoneurons (see Chapter 5). Our goal was to assess the degree of central recovery of individual group Ia afferents. By selecting afferents that exhibited normal Ia afferent behavior in 12 independent tests of their response properties, we hoped to strongly bias our sample toward afferents that were group Ia before nerve injury rather than other stretch activated afferents whose central projections and connectivity are presumably quite different from group Ia even in a control situation. Our original classification scheme used initial bursting activity, 1:1 response to muscle vibration at 100 Hz, and deceleration of firing during muscle contraction to quickly triage an afferent. When these criteria were met, we proceeded with recordings using that particular afferent. Post hoc, we had the ability to scrutinize the similarities and differences between the individual regenerated afferents from which we recorded and our considerable control database.

The range of data points for control Ia afferents was determined for a number of parameters. The properties of regenerated afferents were plotted on an individual basis according to where they fell within the control ranges. These data can be seen in Figure 3.7. Regenerated afferents fell within control ranges for almost all parameters. The only exception was one regenerated afferent which exhibited higher than normal acceleration of firing during stretch ramp (Figure 3.6, E).
Figure 3.7. *Individual regenerated afferents fall within control ranges for a number of parameters of muscle stretch.* Red and blue dots show individual data points for each regenerated and control afferent, respectively. Light gray bars signify the control range. Parameters studied include A. maximum firing rate during the initial burst B. history dependence C. the amount of force produced by the muscle at the time of the first spike D. the dynamic index E. the change in firing rate divided by the change in time (slope of the firing rate acceleration) during the ramp phase of muscle stretch F. the change in firing rate divided by the change in time (slope of the firing rate deceleration) during the hold phase of muscle stretch G. number of spikes during the ramp phase of muscle stretch and H. number of spikes during the hold phase of muscle stretch.
Discussion

The data presented here provided a quantitative assessment of the ability of regenerated rat muscle afferents to encode stretch information. We found that for a number of parameters, regenerated afferents responded to muscle stretch in ways similar to control. In addition, measures of firing properties taken from individual regenerated afferents fell within ranges set by the control database.

In order to understand how well regenerated afferents encode stretch information, it was necessary to have a substantial control database to compare against. While control data are widely available and comprehensive for cat muscle afferents (Matthews, 1972, Banks and Barker, 1989, Barker, 1948, Hulliger, 1984), there are surprisingly few reports of spindle afferent physiology in the rat (Haftel et al., 2004, De-Doncker et al., 2003). The information collected for this chapter provided a wide-ranging set of control data that were certainly useful here, and will also be valuable to exist within the literature.

Control data were acquired from afferents with particular firing behaviors that identified them as group Ia. Several strict criteria were used to gain confidence that these were in fact group Ia afferents rather than group II or group Ib. One criterion used to classify afferents was conduction delay. Afferents with conduction delays of < 2.5 ms were considered in group I range (based on an estimated conduction distance of 95-100 mm). All control and regenerated afferents studied had conduction delays < 2.5 ms with most being < 2.0 ms. This measure was helpful in excluding most, if not all, group II afferents. Another criterion was that afferents decelerated firing during muscle
contraction. This indicated that the receptor lied parallel with the extrafusal fibers and helped exclude group Ib afferents which lie in series with the extrafusal fibers. Group Ia afferents are sensitive to muscle vibration at very high frequencies (100, 167, 250 Hz) and small amplitudes (80 um), while few group II or Ib afferents can follow vibration at any frequency with amplitudes of < 250 um (De-Doncker et al., 2003). Therefore, a third criterion was that afferents must fire 1:1 with 100 Hz vibration (80 um amplitude). Collectively, these criteria gave us assurance that the data analyzed reflected a population of group Ia afferents.

Control data collected for this chapter showed that individual rat Ia afferents encode identical muscle stretch with similar patterns of firing but with variable rates. For example, maximum firing rate during initial bursts ranged from approximately 150 pps in one control afferent up to approximately 550 pps in another, even though the velocity of stretch was constant. Within a given afferent, responses to like muscle stretch were quite consistent when stretches were spaced in time. Interestingly, when one stretch immediately followed a previous stretch, rates and patterns of firing varied. This property was evaluated here as history dependence. All control afferents exhibited some history dependence, but the degree varied by afferent ranging from differences of 3 spikes to 32 spikes in consecutive triangular stretches. In terms of firing rates, control Ia afferents encoded information using a large span of rates. During initial bursts, Ia afferents were capable of firing at rates up to 600 pps but these same afferents encoded static muscle length at rates as low as < 20 pps.
The 12 regenerated afferents sampled here were remarkably consistent with control values. Conduction velocity, force threshold, number of spikes and firing rates at various points during ramp hold and release stretch were all similar in control and regenerated afferents. Out of 12 parameters studied, only the degree of history dependence differed between the two groups. Not only were the groups similar for nearly all measures, the individual values for regenerated afferents fell almost entirely within control ranges. In fact, only one parameter for one regenerated afferent was out of the control range. Change in firing acceleration during the ramp phase of muscle stretch was greater than the largest value for controls. This is not terribly concerning because of the extent and direction for which it was out of range.

There is no doubt a degree of bias in the data represented here. The selection criteria used were rigorous and most probably resulted in a set of data that represented afferents that were the most successful in reinnervating muscle spindles and encoding stretch information properly. The reason for such strict selectivity was that these afferents were used for purposes in addition to solely examining their firing behavior. All of the regenerated afferents were used for either anatomical study of their central projections or for physiologic analysis of their functional connections with motoneurons (data presented in Chapter 5). In performing these experiments, a number of cells with atypical firing patterns were observed, but were not included in the analysis. For example, some cells responded to vibration at 100 Hz, an ability usually reserved for Ia afferents, yet accelerated their firing responses during muscle contraction, a property associated with afferents innervating receptors that lie in series with extrafusal muscle fibers such as
golgi tendon organs. Mismatches like this may be the result of reinnervation of muscle spindles by inappropriate afferents, a topic which must be addressed.

It is unclear how well afferents can reinnervate inappropriate targets (targets other than the original receptor). The data presented here show that afferents reinnervating muscle spindles fire in ways indistinguishable from normal afferents. This may be because original Ia afferents are provided environmental cues to return to their original receptor. Alternatively, these afferents may have previously innervated other receptor types but have reinnervated muscle spindles. A scenario of inappropriate reinnervation would require the regenerated afferent to travel to an unfamiliar target, assume the appropriate structural configuration, functionally associate with intrafusal fibers, express mechanically activated channels, and, as seen here, encode stretch in ways identical to control. This seems to be a daunting task; however, evidence does exist for reinnervation of targets by inappropriate afferents (Collins et al., 1986). Studies of cross reinnervation, in which the proximal end of one nerve is surgically attached to the distal portion of another nerve, indicate that muscle afferents are capable of reinnervating inappropriate tissue and can sometimes even take on characteristics of the afferents originally innervating that tissue. For example, when the medial gastrocnemius muscle nerve is surgically attached to the distal portion of a cut sural cutaneous nerve, muscle afferents respond to cutaneous stimulation in some ways that are characteristic of cutaneous afferents rather than muscle afferents (Lewin and McMahon, 1991a). On the other hand, some characteristics remain unchanged and may be intrinsic to the cell type. Most normal muscle afferents adapt slowly to stretch stimuli and most cutaneous afferents
adapt rapidly to cutaneous stimuli. With cross reinnervation, the original properties of adaptation are often maintained (Lewin and McMahon, 1991a, Lewin and McMahon, 1991c). It is difficult to speculate how similar the responses of muscle afferents innervating inappropriate muscle receptors (e.g. golgi tendon organ afferents innervating muscle spindles or vice versa) would be to normal. But, the capacity for muscle afferents to reinnervate a dramatically different target like skin makes less provocative the suggestion that golgi tendon organs or group II afferents may have the ability reinnervate annulospiral endings.
CHAPTER 4: Reinnervation restores intrinsic motoneuron properties.

Introduction

Regeneration causes notable changes in synaptic efficacy at the Ia-motoneuron synapse. Specifically, muscle stretch is incapable of producing an excitatory synaptic response in many motoneurons. To understand this deficit, it is necessary to be familiar with the factors that control synaptic efficacy. In general, synaptic efficacy is influenced by factors relating to the presynaptic cell, the postsynaptic cell and the interaction between them. Presynaptic neurons influence synaptic activity by their activity (addressed in Chapter 3) and patterns of connectivity (addressed in Chapter 5). The postsynaptic contributions to synaptic efficacy include anatomical characteristics, such as cell size and dendritic structure, and intrinsic membrane properties, including membrane and cytoplasmic resistivity (Burke, 1987). In this chapter, intrinsic properties of the postsynaptic cell known to contribute to synaptic efficacy were examined to determine their role in reducing stretch evoked synaptic activity.

Because their synapses span the dendritic tree (Fyffe, 2001), the electrotonic properties of motoneuron membranes play a crucial role in influencing the efficacy of synaptic information from Ia afferents (Rall et al., 1967). Electrotonic properties are influenced by the geometry of dendrites and their branches, resistivity of the membrane and cytoplasm and membrane capacitance (Rall et al., 1967, Burke and ten Bruggencate,
Using intracellular current injection at the motoneuron soma, the relationship between subthreshold current and voltage (input resistance) can be measured and underlying membrane properties can be inferred. Literature on cat motoneurons suggests that input resistance is a significant determinant of EPSP amplitude (Burke, 1968). A positive correlation between input resistance and EPSP amplitude has also been reported in the rat (Peshori et al., 1998). Input resistance is measured here to determine if it contributes to changes in synaptic efficacy following peripheral nerve regeneration.

In addition to input resistance, afterhyperpolarization (AHP) amplitude and decay time and rheobase current were also studied after reinnervation. These measures were chosen because of their important role in controlling neuronal input-output relations that are important for reflexive behavior, and because they are consistently altered by peripheral nerve injury. Incomplete recovery of these properties could compromise the recovery of the stretch reflex.

In normal systems, motoneurons exhibit systematic variation in their intrinsic cellular properties. For instance, motoneurons innervating fast motor units tend to have higher rheobase current (a measure of cell excitability), lower input resistance, and shorter afterhyperpolarization duration than motoneurons innervating slower motor units. These differences are functionally important for their role in orderly recruitment of motor units, action potential threshold, firing rate modulation, and even synaptic efficacy. Among normal motoneurons, certain relationships exist between properties. For example, motoneurons with larger input resistances tend to have increased membrane time
constants and increased AHP duration (Gustafsson and Pinter, 1984), while rheobase is negatively correlated with AHP half decay time and input resistance (Gardiner, 1993). As mentioned above, motoneurons whose input resistance is large and/or rheobase is small tend to have larger EPSP amplitude (Peshori et al., 1998).

When a motoneuron axon is damaged by a peripheral nerve injury, the motoneuron’s intrinsic properties change. As discussed in chapter 1, axotomy causes an increase in motoneuron input resistance and membrane time constant and a decrease in cell rheobase, cell capacitance, and conduction velocity (Gustafsson and Pinter, 1984, Foehring et al., 1986a). The number of cells exhibiting properties typical of fast type motoneurons shifts towards cells exhibiting properties of slow type motoneurons, creating a much less diverse group of motoneurons. And, typical relationships between given motoneuron properties are lost.

Input resistance
Following axotomy, mean input resistance of cat and rat motoneurons increases, approximately doubling in fast type motoneurons. In normal motoneurons, strong relationships exist between motoneuron type and input resistance. Zengel et al. (1985) showed that fast type motoneurons have much lower input resistances (0.6-0.9 Mohms) than slow type (1.6 Mohms). But, after axotomy, the relationship between input resistance and motoneuron type is lost, with all motoneurons falling within the normal range of slow type motoneurons. Increases in input resistance may reflect alterations in cell geometry occurring as a result of reduced soma size and withdrawal of the dendritic
tree, changes in the specific membrane resistivity, synaptic stripping and/or glial cell arrangement (Titmus and Faber, 1990). A cell’s input resistance is influenced by differences in the regional membrane channels and their leakiness and affects the relationship between current and cell voltage. Therefore, changes in input resistance may well influence the effectiveness of synaptic input. Interestingly, input resistance increases as a result of axotomy and would therefore be expected to contribute to larger EPSPs, exactly the opposite of what we observe in the regenerated Ia-motoneuron circuit.

Cell Excitability

Peripheral nerve axotomy results in an overall reduction in mean rheobase current (Bichler et al., 2007b, Nakanishi et al., 2005). Normally, in the cat, the fast fatiguable and fast fatigue resistant motoneurons have high rheobase values, 20 and 16 nA, respectively, while slow type motoneurons tend to have much lower rheobase values, 5 nA (Gustafsson and Pinter, 1984). After axotomy, the distribution of rheobase currents shifts toward much lower values (mean rheobase value of 4 nA for all types), suggesting that rheobase current is reduced in all cell types, but that fast motoneurons are affected to a greater degree. Reductions in cell rheobase are not trivial as this value is a direct quantification of cell excitability; changes in this neuronal excitability influence if and when an action potential will be generated, and therefore, can have significant functional relevance. Important contributors to altered motoneuron excitability may be changes in subthreshold conductances and changes in tonic synaptic activity occurring as a result of synaptic stripping (Titmus and Faber, 1990).
AHP Decay Times

Afterhyperpolarization potential plays an important role in rhythmic firing behavior. Specifically, the AHP of an action potential is an important determinant in the timing of subsequent action potentials. The effect of axotomy on AHP is less clear than its effects on input resistance and rheobase. Some studies have found no change in AHP duration or amplitude in cat MG motoneurons after axotomy (Foehring et al., 1986a, Gustafsson, 1979, Kuno et al., 1974), while others have reported slight increases in AHP duration (Gustafsson and Pinter, 1984, Pinter and Vanden Noven, 1989). In rat MG motoneurons, there appears to be no change in AHP amplitude or decay time following injury (Seburn and Cope, 1998).

When motor axons are permitted to regenerate and reinnervate muscle fibers, forming functional neuromuscular junctions, intrinsic motoneuron properties return to control levels and relationships between properties are restored in the cat (Foehring et al., 1986a, Foehring et al., 1986b). This process is continuous and occurs over the course of several months. It appears that a functional connection between the motoneuron and muscle fibers is required for properties to return to control levels, as those motoneurons incapable of eliciting muscle force upon activation maintain properties typical of slow type motoneurons (Foehring, 1986a). Furthermore, when motor axons are allowed to regenerate, but not reinnervate peripheral targets, motoneurons properties generally do not return to control levels and correlations between properties fade (Vanden Noven and Pinter, 1989).
Based on the data presented above, we expected that the intrinsic properties for regenerated motoneurons studied here would be comparable to control. However, it was still necessary to obtain and present this data from our database. To date, no published data exist on rat motoneuron properties after long term reinnervation from nerve cut. Because EPSP amplitude correlates with motoneuron properties, a general treatment effect or sampling bias that results in differences between intrinsic properties of control and regenerated motoneurons may obscure the measures of synaptic efficacy. In examining these data, we are able to a) identify and account for unexpected treatment effects and b) check for sampling variability between our control and regenerated groups that could affect the synaptic physiology presented in the next chapter.

**Methods**

*Animals.* Data were collected from adult female Wistar rats using procedures approved by Wright State University Laboratory Animal Care and Use Committee. Animals were housed individually or in pairs and were given food and water ad libitum. Animals were assigned to either the control or nerve treatment group.

*Nerve Treatment.* Animals in the reinnervated group underwent a survival surgery in which the nerves supplying their left medial and lateral gastrocnemius and soleus muscles were cut and immediately surgically rejoined. The specific procedure is detailed in Chapter 2, General Methods, and in the previous chapter.
**Terminal Experiments.** Animals were studied in single terminal recording sessions. Anesthesia was induced (4-5% in 100% O₂, inhalation in induction chamber) and maintained (1-3% in 100% O₂, through tracheal cannula) by isoflurane. Ringer/dextrose solution was injected subcutaneously (~1mL/hour) to maintain mean arterial pressure. End tidal pCO₂ and withdrawal reflexes were used to ensure an adequate level of anesthesia throughout the experiment. Whole body temperature was monitored and maintained at approximately 37°C by radiant heat. Oxygen content and pulse rate were also monitored and maintained above 90% and 300 respectively.

Standard research procedures were used to dissect the spinal cord and one hind limb for the purposes of recording bioelectric signals from motoneurons (Seburn and Cope, 1998). The lumbar spinal segments L3-L6 were exposed dorsally by laminectomy. The left hind limb was dissected such that the medial gastrocnemious muscle nerve was isolated and placed on bipolar hook electrodes. All other hindlimb nerves were crushed. Skin flaps were used to construct mineral oil pools to bathe all exposed tissue. After data had been collected, animals were euthanized by isoflurane overdose (5% in 100% O₂).

**Data Collection.** These data examine the effect of reinnervation on intrinsic motoneuron properties. Physiologic data were collected from motoneurons intracellularly by penetration of sharp glass micropipettes (5-10 MΩ, 2M K-acetate). A depiction of the experimental setup can be seen in Figure 4.1. Motoneurons were identified as medial gastrocnemius by antidromic stimulation of the peripheral nerve. Data collection proceeded when motoneuron action potential amplitude exceeded +60 mV and recording
Figure 4.1. *Experimental paradigm for recording intrinsic motoneuron properties.*

Intracellular recordings were measured from control and regenerated (shown) rat medial gastrocnemius motoneurons using sharp microelectrodes. Input resistance (Rin), rheobase current, and afterhyperpolarization potential (AHP) were measured.
conditions were stable. Cell rheobase, input resistance, and AHP were collected by injecting either depolarizing (rheobase, AHP) or hyperpolarizing (input resistance) current into the cell. A description of these properties and their collection is found in Chapter 2. Records of current and membrane potential were collected, digitized (20 kHz) and stored on a computer for later analysis using Spike2 software.

**Statistics.** Motoneuron properties were compared across treatment groups with consideration for animal to animal variation using Nested Analysis of Variance (Nested ANOVA) (SYSTAT, Systat Software, Point Richmode, CA). Pearson correlations were used to analyze relationships between properties and were performed using Statistica software (Statsoft, Inc., Tulsa, OK). For all statistical tests, significance was reached when $p<0.05$.

**Results**

*Reinnervation restores intrinsic motoneuron properties.*

Measures of intrinsic motoneuron properties were obtained from 167 control and 62 reinnervated motoneurons recorded from 60 total rats. In some cases, not all properties were recorded for a given motoneuron. In these instances, the available data were included in the analysis. Because these data were taken from many animals, the values for each recorded property (rheobase, input resistance, AHP half decay time, and AHP amplitude) have been plotted by experiment in chronological order. Figure 4.2 shows the distribution of individual data points recorded from each motoneuron for every experiment over time. For all properties, variability of values for each experiment is acceptable and no drift in values is seen over time.
Figure 4.2. *Observed values for rheobase current (A), input resistance (B), AHP ½ decay time (C) and AHP amplitude (D) recorded from control (●) and regenerated (○) MG motoneurons during 60 different experiments.*
Our primary objective was to determine whether intrinsic properties differed between control and regenerated motoneurons. Nested ANOVA indicated no significant difference in antidromic action potential amplitude \( (p = 0.934) \), rheobase current \( (p = 0.709) \), input resistance \( (p = 0.145) \), AHP half decay time \( (p = 0.784) \), or AHP amplitude \( (p = 0.177) \) between control and regenerated motoneurons. Table 4.1 shows the mean ± SD for all properties. It should be noted that the control values presented here are similar to previously published values for Wistar rats from our lab (Seburn and Cope, 1998, Bichler et al., 2007a), though they differ somewhat from data collected from Sprague-Dawley rats in another lab (Gardiner, 1993) and from cat (Gustafsson and Pinter, 1984, Foehring et al., 1986b). Cumulative histograms (Figure 4.3) illustrate a similar distribution of values from both control and regenerated motoneurons for all properties.

**Weak correlations exist between properties.**

Relationships among motoneuron properties have been established in cat and rat (Gardiner, 1993, Zengel et al., 1985), and were examined here using Pearson correlations. In control motoneurons, these relationships, though significant and in the expected directions, were surprisingly weak. The strongest relationship found was that between rheobase and input resistance \( (r = -0.36, p = 0.000, \text{see Figure 4.4}) \). Pearson correlations indicate moderate but significant relationships between input resistance and AHP half decay time \( (r = 0.31, p = 0.001, \text{see Figure 4.5}) \) and also between rheobase and AHP half decay time \( (r = -0.32, p = 0.000, \text{see Figure 4.6}) \). In regenerated animals, the correlation between rheobase and AHP half decay time became nonsignificant \( (r = -0.16, p = 0.29, \text{see Figure 4.6}) \) while the correlations between input resistance and rheobase \( (r = -0.31, p \)
Table 4.1. *Pooled data from control and regenerated MG motoneurons.* Values are reported as means ± SD. Number of motoneurons is reported in parentheses. Nested ANOVA indicate no statistical difference (p < 0.05) between control and regenerated values for any measure.
<table>
<thead>
<tr>
<th></th>
<th>Antidromic AP Amplitude (mV)</th>
<th>Rheobase Current (nA)</th>
<th>$R_n$ (MO)</th>
<th>AHP 1/2 Decay Time (ms)</th>
<th>AHP Amplitude (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>69.29 ± 7.39 (167)</td>
<td>9.79 ± 6.42 (165)</td>
<td>1.96 ± 0.97 (131)</td>
<td>12.43 ± 3.75 (153)</td>
<td>1.43 ± 0.82 (153)</td>
</tr>
<tr>
<td>Regenerated</td>
<td>70.72 ± 7.96 (62)</td>
<td>9.42 ± 5.93 (57)</td>
<td>2.13 ± 1.00 (49)</td>
<td>12.32 ± 3.53 (54)</td>
<td>1.45 ± 0.92 (54)</td>
</tr>
</tbody>
</table>
Figure 4.3. *Cumulative histograms of rheobase current (A), input resistance (B), AHP ½ decay time (C) and AHP amplitude (D) for all control (●) and regenerated (○) MG motoneurons.*
Figure 4.4. *Rheobase current plotted against input resistance for both control (●) and regenerated (●) MG motoneurons.* Pearson correlation indicates a moderate relationship for control ($r = -0.36$, $p = 0.00$) and regenerated ($r = -0.31$, $p = 0.043$) motoneurons.
Figure 4.5. *AHP ½ decay time plotted against input resistance for both control (●) and regenerated (●) MG motoneurons.* Pearson correlations indicate a moderate relationship for control ($r = 0.31, p = 0.001$) and regenerated ($r = 0.32, p = 0.032$) motoneurons.
Figure 4.6. *Rheobase current plotted against AHP ½ decay time for both control (●) and regenerated (●) MG motoneurons.* Pearson correlation indicates a weak relationship for regenerated ($r = -0.16$, $p = 0.293$) motoneurons and a moderate relationship for control ($r = -0.32$, $p = 0.00$) motoneurons.
= 0.043, see Figure 4.4) and input resistance and AHP half decay time (r = 0.32, p = 0.032, see Figure 4.5) maintained a moderate level of significance.

**Discussion**

This study examined the effect of reinnervation on motoneuron intrinsic properties and their interrelationships in the adult rat. The purpose was to assess whether reinnervation restored these properties to control levels and determine if the motoneuron intrinsic properties could account for any differences in synaptic efficacy between control and regenerated circuits. These data clearly show that the regenerative process restored all motoneuron properties to pretreatment values, as expected, and suggest that any changes seen at the synaptic level are not influenced by whole motoneuron properties.

Because input resistance is directly correlated with EPSP amplitude, any group differences in input resistance occurring as a result of variability in sampling could affect EPSP size. This was unlikely given the large number of motoneurons sampled, but was considered, nonetheless. Again, because motoneuron properties were not statistically different between control and regenerated samples, the data presented here are comparable and any differences in EPSP size cannot be explained by variability in sampling.

Correlations between motoneuron properties were also examined. While the relationships between properties were consistent in their direction, the magnitude of the correlations was much smaller than previously reported. In cat motoneurons, intrinsic
properties correlate quite strongly with one another (Zengel et al., 1985). Furthermore, Gardiner (1993) reported significant correlations between motoneuron properties in rat tibial motoneurons. The strongest correlation noted by Gardiner in rat motoneurons was that of input resistance and AHP half decay time ($r = 0.62$). Here that relationship is moderate at best ($r = 0.31$). It appears that slow motoneurons contribute greatly to this and other correlations. When “presumptive slow” motoneurons with AHP half decay times of $> 20$ ms were not included in Gardiner’s analysis, all significant correlations became nonsignificant. Because our data are taken from MG motoneurons, few motoneurons are of the slow type (as opposed to tibial motoneurons which include many more slow type motoneurons). Therefore, it is expected that the correlations reported here would be much weaker. Our findings agree with Gardiner’s suggestion that interrelationships among motoneurons are less clear in the rat those described for cat motoneurons.
INTRODUCTION

In the previous chapters, we have clearly established that provisional Ia afferents recover their peripheral connections and are capable of producing and conducting normal responses to muscle stretch and that motoneurons recover their intrinsic properties. Despite this success, abnormalities still exist with the Ia-motoneuron circuit. After peripheral nerve regeneration, muscle stretch is insufficient to depolarize some motoneurons. This observation along with the drastic changes occurring in the central cellular machinery of axotomized and regenerated Ia afferents and motoneurons leave suspicion about their functional capacity. As discussed in Chapter 1, injured Ia afferents retract their terminal axons and synapses between Ia afferents and motoneurons are removed (Mendell et al., 1976). Injured motoneurons undergo a profound stripping of other synaptic input as well (Brannstrom and Kellerth, 1998, Lind et al., 2000, Brannstrom and Kellerth, 1999). It is unknown how well the Ia-motoneuron synapse recovers with regeneration, but recent data published by our lab shows that stimulation of a regenerated peripheral nerve at functionally atypical frequencies (2 pps) always results in a monosynaptic EPSP (Haftel et al., 2005, also see Results). This is important because it indicates that the physical connection made between some afferents and motoneurons is restored and that the synapses are to some degree functional. However, it does not tell us if the system is functional at physiological frequencies when afferents fire at rates up to 600 pps. At these high levels, conduction block may occur within the central processes.
of Ia afferents and/or the synapse may undergo depression. Either deficiency could contribute to the reduced or absent stretch evoked synaptic potentials seen in some regenerated Ia-motoneuron circuits.

Using electrical current at group I strength, we stimulated the peripheral nerve in patterns typical of Ia afferent firing in response to ramp hold and release muscle stretch. This allowed us to examine the function of the regenerated Ia-motoneuron synapse at physiological frequencies without actually stretching the muscle (which activates other muscle afferents and polysynaptic neurons). We hypothesized that the regenerated circuit fails at frequencies typical during muscle stretch, and thus expected to see a substantial decrease in EPSP amplitude throughout the stimulus train. Surprisingly, our results suggest that the opposite is true: electrically activated group I afferents can successfully conduct and transmit at physiologic frequencies. These initial results led us to question whether those afferents responding to muscle stretch were the same afferents that produced synaptic depolarization in motoneurons when activated by electrical stimulation. This is an important question because stretching the muscle often results in no synaptic response while electrical stimulation always results in synaptic depolarization. Using spike triggered averaging, we were able to examine the connectivity between individual afferents whose response to muscle stretch is typical of Ia afferents and motoneurons. Finally, the anatomical central projections of regenerated provisional Ia afferents were examined. We arrive at the surprising yet inescapable conclusion that peripheral reconnection is neither necessary nor sufficient to restore central synapses between regenerated Ia afferents and motoneurons.
**Methods**

**Animals.** Data were collected from adult female Wistar rats using procedures approved by Wright State University Laboratory Animal Care and Use Committee. Animals were housed individually or in pairs and were given food and water ad libitum. Animals were assigned to either the control or nerve treatment group.

**Nerve Treatment.** Animals in the reinnervated group underwent a survival surgery in which the nerves supplying their left medial and lateral gastrocnemius and soleus muscles were cut and immediately surgically rejoined. The specific procedure is detailed in Chapter 2, General Methods.

**Terminal Experiments.** Animals were studied in single terminal recording sessions. The animals’ vitals were measured and maintained as described previously. Standard research procedures were used to dissect the spinal cord and one hind limb for the purposes of recording bioelectric signals from motoneurons (Seburn and Cope, 1998). The lumbar spinal segments L3-L6 were exposed dorsally by laminectomy. The left hind limb was dissected such that the medial gastrocnemious muscle nerve was isolated and placed on bipolar stimulating electrodes. All other hindlimb nerves were crushed. Skin flaps were used to construct mineral oil pools to bathe all exposed tissue. In some animals, stretch evoked synaptic potentials were obtained. For these experiments, the medial and lateral gastrocnemius and soleus muscles were freed from the surrounding tissue. Their tendon was detached from the calcaneous and tied to a servomotor. Resting
length was determined by matching suture ties in the soleus muscle and the surrounding connective tissue that were lined up prior to the tendon being detached. The rats were fixed in a rigid recording frame with knee and ankle joints fixed at 90° angles. After data had been collected, animals were euthanized by isoflurane overdose (5% in 100% O₂).

**Data Collection.** These data examine the effect of reinnervation on motoneuron synaptic potentials in response to several different types of stimuli: (1) low frequency electrical stimulation (1 pps), (2) a train of electrical stimulation mimicking Ia afferent firing patterns in response to ramp hold and release muscle stretch (pulse train), (3) actual ramp hold release muscle stretch, (4) tendon tap muscle stretch, and (5) sustained muscle stretch (for recording spike triggered averaged individual EPSPs). Physiologic data were collected from motoneurons intracellularly by penetration of sharp glass micropipettes (5-10 MΩ, 2M K-acetate). Motoneurons were identified as medial gastrocnemius by antidromic stimulation of the peripheral nerve. Data collection proceeded when motoneuron action potential amplitude exceeded +60 mV and recording conditions were stable. During the collection of all synaptic data, multiple trials were obtained. Typically, motoneuron membrane potential was averaged during 10-15 stimulus trials to enhance the signal to noise ratio. The averages were then used for all measurements.

**Low Frequency Electrical Stimulation**

To determine the functional capacity of the circuit, low frequency Ia-motoneuron EPSPs were recorded. This was done by extracellularly stimulating the entire peripheral nerve using 50 μs pulses delivered at 0.5 Hz (Iso-Flex Stimulus Isolation Unit, AMPI). When
the whole peripheral nerve is stimulated, both afferent and efferent axons are activated. Monosynaptic EPSPs arriving in the motoneuron can be obscured by the antidromic action potential arriving shortly before. To deal with this problem, the stimulus strength was reduced to a level just below the motoneurons firing threshold. At these stimulus strengths, it is likely that some but not all MG Ia afferents were activated.

Muscle Stretch

The basis for these experiments is the observation that some regenerated motoneurons lack stretch evoked synaptic potentials. To verify previous data acquired from decerebrate rats, we collected motoneuron potentials during ramp hold and release muscle stretch (see Figure 5.1). These experiments were performed under similar conditions as were used to collect Ia afferent data for Chapter 3. During ramp hold and release stretches, the muscle was displaced 3 mm with a 150 ms rise and release time (20 mm/s) and 500 ms hold time. Trials began at approximately similar background force (~15 g) and were repeated every 4 seconds. Averages of the motoneuron potentials during stretch were collected and initial amplitude and peak amplitude during the ramp and hold phases were measured. As expected, regenerated motoneurons had stretch evoked synaptic potentials that were reduced in amplitude or altogether absent. One potential contributor to the smaller SSPs is temporal dispersion of Ia afferent input. To minimize this concern, synaptic potentials were recorded during tendon tap muscle stretch. During tendon tap stretch, the muscle was stretched at a much faster velocity (400 mm/s). This type of stretch presumably synchronizes Ia afferent firing and reduces extrinsic influences. Tendon taps displaced the muscle 1 mm from resting length over a
Figure 5.1. **Recording Paradigm for Muscle Stretch.** Stretch evoked synaptic potentials are recorded intracellularly from impaled and antidromically identified rat MG motoneurons. Muscle length over time is shown for A. tendon tap and B. ramp hold and release stretch with an expanded time base shown to the right. Several trials of muscle stretched were performed and averaged to improve resolution.
period of 5 ms including 2.5 ms for the ramp and 2.5 ms for the release. Individual taps were averaged and the averages were used to measure peak amplitude and the time from stretch onset to peak amplitude.

High Frequency Pulse Train Stimulation
The primary aim of this chapter was to determine whether central conduction and/or monosynaptic transmission fail at rates that encode stretch, and thereby could explain why some motoneurons have no synaptic response to muscle stretch. To obtain this information, Ia afferents were stimulated using a pulse train that simulates stereotyped Ia afferent firing in response to ramp hold and release stretches (see Figure 5.2). The pulse train is entirely relevant because it forces afferents to fire in a pattern and at rates that correspond to physiologic activity. The pulse train was used to activate Ia afferents by extracellularly stimulating the whole peripheral nerve (again at levels below motoneuron threshold). Trains were presented in 4 second intervals and motoneuron potentials were averaged over a series of trains (10-15). To examine whether there were any differences throughout the stimulus train, EPSPs were measured at the onset of the stimulus train, maximum stimulus frequency (corresponding to the peak of the ramp of ramp hold and release stretch) and at the end of the steady-state (corresponding to the end of the hold phase of ramp hold and release stretch). The recording paradigm for obtaining motoneuron potentials during electrical stimulation of the peripheral nerve can be seen in Figure 5.2.
Figure 5.2. *Recording Paradigm for Electrical Stimulation.* The spinal cord and medial gastrocnemius nerve of an anesthetized adult female Wistar rat were exposed for electrical stimulation and recording from motoneurons. The peripheral nerve was stimulated extracellularly at a strength below motoneuron threshold and not exceeding 2.5x threshold for the dorsal root volley to activate only group I afferents. The peripheral nerve was stimulated at low frequencies (1 pps, not shown) or using the pulse train stimulus (shown) in which the stimulus mimics typical Ia afferent firing rates and patterns during muscle stretch while the membrane potential is recorded from motoneurons. Membrane potential was continuously recorded while 10-15 trials were run. The trials were averaged together, and the averages were used in the analysis.
Spike Triggered Averaging

These experiments were performed to test the connectivity between regenerated provisional Ia afferents and motoneurons. The spike triggered averaging technique has been useful in examining individual synapses between two cells. This technique uses the presynaptic cell as a trigger for postsynaptic activity, and allowed the collection of individual EPSPs from arising single regenerated Ia afferents synapsing on motoneurons. Dorsal roots were dissected using jewelers forceps (#5) into fine filaments that contained one single afferent that could be discriminated from noise. This filament was placed on small biopolar recording electrodes with care not to damage the root. Using ramp hold and release, triangular and vibrational muscle stretch and firing patterns during muscle contraction, afferents were classified as presumable Ias (afferents reinnervating the annulospiral endings of muscle spindles). Once an afferent was identified as group Ia, motoneuron recordings began. Upon penetration of an MG motoneuron, the muscle was stretched to a length that caused sustained firing (usually ~25 Hz) in the discriminable afferent. Action potentials generated by the afferent were recorded by the extracellular electrodes at the dorsal root, and motoneuron membrane potential was simultaneously recorded by the intracellular recording electrode. A schematic of this design can be seen in Figure 5.3. Triggered by the occurrence of presynaptic action potentials, 10 ms segments of motoneuron membrane potential were recorded, collected and averaged over many sweeps (300-2000). These averages were used to assess the presence/absence of an individual EPSP. When an individual EPSP was present, its amplitude, latency, rise time and half-width were measured.
Figure 5.3 *Recording Paradigm for Spike Triggered Averaging.* Spike triggered averaging was used to characterize the connections between regenerated afferents and motoneurons. Dorsal roots were dissected into fine filaments containing at least one discriminable afferent whose firing properties signify a provisional Ia afferent. Upon impalement of a motoneuron, the triceps surae muscles were stretched to a length that produced continuous firing of the single Ia afferent. Using presynaptic action potentials as a trigger, sweeps of the motoneuron potential were recorded and averaged to identify the presence or absence of an individual EPSP. The resulting individual EPSPs were further analyzed.
In addition, cell rheobase, input resistance, and AHP were collected by injecting either depolarizing (rheobase, AHP) or hyperpolarizing (input resistance) current into the cell. A description of these properties and their collection is found in Chapter 2. In some motoneurons, the quality of the records prevented collection of a complete data set. Records of electrode current, membrane potential, muscle force and muscle length were collected, digitized (20 kHz) and stored on a computer for later analysis using Spike2 software.

Intracellular Axon Fills

Anatomic data were collected from 17 rats to examine the spinal cord projections of regenerated provisional Ia afferents. Orthodromically identified provisional Ia afferents were impaled with glass micropipettes filled with 10% Neurobiotin in 3M K-acetate. Following collection of responses to muscle stretch as described in Chapter 3, afferents were injected with Neurobiotin. Depolarizing pulses (5 to 25 nA, 400ms) were delivered at 2 Hz for 5 to 25 minutes. Animals were perfused no fewer than 6 hours after an afferent was filled to allow time for transport of Neurobiotin into the spinal cord.

Animals were fixed using 4% paraformaldehyde. Tissue was processed, and Neurobiotin-filled afferents were visualized using FITC-conjugated streptavidin (1:100, Jackson ImmunoResearch). Low magnification images were obtained using an epifluorescence microscope. These data were collected in collaboration with the lab of Dr. Francisco Alvarez.
Statistics. Synaptic properties were compared across treatment groups with consideration for animal to animal variation using Nested Analysis of Variance (Nested ANOVA) (SYSTAT, Systat Software, Point Richmode, CA). For all statistical tests, significance was reached when $p < 0.05$.

Results

The initial data presented here show that low frequency EPSPs are always present in regenerated motoneurons while stretch evoked synaptic potentials are only present in some motoneurons. These findings corroborate previous data collected from decerebrate rats in our laboratory in the only other study of stretch evoked synaptic potentials following peripheral nerve regeneration. In addition, these data extend the findings to the anesthetized rat preparation.

Low Frequency EPSPs were present in all regenerated motoneurons.

Data were collected from 132 control motoneurons and 39 regenerated motoneurons. The intrinsic motoneuron properties did not differ statistically between groups, as discussed in the previous chapter. Low frequency EPSPs were present in all control and regenerated motoneurons. Measures of EPSP latency allowed us to be certain that input was arriving in the motoneuron at a time consistent with monosynaptic transmission. Rise time was used to assess whether regenerated afferent input was abnormally dispersed over time (see Table 5.1). These measures did not statistically differ between control and regenerated conditions (nested ANOVA, $p > 0.05$).
Table 5.1. *Low frequency EPSP characteristics were comparable in control and regenerated circuits.* Values are reported as mean ± SD. No significant differences were observed.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Amplitude (mV)</th>
<th>Latency (ms)</th>
<th>Rise time (ms)</th>
<th>Half width (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 132)</td>
<td>1.18 ± 1.66</td>
<td>2.03 ± 0.24</td>
<td>0.91 ± 0.32</td>
<td>2.10 ± 0.77</td>
</tr>
<tr>
<td>Regenerated (n = 39)</td>
<td>1.35 ± 1.33</td>
<td>2.09 ± 0.37</td>
<td>0.91 ± 0.42</td>
<td>1.92 ± 0.90</td>
</tr>
</tbody>
</table>
Ramp hold and release Stretch evoked Synaptic Potentials (SSP) were present in some but not all regenerated afferents.

The basis for studying the regenerated Ia-motoneuron circuit was the absence of stretch evoked synaptic potentials in some motoneurons, making it necessary to verify the presence of this effect. Ramp hold and release stretch evoked synaptic potentials were collected from 65 control motoneurons and 41 regenerated motoneurons. 12/41 regenerated motoneurons had no SSP (for example, see Figure 5.4) compared to 1/65 control. One control motoneuron did not have a stretch evoked synaptic potential. This is quite unusual and may have been the result of mis-identification as an MG motoneuron due to current spread to a nerve other than MG or the muscle may not have been stretched sufficiently to activate Ia afferents. In addition, some SSPs in regenerated animals had an inhibitory component (4/41), typically expressed during the hold phase of muscle stretch. This response was not seen in any control motoneurons.

Regenerated motoneurons from the same animal had different synaptic responses to muscle stretch. Synaptic responses were acquired from multiple motoneurons in 10 treated animals. In 5 of those 10 animals, either no synaptic response or a negative synaptic response was seen in at least one motoneuron while an excitatory postsynaptic response was seen in another.

SSP amplitude was measured at the peak of the ramp and compared between the groups. In those motoneurons in which SSPs were present, SSP amplitude at the ramp peak was significantly smaller in regenerated motoneurons (p = 0.000, nested ANOVA). The
Figure 5.4. *Some regenerated motoneurons have no synaptic response or even inhibitory synaptic responses to muscle stretch despite stretch induced activity in the dorsal roots.*
cumulative histograms in Figure 5.5 show a shift toward much smaller amplitudes in regenerated animals. Mean SSP amplitude at the ramp peak in control motoneurons was $1.5 \pm 1.0 \text{ mV}$ compared to $0.5 \pm 0.6 \text{ mV}$ in regenerated motoneurons. While not shown here, SSP amplitudes at other points during muscle stretch were similarly decreased.

One potential explanation for the decrease in SSP peak amplitude in regenerated motoneurons is that afferents input becomes temporally dispersed and therefore does not summate. To test this possibility, we first examined the rise time of low frequency EPSPs and secondly used tendon tap muscle stretch, in which the muscle is quickly stretched and released (see Figure 5.1). The advantage to studying stretch evoked synaptic potentials elicited by tendon tap stretch is it has the ability to a) selectively activate Ia afferents, b) synchronize the activated Ia afferents, and c) further test the cellular components of the regenerated Ia-motoneuron circuit using the very stimulus in which we find abnormalities: stretch. Tendon tap stretch is particularly effective at activating Ia afferents because of their sensitivity to detect changes in muscle velocity in addition to muscle length. The tendon tap stretch evoked synaptic potential, therefore, reflects input nearly entirely from Ia afferents rather than other afferent types. Tendon tap stretch evoked synaptic potentials (TTSSP) were recorded from 53 control motoneurons and 20 regenerated motoneurons. Regenerated motoneurons ($0.51 \pm 0.97 \text{ mV}$) had reduced peak amplitude during tendon tap stretch compared to control ($1.33 \pm 0.96 \text{ mV}$, $p = 0.000$, nested ANOVA, see Figure 5.6). Of the 20 regenerated motoneurons, eight had no TTSSP. Five of those eight did not have SSPs elicited from ramp hold and release stretch either (see Figure 5.7). In three cases, a TTSSP was not present but a small
Figure 5.5. *Stretch evoked synaptic potentials recorded from regenerated motoneurons have reduced amplitudes at the ramp peak of muscle stretch compared to control.*
Figure 5.6. *The peak amplitude for synaptic potentials occurring in response to tendon tap muscle stretch is reduced in regenerated motoneurons.* Insert shows a tendon tap synaptic potential recorded from a regenerated motoneuron.
Figure 5.7. *Tendon tap SSP peak amplitude vs. ramp hold and release SSP peak amplitude for control and regenerated motoneurons*. Both types of muscle stretch produced reduced synaptic responses in regenerated motoneurons. The dark red data point marks 5 regenerated cells which had no SSP or TTSSP.
positive SSP (< 1 mV) was present. On the other hand, 8/20 regenerated animals had no SSP. There were three regenerated motoneurons that had no detectable SSP but a slight yet measurable TTSSP. Interestingly, tendon tap stretch produced a negative potential in one motoneuron. This was surprising because its synaptic response to ramp hold and release stretch was positive, though small.

The time from the onset of muscle stretch to the peak amplitude of synaptic potentials produced by tendon tap muscle stretch was also measured. TTSSPs recorded from regenerated motoneurons peaked on average 9.01 ± 5.21 ms after stretch onset, nearly 4 ms longer than the time to peak in controls (5.25 ± 2.63 ms, see Figure 5.8). This difference was statistically significant (nested ANOVA, p = 0.002).

*Regenerated Ia-motoneuron circuits transmit at physiologic rates and patterns.*

Of primary interest was the synaptic potential recorded in response to high frequency nerve stimulation simulating Ia afferent firing in response to muscle stretch. Data were taken from 50 control and 21 regenerated afferents. Figure 5.9 shows the typical response for both a control and regenerated circuit. In both cases, the result is a series of EPSPs corresponding to each stimulus in the train. These experiments compare composite EPSPs consisting of multiple individual EPSPs. In order to determine whether any individual Ia afferents were unable to follow the stimulus train, EPSP amplitudes were compared throughout the train. The EPSP amplitude during the onset of the stimulus train was compared with the amplitude at significant points throughout the train. Rather than using the first EPSP of the train for the data point at the onset of the stimulus
Figure 5.8. *The time from muscle stretch onset to TTSSP peak amplitude was increased in regenerated circuits.* Each dot represents the time to peak amplitude for the TTSSP recorded from one motoneuron. Measures are reported for synaptic potentials recorded from 53 control motoneurons and 12 regenerated motoneurons.
train, we chose to use the second EPSP to avoid any effect of potentiation (during repeated trials of high frequency stimulation, the initial EPSP is often enhanced (Davis et al., 1985). We expect that points in which some individual afferents were not able to follow the stimulus train should have reduced EPSP amplitudes. Figure 5.10 shows that very little depression occurs throughout the stimulus train indicating an ability of afferents to fire at these high frequencies. Our results clearly show that regenerated Ia afferent-motoneuron circuits are competent to conduct and transmit at physiological frequencies (c.f. Mendell et al., 1995). While a slight depression was seen in frequency modulation at the maximum stimulus frequency, this depression is insufficient to account for the lack of any synaptic response seen in many regenerated Ia-motoneuron circuits.

Very few stretch responsive regenerated spindle afferents make monosynaptic connections with motoneurons.

The present data reveal a conundrum in which muscle stretch and electrical activation of group Ia afferents produce dramatically different synaptic response in some regenerated motoneurons (see Figure 5.11). To understand whether those afferents making synaptic monosynaptic connections with motoneurons (and successful in producing electrically evoked synaptic potentials) were actually the same afferents that were responding to muscle stretch, individual connections between single provisional Ia afferents and motoneurons were examined. Control data were collected from 8 afferents and 29 Ia-motoneuron pairs. Whenever possible, one afferent was sampled with multiple motoneurons. In total, 27/29 (93%) of control afferent-motoneuron pairs were determined to be connected by the presence of a spike triggered averaged individual
Figure 5.9. *EPSPs sustained amplitude throughout high frequency stimulus train in both control and regenerated Ia-motoneuron circuits.*
Stimulus Train

Control
la-motoneuron circuit

Regenerated
la-motoneuron circuit
Figure 5.10. *Intratrain variation in EPSP amplitude.* Relationships between initial EPSP in train vs. (A, C) EPSP at maximum stimulus frequency (inset in A) or vs (B, D) EPSP at end of steady-state firing (inset B) for control (n = 50) and regenerated (n = 21) motoneurons. These measures show little deviation from the initial EPSP: points distribute evenly about the line of identity in A&B and ratios with EPSPs at maximum and steady-state firing rates approximate 1 (dashed line) in C&D.
5.11. *Some regenerated Ia-motoneuron circuits have no stretch-evoked synaptic responses despite the ability of their synapse to transmit at high frequencies.*  

A. Synaptic potentials resulting from electrical stimulation and muscle stretch (3mm) were recoded in two different motoneurons. In the control Ia-motoneuron circuit, EPSPs are seen in response to both electrical and stretch stimuli. In the regenerated circuit, however, only electrical stimulation is successful in producing excitation. Muscle stretch is completely ineffective in eliciting a synaptic response even when sensory afferents are actively responding (as seen in the extracellular dorsal root record, B).
EPSP with a monosynaptic latency (0.703 ± 0.296). Individual control EPSPs had amplitudes ranging from 14 μV to 385 μV with a mean value of 77.22 ± 73.57 μV. Figure 5.12 shows a representative Ia-motoneuron pair in which afferent firing was typical of group Ia and an SSP was present. This control Ia-motoneuron pair produced the largest STA EPSP (385 μV). Connectivity in regenerated Ia-motoneuron circuits was quite different from control (see Table 5.2). Connections between 7 afferents and 18 motoneurons were studied. Only 1/18 (5.6%) Ia-motoneuron pairs had an STA EPSP (167 μV) with a monosynaptic latency (0.72 ms). Two other STA EPSPs were observed, both smaller in amplitude (12 and 68 μV) and having latencies that fell within the polysynaptic range (1.5 and 1.5 ms, see Figure 5.13, (Watt et al., 1976). Interestingly, the same afferent was capable of producing quite different responses in various motoneurons (see Figure 5.14). For example, one provisional afferent produced a large monosynaptic STA EPSP, a small polysynaptic STA EPSP, and no STA EPSP in three different regenerated motoneurons.

**Regenerated provisional Ia afferents have reduced projections into Lamina IX.**

The central projections of 12 control and 5 regenerated afferents were examined. In all control cases, afferents projected into laminas V, VII, and IX. All regenerated afferents also had collaterals in lamina V and lamina VII, though two of the five afferents had few collaterals in lamina VII. In contrast, four of the five regenerated afferents had no projections into lamina IX (see Figure 5.15). The remaining regenerated afferent had only one collateral in lamina IX.
Figure 5.12. *Stretch evoked activity recorded from the control Ia-afferent and motoneuron pair.*
Table 5.2. *Levels of connectivity are drastically different in control and regenerated (provisional) Ia-motoneuron pairs.* Table shows the afferent number, absolute connectivity and percent connectivity between individual (provisional) Ia afferents and motoneurons. Connectivity is reported as the number of motoneurons that particular afferent made a functional monosynaptic connection with out of the total number of motoneurons sampled. Data are reported separately for control and regenerated circuits. Though individual spike triggered averaged EPSPs were present in three provisional Ia-motoneuron pairs, only one EPSP had a monosynaptic latency and is reported here.
<table>
<thead>
<tr>
<th>Afferent</th>
<th>Control Connectivity</th>
<th>%</th>
<th></th>
<th>Afferent</th>
<th>Regenerated Connectivity</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/1</td>
<td>100</td>
<td></td>
<td>1*</td>
<td>1/3</td>
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<td>93</td>
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</table>

* Same Animal
Figure 5.13. 2/3 individual EPSPs obtained by spike triggered averaging had polysynaptic latencies. Shown here are the latencies for electrical and spike triggered averaged EPSPs. The dorsal root recording, monosynaptic composite EPSP and polysynaptic individual EPSP were all collected from the same motoneuron. The dorsal root recording shows the action potential corresponding with the polysynaptic individual EPSP. The monosynaptic individual EPSP was collected from a different motoneuron in the same rat but was included here for comparison with the polysynaptic individual EPSP.
One regenerated afferent produced vastly different individual EPSPs in three motoneurons. Top traces show muscle length, instantaneous firing rate, and extracellular dorsal root recording during triangular, ramp hold and release and vibrational muscle stretch which were used to classify the afferent as probable group Ia. The lower traces show the spike triggered averaged motoneuron records averaged over the number of sweeps reported to the right. In the first motoneuron, no individual EPSP was found. In motoneuron 2, a small (12 μV) EPSP with a polysynaptic latency (1.5 ms) was observed. The third motoneuron was the only case in which a monosynaptic (0.72 ms) individual EPSP (167 μV) was seen.
Regenerated Ia afferent Data:

Individual EPSPs

Mn1

Mn2

Mn3

Sweeps:

822

536

351
Figure 5.15. *Low magnification images of the central projections from a Neurobiotin filled control Ia afferent and regenerated provisional Ia afferent.* Fiber collaterals from the control Ia afferent branch into terminal arborizations in lamina V (L V), L VII and in L IX, a pattern typical for normal Ia afferents. In contrast, no collaterals are seen in LIX and fewer projections were noted in lamina VII for the regenerated fiber. The border for lamina IX is denoted by the dotted yellow line, and the parent axon for each is indicated by the yellow arrows. The afferents’ responses to muscle stretch can be seen below.
**Discussion**

These results provide insight into the function of the regenerated Ia-motoneuron circuit and its underlying connectivity. We found that failure of the regenerated circuit is not the result of either cell’s ability to function properly, but rather a mismatch in peripheral reinnervation and central connectivity.

**SSPs are absent in some motoneurons**

Excitatory SSP were absent in approximately 40% of regenerated motoneurons even when muscle stretch was capable of activating muscle afferents. This absence clearly indicates that the Ia-motoneuron circuit experiences central deficits despite successful nerve regeneration and reinnervation. The data collected here support and extend earlier findings from our lab using a decerebrate rat preparation in which two thirds of motoneurons were found to have no SSP. In both the previous and present study, inhibitory synaptic responses were recorded from a small portion of motoneurons during muscle stretch. This finding strongly suggests that either synaptic connections are disrupted in a way that stretch information is actually communicated by IPSPs or that injury or regeneration activates central mechanisms that actively suppress SSPs. In the majority of abnormal Ia-motoneuron connections, SSPs were simply absent. To understand the basis for reduced synaptic activity in regenerated circuits, several potential contributors were considered.
**Temporal Dispersion/Inhibitory Networks**

First, for records of synaptic activity to be discriminable from noise in a small number of sweeps, some degree of synchrony among the input is required. Desynchronization of input in regenerated animals could result in reduced or absent SSPs. Second, the few instances of inhibitory SSPs suggest that inhibitory external networks may be acting on the regenerated Ia-motoneuron circuit. Ramp hold and release stretch, due to the slow change in muscle length, has the capacity to activate group Ia, Ib, II, III, and even IV afferents and, as a result of sustained afferent firing, turn on extrinsic networks that may influence the Ia-motoneuron circuit. Here, we utilized a different type of stretch, tendon tap, in which the muscle was quickly stretched and released. The advantage to studying stretch evoked synaptic potentials elicited by tendon tap stretch is its ability to simultaneously address the role of both dispersion of input and external networks.

Tendon tap stretch is particularly effective at activating Ia afferents because of their sensitivity to detect changes in muscle velocity in addition to muscle length. The tendon tap stretch evoked synaptic potential, therefore, reflects input primarily from Ia afferents rather than other afferent types. In addition, tendon tap stretch, because of its brevity (2.5 ms), is effective in synchronizing afferents and is less likely to activate spinal networks than is the long lasting ramp hold release. Specifically, this method of stretch should be effective in correcting temporal dispersion among regenerated afferents and reducing any potential inhibitory networks that influence the circuit and may have formed as a result of injury and/or regeneration. The ability of the tendon tap stretch to reduce extrinsic activity lies in the precise timing of neural activity. Network activity is necessarily polysynaptic, and the delay time for polysynaptic activity is 2-3 ms greater than the time
required for monosynaptic activity to reach the motoneuron. Because the ramp of muscle stretch is only 2.5 ms long, Ia afferents begin and terminate firing before any polysynaptic activity influences the monosynaptic potential. This is different from the much slower ramp hold and release stretch where inhibitory networks have the ability to influence the synaptic potential very early during the ramp phase. We expected, then, that tendon tap muscle stretch would be successful in eliciting an excitatory synaptic response even when ramp hold release stretch was not. Interestingly, when muscles were stretched with the tendon tap, their synaptic responses largely paralleled the responses to ramp hold and release stretch suggesting that neither temporal dispersion nor external inhibitory networks are large contributors to the loss of SSPs in some motoneurons. There were some instances in which tendon tap stretch was capable of producing stretch evoked synaptic responses while ramp hold and release stretch was not. In these cases, temporal dispersion and/or inhibitory networks may have a more significant role. But, their overall contribution seems weak at best.

Conduction Block/Synaptic Transmission

The potential roles of conduction block and/or monosynaptic transmission in reduced synaptic activity were also studied using electrical stimulation of the peripheral nerve at physiologic frequencies. As opposed to the relatively poor synaptic responses to muscle stretch, electrical stimulation of the Ia-motoneuron circuit produced normal synaptic responses. Synaptic potentials collected from regenerated motoneurons during electrical stimulation at physiologic frequency were quantitatively indistinguishable from control. Examination of these data was critical because it allowed us to test central conduction of
action potentials in regenerated afferents and monosynaptic transmission using the frequencies and patterns in which stretch evoked synaptic potentials are absent. The data clearly show that both central conduction and monosynaptic transmission of regenerated muscle afferents are successful, ruling out two of the most likely contributors to the loss of stretch evoked synaptic activity. Given that Ia afferents and motoneurons functionally disconnect after injury (Mendell et al., 1976), this result is quite impressive. It signifies that connections between regenerated afferents and motoneurons do in fact reform after their initial disconnection and are functional not only at low frequencies but also at the much higher physiological frequencies (c.f. Mendell et al., 1995). Furthermore, central conduction of action potentials is also successful at these high frequencies. Newly formed branch points and boutons do not interrupt action potential propagation and frequency-dependent conduction block is not observed. All in all, the cellular machinery appears entirely functional after regeneration and points to other central factors that must be responsible for the lack of the stretch reflex.

A less likely interpretation of these data is that the spinal cord has the remarkable capacity to compensate for deficits in the circuit and, through other pathways, produces high frequency synaptic potentials nearly identical to controls. To address this possibility, the composition of the composite EPSP was considered. Specifically, it is possible to have measured EPSPs resulting from input of cell types (group II, polysynaptic primary afferent input, etc.) other than Ia afferents. As discussed in the general background, group II afferents and polysynaptic primary afferent input may contribute to composite EPSPs (Cope et al., 2001) especially in the rat where the
distribution of group I and group II afferent conduction velocities overlap (De-Doncker et al., 2003). It is unlikely that the composite EPSPs recorded are contaminated by these inputs. First, the stimulus strength used to activate group Ia afferents was reduced to levels below motoneuron firing threshold. At these stimulus strengths, only the largest diameter Ia afferents should be activated. Second, the EPSP properties were measured and their values were comparable to control. It is expected that a larger contribution by group II and polysynaptic input, because of their slower conduction velocity and polysynaptic delay, would increase both the rise time and EPSP half-width. Because nearly all EPSP properties were similar in control and regenerated circuits, their input is likely the same.

**Question of Connectivity**

Upon self reinnervation of a muscle, some afferents respond to muscle stretch by firing in patterns nearly identical to untreated Ia afferents (Haftel et al., 2005). Their firing responses and orthodromic action potential latencies place them in group Ia, but the original characterization of these regenerated afferents is unknown as cut afferents may reinnervate different end organs. For example, an afferent previously innervating a golgi tendon organ may reinnervate a muscle spindle. In cases where the new target differs from the original end organ, synapses arising from the afferent’s central projections must be rewired in order for the system to regain original function. Inappropriate reinnervation without adjustments in spinal cord circuitry could result in functional failures. This was the final potential contributor we tested.
Both physiologic and low frequency electrical stimulation of a regenerated peripheral nerve at group I strength results in EPSPs with monosynaptic latencies. However, muscle stretch often results in either no synaptic potential or an inhibitory synaptic potential (see Figure 5.11). This conundrum could be explained by inappropriate reinnervation of target organs. The strength of electrical stimulation throughout these and previous experiments is set 2.5X dorsal root threshold to activate only the largest diameter axons. The possibility exists that afferents activated by electrical stimulation are not the same afferents that respond to stretch. Large diameter afferents easily activated by electrical stimulation and forming central synapses with motoneurons may no longer innervate muscle spindles, while afferents reinnervating muscle spindles may have higher electrical thresholds and may not make monosynaptic connections with motoneurons. This could easily account for a situation in which a stretch evoked synaptic potential is not present while electrical stimulation at group I strength produces an EPSP. Therefore, it was essential to illustrate that those regenerated afferents firing in patterns typical of Ia afferents make monosynaptic connections with motoneurons.

Surprisingly, we found the opposite to be true. Only 1/18 provisional Ia-motoneuron synapses were functionally connected, as evidenced by the presence of an individual spike triggered averaged EPSP (compared to 93% connectivity in the control). Connectivity at levels as dramatically reduced as were seen here could entirely account for both the reduced and absent synaptic potentials in regenerated Ia-motoneuron circuits.

Why was only one provisional Ia-motoneuron pair connected? It is clear that nonspecific reinnervation may explain why a number of regenerated Ia-motoneuron pairs are
centrally disconnected, but the probability that only 1 of 18 afferents reconnecting with a muscle spindle was originally a Ia afferent seems unrealistic. According to our calculations, assuming equal survival of all afferent types and assuming that muscle spindles were reinnervated by either group I or group II afferents, there is a 41% chance that a reinnervated muscle spindle was reinnervated by an original Ia afferent.

Our rationale is the following. The rat medial gastrocnemius contains approximately 20 Ia afferents (Sekiya et al., 1986). The number of GTOs in the rat MG is unknown, and estimated based on ratio of Ia afferent to GTO afferent in other muscles/species. Given that in the cat medial gastrocnemius, the ratio of GTO afferent to Ia afferent is 62%, and that this number falls between the projected value based on the rat EDL (85%) and soleus (40%) values (Zelena, 1994, Soukup et al., 1977), it is estimated that the rat medial gastrocnemius contains approximately 13 Ib afferents. The number of group II afferents innervating the rat medial gastrocnemius muscle is also unknown, and is therefore estimated. The number of expected group II afferents innervating a given spindle in the rat is between 0.6 and 1.0 depending on the muscle (these ranges are taken from lumbricle, EDL, and soleus) (Zelena, 1994). Using the ratio of 0.8 group IIs per spindle, the number of group II afferents in the medial gastrocnemius is estimated at 16.

Using these values, it is estimate that the rat medial gastrocnemius muscle contains a total of approximately 49 group I and group II afferents. Assuming equal potential to regenerate and no specificity during reinnervation, it is expected that an afferent successfully innervating a muscle spindle, whose conduction velocity is in the group I/II
range, would mostly likely have been an original Ia (41% chance as opposed to 33% for GTO or 26% for group II). In other words, 41% of afferents reinnervating muscle spindles should be original Ia afferents.

The finding that only 5% (1/18) of regenerated afferents responding to stretch in patterns and at rates typical of Ia afferents make monosynaptic connections with motoneurons (as opposed to 93% (27/29) of controls), almost certainly suggests that inappropriate peripheral reinnervation is not the only abnormality.

*Evidence for additional central deficits*

It is likely that the central connections between original Ia afferents and homonymous motoneurons are altered after axotomy and do not recover even when appropriate reinnervation occurs. Strong evidence shows that motoneurons undergo a stripping of synapses after peripheral nerve injury (Brannstrom and Kellerth, 1998, Lind et al., 2000, Alvarez et al., 2000). What is not clear, however, is how much of that stripping involves group Ia afferents, and how well it recovers. The some of the most compelling evidence for a functional stripping of Ia-motoneuron synapses comes from Mendell et.al (1976) who studied individual synapses between axotomized group I afferents and homonymous motoneurons in the cat. Their data show a progression in which conduction velocity was reduced, followed by an elongation of EPSP rise time and half-width and finally a disconnection of synapses (individual EPSPs were absent). There may be multiple explanations for why EPSPs were not seen in many axotomized Ia-motoneuron pairs: changes in motoneuron properties, redistribution of synapses distally, actual synaptic
detachment, etc. In any case, they provide clear evidence that axotomy produces significant changes in functional connectivity of the Ia-motoneuron synapse.

Anatomic data support the idea that the Ia-motoneuron synapse is compromised after axotomy and reinnervation. Current unpublished data from our colleagues (Alvarez and Titus) shows a reduction in Vglut1 on motoneuron soma and proximal dendrites, but not distal dendrites, after peripheral nerve cut. Interestingly, muscle reinnervation does not restore somatic/proximal Vglut1 levels to normal. Vglut1 is present in proprioceptive afferents but also in some descending axons (Persson et al., 2006). Whether the changes in Vglut1 contacts reflect a stripping of Ia afferent input is unknown but these data fit remarkably well with the physiologic finding that synaptic connections are reduced upon axotomy. The anatomic data shown here using intracellular Neurobiotin to label provisionary Ia afferents shows labeling ending in lamina VII in 4/5 studied afferents (compared to 12/12 controls where labeling extended into lamina IX). A likely reason for this is that provisionary Ia afferents do not project any further than lamina VII, a significant structural change that no doubt could impact synaptic function.

The possibility exists that Ia afferents remain structurally associated with motoneurons on the distal dendrites, where Vglut1 levels are maintained. Motoneuron dendritic trees are extensive, easily reaching into lamina VII where distal contacts could be formed with regenerated Ia afferents. It seems unlikely that we would be unable to detect STA EPSPs in these connections, but, because the records were taken from the soma, consideration must be given to the amount of electrotonic decay that occurs. Since the individual
EPSPs are small events to begin with, even a slight amount of decay could be problematic. In addition, it is difficult to predict that all contacts from a given afferent are preserved. A reduction in proximal boutons with preservation of distal boutons for an individual afferent in combination with some degree of decay may make detection of individual EPSPs nearly impossible.

Monosynaptic vs. Polysynaptic Input

The relative roles of monosynaptic and polysynaptic pathways in synaptic activity evoked by muscle stretch are unclear. Because of their known monosynaptic connections with motoneurons, it has been assumed that monosynaptic input from Ia afferents makes a large contribution to SSPs. However, no direct evidence supports or opposes this. Consider that polysynaptic pathways have a far greater role in producing SSPs. If primary input to polysynaptic interneurons is reduced, as is expected with axotomy due to cell death and an inability of some cells to reinnervate any target, this weaker signal may be insufficient to activate polysynaptic cells, which in turn would reduce SSP amplitude. Depending on the contribution of polysynaptic pathways, this reduction could be minimal or substantial. Spike triggered averaging data presented here support the idea that, in reinnervated circuits, polysynaptic pathways are significant contributors to SSPs as two out of three spike triggered averaged EPSPs had latencies in the polysynaptic range (1.5 ms).
CHAPTER 6: General Discussion

Reinnervated muscles respond to muscle stretch with no reflexive contraction. To gain an understanding of why this behavioral abnormality persists long after the peripheral nerve is successful in reinnervating muscle, Haftel et. al. (2005) studied the synaptic responses to ramp hold and release muscle stretch in adult decerebrate rats. They reported a striking finding in which muscle stretch was completely ineffective in eliciting synaptic responses in 2/3 regenerated rat motoneurons despite its ability to activate muscle afferents. This problem is significant because it may explain abnormal motor behavior in individuals experiencing peripheral nerve injury and subsequent regeneration (as with trauma, Guillain Barre, etc.) and determining its cause may aid in our understanding of how the central nervous system responds to injury. This dissertation is devoted to understanding what factors cause deficits in synaptic efficacy and in turn may be responsible for limiting full functional recovery. In order to understand deficits at the synapse, we carefully examined factors normally responsible for controlling synaptic efficacy: presynaptic afferent activity, postsynaptic motoneuron intrinsic properties, synaptic transmission, and connectivity.

Our results collectively showed that function is restored in both the presynaptic provisional Ia afferents and postsynaptic motoneurons, but that the connectivity between them is altered. Provisional Ia afferents fired at normal rates and in normal patterns, and intrinsic motoneuron properties returned to control levels. In addition, electrical
stimulation at both physiologic and low frequencies was always capable of eliciting an excitatory postsynaptic potential in regenerated motoneurons whereas muscle stretch was only sometimes successful in eliciting synaptic responses (see Figure 6.1). This finding suggests that either recovery of central synaptic function is unrelated to the ability of an axon to reinnervate peripheral receptors or that central synaptic function may actually be strengthened when peripheral reinnervation is unsuccessful. On the other hand, most of the afferents reinnervating muscle spindles did not make central connections with motoneurons. This indicates that peripheral reinnervation of a muscle spindle is not sufficient to restore central connectivity.

Central connections are maintained and/or restored by afferents unsuccessful in reinnervating peripheral receptors.

The notion that electrically evoked synaptic potentials are generated by afferents that do reinnervate muscle spindles is supported by electrophysiologic studies of regeneration in the cat. One such study was performed by Gallego et al (1980) who were interested in understanding increases in EPSP amplitude after nerve crush. They studied homonymous monosynaptic EPSPs in cats whose MG muscle nerve was crushed. 8 weeks after crush, composite EPSP amplitude was increased to 1.5 times normal. To determine the cause of this increase, they used the spike triggered averaging technique to assess the amplitude of individual EPSPs produced by single MG Ia afferents. In their report, this was feasible because connectivity of regenerated afferents was much greater than reported here. This is probably a combination of a species difference and the effect of nerve crush rather than cut. The expectation was that individual synapses from functionally reinnervated
Figure 6.1. **Potential scenarios of motoneuron connectivity in relation to electrical and stretch EPSPs.** A shows a normal untreated control motoneuron with contacts from Ia afferents. Motoneurons represented in B, C, and D all represent cases in which the peripheral nerve has been cut and regenerated. Synaptic stripping is represented in B, C, and D by a reduction in the number of synapses on the soma and proximal dendrites. Nonspecific reinnervation is represented by the different color contacts. Those in green indicate appropriate reinnervation by Ia afferents (reinnervated annulospiral endings of muscle spindles) and orange dots indicate synapses formed by original Ia afferents reinnervating inappropriate targets or being unsuccessful at reinnervation. In the control motoneuron, electrical EPSPs (top trace) and SSPs (bottom trace) are produced by Ia input. In B, C, and D, electrical EPSPs are dominated by afferents unsuccessful (orange) in reinnervating muscle spindles and may receive minimal contributions from afferents successful in reinnervating muscle spindle (green). Stretch evoked synaptic potentials may be absent in cases in which no original Ia contacts are maintained (not shown) or contacts are maintained distally and do not generated enough current to be effective somatically, B. SSPs may be present in cases where small amounts of original Ia input near the motoneuron soma are maintained, C, or where polysynaptic input contributes, D. Individual EPSPs would be present for green synapses, but may not be detected at remote locations due to their potential for electrotonic decay.
afferents were increased in strength and could account for the increase in composite EPSP strength. Interestingly, the amplitudes of the individual EPSPs were similar in control and regenerated circuits, and therefore could not explain why monosynaptic composite EPSPs were greater in regenerated circuits. They concluded that connections between motoneurons and afferents that do not achieve functional reinnervation with peripheral receptors were strengthened. EPSPs from rat Ia-motoneuron synapses also become larger when peripheral nerve activity is blocked by tetrodotoxin (Manabe et al., 1989). These data strengthen our conclusion that the electrical EPSPs we observed were produced, in large part, by afferents that do not reinnervate muscle spindles.

That afferents unsuccessful in regenerating muscle spindles make monosynaptic connections with motoneurons is further supported by a study of H-reflexes. H-reflexes were examined in adult rats whose sciatic nerves had been cut and allowed to reinnervate muscle. The H reflex uses electrical stimulation of the peripheral nerve to reflexively activate muscle tissue, effectively bypassing the peripheral receptor. Despite an inability of muscle stretch to produce reflexive force (Haftel et al., 2005), electrical stimulation was successful in producing an H wave in homonymous muscle (Valero-Cabre and Navarro, 2001). The reflexive behavior of the regenerated circuit is extremely consistent with the synaptic potentials observed here: electrical stimulation is successful at activating the circuit while muscle stretch is either less successful or not successful. Study of the H-reflex confirms that electrically activated afferents maintain connections with homonymous motoneurons.
Central connections are not recovered by successful reinnervation of peripheral receptors.

The other major finding was that those afferents successfully reinnervating muscle spindles did not make functional connections with motoneurons. This finding is of huge importance because it explains why stretch evoked synaptic potentials are absent in some motoneurons. Furthermore, it indicates that any peripheral cues indicating reinnervation has occurred are ineffective at maintaining central synapses. Interestingly, almost none of the regenerated provisional Ia-motoneuron pairs were connected. The finding that only 1/18 regenerated afferents were monosynaptically connected to motoneurons causes some trouble when trying to explain why nearly 60% of the motoneurons sampled here did have SSPs. The one regenerated Ia-motoneuron pair with a monosynaptic latency had an amplitude of 167 uV. Because only one monosynaptic individual EPSP was seen following regeneration, it is impossible to know whether the synaptic efficacy is enhanced, depressed or unchanged at the few existing functional synapses. Provided other individual EPSPs were as large as the one observed here, very few would be required to produce discriminable SSPs. It seems unlikely that individual EPSPs from regenerated circuits would be larger than control given the scenario that synaptic stripping on the motoneuron could eliminate some synaptic contacts from a particular Ia afferent while sparing others. In that case, one might expect the size of individual EPSPs to be reduced unless dendritic synapses were compensating to make the few Ia connections stronger.
STA EPSPs from regenerated Ia-motoneuron pairs had polysynaptic latencies indicating that, least in regenerated circuits, polysynaptic inputs have the ability to contribute to SSPs. Because polysynaptic inputs would, by definition, require the activation of an additional cell, the input from few cells (as with sustained stretch during spike triggered averaging) might not be able to bring the interneuron to threshold, whereas ramp hold and release muscle stretch which activates many more afferents could. This could explain why stretch evoked synaptic activity is sometimes seen when STA EPSPs are not. A situation like this may occur when group II afferents, many of whom do not make monosynaptic connections with motoneurons, inappropriately reinnervate the annulospiral endings of a muscle spindle.

**Evidence for motoneuron role in specifying connectivity**

When peripheral nerves reinnervate muscle, synaptic responses to muscle stretch can be recorded from some motoneurons but not others even within the same animal. This observation provides clear evidence that factors other than nonspecific reinnervation contribute to reduced/absent synaptic activity as the degree of nonspecific reinnervation remains the same for any motoneuron sampled within a given animal. Therefore, additional factors influencing central connectivity appear to be motoneuron specific.

One major factor impacting motoneurons after axotomy is synaptic stripping. Unpublished data from our colleagues (Alvarez and Titus) indicate that axotomy results in major synaptic remodeling of the motoneuron membrane. Specifically, VGLUT1 contacts were dramatically reduced on the motoneuron soma and proximal dendrites and
did not recover with muscle reinnervation. Provided decreases in VGLUT1 reflect removal of Ia afferent input, this stripping process causes motoneurons to lose their most powerful synaptic contacts from Ia afferents (Rall et al., 1967). In contrast to the soma and proximal dendrites, distal dendrites were relatively unaffected by axotomy. The variation in the original distribution of Ia synapses and the extent to which stripping on one motoneuron differs from another could determine the difference in stretch evoked synaptic potentials.

On the whole, regenerated Ia-motoneuron synapses are less responsive to muscle stretch than control. The data presented here give good reason for this: many provisional Ia-afferents are not functionally connected to motoneurons. What these data do not explain is why muscle stretch is entirely ineffective in producing reflexive force even when applied during reflexive contraction from other stimuli. Many motoneurons sampled did have synaptic responses to muscle stretch. When depolarization is raised to a level by other stimuli (cutaneous or electrical) sufficient to activate some motoneurons, synaptic activity produced by muscle stretch, albeit reduced, would be expected to activate some additional motoneurons. Activation of additional motoneurons would be reflected in the amount of reflexive force produced. However, stretch of a reinnervated muscle is never effective in producing additional force (Haftel et al., 2005). This leaves open the possibility that additional unidentified mechanisms (possibly including spinal networks that impinge on the Ia-motoneuron synapse or abnormalities in persistent inward currents) contribute to the loss of stretch reflex after peripheral nerve injury.
Summary

The data presented here demonstrated that reduced and absent synaptic activity occurs as a result of dramatically reduced connectivity between regenerated provisional Ia afferents and their homonymous motoneurons. These data illustrated that while many regenerated provisional Ia afferents and motoneurons are not centrally connected, afferents unsuccessful in reinnervating muscle spindles are capable of maintaining their monosynaptic connections with these same motoneurons. It is unclear what factors are responsible for maintaining central synapses formed by peripherally disconnected afferents, but a variety of factors may be responsible for reduced connectivity between provisional Ia afferents and motoneurons. Inappropriate reinnervation of peripheral targets and stripping of central synapses are both likely to play an important role in preventing functional recovery at the Ia-motoneuron synapse and may in turn, cause stretch areflexia seen after nerve section and regeneration. The recovery of the stretch reflex and proprioceptive sense would be possible if afferents making appropriate peripheral connections could recover central synapses or if afferents maintaining central synapses could be directed to reinnervate muscle spindles.
APPENDIX A: Commonly Used Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ia</td>
<td>Group Ia primary afferent</td>
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<tr>
<td>AHP</td>
<td>Afterhypolarization potential</td>
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<tr>
<td>EPSP</td>
<td>Excitatory post synaptic potential</td>
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<tr>
<td>GTO</td>
<td>Golgi tendon organ</td>
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<tr>
<td>MG</td>
<td>Medial gastrocnemius</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>L</td>
<td>Rexed lamina</td>
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<tr>
<td>LG-S</td>
<td>Lateral gastrocnemius-soleus</td>
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<tr>
<td>SSP</td>
<td>Stretch evoked synaptic potential</td>
</tr>
<tr>
<td>STA</td>
<td>Spike triggered averaged</td>
</tr>
<tr>
<td>TTSSP</td>
<td>Tendon tap stretch evoked synaptic potential</td>
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