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Neuromuscular activity, axonal sprouting and stability of chronically enlarged motor units in an animal model of motoneuron disease

by

SIU LIN TAM 

A thesis submitted to the faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Master of Science

Department of Pharmacology

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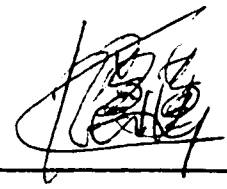

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Neuromuscular activity, axonal sprouting and stability of chronically enlarged motor units in an animal model of motoneuron disease** submitted by **Siu Lin Tam** in partial fulfillment of the requirements for the degree of **Master of Science**.

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26th May 1999

Abstract

Partial nerve injuries and motoneuron diseases like poliomyelitis, post-polio syndrome and amyotrophic lateral sclerosis (ALS) are some of the neuromuscular conditions resulting in compensatory motoneuron sprouting to form enlarged motor units (MUs). Despite the compensatory process, the prognosis of these conditions is rather disappointing. In cases like ALS, the consequence is even deadly. In some cases such as post-polio syndrome, even the etiology is unclear. While management of patients with these conditions is still unclear, exercise regimen has been advocated because of the strong association of muscle exercise with strength and endurance. Although some positive effects have been reported, the issue remains contentious. We, therefore, sought to re-examine the effect of exercise on axonal sprouting and stability of chronically enlarged MUs. We have used for the first time both electrophysiological and morphological quantitation of MU enlargement and number of collateral sprouts in four functionally different and partially denervated hindlimb muscles in the rat.

We partially denervated the rat hindlimb muscles by evulsion of either L4 or L5 spinal root. Immediately or 11 months later, the rats were subjected to 4 week-program of 1) normal caged activity as control, 2) physiological (running on exercise wheels, 8hrs daily) neuromuscular activity, 3) imposed (functional electrical stimulation of sciatic nerves, FES, 20 Hz, 8hrs daily) neuromuscular activity or 4) muscle paralysis using tetrodotoxin blockade. We then determined

Abstract

the effects of increased neuromuscular activity and muscle paralysis on MU enlargement and axonal sprouting in the hindlimb muscles.

Both MU force measurement and morphological examination of collateral sprouts using combined silver/cholinesterase histochemistry showed that both muscle paralysis, induced by TTX, and increased neuromuscular activity, by either daily exercise on wheels or FES, significantly reduced axonal sprouting and MU size under conditions where partially denervated muscles contained less than 20% of their normal MU complement. MU enlargement after normal caged activity was significantly reduced at 12 month in extensively but not moderately denervated muscles as compared to 1 month, indicating that the stability of chronically enlarged MUs in the extensively denervated muscles was compromised. A one month period of increased neuromuscular activity by either wheeling exercise or FES further exacerbated the time-related reduction in MU size in the extensively but not moderately denervated muscles. A one month period of TTX-induced muscle paralysis did not have significant effect on the time-related reduction in MU size in either the extensively or the moderately denervated muscles.

Therefore, our findings indicate that only moderate levels of neuromuscular activity are appropriate for patients suffering motoneuron disease and/or injury which results in extensive MU loss. Our ultimate goal is to establish a better understanding of how to optimize axonal sprouting to improve muscle reinnervation after partial nerve injuries and motoneuron diseases.

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List of Abbreviations

α-BTX	α-Bungarotoxin
Ach	Acetylcholine
AchE	Acetylcholinesterase
Ag/AchE	Combined silver/cholinesterase
ALS	Amyotrophic lateral sclerosis
ANOVA	One-way analysis of variance
ARIA	nAChR-inducing activity
BuTX	Botulinum toxin
CGRP	Calcitonin gene-related peptide
CNTF	Ciliary neurotrophic factor
CSA	Cross-sectional area
DAG	Dystrophin-associated glycoproteins
ECM	Extracellular matrix
EDL	Extensor digitorum longus
EMG	Electromyography
FES	Functional electrical stimulation
IGF-II	Insulin-like growth factor II
IR	Innervation ratio
LG	Lateral gastrocnemius
LRE	Leuine-arginine-glutamate
MASC	Myotube-associated specificity complex
MG	Medial gastrocnemius
MU	Motor unit
MuSK	Muscle-specific kinase
nAChR	Nicotinic acetylcholine receptor
NCAM	Neural cell adhesion molecule
PD	Partial denervation
PL	Plantaris

List of Abbreviations

RAPSYN	Receptor associated protein of the synapse
S-laminin	Synaptic laminin
SNAP	Soluble N-ethylmaleimide-sensitive factor attachment proteins
SOL	Soleus
TA	Tibialis anterior
TTX	Tetradotoxin

CHAPTER 1

1. General Introduction

Attrition of motoneurons resulting in partial muscle denervation and axonal sprouting from remaining motoneurons occur in partial nerve injuries and many pathological conditions such as poliomyelitis and amyotrophic lateral sclerosis (ALS). The surviving motoneurons increase their motor unit (MU) size up to a limit of 5-8 times by axonal sprouting to reinnervate denervated muscle fibers in order to compensate for more than 80% of muscle function loss. Sprouting capacity is, however, limited even under normal conditions and declines with age.

Although discovery of the polio vaccine has effectively reduced the incidence of poliomyelitis, a population of patients who were afflicted in the epidemics of the 1950's and early 1960's and, in some cases by the vaccine itself were left with the post-polio syndrome where patients experience muscle weakness and fatigue decades after the initial motoneuron death. Both the etiology and management of this syndrome still remain an issue.

The strong association of muscle exercise with strength and endurance has led to attempts to optimize the muscle function in motoneuron diseases and

injuries. A number of studies using animal model of partial denervation has been carried out in the past to examine whether neuromuscular activity is good or bad for axonal sprouting. But to date, the evidence for any beneficial effect is controversial. These studies are described in sections 1.3.5. and 1.4.2.. The question of whether neuromuscular activity is good or bad is a particular concern for chronic denervation syndromes like post-polio syndrome because of the possibility that muscle weakness and fatigue result from nerve terminal withdrawal when surviving motoneurons can no longer maintain their chronic MU enlargement.

The ability of motoneurons to expand their peripheral innervation field when skeletal muscles become partially denervated is an example of neuromuscular plasticity. During the early stage of postnatal development, immature skeletal muscle fibers are innervated by several motor axons (polyneuronal innervation) and motoneurons on average innervate about 5 times as many muscle fibers as in mature muscles. Within about two weeks after birth, excessive synapses and then axons are withdrawn (synapse elimination) and subsequently the size of MUs is reduced and, finally the mature pattern of innervation where one motor endplate is innervated by one single axon (mononeuronal innervation) is established. When muscles become partially denervated, motoneurons appear to relive the developmental stage where they expand their MU size by axonal sprouting and thereby synapses are re-

established on the denervated muscle fibers. In the light of this relevance, it is important to look at the effect of activity on the polyneuronal innervaton and synapse elimination, and on the synapse regeneration and remodeling so as to understand how neuromuscular activity might affect axonal sprouting and muscle-nerve reconnection and stability. These are described in sections 1.1.3.2. and 1.1.5.

Phenomenon of axonal sprouting has been well documented for years. However, its underlying mechanism is much less understood and has been postulated at one time or another to involve sprouting stimuli. Five potential sprouting stimuli are discussed in sections 1.3.3.. Any possible perturbation to these stimuli by activity would provide insight about the mechanistic basis underlying the effect of neuromuscular activity on axonal sprouting.

1.1. Development of neuromuscular junction

1.1.1. Synaptic function and structure

The neuromuscular junction is a chemical synapse and a very specialized functional and structural connection between a motoneuron (pre-synaptic domain) and a muscle fiber (post-synaptic domain). Depolarization of the pre-synaptic nerve terminal causes voltage-gated calcium channels in the terminal membrane to open, leading to calcium influx. Increased intracellular calcium then triggers release of acetylcholine (ACh, neurotransmitter) from vesicular stores, and released ACh diffuses the across synaptic cleft and binds to nicotinic

acetylcholine receptors (nAChRs) in the post-synaptic muscle membrane. Binding of ACh to receptors leads to the opening of ion channels and influx of ions, resulting in excitation-contraction coupling of the muscle (Kendel et al, 1991).

Electron micrographs of neuromuscular junction show clearly that both nerve terminal and muscle fiber are highly specialized in their region of contact (Engel, 1986). The nerve terminals are filled with synaptic vesicles containing ACh and clustering next to active zones, which are the sites at which synaptic vesicles fuse with the pre-synaptic membrane to release their stored ACh. Molecules such as SNAP (soluble N-ethylmaleimide-sensitive factor attachment proteins), synaptotagmin, synaptobrevin (also known as VAMP), synaphin and syntaxin, associated with the active zone have been characterized and shown to be involved in the process of neurotransmitter release (Littleton and Bellen, 1995). Pre-synaptic motoneurons also synthesize and release at the nerve terminal molecules such as calcitonin gene-related peptide (CGRP), agrin and nAChR-inducing activity (ARIA) which have been demonstrated to be important for post-synaptic differentiation (Fontaine et al., 1987; Reist et al., 1992; Lu et al., 1993; Deyst et al., 1995; Jo et al., 1995).

The specialized post-synaptic domain includes the thickened membrane which covers about 1μm deep junctional gutters on the muscle fiber surface into which the nerve terminals fit. This specialized region is generally referred to as

the "motor endplate". nAChRs are densely packed at the crests of the gutters (about 10^4 per μm^2), but their levels fall by more than 90% in the tough, and there are fewer than 10 nAChRs per μm^2 in the extrasynaptic membrane (Salpeter et al., 1983). The synaptic nuclei are morphologically different from their nonsynaptic counterparts and selectively transcribe genes which encode proteins such as nAChRs, acetylcholinesterase (AChE), and a group of nAChR-clustering molecules including rapsyn (receptor associated protein of the synapse), dystrophin-associated glycoproteins (DAG), muscle-specific kinase (MuSK) and myotube-associated specificity complex (MASC) (Campanelli et al., 1994; Fallon and Hall, 1994; Gee et al., 1994; Glass et al., 1996; Glass and Yancopoulos, 1997; Matsumura et al., 1997; Colledge and Froehner, 1998). Cell adhesion molecules such as neural cell adhesion molecule (NCAM) and N-cadherin, extracellular matrix (ECM) such as synaptic laminin (s-laminin) and growth factors such as insulin-like growth factor II (IGF-II) are also found to be expressed by muscle fibers and suggested to be important for synaptogenesis during development and reinnervation (Sanes et al., 1986; Covault and Sanes, 1986; Ishii, 1989; Reichardt, 1991; Cifuentes-Diaz et al., 1994; Marsh et al., 1994; Martini, 1994; Glazner and Ishii, 1995; Moscoso et al., 1995; Porter et al., 1995). Muscle-derived neurotrophins including neurotrophin 3 and 4 have also been suggested to be essential for synaptogenesis by strengthening and

stabilizing the nerve-muscle connections in an activity-dependent manner (Funakoshi et al., 1995; Xie et al., 1997).

The 50nm wide synaptic cleft between nerve terminal and muscle fiber is transversed by a basal lamina which extends into the junctional gutters. It is part of the continuous sheath which encircles the muscle fiber and is fused to the basal lamina of the Schwann cells which wrap the nerves. Synaptic basal lamina contains AChE which inactivates ACh after it is released from nAChRs, and ECM such as s-laminin. Unmyelinated terminals at the neuromuscular junction are associated with specialized non-myelinating Schwann cells. These terminal Schwann cells exhibit a calcium transient in response to axonal action potentials, indicating that they can sense electrical signals (Jahromi et al., 1992; Reist and Smith, 1992; Lev-Ram and Ellisman, 1995; see section 1.3.3.1.). They elaborate extensive processes, following axonal damage, which have been found to induce and guide axonal sprouting and regeneration (Reynolds and Woolf, 1992; Son and Thompson, 1995a,b; Son et al., 1996).

1.1.2. Synapse formation

Motoneurons in the spinal cord extend their axons into the mesenchymal tissue of the embryonic trunk and limb at about the time when myoblasts begin fusing to form myotubes which eventually derive into mature muscle fibers. Neuronal growth cones navigate along specific pathways to their final targets and appear to be guided by at least four different mechanisms: contact

attraction, chemoattraction, contact repulsion, and chemorepulsion (for detailed description, see Tessier-Lavigne and Goodman, 1996).

Upon contact with myotubes, nerve terminals begin to differentiate both structurally and functionally. Synaptic vesicles start accumulating at the active zones, both pre-and post-synaptic membranes thicken, the synaptic cleft widens, and basal lamina materials are made. Features and elements characterizing growth cones are eventually lost and terminal arborizations of axons take place Kelly and Zacks, 1969). Following the establishment of the synaptic contact between the first axon and myotube, other axons converge on the same motor endplate, resulting in polyneuronal innervation. The stage of polyneuronal innervation is transient and lasts until about 2 weeks after birth in mammals (see section 1.1.3.). Synapse elimination, where all axons except one are later withdrawn, soon occurs afterward and brings the immature form of innervation, polyneuronal innervation, to the mature form of innervation, mononeuronal innervation (Brown et al., 1976). More detailed elaboration of synapse elimination is described in section 1.1.4.. Synaptic transmission begins very quickly after nerve contacts the muscle. Morphological changes are soon accompanied by increases in the frequency and size of spontaneous synaptic potentials (Dennis, 1981).

Differentiation of the nerve terminal begins as polyneuronal innervation occurs, proceeds even in nerve terminals which will be withdrawn later, and is

completed only after synapse elimination has occurred. Evidence shows that muscle-derived s-laminin plays a key role in triggering pre-synaptic differentiation (Moscoso et al., 1995; Noakes et al., 1995; Porter et al., 1995). S-laminin, a homolog of the laminin B1 subunit, is concentrated in the synaptic portion of the basal lamina sheath of a muscle fiber and bears a site which is selectively adhesive to motoneuron-like cells (Hunter et al., 1989a,b). A major determinant of this site is the tripeptide, leuine-arginine-glutamate (LRE). It has been demonstrated that LRE site in s-laminin provides a signal to stop neurite outgrowth and to instruct axonal differentiation (Porter et al., 1995). There is evidence showing that differentiation of neuromuscular junction becomes aberrant in mice lacking s-laminin (Noakes et al., 1995). It has also been shown that s-laminin might be responsible for signalling the preferential reinnervation of the original synaptic sites by regenerating axons (Sanes et al., 1978; Kuffler, 1986). Other muscle-derived molecules including NCAM, N-cadherin and IGF-II have also been shown to be important for synapse formation. They are present at very high concentration in embryonic and denervated muscles, and become downregulated in innervated muscles (Covault and Sanes, 1986; Covault et al., 1986; Sanes et al., 1986; Ishii, 1989; Cifuentes-Diaz et al., 1994; Baldwin et al., 1996). Their spatial and temporal expression indicate that they act as a chemoattractant to direct growing axons to the target muscle where innervation is needed. Particularly, NCAM and N-cadherin are found to remain at the mature

synapses while downregulated at the extrasynaptic sites, suggesting a role of these molecules in stabilizing mature synapses.

Initial contact between growth cone and myotube also initiates a series of changes in both synaptic and extrasynaptic areas of muscle fibers (Sanes, 1997). Among these changes, aggregation of nAChRs has been most extensively studied. The striking concentration of nAChRs at the synaptic area is regulated by activity independent and dependent neural mechanisms: 1) local synthesis of new nAChRs, a process triggered potentially by CGRP and ARIA, and 2) activity-dependent, selective clustering of nAChRs initially present on myotube membrane. The initial formation of nAChRs in myotubes is a nerve-independent process, occurs prior to nerve-muscle contact, and is believed to be part of the general program of muscle differentiation controlled partly by a family of muscle-specific transcription factors (Witzemann and Sakmann, 1991). As the ingrowing axon approaches, the nAChRs start to cluster near the region of nerve contact, and synaptic nuclei are stimulated to synthesize new nAChRs. Studies using myotube culture show that exogenous administration of CGRP or ARIA leads to increases in both nAChR subunit mRNA and protein levels (Fontaine et al., 1987; Jo et al., 1995). Clustering of nAChRs is an activity-dependent process. Nerve-induced activity during development suppresses the expression of nAChR subunit mRNAs in extrasynaptic nuclei while the absence of muscle activity due to either muscle paralysis or denervation increases their expression

(Merlie et al., 1984; Moss et al., 1987; Goldman et al., 1988; Martinou and Merlie, 1991). Furthermore, clustering of nAChRs involves a number of clustering molecules. Agrin, the first synapse-organizing protein being identified, is just one of the many molecules involved in the process and regulation of nAChR clustering; but it is the most well studied (Reist et al., 1992; Deyst et al., 1995). Recently, a list of nAChR-clustering molecules has been identified and suggested to participate, together with agrin, in the process and regulation of nAChR clustering. They include rapsyn, DAG, MuSK and MASC (Campanelli et al., 1994; Fallon and Hall, 1994; Gee et al., 1994; Glass et al., 1996; Glass and Yancopoulos, 1997; Matsumura et al., 1997; Colledge and Froehner, 1998). Following synaptic clustering of nAChRs, extrasynaptic nAChRs are downregulated. Upon denervation, nAChRs reappear at extrasynaptic area due to new synthesis of nAChR subunit mRNAs and protein, but are downregulated again after muscle reinnervation (Brockes and Hall, 1975).

1.1.3. Polyneuronal innervation

The duration of polyneuronal innervation is transient and varies in different species. In rats, for example, it lasts for about 2 weeks before synapse elimination occurs (Thompson and Jansen, 1977). The significance of this developmental stage, which is also a feature of neuronal synaptic development, is not clear. Perhaps, it is to ensure that no post-synaptic target cells lacks

innervation, and that target cells are equitably distributed among incoming axons.

1.1.4. Synapse elimination

The functional reasons for synapse elimination are not known, but several possibilities have been proposed. Some proposed that the reason for synapse elimination was to remove synapses which had formed in the wrong target sites. This proposal now seems unlikely since growing axons generally make synapses in the appropriate target sites due to growth cone pathway guidance mechanisms (Tessier-Lavigne and Goodman, 1996). In many studies of retino-visual system, it appears that synapse elimination serves a functional purpose because the refinement of afferent projections to a target population segregates these afferents into functionally-appropriate domains (Goodman and Shatz, 1993). There is also a suggestion that synapse elimination may play a role in controlling MU size (Brown et al., 1976; Bixby et al., 1980). In the case of adult rat soleus (SOL) muscle, for example, MU sizes fall into a narrow range. In newborn rat, however, there appears to be a greater variability in the number of muscle fibers in each units. Synapse elimination appears to favor synapse loss more from the large MUs than from the small MUs. In other muscles where MU sizes are diverse, synapse elimination may play a role in ensuring that each

motoneuron innervates an appropriate number of muscle fibers (Balice-Gordon and Thompson, 1988; Stollberg, 1995).

It is generally believed that competition between converging axons on the same synaptic site underlies the mechanism of synapse elimination. Direct evidence comes from the findings that the size of the remaining MUs in developing muscles remains large when the muscles have been partially denervated prior to the period of synapse elimination (Thompson and Jansen, 1977; Betz et al., 1980; Fisher et al., 1989; Gates and Ridge, 1992). These findings demonstrate that a motoneuron can maintain innervation with more muscle fibers when there are fewer other motoneurons innervating the same muscle, indicating that competition between the innervating axons is necessary for synapse elimination. In addition to the active role of pre-synaptic terminals in synapse elimination, there is also evidence indicating that post-synaptic muscle cells also actively participate in the synapse elimination process. Five mechanisms underlying synapse elimination have been proposed. 1) Loss of nAChRs in the post-synaptic membrane provides driving force for the elimination of the overlying synaptic terminals (nAChR removal). 2) Ability of innervating synaptic terminals to compete for target site depends on their neural activity (Relative terminal activity). 3) Innervating synaptic terminals compete for a limited amount of trophic factor supplied by the target muscle cells (Competition for "trophic" factor(s). 4) There is a spatial competition between the innervating

synaptic terminals on the same target site (Spatial competition). 5) An activity-dependent release of protease appears to be a possible mechanism (Protease release).

1.1.4.1. nAChR removal

Using a system in which mouse sternomastoid muscle neuromuscular junction can be repeatedly observed during many days, and by separately labeling pre-synaptic terminals and post-synaptic nAChRs at each observation, Balice-Gordon and Lichtman (1993) observed that nAChRs underlying nerve terminals which were destined to be eliminated were removed prior to nerve terminal retraction. This observation indicates that muscle cell may actively select, by removal of underlying nAChRs, the terminals to be eliminated. It is also possible that small random changes in the distribution of the nAChRs, and the resultant changes in efficacy of the multiple terminals, produce sufficient asymmetry to trigger synaptic competition and elimination. The physiological consequences of a decrease in receptor density which precedes nerve terminal elimination have been investigated by recording intracellularly from multiply innervated muscle fibers during the period of synapse elimination (Nabekura and Lichtman, 1989). Nabekura and Lichtman (1989) found that in a subset of multiply innervated muscle fibers, one of the innervating axons had smaller than normal miniature endplate potentials of evoked endplate potentials. They suggested that changes in post-synaptic receptor density could alter the relative

synaptic efficacies of different inputs at multiply innervated endplates underlying synapse elimination.

It is not known whether eliminated synaptic terminals degenerate or are resorbed by the axon. In central nervous system, the presence of certain types of glia has been implicated in the elimination of transient projections. In developing cat visual cortex, macrophages appear to phagocytose groups of transitory callosal axons, although it is not clear whether these macrophages actively select the axons to eliminate or simply clear those nerve terminals already eliminated (Innocenti et al., 1983). At the neuromuscular junction, however, it appears that eliminated synaptic terminals are retracted into the main axon rather than degenerating, since "retraction bulbs" are found in muscles during this process (O'Brien et al., 1978; Riley, 1981; Balice-Gordon et al., 1993). Balice-Gordon et al. (1993) also observed that viable synapses were lost one after another until no synaptic territory was left for the axon before the axon was retracted.

1.1.4.2. Relative terminal activity

In addition to the demonstration that post-synaptic target cell plays an active role in synapse elimination, it is generally believed that competition between axons innervating the same muscle fiber is also important to determine which axons to be remained or eliminated. The fact that activity influences synapse elimination (Thompson, 1985) leads to attempts to investigate how

activity patterns of an individual motoneuron could affect its chances or those of other axons to remain or be eliminated. These experiments have reached quite different conclusions. Balice-Gordon and Lichtman (1994), by locally puffing α-bungarotoxin (α-BTX) onto a portion of the motor endplate to induce an imbalance in synaptic effectiveness between separate boutons in a singly innervated adult neuromuscular junction, showed that the pre-synaptic terminals overlying the α-BTX blocked receptors were eventually withdrawn. In contrast, the pre- and post-synaptic regions containing unblocked receptors remained relatively unchanged. Ridge and Betz (1984) showed that chronic electrical stimulation of one of the two innervating nerves of lumbrical muscle in rat foot sustained its enlarged MU size during the postnatal period of synapse elimination. There are a number of other studies supporting the idea that active synapses have competitive advantage over the inactive synapses (Ribchester and Taxt, 1983,1984; Ribchester, 1988). Ribchester and his colleagues have repeatedly shown that active nerve terminals have a competitive advantage over ones which were inactivated by tetradotoxin (TTX), for making synaptic connections with motor endplates in lumbrical muscle in rat foot (Ribchester and Taxt, 1983,1984; Ribchester, 1988). In these experiments, the competition is between active and inactive terminals and the active ones win. If, on the other hand, there are only relative differences in MU activity as there are normally, less active motoneurons are at a competitive advantage (Callaway et al., 1987;

1989). This is consistent with the size principle where smallest and most active MUs innervate fewer muscle fibers than the large and less active MUs. But, if during regeneration, all MUs are chronically stimulated, the size principle stays and MU size remains correlated with motoneuron size. Recent findings that motoneurons are electrically coupled by gap junction during development and the loss of gap-junctional proteins coincides with the time course of synapse elimination further suggest that synapse elimination is very likely associated with the loss of synchronized activity between innervating axons (Balice-Gordon et al., 1995). These also indicate that differential activity patterns lead to the size principle of MUs.

1.1.4.3. Competition for "trophic" factor(s)

Another proposed mechanism, derived from studies of target-dependent neuronal survival (Oppenheim, 1991), is that active nerve terminals compete more successfully for soluble, muscle-derived "trophic" factors present in limited quantities. The availability of these factors is believed to be activity-dependent with more available in inactive muscle (Slack et al., 1983; Thompson, 1985). Thus, the idea is that when there is a fixed, limited amount of trophic factor, the stronger or more active nerve terminals are more effective in capturing the factor and thereby more capable of winning the competition. This may explain the findings of Ribchester's studies that active terminals are at the advantage over the inactive ones. There is another suggestion that certain receptor present in

the terminals may be involved in capturing this trophic factor. The idea is that each motoneuron makes certain amount of the receptor in its soma for a trophic factor released by the target muscle cell. The receptors are then distributed to the collateral terminals of the motoneuron. The receptors on different terminals on a muscle cell then compete for the trophic factor. The more receptor-factor bonds made, the better chance there is for terminal growth and stabilization (Bennett and Robinson, 1989). Two candidates of such trophic factor, ciliary neurotrophic factor (CNTF) and leukemia inhibiting factor, have been implicated in the regulation of the timing or extent of neuromuscular synapse elimination (English and Schwartz, 1992, 1993; Jordan, 1993; Kwon et al., 1994).

1.1.4.4. Spatial competition

In addition to the trophic factor component to the competition theory, there is some limited evidence suggesting a possibility of having a spatial component to the competition theory. The idea is that each terminal at a motor endplate has a probability of growing or repressing others. The competition is therefore, played out on a scenario that attempts of a terminal to grow will be often impeded by the physical presence of others, and the winning terminal finally crowds out the loser terminals (Van Essen et al. 1990). There are studies showing that once established, a synapse is eliminated only if it is locating very close to another synapse, but not necessarily in direct contact to (Bennett and Pettigrew, 1976; Kuffler et al., 1977). However, competition for space is more a

proposed theory, and little evidence is available. More studies, therefore, would be needed to explore this theory.

1.1.4.5. Protease release

Finally, there is a line of evidence suggesting the possibility that an activity-dependent release of protease could be a signalling mechanism in synapse elimination. O'Brien et al. (1978) showed that sciatic stimulation at 8Hz promoted synapse elimination in neonatal SOL muscle and that ACh-treatment of muscle elicited release of proteolytic enzymes. Therefore, they suggested that the effect of activity in promoting synapse elimination was mediated by the release of proteolytic enzymes from active muscles which causes the withdrawal of nerve terminals. This is supported by the early findings that sciatic nerve stimulation *in vivo* caused a significant increase in proteolytic enzyme activity at the neuromuscular junctions of adult rats (Poberai et al., 1972; Poberai and Sávay, 1976). Direct evidence supporting the possibility that synapse elimination results from an action of proteolytic enzyme which is likely to be calcium-dependent, include the findings that synapse elimination *in vivo* was reduced by calcium reduction and chronic application of protease inhibitor (O'Brien et al., 1984; Connold et al., 1986). Further, Liu et al. (1992), using nerve-muscle co-culture, showed that protease inhibitor prevented stimulation-induced synapse elimination. Further extending the findings of the studies of Connold et al. (1986), the same authors, using an *in vitro* mammalian neuromuscular preparation,

demonstrated that a potent and specific thrombin inhibitor, hirudin, completely blocked the activity-dependent synapse reduction (Liu et al., 1994). These findings suggest that thrombin could be a potential candidate for this calcium-activated protease. Liu et al. (1994) proposed that post-synaptic activity caused the release of protease which might then act to reduce the efficacy of pre-synaptic inputs, resulting in terminal withdrawal.

1.1.5. Role of activity in synapse elimination

It has become clear that synapse elimination, like other developmental stages, is plastic. Increasing or decreasing the overall level of neuromuscular activity during the period of synapse elimination alters the rate of synapse elimination.

Various experiments aimed at reducing overall neuromuscular activity levels through a variety of means have generally reached similar conclusions: diminished activity results in a reduction or cessation of synapse elimination. Benoit and Changeux (1975) tenotomized SOL muscle in 4-day-old rats and later examined the muscle by recording endplate potential intracellularly. Tenotomy dramatically reduces activity of the muscle, presumably by reducing afferent excitation to the spinal motoneurons from muscle spindle. They found that the tenotomized muscles had higher levels of polyneuronal innervation than control muscles, and concluded that neuromuscular inactivity resulted in disappearance of synapse elimination. However, Riley (1978) argued that the effect of tenotomy

in synapse elimination might not be due to the reduction in neuromuscular activity because tenotomy in adult cat SOL muscle did not appear to alter its activity.

Using an experimental paradigm of post-synaptic activity blockade by α-BTX, Greensmith and Vrbová (1991) showed that synapse elimination was completely arrested by α-BTX in SOL muscle of 10-day-old rats. Thompson and his collaborators (Thompson et al., 1979) examined the effect of TTX blockade of the sciatic nerve on synapse elimination in SOL muscle of 9-10 day-old rats by intracellular recording of endplate potential, and found that synapse elimination was delayed by TTX blockade of pre-synaptic activity. Barry and Ribchester (1995) found similar effect of TTX in delaying synapse elimination even in reinnervated mature muscles. One of two nerves innervating rat lumbrical muscle was crushed and allowed to regenerate to reinnervate the muscle fibers which had already been reinnervated by axonal sprouting of the remaining nerve. During the period of polyneuronal innervation, TTX blockade of both regenerated and intact sprouted nerves sustained the convergent synaptic terminals of both nerves. Another pre-synaptic blocking agent, botulinum toxin (BuTX), has also been employed in the study of activity and synapse elimination. It appears that, with BuTX, its effect on synapse elimination varies depending upon the types of muscle used. Brown et al. (1982b) injected BuTX into newborn mouse hindlimb muscles. In the case of the gluteus muscle, they showed by

endplate morphology and electrophysiology that BuTX-induced muscle paralysis did not prevent synapse elimination. In contrast, it completely prevented synapse elimination in another muscle, tensor fasciate latae muscle, even after the paralysis had worn off.

The alternative approach to the induction of muscle paralysis is to increase neuromuscular activity. Attempts to artificially increase levels of neuromuscular activity, collectively, result in an acceleration of synapse elimination. O'Brien and his colleagues (1978) examined the effect of increased neuromuscular activity using electrical stimulation of sciatic nerves of rat pups 6-7 days old at 8 Hz for 4-6 hours per day for 2-4 days. They examined the levels of polyneuronal innervation in the stimulated SOL muscle using intracellular recording and endplate morphology, and found that synapse elimination was significantly accelerated. Another effort at examining the effect of activity on synapse elimination was conducted by Thompson (1983). He directly stimulated SOL muscle of 7 day-old rats for 3-4 days at two different patterns of muscle stimulation, and measured the level of polyneuronal innervation by intracellular recording. He found that brief stimulus trains containing 100Hz bursts of stimuli produced an acceleration of synapse elimination, whereas the same number of stimuli presented continuously at 1 Hz did not produce such effect. These results indicate that effect of activity on synapse elimination depends upon not only the presence of stimulation, but also the patterning of stimuli.

1.2. Neuromuscular plasticity

1.2.1. Remodelling of neuromuscular junction

Neuromuscular junctions, after complete differentiation and maturation, do not just stay static. Adult neuromuscular junctions are dynamic and undergo synaptic remodelling in response to several factors, including altered neural activity, injury, aging and diseases.

1.2.1.1. Effect of neural activity

As an organism develops, neural activity-related mechanisms can induce plastic modifications in the neuromuscular junctions. This process of activity-dependent synaptic plasticity seems to continue throughout the life-time of the organism. One example of this ongoing activity-dependent synaptic plasticity would be the activity-related seasonal remodelling changes observed in frog neuromuscular junctions (Wernig et al., 1980). Amphibian neuromuscular junctions exhibit less complex junctional morphology in summer where they are subjected to higher neuromuscular activity.

Less complex junctional morphology was also observed in mammalian neuromuscular junctions in response to increased neuromuscular activity (Tomas et al., 1989). Tomas and his colleagues found, using morphometric analysis, that the mean length of terminal arborization and mean number of branch points of terminals significantly increased at neuromuscular junctions in 2 month-old rats whose locomotor activity was decreased by being constricted in

very small cages for 28 days. On the contrary, at the junctions in rats trained on treadmill for the same length of time, mean length of terminal arborization and mean number of branch point of terminals were found even lower than the values in control rats. This significant decrease in the complexity of branching patterns is in good agreement with the recent findings of greater incidence of post-synaptic vacant sites at extensor digitorum longus (EDL) neuromuscular junction in rats which had 4-week walking training (Tomas et al., 1997). Tomas and his co-workers also reported a significant enlargement of active zone at the pre-synaptic terminals. Other ultrastuctural signs such as pre-synaptic terminal shortening after increasing activity was also reported in rat EDL neuromuscular junction when they were subjected to 10 day continuous low frequency (10Hz) electrical stimulation via implanted electrodes on the sciatic nerve (Mussini and Carraro, 1991). Despite the decrease in complexity of branching patterns (Tomas et al., 1989) and pre-synaptic nerve terminal shortening (Mussini and Carraro, 1991), the enlargement of pre-synaptic active zone suggests that there is a compensatory adaptation which tends to optimize synaptic efficacy in response to increase in neuromuscular activity.

There is also a suggestion that the morphological changes at neuromuscular junctions in EDL muscle in response to altered neuromuscular activity are simply a reflection of fast-to-slow transformation of skeletal muscles (Somasekhar et al., 1996). Somasekhar and colleagues (1996) showed that low

frequency (10Hz) stimulation, which is known to induce fast-to-slow phenotype transformation (Delp and Pette, 1994; Gordon et al., 1997), caused significant alternations in the morphology of neuromuscular junctions in both EDL and tibialis anterior (TA) muscles. In fast twitch muscles such as EDL and TA muscles which compose of mainly fast muscle fibers, motor endplates are larger and have more junctional folds, and length of terminal branches are longer and the number of branches are higher, as compared to those on slow muscle fibers in slow twitch muscles such as soleus muscle (Sieck and Prakash, 1997). Fast motor units innervating fast muscle fibers are less active and are recruited later than slow units. Somasekhar et al. (1996) using light and electron microscopy showed that 3 week low frequency (10Hz) stimulation of sciatic nerve via implanted electrodes significantly reduced size of motor endplates, number of post-synaptic junctional folds, number of pre-synaptic terminal branches, and length of the terminals in TA and EDL muscles. The dimensions of neuromuscular junctions of fast-to-slow transformed muscles partially resembled those of slow soleus muscle.

1.2.1.2. Effect of aging

There are intriguing findings indicating that organization of pre- and post-synaptic structures of neuromuscular junction change with age (Fahim et al., 1983; Wernig et al., 1984; Elkerdany and Fahim, 1993). Using electron and light microscopy, aged and young neuromuscular junctions of different muscles have

been examined and compared in regards to their structures in a number of studies, and similar observations have been reported. Aged neuromuscular junctions have been found to be generally more complicated, as compared to neuromuscular junctions in young animals. In mouse EDL muscle, for example, aged neuromuscular junctions have been reported to have higher number of unmyelinated branches of the same terminal axon entering the junction (intrasynaptic terminal branches), longer intrasynaptic terminal branches and larger endplate area (Fahim et al., 1983). Similar observations of aged neuromuscular junctions were also reported in SOL (Wernig et al., 1984) and masseter (Elkerdany and Fahim, 1993) muscles in mice. Synaptic vesicle density was found to decline significantly in the SOL muscle of old CBF-1 mice (Fahim and Robbins, 1982). Interestingly, in the SOL and EDL muscles of old mice of the same strain, quantal content of neurotransmitter release was found to increase (Banker et al., 1983; Kelly and Robbins 1983). In addition, the rate of transmitter turnover in old mice was found, by measuring precursor uptake and release, to be more than twice that in young mice (Kelly and Robbins, 1986).

Many of these synaptic remodeling have been generally believed to be the result of adaptation or compensation for synaptic deficits associated with aging. To maintain a sufficient synaptic transmission, for instance, increases in quantal content and turnover rate of neurotransmitter would be likely to compensate for the reduced number of available synaptic vesicles (Fahim et al.,

1983). Other changes such as an increase in synaptic contact area by increasing intrasynaptic terminal branches and correspondingly the motor endplate area, may very well be serving similar compensatory purpose. Greater complexity of terminal arborization at older junctions is often associated with dispersion of terminals into more synaptic regions than in the young animals. Using combined fluorescent staining for nerve terminal membrane and nAChRs, Robbins et al. (1990) made repeated *in vivo* observations of neuromuscular junctions in young and old mice. They observed an ongoing swing between outgrowth of nerve terminals and retraction of these terminals at the perisynaptic regions of the junction in old mice. The junction appeared to be a zone susceptible to the formation of additional synaptic contacts with the size of the zone increasing with age. Similar ongoing outgrowth and retraction of nerve terminals at neuromuscular junctions have also been reported in aged cat hindlimb muscles (Tuffery, 1971) and human skeletal muscles (Oertel, 1986).

Although age changes at neuromuscular junction appear to be rather successful compensations in reaction to cellular deficits, the increasing extent of adaptation entails progressively more fragility. This fragility may account in part for the greater sensitivity of aging neuromuscular junctions to activity and/or pathological conditions.

1.2.2. Synapse reformation after complete and/or partial nerve injuries

Following nerve injuries, the distal stump of the severed nerve undergoes Wallerian degeneration which is the degeneration of cut axons and the removal of myelin debris by macrophages and Schwann cells (Fu and Gordon, 1997). Synaptic sites on target cells, therefore, become deprived of nerve terminals and thereby target cells become denervated. Unlike neurons in the central nervous system, surviving neurons are capable of regenerating axons to reinnervate targets in peripheral nervous system (Bähr and Bonhoeffer, 1994). Synapse regeneration and thereby functional recovery of the targets become possible.

After a nerve is cut, surviving neurons undergo "chromatolytic changes", and switch from a "transmitting mode" to a "growth mode" where they express many molecules similar to those found in development (Fu and Gordon, 1997). Examples of these molecules include the fast transported growth-associated protein, GAP-43, and the slow transported cytoskeletal proteins, actin and tubulin (Tetzlaff et al., 1991; for review, see Bisby and Tetzlaff et al., 1992).

There are similar changes in the distal stumps of the severed nerve. The endoneurial tubes, which are now empty of their axons which have undergone Wallerian degeneration, are lined by Schwann cells. Schwann cells proliferate very rapidly, revert to a "growth mode" and recapitulate development in the sense of expressing many of the same molecules found in development (Fu and Gordon, 1997). Some of these molecules include IGF-II and NCAM. Neurotrophic factors of the cytokine family, such as CNTF which are released

from Schwann cells have also been suggested to play a role in nerve regeneration. CNTF has been regarded as a postnatal survival promoting factor because of its abundance in Schwann cells of the intact nerve and the observation that its deficiency in transgenic mice does not affect neuronal survival until neurons attain maturity (DeChiara et al., 1995). Without a secretory signal sequence, CNTF is not likely to be released under physiological conditions. Damage of Schwann cells following nerve injury may trigger the release of CNTF to the extracellular matrix where it could serve as a "lesion factor" (Stöckli et al., 1989; Dobrea et al., 1992; Rabinovsky et al., 1992; Sendtner et al., 1992; Smith et al., 1993; Lee et al., 1995).

Regenerating axons preferentially reinnervate the original synaptic sites over the remaining post-synaptic folds (Bennet and Pettigrew, 1974). Signals, which direct the accuracy of site of reinnervation by regenerating axons, are believed to come from the synaptic basal lamina sheath. This conclusion derives from experiments in which muscle fibers were damaged and denervated *in vivo*, leaving behind "ghosts" of basal lamina. The reinnervation which then occurred was as topographically precise in the absence of muscle fibers as in their presence: more than 90% of axonal contacts with basal lamina occurred at former synaptic sites (Sanes et al., 1978; Kuffler, 1986). One of these signals is suggested to be s-laminin. S-laminin composes of a LRE site which is selectively adhesive to motoneuron-like cells (Hunter et al., 1989), and signals neurites to

stop outgrowth and to instruct axonal differentiation (Moscoso et al., 1995; Noakes et al., 1995; Porter et al., 1995). Direct evidence for a role of s-laminin in preferential reinnervation of the original synaptic sites by regenerating axons comes from studies which demonstrated that anti-s-laminin monoclonal antibody blocked the reinnervation of original synaptic sites on skeletal muscle sections by ciliary ganglion neurites (Iglesias et al., 1995).

It has been well known that denervated muscle fibers provide essential guidance cues for nerve regeneration and muscle reinnervation (Covault et al., 1987; Kuffler, 1989; Kuffler and Luethi, 1993). In addition to s-laminin, other cues include NCAM, N-cadherin and IGF-II which are expressed on denervated muscle fibers and are downregulated upon muscle reinnervation (Sanes et al., 1986; Covault and Sanes, 1986; Ishii, 1989; Near et al., 1992; Cifuentes-Diaz et al., 1994; Marsh et al., 1994; Martini, 1994; Glazner and Ishii, 1995). Expression of these guidance molecules by denervated muscle fibers is accompanied by the denervation changes such as hypersensitivity to ACh (Lömo, 1976; Cangiano et al., 1984).

Synapse reformation, an example of neuromuscular plasticity, is not static, but can be affected by neuromuscular activity (Gutmann and Jakoubek, 1963; Soucy et al., 1996). The findings that increased motor activity via a daily swimming regime significantly reduced size of regenerated axons and prevented the final synapse maturation after nerve crush indicates increased

neuromuscular activity is detrimental to synapse formation and maturation (Gutmann and Jakoubek, 1963). This is further supported by the recent findings that increased neuromuscular activity by 30-day treadmill running impaired reinnervation in lateral gastrocnemius (LG) muscle by destabilizing the newly formed synapses as indicated by the exacerbated "tetanic fade" phenomenon (Soucy et al., 1996).

1.2.3. Axonal sprouting

Edds (1949) and Hoffman (1950) used histological techniques to first demonstrate conclusively that in partially denervated muscles new nerve sprouts may grow out from surviving axons to reinnervate denervated muscle fibers. Since then, axonal sprouting has been studied extensively in animal models. Axonal sprouting is a process where fine nerve processes--collateral sprouts--grow out from the intact axons at either the nerve terminals or the nodes of Ranvier. It takes place as a natural compensatory mechanism following motoneuron diseases and/or trauma such as poliomyelitis and ALS, and partial nerve injuries, where muscles often become partially denervated due to the death of some of the innervating motoneurons. Axonal sprouting can also be induced under controlled experimental conditions.

1.2.3.1. Motoneuron diseases and/or trauma

Poliomyelitis is an acute viral disease which attacks the lower motoneurons in the ventral horn of the spinal cord, resulting in partial denervation of the hindlimb muscles (Halstead and Wiechers, 1987; Halstead and Grimby, 1995). The prognosis for functional recovery of the affected muscles depends strongly on the extent of the motoneuron loss and the sprouting capacity of the remaining motoneurons. The surviving motoneurons in the affected muscles sprout and reinnervate denervated muscle fibers and thereby compensate for the loss of motor function (Wohlfart, 1957; McComas et al., 1971; reviewed by Miller, 1984; Dengler et al., 1989; Grimby et al., 1989; Trojan et al., 1991). Limitation on the sprouting capacity of remaining motoneurons prevents complete functional recovery. When motoneuron loss is extensive, MU enlargement cannot fully compensate leading to muscle paralysis and deformity.

Acute poliomyelitis has been rare in developed countries since the introduction of effective immunization in 1955. However, new concerns have arisen to acute poliomyelitis survivors who experience progressive muscle weakness and fatigue decades after the initial motoneuron death. These progressive muscle weakness and fatigue have been generally referred as "post-polio syndrome" (Halstead and Wiechers, 1987; Windebank et al., 1991; Halstead and Grimby, 1995). Although the etiology of post-polio syndrome is still unclear, it has been suggested that post-polio syndrome is very likely a result of nerve terminal withdrawal due to over-exhaustion of the sprouted motoneurons

and/or natural motoneuron attrition with age (Tuffery, 1971; Fahim and Robbins, 1982; Slack and Hopkins, 1982; Dalakas et al., 1986; Cashman et al., 1987; Halstead and Wiechers, 1987; Banker et al., 1983; Kelly and Robbins, 1983; Kelly and Robbins, 1986; Oertel, 1986; Hasizume et al., 1988; Rocel and Robbins, 1988; Lange et al., 1989; Emeryk et al., 1990; Jacob and Robbins, 1990a,b; Robbins et al., 1990; Agre et al., 1991; Trojan et al., 1991; Maselli et al., 1992; Dalakas, 1995). It should be noted that clinical observations indicate that symptomatic post-polio syndrome is more commonly experienced in patients who suffered severe motoneuron loss in prior poliomyelitis.

Axonal sprouting also occurs as a compensatory mechanism for functional loss in the early stage of ALS (Wohlfart, 1957; Halstead and Wiechers, 1987). ALS is also called Lou Gehrig's disease, and is an incurable motoneuron disease of unknown etiology. Patients at the early stage of ALS suffer a certain extent of motoneuron loss, and could be able to regain some functional control due to axonal sprouting. However, unlike poliomyelitis, motoneuron loss continues and functional loss of ALS patients worsens as they proceed to the end of the course of the disease. Chronically enlarged MUs become overstressed and cannot be maintained over a long period of time, and already sprouted and exhausted motoneurons cannot keep up with the continuous motoneuron loss. Therefore, functional disability becomes very severe at the

later stage of ALS, and eventually complete paralysis of the respiratory muscles results in patient's death.

1.2.3.2. Experimental manipulations of inducing axonal sprouting

The study of axonal sprouting and the search for sprouting stimuli involved in the mechanism underlying axonal sprouting have motivated researchers to design different experimental conditions where axonal sprouting can be induced artificially. One of these experimental conditions is partial denervation of hindlimb muscles (Hoffman, 1950; Brown and Ironton, 1978; Pachter and Eberstein, 1992; Mehta et al., 1993; Son and Thompson, 1995b). Partial denervation of hindlimb muscles can be achieved commonly by section of spinal roots (Hoffman, 1950; Brown and Ironton, 1978; Pachter and Eberstein, 1992). Each hindlimb muscle receives different degrees of innervation from motoneurons in the spinal cord through up to 3 spinal roots. Therefore, section of any one of the spinal roots supplying innervation to the hindlimb muscles results in different extent of partial denervation in each hindlimb muscle. Axonal sprouting in response to different extent of partial denervation can be studied. Partial denervation of hindlimb muscles can also be achieved by cutting one of the innervating nerve branches at the entry point to the muscle (Mehta et al., 1993; Son and Thompson, 1995b). The advantage of this type of partial denervation is that axonal sprouting around the boundaries of compartments in compartmentalized muscles such as medial gastrocnemius (MG) muscle can be

studied. It is because axons coming from the same nerve branch innervate muscle fibers close together (in the same muscle compartment).

Axonal sprouting can also be induced by muscle paralysis (Brown and Ironton, 1977; Holland and Brown, 1980; Pamphlett, 1989). The procedures that produce muscle paralysis and that have been shown to induce axonal sprouting include pre-synaptic blockade with BuTX (Pamphlett, 1989), nerve conduction block with TTX (Brown and Ironton, 1977), and post-synaptic blockade with α-BTX (Holland and Brown, 1980).

All of the aforementioned conditions which promote axonal sprouting have muscle fiber denervation and/or inactivity in common. Although studies of axonal sprouting using these conditions have given indisputable evidence that muscle fiber inactivity and/or denervation are stimuli for axonal sprouting, other interpretations of these results have engendered different hypotheses (O'Brien et al., 1978; Pestronk and Drachman, 1978). In addition, because of the inability of any of these sprouting-induced methods to selectively inhibit a single physiological parameter, it is very difficult to assess whether the primary cause of axonal sprouting is muscle fiber inactivity alone or impaired neuromuscular functions and/or the presence of denervation and degenerating nerve tissue. To overcome this difficulty, investigators have come up with other ways of inducing axonal sprouting which allow sprout-inducing components to be assessed independently. One of these ways is to use sprout-inducing chemicals such as

formamide. Repetitive formamide treatment inhibits muscle contractile activity without affecting intact neuromuscular innervation, functional synaptic transmission or muscle fiber electrical activity (Wines and Letinsky, 1988). Colchicine is another sprout-inducing chemical used in the study of mechanisms underlying axonal sprouting. The findings that colchicine blocks axonal transport of cytoskeletal proteins, and that it induces axonal sprouting without affecting muscle activity or innervation, suggests the involvement of motoneuron-derived sprout-inhibiting factors, CGRP for example (Diamond et al., 1976; Riley and Fahlman, 1985; Schlumpf and Davis, 1986).

1.3. Axonal sprouting

1.3.1. Sprout growth and types

Three types of collateral sprouts can be generated through the process of axonal sprouting. They are intranodal, preterminal and ultraterminal sprouts. Intranodal sprouts originate from a node of Ranvier. Preterminal sprouts originate from unmyelinated region of an axon at the entry point to the motor endplate. Ultraterminal sprouts grow out from the motor endplate region. Preterminal and ultraterminal sprouts, together, are generally referred as terminal sprouts.

Silver-stained preparations of partially denervated muscle show that intranodal sprouts arise from sharply defined constrictions in the axons which are almost certainly nodes of Ranviers (Hoffman, 1950). However, not all nodes in a

nerve of a partially denervated muscle produces sprouts. Intranodal sprout arises from nodes only within the intramuscular nerves, but not the extramuscular nerve (Edds, 1949; Slack and Williams, 1981). In addition, Hoffman (1950) observed that the majority of intranodal sprouts at the early time point after partial denervation arose from the nodes about 100 to 200 μm from the reinnervated motor endplates. Slack et al. (1979) later observed that intranodal sprouts arose only from nodes close to the branch points of denervated perineural nerve sheath through which they reinnervated denervated motor endplates. As in muscles inactivated by BuTX, intranodal sprout develops mostly at the last node of Ranvier before the nerve terminal (Hopkins et al., 1981). These observations indicate that the distance of the node of Ranvier from a denervated motor endplate and the location of the vacant nerve perineural sheath through which an intranodal sprout grows, are important determinants of which nodes are involved in axonal sprouting.

The observation that terminal sprouts grow erratically on the surface of muscle fibers, resembling "escaped" reinnervating axons described by Gutmann and Young (1944) suggests that both terminal sprouts and "escaped" reinnervating axons are probably mediated by similar mechanisms (Hoffman, 1950). There has recently been evidence showing that Schwann cell processes formed at the neuromuscular junctions induce and guide axonal sprouting (Son and Thompson, 1995b). Evidence also indicates that diffusible sprouting factors

released from denervated or otherwise inactive muscle fibers may be involved in axonal sprouting (Brown et al., 1978a,b, 1980a; Slack and Pockett, 1981; Pockett and Slack, 1982; Gurney, 1984; Gurney et al., 1986).

The method of inducing collateral sprouts is a major determinant of what types of collateral sprouts would be produced. While terminal sprouts can always be produced as a result of partial denervation and muscle paralysis, intranodal sprout has rarely been seen in paralyzed muscles if any (Duchen and Strich, 1968; Ironton et al., 1978; Brown et al., 1980b). The failure to see intranodal sprout in paralyzed muscles may be due to the reasons that inactive muscle is the major stimulus for terminal sprouts and intranodal sprouts are induced mainly by nerve degeneration products. This is because intranodal sprouts are not prevented by direct muscle stimulation which reduces terminal sprouts (Ironton et al., 1978). Other possible reasons could be that the intact and nerve-filled perineural nerve sheaths in activity-blocked muscles might act as a barrier to sprouting factors liberated from muscle, and that appropriate pathways--vacant perineural nerve sheaths--for intranodal sprout are not available in paralyzed muscles.

In addition to the method of inducing axonal sprouting, muscle types have also been suggested to affect the types of collateral sprouts being produced (Brown et al., 1980b). Brown and his colleagues reported that partial denervation by cutting L4 or L5 sciatic rami caused relatively more intranodal

sprouting in the fast peroneus tertius and EDL muscles than in the slower SOL muscle, which itself had considerably more terminal sprouting than the others (Brown et al., 1980b). They suggested that fast muscles might be less susceptible to denervation-like changes associated with muscle inactivity and "inflammatory effect" inflicted by nerve degeneration products on muscle fiber properties, which have been suggested to be the two major sprouting stimuli for terminal sprouts. Substantial evidence supporting the view of Brown et al. (1980b) has not been available yet, and therefore further investigation will be needed to evaluate their view.

1.3.2. Sprouting capacity

It is indisputable that adult motoneurons can maintain their capacity to increase the number of their axonal branches and supply more muscle fibers by extending collateral sprouts by axonal sprouting to reinnervate denervated muscle fibers. It has been generally accepted that sprouting motoneurons have the sprouting capacity of increasing the number of muscle fibers in their MUs by maximum of 4 to 5-fold (Thompson and Jansen, 1977; Brown and Ironton, 1978; Yang et al., 1990). There are also examples of increases of as much as 20 times the normal size in cat hindlimb muscles (Luff et al., 1988). The wide range of differences has been attributed to the use of different methods of measuring MU size and muscle force recovery and different sample size. Brown and Ironton (1978) have shown that the ability of motoneurons to increase their MU size upon

partial denervation is independent of the original MU size and type. Rafuse and his colleagues (1992) have further elaborated this view by examining MU enlargement of cat triceps surae muscle after partial denervation using muscle force measurement. They showed that the force distribution of MUs in partially denervated muscles was similar to normal but was shifted to larger force values in direct proportion to the extent of partial denervation, indicating that all MUs increased in size by the same factor to preserve the normal distribution of MU force. These findings indicate that all motoneurons within a motoneuron pool compensate for partial nerve injuries by axonal sprouting, and that enlargement of MU size is a function of motor axon size consistent with Henneman's size principle.

A number of possible factors has been suggested to account for the up-to-5 fold limit of sprouting capacity of motoneurons. The findings in the studies of Brown and Ironton (1978) that MU enlargement was independent of the original MU size and type has led them to speculate that limit in sprouting capacity might represent an intrinsic growth limit or eventual failure of the motoneuron to supply more than a 5-fold increase in the number of functional terminals. The limit of MU enlargement after partial denervation has also been attributed to an inability of remaining MUs to increase their territories by branching from the large proximal intramuscular nerve trunks (Gordon et al., 1991, 1993; Rafuse et al., 1996a,b). The expansion of the most proximal branches of the axons in a muscle is reflected by the size of MU territory in a muscle. This branching style distributes nerve fibers

to the many muscle fascicles where the more distal terminal branches establish tne mosaic distribution of the unit fibers within the MU territory. Because the extent of proximal branching delineates the area of muscle fibers being supplied, it limits MU size to the number of muscle fibers within the MU territories which are available for reinnervation by axonal sprouting. The number of muscle fibers within this area may therefore determine the upper limit in MU size. Finally, distal branches of sprouting axons are ultimately obstructed from branching out to innervate more muscle fibers by structural barriers such as the perimysial sheets of connective tissue in the muscles (Kugelberg et al., 1970).

Capacity of motoneurons to sprout appears to depend also upon the age of the animal. The motor nerves of newborn rats seem reluctant to sprout (Thompson and Jansen, 1977). The immature SOL muscle was partially denervated while MUs were still in the neonatal expanded state, that is an average of 4 to 5 times their normal mature size. Muscle force measurement revealed that reduction in MU size which takes place during normal development went on despite the extensive denervation, and that the MUs ultimately formed after the phase of synapse elimination showed only two-fold increase in size instead of the normal 5-fold increase that follows partial denervation of adult muscle. Recent experiments have shown that Schwann cells regulate axonal sprouting of adult motoneurons by inducing and guiding axonal outgrowth through the processes they extend (Son and Thompson, 1995b). In light of the

roles of Schwann cells, Trachtenberg and Thompson (1996) examined the Schwann cells in neonatal SOL muscle in response to denervation. Using immunocytochemistry, they showed that Schwann cells at the neuromuscular junction and in the pre-terminal nerve branches disappeared, and decreased in intensity in the intramuscular nerves. To show that disappearance of Schwann cells was not due to the loss of Schwann cell marker, S-100, they applied the TUNEL technique to identify the nuclei of cells undergoing the DNA fragmentation characteristic of apoptosis, and showed that neonatal denervation led to the rapid, apoptotic cell death of Schwann cells at the neuromuscular junctions. These experiments indicate that apoptotic death of Schwann cells, at least in part, accounts for the inability of neonatal motoneurons to sprout upon denervation.

The findings that partial denervation by severing spinal root L5 induced a more profound increase in the percentage of motor endplates exhibiting ultraterminal sprouting in EDL muscle of 10- compared with 25-month-old rats, also show that the ability of motoneurons to sprout is compromised by aging (Rosenheimer, 1990). In addition, Jacob and Robbins (1990a,b) found that physiological reduction in miniature endplate potential amplitude and frequency, transmitter release, and especially latency at terminals of enlarged MUs, after partial denervation, were much more pronounced in old than in adult mice. These deficits were found not only at the reinnervated junctions, but also at the

original non-denervated junctions. These findings indicate a reduced capacity of old motoneurons to adapt to, generate, and maintain an enlargement of MUs. Age differences in response to partial denervation could, therefore, become clinically important when further MU enlargement is required as in severely affected muscles in older patients with ALS or post-polio syndrome. The mechanisms underlying the inability of aged motoneurons to sprout to full extent is unclear. The role of Schwann cells, which has been implicated in axonal sprouting of neonatal motoneurons, has not been investigated in axonal sprouting of aged motoneuerons. Reduced slow or fast axonal transport in old animals has, however, been suggested to be a factor limiting the capacity of aged motoneurons for expanding their field of innervation (McMartin and O'Connor, 1979; McQuarrie et al., 1989).

1.3.3. Sprouting stimuli

Axonal sprouting was first observed in response to partial denervation. The possible consequences that follow partial denervation have all been suggested at one time or another as the sprouting stimuli. Six potential sprouting stimuli have been suggested: 1) terminal Schwann cell processes, 2) peripheral nerve degeneration products, 3) inhibition of release of anti-sprouting factor, 4) signals from denervated muscle, 5) signals from sprouting motoneurons, and 6) loss of afferent input.

1.3.3.1. Role of terminal Schwann cell processes

Duchen (1971) was first to have suggested that collateral sprouts might be acompanied by Schwann cell processes. Recent elegant immunohistochemical studies have verified this idea by demonstrating that terminal Schwann cells induce and guide axonal sprouts by forming Schwann cell processes at both the innervated and the denervated endplates, and Schwann cell processes bridge between both types of endplates in partially denervated SOL muscle (Reynolds and Woolf, 1992; Son and Thompson, 1995a,b; Son et al., 1996; Thompson and Kopp, 1996). How these Schwann cell processes are formed from terminal Schwann cells is not clear. Since inhibition of Schwann cell mitosis by nitrogen mustard had no effect on axonal sprouting in partially denervated muscles (Hoffman, 1952), it is unlikely that Schwann cell processes implicated in the process of axonal sprouting are formed through Schwann cell proliferation. In view of the close association of Schwann cells and axonal sprouting, candidates which either promote or inhibit axonal sprouting might be expected to perturb this association or even be the result of such a perturbation. Love and her colleagues (1997) have recently shown that direct muscle stimulation prohibited bridge formation of terminal Schwann cell processes and thereby reduced axonal sprouting in SOL muscle 7 days after partial denervation. These findings could explain the early findings of Brown & Holland (1979) that direct muscle stimulation for 6-11 days significantly reduced axonal sprouting and, in turn, MU enlargement in

partially denervated SOL muscle. From our own laboratory (Tam et al., 1998), studies of extensive denervated TA muscle 4 weeks after avulsion of L4 spinal root using triple fluorescent labeling for neurofilament, Schwann cells and nAChRs also showed that increased neuromuscular activity by having experimental rats run on exercise wheel significantly reduced number of collateral sprouts and bridge formation over the entire period of 4 weeks. Interestingly, we, in addition, found that wheeling exercise actually increased the length of processes but complicated the orientation of these navigating processes. We suggested that this complication might account for the reduction of their bridge formation. Another possibility is suggested by recent findings that Schwann cells respond to neuromuscular activity by electrical or receptor-mediated elevations of intracellular calcium level (Jahromi et al., 1992; Reist and Smith, 1992; Reynolds and Woolf, 1993; Lev-Ram and Ellisman, 1995). The responses of terminal Schwann cells to ACh released from nerve terminals may be essential for their normal function; but high level of neuromuscular activity in partially denervated muscles may cause excessive Schwann cell activation which in turn may perturb the ability of Schwann cells to migrate, form processes and guide sprouting axons to denervated endplates.

1.3.3.2. Peripheral nerve degeneration

After nerve injury, injured axons in the distal stump undergo Wallerian degeneration with an initiation of a series of inflammatory responses. Denervated Schwann cells proliferate, line vacant edoneurial tubes, and convert to "growth"

mode for supporting axonal regeneration (Fu and Gordon, 1997). After partial denervation, some of the axons degenerate and Schwann cells which formally accompanied them are denervated in the vacant nerve sheaths. It is, therefore, plausible that these denervated Schwann cells in the vacant nerve sheaths in partially denervated muscles and inflammatory reactions associated with nerve degeneration may resemble those after nerve injury in the sense of enhancing nerve regeneration. This view is supported by the observations that intranodal sprouts arose only from nodes close to the points from which denervated perineural nerve sheath branched, and entered the vacant perineural nerve sheath in which denervated axons had been removed by Wallerian degeneration (Slack et al., 1979). In addition, it has been shown that terminal Schwann cells play an active role in inducing and guiding axonal sprouting by forming Schwann cell processes upon denervation. The findings that terminal Schwann cell "sprouting" in response to nerve lesion was delayed in mice with slowed Wallerian degeneration (Barry et al., 1997), indirectly implicate a possible role of nerve degeneration in axonal sprouting.

Evidence that damaged and degenerating nerves produce sprouting stimuli has been provided by a number of investigators. David and Aguayo (1985) demonstrated that fragments of periperal nerve implanted in central nervous system induced axonal sprouting of central axons into the nerve graft. A similar demonstration was also shown later by Diaz and Peot-Dechassine (1990) in their

experiment in which axonal sprouting was induced by motor axons implanted over frog pectoral muscle. Hoffman (1950) first suggested that intranodal sprout was guided to denervated endplates by some "elements" supplied by the denervated Schwann cells in the vacant nerve sheaths in partial denervated muscles. The source of sprouting stimuli, therefore, includes the non-neural cells of the distal nerve stumps, terminal Schwann cells.

CNTF, which is abundantly expressed in myelinating but not non-myelinating Schwann cells, is believed to serve as a lesion factor which may be released after injury (Stöckli et al., 1989; Dobrea et al., 1992; Rabinovsky et al., 1992; Sendtner et al., 1992; Smith et al., 1993; Lee et al., 1995). CNTF has also been suggested to be a sprouting factor. Supporting evidence includes the findings that exogenous administration of CNTF induced axonal sprouting in normal gluteus muscle in adult mice (Gurney et al., 1992; Kown and Gurney, 1994), and sustained polyneuronal innervation in neonatal LG muscle (English and Schwartz, 1995). Direct evidence showing that CNTF is an endogenous sprouting factor, comes from the recent studies of Siegel and English (1997). A partial denervation by transection of one of the two innervating nerves was produced in LG muscle of normal and mutant mice which lack the gene for CNTF. Denervation-induced axonal sprouting found in normal LG muscle was not detected in LG muscle of mutant mouse. Denervation-induced axonal sprouting, was however, detected in

another group of mutant mice in which CNTF had been administrated exogeneously following partial denervation.

1.3.3.3. Inhibition of release of anti-sprouting factor

It is conceivable that nerves which innervate a particular region of the periphery interact directly in some way to mutually inhibit each other's growth. Diamond and his colleagues (1976) suggested that the remaining nerves would be released from this inhibition and allowed to sprout to restore the lost innervation if some of the nerves were removed by partial denervation. Indirect evidence supporting his view is the finding that colchicine blocks axonal transport of cytoskeletal proteins, and that it induces axonal sprouting without affecting muscle activity or innervation (Diamond et al., 1976; Riley and Fahlman, 1985; Schlumpf and Davis, 1986). Diamond suggested that the release of anti-sprouting factors from motoneurons might be blocked following partial denervation. CGRP has been suggested to be a candidate anti-sprouting factor. CGRP is released from nerve terminal at developing neuromuscular junction for synaptogenesis, and has been shown to prevent disuse-induced axonal sprouting in rat hindlimb muscles in a dose-dependent manner when exogeneously administrated (Tsujimoto and Kuno, 1988).

1.3.3.4. Signal from denervated muscle

Muscle fibers are known to undergo profound changes in the their properties when they are denervated or otherwise inactivated (Lömo, 1976; Cangiano et al., 1984; Rabben et al., 1997). These profound changes referred as "denervation-like changes" include ACh supersensitivity and TTX resistance. A line of evidence has strongly indicated that denervation-like changes in inactive muscle are very likely the sources for sprouting stimuli. Firstly, it has been well known that denervated muscle fibers accompanied with these denervation-like changes provide essential guidance molecules for nerve regeneration and muscle reinnervation (Covault et al., 1987; Kuffler, 1989; Rassendren et al., 1992; Kuffler and Luethi, 1993). Secondly, denervation-like changes in innervated muscle can also be induced by partial denervation which has been well known to induce axonal sprouting (Cangiano and Lutzemberger, 1977). Thirdly, topological analysis of positions of collateral sprouts upon partial denervation have shown that they occur in a very close range, up to 200μm from denervated muscle fibers, suggesting that a short-range diffusible sprout-inducing factor may be released from denervated or inactive muscle fibers (Brown et al., 1978b, 1980a; Slack and Pockett, 1981; Pockett and Slack, 1982). More evidence supporting this view comes from the findings that BuTX-induced axonal sprouting at neuromuscular junction was inhibited by monoclonal antibodies against a denervated muscle-derived antigen of 56KDa (Gurney, 1984; Gurney et al., 1986). Finally, direct electrical stimulation of inactive or

denervated muscle fibers inhibits both the denervation-like changes (Lömo, 1976; Eberstein and Pachter, 1986) and axonal sprouting (Brown and Holland, 1979), whereas axonal sprouting is stimulated in normally innervated muscle fibers in which denervation-like changes have been induced by an inflammatory effect of nerve degeneration (Brown et al., 1978a) or blockade of muscle activity by BuTX (Brown et al., 1982a).

A number of sprouting stimuli released from denervated or inactive muscles has been suggested and includes neurocrescin (Nishimune et al., 1997), NCAM (Gurney et al., 1986), and IGF-II (Caroni and Grandes, 1990; Caroni et al., 1994; Thompson and Kopp, 1996). Among them, IGF-II has been relatively well studied with respect to its role of being a potential sprouting stimulus. Supporting evidence includes the findings that IGF-II is upregulated in denervated muscles and Schwann cells (Near et al., 1992; Glazner et al., 1994; Glazner and Ishii, 1995; Marsh et al., 1994; Pu et al., 1995). More direct evidence comes from the findings that exogeneous adminstration of IGF-II induced axonal sprouting in normally innervated mouse gluteus and rat levator auris longus muscles (Caroni and Grandes, 1990; Thompson and Kopp, 1996), and that axonal sprouting in BuTX-paralysed muscle was suppressed by IGF-binding proteins infused via implanted minipump before application of BuTX (Caroni et al., 1994).

1.3.3.5. Signal from sprouting motoneurons

Chromatolytic changes associated with nerve regeneration were also found in cell bodies following BuTX injection into muscles, a situation where axonal sprouting is known to occur (Watson, 1969; Duchen and Strich, 1968). However, it is not clear whether remaining intact motoneurons also undergo such changes after partial denervation although some of the physiological changes which occur in axotomized motoneurons such as shorter duration of after-hyperpolarization have been seen (Huizar et al., 1977). However, how sprouting motoneurons are signalled to elicit chromatolytic changes associated with nerve regeneration, is not clear. The possibility that some signal spreads from axotomized, chromatolyzing motoneurons to remaining intact motoneurons following partial denervation, a signal perhaps similar to that which causes axotomized motoneurons to regenerate axons, has been proposed (Rotshenker and McMahan, 1976; Pachter and Eberstein, 1991). However, this proposal has yet to be proven.

Nevertheless, sprouting motoneurons exhibit similar changes which motoneurons are known to have during nerve regeneration. Indeed, it has been most recently reported that GAP-43 mRNA was transiently upregulated in sprouting motoneurons in mice at 4 days after partial denervation (Bisby et al., 1996). It has also been reported that there was a delayed increase in immunoreactivity of GAP-43 at 2 weeks after BuTX-induced axonal sprouting (Hassan et al., 1994). More evidence supporting the involvement of GAP-43 in

axonal sprouting comes from the findings that sprouting axons and terminal Schwann cells also become GAP-43 immunoreactive in partially denervated muscles (Woolf et al., 1992; Mehta et al., 1993), and that overexpression of GAP-43 induces axonal sprouting and potentiates paralysis-induced axonal sprouting (Aigner et al., 1995; Caroni et al., 1996).

1.3.3.6. Loss of afferent input

When a muscle is partially denervated by section of a mixed nerve, for example, any branches of the sciatic nerves or any spinal roots, which contain both sensory and motor nerves, afferent input to the spinal cords is also removed by section of sensory nerve fibers. Therefore, conceivably some degeneration of central sensory nerve fibers could also occur. Since motor nerve degeneration has a local effect in triggering axonal sprouting in a partially denervated muscle, one might ask whether degeneration of central sensory nerve fibers might also have some influence in triggering motoneurons to sprout. This is, however, unlikely to be the case. Brown and his colleagues (Brown et al., 1978a) investigated the incidence of axonal sprouting in hindlimb muscles of mice in which the dorsal roots central to the ganglia were cut with afferent axons remained intact (rhizotomy), and found no evidence of axonal sprouting.

1.3.4. Remodelling in reinnervated muscles after partial denervation

It has been established that intact motoneurons in response to partial denervation enlarge their peripheral innervation fields and thereby their MU size through axonal sprouting and reinnervate denervated muscle fibers. As a result, enlarged MUs now innervate muscle fibers which previously belonged to other units with potentially very different properties. It has been reported that the size and metabolic properties of MU fibers in plantaris (PL) muscle appear to be more variable at 7 days after partial denervation, as compared to control (Gardiner et al., 1987). In another study which MU fibers in TA muscle were examined at a period up to 12 months after partial denervation, MU fibers appeared to be more uniform in size and homogenous in metabolic properties (Bell et al., 1992) with time. Therefore, MU fibers in reinnervated muscles following partial denervation appear to proceed through a phase of reorganization which alters the properties of the new fibers to those defined by the confines of the original composition of the MU. Features of this reorganization in reinnervated muscles following partial denervation include MU fiber type grouping, changes in mechanical, morphological and metabolic properties, and muscle fiber size. To discuss all of them is beyond the scope of this thesis. Therefore, only MU fiber type grouping and changes in muscle fiber size in response to partial denervation are discussed.

1.3.4.1. MU fiber type grouping

In skeletal muscle, 2 or 3 muscle fiber types can typically be demonstrated on the basis of the pH sensitivity of myosin ATPase (Tötösy de Zepetnek et al., 1992). Normally, different muscle fiber types are randomly distributed in a characteristic mosaic pattern. Spatial analysis of MU fiber distribution using glycogen depletion technique followed by periodic acid Schiff reaction, shows that MU fibers are normally scattered in a mosaic pattern and among fibers of many different MUs within a defined territory whose size increases with MU size (Edstrom and Kugelberg, 1968). Spatial analysis of MU fiber distribution in normal and reinnervated muscles, shows further that location and size of MU territories, and number of MU fibers within MU territory are respectively determined by the proximal intramuscular nerve and distal axonal branching (Rafuse et al., 1996b). Partial denervation which leads to axonal sprouting of distal axonal branches, can therefore increase the number of MU muscle fibers within a defined territory without significantly changing the size of the MU territory (Gordon et al., 1993). As the number of innervating MUs is reduced and thereby the extent of partial denervation increases, MU fiber clumping becomes increasingly more visible and the mosaic distribution of fibers within the MU territory becomes more dense with muscle fibers (Gordon et al., 1991).

MU fiber type grouping can be demonstrated in reinnervated muscles in experimental animal models of partial denervation and/or peripheral nerve

section and repair (Gordon et al., 1991; Tötösy de Zepetnek et al., 1992; Gordon et al., 1993; Rafuse et al., 1996a,b). It is also a general finding in muscle biopsy for a number of neuromuscular disorders in which muscle denervation and reinnervation occur. These include poliomyelitis, ALS, nerve and/or spinal cord injuries where muscles become completely or partially denervated (Dubowitz, 1967; Sunderland, 1978).

1.3.4.2. Changes in muscle fiber size

Pachter and Eberstein (1992) reported that the mean cross-sectional areas (CSA) of both type I and II muscle fibers in PL muscle at 1 month after partial denervation were significantly lower than control. They also found evidence of pronounced muscle fiber atrophy as well as hypertrophy. The presence of atrophic muscle fibers was also found in the partially denervated muscles in the studies of Jacob and Robbins (1990b). Pachter and Eberstein (1992) explained the presence of atrophic muscle fibers as a situation when all muscle fibers had not yet been completely reinnervated by axonal sprouting. However, it has been found that the process of axonal sprouting would generally be complete in about 1 month (for review, see Brown, 1981). In addition, it has been shown that MU size increases as a function of the extent of partial denervation with a maximal capacity of 4 to 5-fold such that MU enlargement by axonal sprouting compensates for functional loss of as much as a 80% loss of the normal number of MUs (Coers and Woolf, 1959; Thompson and Jansen,

1977; Brown and Ironton, 1978; Fisher et al., 1989; Jansen and Fladby, 1990; Yang et. al., 1990; Rafuse et al., 1992 and Gordon and Pattullo, 1993). It is, therefore, when denervation is extensive, axonal sprouting cannot reinnervate all denervated muscle fibers and muscle weakness becomes evident (Luff et al., 1988; Rafuse et al., 1992; Rafuse and Gordon, 1996a,b). On this basis, another possibility explaining the presence of atrophic muscle fibers in the studies of Pachter and Eberstein (1992) could be that some muscle fibers might never be reinnervated due to possibly extensive denervation and the limit of sprouting capacity. It is very likely especially as they did not measure the extent of denervation of PL muscle.

Nine months after partial denervation, Pachter and Eberstein (1992) found that the mean CSA of muscle fiber type I and II were significantly decreased after they had been comparable to control prior to the decrease. This is a characteristic commonly seen in many motoneuron diseases such as post-polio syndrome. It appears that the remaining, chronically enlarged MUs are unstable due to long-term overuse, overstress, and attribution of age. They are unable to maintain the metabolic demands for their peripheral innervation field and ongoing denervation. Unstable terminals withdraw and some muscle fibers thereby become denervated. At 12 months after partial denervation, muscle fiber CSA was significantly decreased and muscle fibers became very atrophic, supported by the decrease in the number of collateral sprouts to the level comparable to

control, and the presence of many degenerating and denervated motor endplates.

Changes in muscle fiber size is one of the many essential components to be concerned with especially when one desires to make an accurate physiological assessment of MU enlargement. Partial denervation leads to increase in MU size of intact motoneurons by axonal sprouting. In other words, the number of muscle fibers innervated by the intact motoneuron (i.e. innervation ratio, IR) increases. IR can be estimated directly using glycogen-depletion technique, and indirectly using MU force measurement. The most direct estimate of IR can be obtained by counting muscle fibers in an isolated and glycogen-depleted MU. The validity of the glycogen-depletion technique for enumeration of MU fibers, however, depends critically on the reliability of the glycogen-depletion protocol and the muscle architecture such as muscle fiber length and pinnation angle. For instance, rat TA muscle fibers are long and extent for more than 50% of the muscle length, and the fibers have rather small pinnation angle (Tötösy de Zepetnek et al., 1992). Therefore, depleted fiber counts in cross sections which contain the highest number of glycogen-depleted muscle fibers provide a good estimate of IR. On the contrary, cat MG muscle fibers are short relative to the muscle length, and the pinnation angle is relatively large (Burke and Tsairis, 1973). Therefore, depleted fiber counts in the cross sections which contain the

highest number of glycogen-depleted muscle fibers represent probably only 50-67% of the total number of MU fibers.

The glycogen depletion technique can directly estimate IR of an individual MU and is an excellent tool for studying MU fiber type grouping. To study MU enlargement, however, MU force must be known. Since muscles, especially large muscles, often cover a wide range of MU force, accurate measurement of MU enlargement requires large representative samples of MU force (such as the overall average of the whole population of MU force). MU force is the product of IR, CSA and specific force of muscle fiber. Several studies have shown that MU force varies systematically with IR and CSA in both normal and reinnervated muscles (Tötösy de Zepetnek et al., 1992) and specific force does not change after reinnervation (Tötösy de Zepetnek et al., 1992; Fu and Gordon, 1995a,b). MU force corrected for muscle fiber CSA, therefore, reasonably reflects IR. Comparison of MU force normalized for muscle fiber CSA between normal muscle and reinnervated muscle following partial denervation, therefore, provides accurate assessment of MU enlargement.

1.3.5. Neural activity and axonal sprouting

The effect of increased neuromuscular activity on axonal sprouting has been controversial. Hoffman (1952) using histological techniques demonstrated that after partial denervation by section of spinal root L5, electrical stimulation of intact neurons accelerated the growth of collateral sprouts. Herbison et al.(1986)

later demonstrated that partially denervated SOL muscle force was increased by a 6 month period of sciatic nerve electrical stimulation. Without the evidence of muscle hypertrophy, they concluded that the effect of electrical stimulation in enhancing muscle force was due to enhancement of axonal sprouting. A preferential effect of prolonged treadmill exercise of promoting MU enlargement of fast-fatiguable and fast-intermediate units was reported in MG muscle (Einsiedel and Luff, 1994). However, evidence from animal studies has also indicated that exercise could be detrimental to axonal sprouting or have no effect at all. A short period of wheeling exercise or functional overload by the removal of synergistic muscles did not affect axonal sprouting in PL muscle (Gardiner and Faltus, 1986; Michel and Gardiner, 1989). A ten week period of daily treadmill exercise (Gardiner et al., 1984) and direct muscle stimulation (Brown and Holland, 1979), however, reduced axonal sprouting in partially denervated SOL muscles.

The effect of neuromuscular inactivity on axonal sprouting is also unclear. Short-term muscle paralysis induced either by TTX (Brown and Ironton, 1977) or α-BTX always promotes axonal sprouting (Holland and Brown, 1980). However, in these experiments, the muscles were studied for only a short period of time after axonal sprouting was induced. Since it is the final expansion of MU territory in partially denervated muscles which determines the functional recovery of the muscles, the ultimate effect of neuromuscular inactivity on axonal sprouting could

not be assessed accurately in the paralyzed muscle in these studies. Supporting evidence on this theory are the studies by Connold and Vrbová (1991) in which axonal sprouting was significantly reduced in the partially denervated SOL muscles after 2-10 month period of post-synaptic blockade of neuromuscular activity using α-BTX. Together with their findings that the large neonatal MU size was not maintained after the application of α-BTX in spite of partial denervation (Connold and Vrbová, 1990), they suggested that neuromuscular inactivity reduced MU enlargement by reducing the ability of newly formed sprouts to maintain the synaptic contacts with muscle fibers. Another possibility is that long-term neuromuscular inactivity may have prevented nerve outgrowth simply due to lack of calcium influx at the nerve terminals. It has been demonstrated in numerous studies that neurite outgrowth depends significantly on a narrow range of intracellular calcium concentration (Cohan et al., 1987; Mattson and Kater, 1987; Mattson et al., 1988; Kater et al., 1988, 1989; Connor et al., 1990; Collins et al., 1991; Kater and Mills, 1991; Rehder and Kater, 1992). Too low or too high intracellular calcium level results in neurite outgrowth arrest.

1.4. Stability of enlarged motor units

Chronic denervation syndromes such as post-polio syndrome and ALS are associated with enlarged MUs to compensate for motoneuron death and progressive muscle weakness and fatigue. The etiology of these chronic denervation syndromes is not known as yet. Although the ability of motoneurons

to maintain a chronically enlarged peripheral innervation field has not been systematically and extensively examined, some evidence from electrophysiological studies and muscle biopsy seem to suggest that chronically enlarged MUs undergo a progressive loss of functional nerve terminals. Furthermore, management of these chronic denervation syndromes is also unclear.

1.4.1. Proposed mechanism

1.4.1.1. Post-polio syndrome

The favorite theory for chronic denervation syndromes has been that the chronically enlarged MUs have been overstressed and cannot be maintained over a long period of time. Supporting evidence coming from muscle biopsy and quantitative electromyographic (EMG) studies of patients with prior poliomyelitis has suggested a link between this progressive muscle weakness and fatigue and increased 'jitters' indicative of synaptic instability and impaired neuromuscular transmission (Dalakas et al., 1986; Cashman et al., 1987; Lange et al., 1989; Maselli et al., 1992). It has also been reported that whole muscle force decreases with prolonged periods of partial denervation of rat PL muscles (Gardiner and Faltus, 1986; Eberstein and Pachter, 1992). These data have been interpreted as the inability of sprouted motoneurons to support and maintain their enlarged peripheral innervation fields. Histological examination of the partially denervated PL muscle showed that the number of collateral sprouts

was significantly reduced at 12 months after partial denervation, as compared to earlier periods of partial denervation, and became comparable to the level of control muscle (Pachter and Eberstein, 1992). These findings indicate that withdrawal of unstable nerve terminals accounts for, at least in part, the progressive muscle weakness and fatigue.

In addition, quantitative EMG studies of frog neuromuscular transmission and muscle reinnervation have shown that maximally enlarged MUs form less effective synapses, and that the efficiency of neuromuscular transmission can be enhanced by reducing MU size (Herrera and Grinnell, 1980, 1985). Later studies of neuromuscular transmission in patients with prior poliomyelitis by measuring single fiber EMG jitters demonstrated a correlation between the extent of neuromuscular transmission impairment and MU enlargement in response to the initial poliomyelitis (Maselli et al., 1992). These findings suggest that impaired neuromuscular transmission is most common in patients with prior poliomyelitis whose MUs have been maximally enlarged by axonal sprouting. These findings, therefore, may explain the clinical observation that symptomatic post-polio syndrome is experienced mainly in patients who suffered severe motoneuron loss in the prior poliomyelitis.

1.4.1.2. Aging effect

Another problem involved in the condition of chronic denervation syndromes is the reduced ability of aging motoneurons to sprout. Rosenheimer

(1990) demonstrated that partial denervation by severing spinal root L5 induced a more profound increase in the percentage of motor endplates exhibiting ultraterminal sprouting in EDL muscle of 10- compared with 25-month-old rats. In addition, reduction in miniature endplate potential amplitude and frequency, transmitter release, and especially latency at terminals of enlarged MUs, after partial denervation, have been shown to be more pronounced in old mice, as compared to adult (Jacob and Robbins, 1990a,b). These findings indicate a reduced capacity of old motoneurons to adapt to, generate, and maintain an enlargement of MUs.

Furthermore, it is known that even under normal conditions, aged motoneurons exhibit significant synaptic deficits including reduced efficacy of synaptic transmission, and spontaneous denervation and insufficient reinnervation (Tuffery, 1971; Fahim and Robbins, 1982; Banker et al., 1983; Kelly and Robbins, 1983; Kelly and Robbins, 1986; Oertel, 1986; Robbins et al., 1990). Therefore, even prior to loss of motoneurons inflicted by diseases such as post-polio syndrome and ALS, the capacity of aged motoneurons to maintain motor innervation has already been compromised. Thus, as a result of continuous maintenance of MU enlargement and/or natural motoneuron attribution with age, aged motoneurons with chronically enlarged MU size cannot simply bear the undue functional demands and therefore lose their nerve terminals.

1.4.2. Effect of neural activity

Exercise regimes have been advocated and some positive effects on muscle strength and endurance have been reported in patients with post-polio syndrome (Einarsson and Grimby, 1987; Feldman and Soskolne, 1987; Milner-Brown and Miller, 1988; Einarsson, 1991). Positive effects may, however, arise from several possible adaptations including increased central drive, MU and muscle recruitment, MU firing and/or muscle fiber hypertrophy. The danger lies in whether these adaptations mask possible detrimental effects of the neuromuscular activity on the enlarged MUs by provoking nerve terminal withdrawal. Other problems with most of these studies include the lack of a clear definition of post-polio syndrome, and the lack of well defined and quantifiable measures of muscle strength and endurance. It is also important to know the degree of involvement of initial polio and the severity of post-polio syndrome before the effect of muscle exercise can be accurately assessed.

1.5. Objectives of the present studies

The present thesis project concerns one of the major aspects of neuromuscular plasticity: MU enlargement by axonal sprouting. The purpose of the present thesis project is to systematically and thoroughly investigate how neuromuscular activity affects axonal sprouting and the stability of enlarged MUs, using animal models of motoneuron diseases, and both electrophysiological and histochemical techniques.

The specific objective of the experiments described in the present thesis are as follows:

1) to document the extent of denervation for functionally different muscles;

2) to systematically quantitate MU enlargement and axonal sprouting using complementary force measurement and histochemical methods;

3) to determine and compare the effect of natural and artificial means of increasing neuromuscular activity on axonal sprouting;

4) to determine whether enlarged MUs will destabilize with time in the animal model as predicted if the weakness of post-polio syndrome involves MU destabilization;

5) to determine whether enlarged MUs become further destabilized by increased neuromuscular activity in an animal model of chronic denervation;

6) to determine the effect of muscle paralysis on MU enlargement and axonal sprouting, and stability of enlarged MUs.

The ultimate objective of these studies is to establish optimum rehabilitation regimes for acute and chronic denervation syndromes which are associated with progressive muscle weakness and for paralyzed muscles in spinal cord injury patients.

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CHAPTER 2

2. General Methods and Materials

2.1. Surgical procedures

A total number of 137 female Sprague-Dawley (body weight 180-200g) rats was used for these studies. Rats were fed normal rat food and supplied with water. Surgery was performed under surgical anaesthesia (sodium pentobarbital administered intraperitoneally as 0.07ml/g body weight) and aseptic conditions. A small insertion near L4 and L5 spinal roots was made on the back of the rats. Either the right L4 (ACUTE: n=37; CHRONIC: n=27) or L5 (ACUTE: n=28; CHRONIC: n=29) spinal root was then exposed and evuled to partially denervate the hindlimb muscles including tibialis anterior (TA), medial gastrocnemius (MG), soleus (SOL) and plantaris (PL) muscles. A group of 16 unoperated rats was used as controls (ACUTE: n=10; CHRONIC: n=6).

2.2. Experimental groups

Immediately (ACUTE) or 11 months (CHRONIC) after the surgery, rats were divided into 4 different groups and subjected to experimental treatment for 4 weeks:

1) Control normal cage activity. Rats were put back into their normal rat cages and allowed to move freely (ACUTE: n=24; CHRONIC: n=20).

2) Natural wheeling exercise. Rats were allowed to run on exercise wheel voluntarily for 8 hours per day (ACUTE: n=16; CHRONIC: n=14).

3) Functional electrical stimulation (FES). A 2 cm of insulated stainless steel wire was bared on two wires and was implanted on either side of the sciatic nerve in the experimental leg for chronic stimulation. The insulated wires were either externalised for attachment to an external stimulator (Grass SD9) or remained internalised and attached to an implantable stimulator. The stimulator was turned on at 20Hz for 8 hours per day (ACUTE: n=12; CHRONIC: n=10).

4) Muscle paralysis by tetrodotoxin (TTX). A miniosmotic pump was implanted which was attached to a centimeter cuff around the sciatic nerve via a loosely fitting silastic tube. TTX at a concentration of 80μg per ml (Brown and Ironton, 1977) was administered via this implanted miniosmotic pump to sciatic nerve at the rate of 2.5μl per hour (ACUTE: n=13; CHRONIC: n=12).

The unoperated left side was used to serve as contralateral control. A group of unoperated rats (ACUTE: n=10; CHRONIC: n=6) was used to serve as normal control.

2.3. Muscle and MU force recordings

At the end of the four-week experimental treatment, muscle force recovery and MU enlargement were evaluated by muscle and MU isometric force measurement in the final experiment. Rats were again anaesthetised using sodium pentobarbital. The trachea was canulated for mechanical ventilation. TA, MG, PL and SOL muscles were isolated bilaterally by denervating all other hip, tail and hindlimb muscles (Fig. 2.1A). Bipolar silver wires were placed on either side of the sciatic nerve for stimulation. Braided silk threads, 2.0 gauge, were tied to the distal tendons for attachment to a force transducer and the skin around the incisions was closed loosely. A laminectomy from L3 to L6 spinal processes was performed in order to isolate the L4 and L5 spinal ventral roots bilaterally. Over the 8 to 10 hour period of muscle and MU force measurement, rats were continuously anaesthetised, and hydration and their blood volume were maintained by hourly intravenous injection of sodium pentobarbital and saline solution, respectively, via external jugular cannula.

The skin flaps of the laminectomy were used to create a spinal pool which was filled with medical grade paraffin oil. The knees and ankles were clamped bilaterally to allow for attachment of the distal tendon of each muscle to the strain gauge. The tendons were kept moist as they exited the opening of the skin at the ankle.

For each muscle in the experimental partially denervated hindlimb, contralateral unoperated control and unoperated normal control muscle isometric

twitch and tetanic forces were recorded in response to suprathreshold (2X threshold) sciatic nerve stimulation. Maximal evoked muscle twitch and tetanic forces were measured in response to 1, 5 and 21 pulses at 100 Hz at a repetition rate of 0.5-1 Hz. Recordings were made sequentially in the TA, MG, SOL and PL muscles. In each case, the muscle length for maximal twitch force was determined prior to force recordings. Muscle tetanic force elicited by stimulation of each ventral root (L4 or L5) was recorded. Ventral roots were then teased into small filaments, each of which contained about 5-10 motor axons. Ventral root filaments were stimulated by gradually increasing voltage to progressively recruit single MUs as judged by all-or-none increments in twitch force (Fig. 2.1B).

2.4. Histochemistry

Acid or alkaline-myosin ATPase. After muscle and MU force recordings, the TA and MG muscles were quickly removed, cut into three cross-sectional blocks, and frozen in isopentane cooled in liquid nitrogen. Cryostat cross sections at 12μm were cut and stained for acid or alkaline-myosin ATPase (Tötösy de Zepetnek et al., 1992b). Muscle fiber cross-sectional area was subsequently measured from these sections.

Combined silver/cholinesterase (Ag/AChE) histochemical staining. PL and SOL muscles were also removed and then fixed in 4% buffered formalin overnight and cryo-protected by subsequent overnight incubation in gum sucrose

solution. Muscles were then frozen in isopentane at -74°C. Cryostat longitudinal sections at 100µm were cut and stained using combined Ag/AChE histochemical staining (method modified from Pestronk and Drachman, 1978) in order to visualize the motor axons and sprouts and endplates. Briefly, for cholinesterase staining, 100µm cryostat longitudinal sections were collected in distilled water and incubated for 25 minutes at room temperature in a mixture of 0.01M Tris-HCl buffer pH 7.2, bromoindoxyl acetate, 1.65% potassium ferricyanide, 2.11% potassium ferrocyanide and 1.11% calcium chloride. For silver staining, sections from acetylcholinesterase staining were 1) incubated in 20% silver nitrate for 15 minutes, then 2) incubated in 3% sodium sulphite for 10 minutes, and finally 3) developed in a mixture of silver nitrate and physical developer. Washing in distilled water was carried out between steps. Number of sprouts and endplates were subsequently counted from the sections.

2.5. Data analysis

Muscle fiber cross-sectional area (CSA). With the exception of atrophic denervated muscle fibers in partially denervated muscles, CSA of all muscle fibers in TA and MG muscles from control (TA: n=11; MG: n=7) and experimental animals with caged activity (TA: n=7; MG: n=6), wheeling exercise (TA: n=5; MG: n=6), FES (TA: n=4; MG: n=7) and TTX (TA: n=5; MG: n=5) was measured using

a microcomputer digitizing software program (JAVA, Jandel Scientific). Mean muscle fiber CSA was then calculated.

MU number and size. All MUs in each partially denervated muscle, and at least 40% of MUs in contralateral control muscles and unoperated normal muscles were sampled to obtain a representative mean MU twitch force in each case. The total number of MUs in each muscle was estimated by dividing the whole muscle twitch force by the mean MU twitch force. For TA and MG, in which their muscle fiber CSA was measured, MU twitch force was corrected for the changes in the muscle fiber CSA. MU force is the product of innervation ratio (IR, number of muscle fibers innervated by one motoneuron), CSA and specific force of muscle fiber. Several studies have shown that MU force varies systematically with IR and CSA in both normal and reinnervated muscles (Bodine et al., 1987; Kanda and Hashizume, 1992; Tötösy de Zepetnek et al., 1992a) and specific force does not change after reinnervation (Tötösy de Zepetnek et al., 1992a; Fu and Gordon, 1995a,b). MU force corrected for muscle fiber CSA, therefore, reasonably reflects IR.

$$\text{Corrected MU twitch force (mN)} = \text{Recorded MU twitch force (mN)} \times \frac{\text{Mean muscle fiber CSA (Control)}}{\text{Mean muscle fiber CSA (Experimental)}}$$

Extent of partial denervation. There is bilateral symmetry in the relative contribution of L4 and L5 spinal roots to the motor innervation of TA, MG, PL and SOL muscles (Buller and Pope, 1977; Gordon et al., 1986). Therefore, the relative contribution of each spinal root to the innervation of a muscle on one side provides a relatively reliable estimate of its contribution on the contralateral side. We, therefore determined the contributions of L4 and L5 spinal roots to the muscles in the contralateral side to obtain a reasonable estimate of extent of partial denervation of the muscles on the experimental side. Muscle tetanic force elicited by the stimulation of each spinal root and sciatic nerve on the contralateral side were measured. The ratio of muscle tetanic force elicited by the stimulation of each spinal root to that elicited by the stimulation of sciatic nerve was determined and used to represent the extent of partial denervation of the muscles on the experimental side.

Analysis of axonal sprouts and free endplates. At first, up to 3500 endplates per muscle were examined in 2 muscles from each experimental group. Since examining 3500 endplates was very time-consuming and it was impossible to examine that many endplates for the rest of all the muscles for our studies, subsequently about 500 endplates from a total of 6 sections obtained from the middle portion of the muscle was examined for the rest of all the muscles in the studies. Endplates from sections stained for combined Ag/AChE histochemical staining were examined under light microscopy at a total

magnification of 160X or 400X and classified as one of the following: 1) free endplates which are the endplates having no axonal attachment and 2) endplates reinnervated by either a) intranodal sprouts, axonal outgrowth coming out from a node of Ranvier, b) preterminal sprouts, axonal outgrowth originating from the myelin-free region of an axon at the entry point to the motor endplates , or c) ultraterminal sprouts, axonal outgrowth from the myelin-free axons within the motor endplate region. The percentage of endplates which are reinnervated by each type of sprout was determined. The quantity of free endplates was determined by the percentage of free endplates from the total numbers of endplates sampled. All counting was "blind" in the sense that the investigator was not aware of the nature of the tissue samples being examined. The identity of muscles from the different experimental groups was unknown at the time of counting and encoded. The identity of muscles was revealed only after the counting was completed.

2.6. Statistics

Throughout this paper, mean with standard errors was given. The statistical significance of differences in mean muscle twitch force, MU number and twitch force, number of different sprouts and free endplates between control, partially denervated muscles with and without neural activation was determined using one-way analysis of variance (ANOVA) and subsequently Tukey HSD

(Honestly Significant Difference) test. One-way ANOVA was used to determine whether there was any of the differences among the mean scores between and within experimental groups. When significant differences existed, Tukey HSD test was carried to determine the locus of the significance. Tukey HSD test is one of the five post hoc tests and has the second lowest Type 1 error among the five tests. The Kolmogorov-Smirnov test (Daniel, 1995) was applied to examine the statistical significance of differences in cumulative distribution of MU twitch force between control, partially denervated muscles with and without neural activation. For all above statistical analyses, p values of less than 0.05 were regarded as significant.

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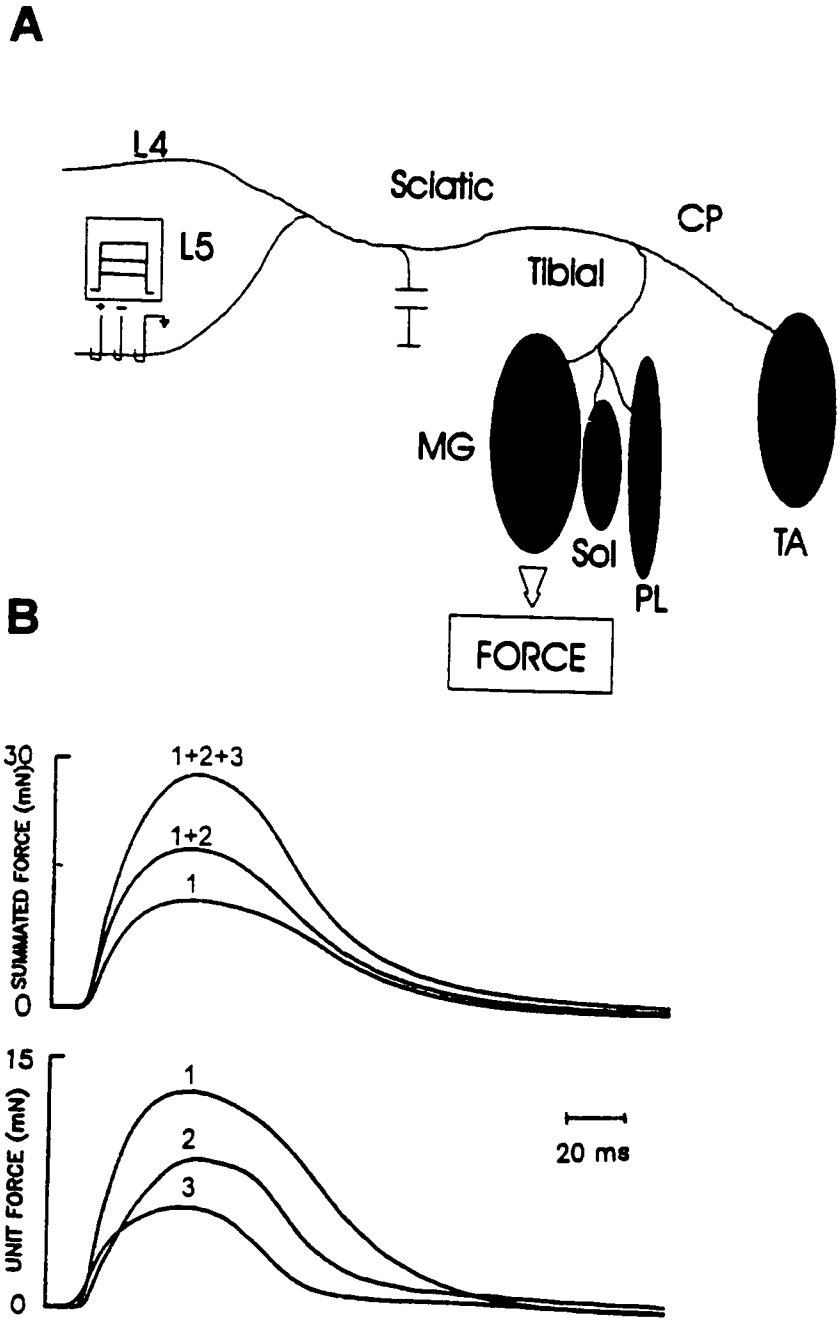
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CHAPTER 3

3. Effect of Neuromuscular Activity on Acute Phase of Axonal Sprouting

3.1. Introduction

Poliomyelitis, the early stages of amyotrophic lateral sclerosis (ALS), and partial nerve injuries are only some of the neuromuscular conditions resulting in compensatory motor axon sprouting to form enlarged motor units (MUs) (McComas et al., 1971; Brown et al., 1981 and Wiechers, 1987a,b). Surviving motoneurons in the affected muscles increase their MU size by axonal sprouting to reinnervate denervated muscle fibers in order to compensate for the motor function loss (reviewed by Miller, 1984; Grimby et al., 1989; Trojan et al., 1991). MU enlargement is unfortunately restricted to a limit of 5-8 fold such that axonal sprouting compensates for functional loss of as much as an 80% loss of the normal number of MUs (Coers and Woolf, 1959; Thompson and Jansen, 1977; Brown and Ironton, 1978; Fisher et al., 1989; Jansen and Fladby, 1990; Yang et al., 1990; Rafuse et al., 1992 and Gordon and Pattullo, 1993). It is only when less than 20% of functional MUs remain such that the axonal sprouting cannot

reinnervate all denervated muscle fibers and muscle weakness becomes evident (Luff et al., 1988; Rafuse et al., 1992; Rafuse and Gordon, 1996a,b).

The strong association of muscle exercise with muscle strength and endurance has led naturally to attempts to optimize muscle function with exercise. However, a role of neuromuscular activity in the acute phase of axonal sprouting is unclear and controversial. Muscle force in partially denervated soleus (SOL) muscle force was increased by a 6 month period of nerve electrical stimulation (Herbison et al., 1986). A preferential effect of prolonged treadmill exercise of promoting MU enlargement of fast-fatiguable and fast-intermediate units was reported in medial gastrocnemius (MG) muscle (Einsiedel and Luff, 1994). The observation that normally active motor axons were more successful in reinnervating partially denervated muscles than motor axons whose activity was blocked locally by infusion of tetrodotoxin was also attributed to a facilitatory role of neural activity on axonal sprouting (Ribchester, 1988).

On the other hand, there are several lines of evidence which argue against a positive role of activity in the injured neuromuscular system. Findings that all MUs increased their innervation ratio (IR: number of muscle fibers per motoneuron) by the same factor in partially denervated muscles showed that the absolute number of sprouts in the most active small slow MUs was less than that in the more forceful larger fast MUs (Rafuse et al. 1992). Furthermore, electrical stimulation promotes withdrawal of supernumerary axons in polyneuronally

innervated neonatal SOL muscle (O'Brien et al., 1978). Evidence from animal studies has also indicated that exercise could be detrimental to axonal sprouting or have no effect at all. A short period of wheeling exercise or functional overload by the removal of synergistic muscles did not affect axonal sprouting in plantaris (PL) muscle (Gardiner and Faltus, 1986; Michel and Gardiner, 1989). Moreover, 30-day running exercise did not affect MU enlargement in partially denervated lateral gastrocnemius (LG) muscle (Seburn and Gardener, 1996). A ten week period of daily treadmill exercise (Gardiner et al., 1984) and direct muscle stimulation (Brown and Holland, 1979), however, reduced axonal sprouting in partially denervated SOL muscle. In most of these studies, the extent of partial denervation was moderate or average, possibly contributing to the variability in the effects of increased neuromuscular activity on MU enlargement.

We have re-examined the issue in extensively denervated muscles using two functionally different fast-twitch muscles, tibialis anterior (TA, flexor) and MG (extensor) muscles, and compared the effectiveness of neuromuscular activity in modulating MU enlargement in the most frequently studied extensor SOL (slow) and PL (fast) muscles. A further modification in the experimental approach has been to 1) document the extent of denervation for each muscle (TA, MG, PL and SOL muscles), 2) use complementary force measurement and histochemical methods to quantitate MU enlargement and axonal sprouting, respectively, and 3) compare the effect of natural (running on exercise wheel) and artificial

(functional electrical stimulation, FES) means of increasing neuromuscular activity on axonal sprouting. In the present study, we show that increased neuromuscular activation during the acute phase of axonal sprouting is not beneficial for axonal sprouting. In fact, in partially denervated muscles in which MUs enlarge by a factor of 2 or more, increased neuromuscular activity is detrimental and the MUs are smaller than under conditions in which the muscles experience normal physiological levels of activation.

The present results have been presented and published in abstract form (Tam et al. 1995, 1996, 1997; Archibald et al., 1996).

3.2. Experimental design

In 44 female Sprague Dawley rats, either the L4 (n=23) or L5 (n=21) spinal roots was evulsed unilaterally to extensively (>80% partial denervation) denervate TA or MG muscles, respectively and moderately (~50% partial denervation) denervate PL and SOL muscles (see section 2.1.). Immediately after partial denervation, the rats were divided into three groups: 1) normal caged activity (n=20), 2) natural (running on exercise wheel, 8hrs daily; n=12) and 3) artificial (FES of sciatic nerves at 20Hz, 8hrs daily; n=12) neuromuscular activity (see section 2.2.). The unoperated muscles of the left side hindlimb of the experimental rats was used to serve as contralateral control and 6 unoperated rats were used as normal control. One month later when enlarged MUs had stabilized, functional

recovery in muscle force and weight, and MU enlargement were evaluated in all four muscles using MU and muscle force measurements (see section 2.3). Twelve micrometer thick cryostat cross sections of fresh TA and MG muscles were stained for acid or alkaline-myosin ATPase (see section 2.4.) and muscle fiber cross-sectional area was measured from these sections (see section 2.5.). PL and SOL muscles were fixed in 4% formalin and cut into 100μm thick cryostat longitudinal sections on which combined silver/acetylcholinesterase (Ag/AChE) histochemical staining was done (see section 2.4.). Number of collateral sprouts and free endplates were counted from the sections.

In the present study, both electrophysiological and histochemical evaluation were able to be done for PL and SOL muscles where L4 or L5 evulsion resulted mainly in moderate denervation with few exceptions of partial denervation which exceeded 75%. For TA and MG muscles, L4 and L5 evulsion, respectively, resulted mainly in extensive denervation with virtually no exceptions. Only electrophysiological evaluation was done in the TA and MG muscles because they were prepared for cross-sectional histochemical analysis (myosin ATPase) and CSA measurement. In order to obtain morphological histochemical evidence of sprouting to complement the electrophysiological data in extensively denervated TA muscles, a further set of experiments was carried out in a group of rats (n=12). Evulsion of L4 spinal root was performed to extensively denervate the hindlimb flexor muscles (n=8). Equal numbers of rats

were subjected to either normal cage activity (n-4) or natural wheeling exercise (n=4, 8hrs daily). Unoperated rats (n=4) were used as normal control. At four weeks, rats were perfused transcardially with 4% buffered formalin, and fixed TA muscles were removed and prepared for Ag/AChE histochemical staining.

3.3. Results

Throughout this study, no significant difference was found between the contralateral control and normal control for all parameters examined. Thus, the results from both groups were collated and used as the overall control.

3.3.1. MU numbers and reduction by partial denervation

By evulsion of either L4 or L5 spinal roots, we reduced the number of MUs in TA, MG, PL and SOL muscles. Because of the bilateral symmetry of the contribution of each spinal root to the motor innervation of the muscles on the contralateral unoperated side (Buller and Pope, 1977; Gordon et al., 1986), the extent of partial denervation of the muscles was estimated by using the ratio of muscle tetanic force elicited by the stimulation of each spinal root to that elicited by the stimulation of sciatic nerve on the contralateral unoperated control side. Stimulation of L4 and L5 ventral roots evoked more than 80% of the force in TA and MG muscles, respectively. In view of an equal distribution of small and large MUs via motoneurons in each root, the L4 and L5 ventral roots carry the majority

of motor axons to the TA and MG muscles respectively. In comparison, PL and SOL muscles demonstrate similar preferential distribution of motor axons in L4 and L5 ventral roots respectively, but the differences are much more modest. Hence, evulsion of either L4 or L5 spinal roots resulted in more than 80% denervation in TA (average 89±3%) and MG (average 90±2%) muscles, respectively, with little variation (Fig. 3.1A). Partial denervation of PL and SOL muscles, on the other hand, ranged from 4% to 92% (average 45±4%) and 2% to 95% (average 47±4%), respectively. Also shown in figure 3.1 are the differences in the muscles with respect to the number of MU's derived from division of muscle twitch force by mean MU twitch force in each muscle. Muscle twitch force correlates more directly with MU number than mean MU force which is very similar in fast-twitch muscles and smaller in the slow twitch SOL muscle which contains predominantly slow MUs.

3.3.2. MU enlargement by axonal sprouting in extensively denervated muscles

MU twitch force in TA muscle normally varied over a 20-fold range. The distribution of MU twitch forces in TA muscle was skewed to the right because there were many more small MUs than large in uninjured muscles (Fig. 3.2A, open histograms). After partial denervation, the distribution was shifted to the larger force values; but the range and shape of the frequency histograms were

very similar (Fig. 3.2A, filled histograms). The arithmetic mean MU twitch force increased significantly from 22.1±0.9mN for normal TA muscle to 67.4±7.0mN for partially denervated TA muscle. The logarithmic values of MU twitch force were more normally distributed (Fig. 3.2B). This is more clearly demonstrated when the distribution of MU twitch forces was replotted as cumulative frequency histograms (Fig. 3.2C), where a parallel shift in the distribution to the right after partial denervation indicates that all MUs in the population enlarged by the same factor. The geometric mean MU twitch force, after normalization for CSA, increased significantly from 19.1mN for normal TA muscle to 52.5mN for partially denervated TA muscle for a reduction of average MU number from 133±14 to 7±2 (Fig. 3.3).

Similarly, mean MU twitch force in partially denervated MG muscle increased about 3 fold on average with a significant increase in mean MU twitch force from 18.6±0.8mN to 60.2±4.5mN for a reduction of average MU number from 88±1 to 7±2 (Fig. 3.3). All MUs expanded to the same extent as illustrated when the MU twitch forces, corrected for muscle fiber CSA, were plotted as cumulative frequency histograms on semi-logarithmic scale and all MU's increased to the same extent for the parallel rightward shift to the larger force values (Fig. 3.3). This maximum 2 to 3 fold enlargement of MUs did not compensate for the greater than 80% denervation so that whole muscle twitch forces after normalization for CSA were significantly reduced (from 1897±64mN

to 483±130mN for TA muscle and 1744±78mN to 401±71mN for MG muscle) (Table 3.1).

3.3.3. MU enlargement by axonal sprouting in moderately denervated muscles

PL and SOL muscles suffered less severe partial denervation. The mean number of MUs was reduced from 48±5 to 16±2 for PL muscles and 26±1 to 12±2 for SOL muscles (Fig. 3.3). MU twitch force in PL and SOL muscles after moderate denervation increased less than 2-fold and the rightward shift of distribution was much less than for the TA and MG muscles (Fig. 3.3). Nevertheless, the mean of MU twitch force for both partially denervated PL and SOL was statistically higher as compared to normal (from 23.9±1.9mN to 28.0±1.2mN for PL muscle and from 11.4±0.7mN to 16.8±1.7mN for SOL muscle) (Fig. 3.3). Although partial denervation of PL and SOL muscle was not severe, the less than 2-fold increase in MU size did not compensate for the average 50% loss of MUs and the whole muscle twitch forces did not fully recover. The mean whole muscle twitch forces were, therefore, significantly decreased from 747±38mN to 521±69mN for PL muscle and from 256±13mN to 194±22mN for SOL muscle (Table 3.1).

3.3.4. Effect of neural activity on MU enlargement in extensively denervated muscles

Effect of wheeling exercise or FES on axonal sprouting were examined in the extensively denervated TA and MG muscles where numbers of remaining MUs were 4±2 to 8±2 in TA muscles and 11±3 to 19±8 in MG muscles which were not statistically significant (Fig. 3.4). Increased neuromuscular activity, either by wheeling exercise (average 1757±310m/day) or FES (20Hz, 8hrs daily), severely reduced MU enlargement in extensively denervated but not moderately denervated muscles. The cumulative distribution histograms were shifted far to the left of the partially denervated muscles in rats which experienced normal caged activity with a corresponding significant reduction in mean MU twitch force (Fig. 3.4). There was a relative sparing of the fastest and largest MUs for the detrimental effects of neuromuscular activity, consistent with previous reports of preferential enlargement of the largest and fastest MUs by neural activity (Einsiedel and Luff, 1994). More detailed analysis of the cumulative frequency histograms which compare MUs from active and normal activity showed that the detrimental effect was most pronounced for the smaller MUs and less so for the MUs which developed larger forces. As shown in figure 3.4, the neuromuscular activity may even have a small beneficial effect. The possibility that the larger MUs might have been spared because they were less readily recruited and hence not activated during running exercise was discounted. It is because the

same effects were seen with FES which stimulated all the remaining MUs in partially denervated muscles (Fig. 3.4, C and D).

Evulsion of L4 or L5 spinal root resulted in significant reduction in the number of MUs in all experimental groups of partially denervated TA and MG muscles with no significant difference between groups (Fig. 3.4). Because whole muscle force is the product of MU number and MU force, whole muscle twitch force was expected to be significantly reduced as there was significant reduction in MU twitch forces by increased neuromuscular activity. The whole muscle twitch force of partially denervated TA and MG muscles after neural activation, however, was not found to be significantly reduced as compared to normal caged activity with the exception of partially denervated TA after wheeling exercise (Table 3.1). It could be explained by the fact that the larger and more forceful MUs were relatively spared and therefore, the detrimental effect of neuromuscular activity on axonal sprouting was not always detected by measuring the whole muscle twitch or tetanic force. This explanation becomes more convincing as the absence of sparing effect of the larger MUs in partially denervated TA muscle for increased neuromuscular exercise by wheeling exercise resulted in significant decrease in whole muscle twitch force.

Since MU force is the product of IR, CSA and specific force of muscle fiber, and specific force does not change after reinnervation (Tötösy de Zepetnek et al., 1992a; Fu and Gordon, 1995a,b), we normalized MU twitch force values

by CSA in order for MU force to reflect IR. All muscle fiber CSA were measured and corrected for in TA and MG muscles. Therefore, the detrimental effect of increased neuromuscular activity on MU enlargement in extensively denervated TA and MG muscles could not be accounted for by the reduced muscle fiber size. The remaining effect was due to decrease in axonal sprouting

3.3.5. Effect of neural activity on MU enlargement in moderately denervated muscles

For the partially denervated PL and SOL muscles where the extent of partial denervation was moderate and enlargement of the remaining MUs was less than 2 fold (Fig. 3.3), increased neuromuscular activity, either by wheeling exercise (average 1757±310 m/day) or FES (20Hz, 8hrs daily), did not appear to significantly affect MU enlargement. This is clearly demonstrated in the cumulative frequency histograms (Fig. 3.5). The cumulative distribution histograms almost completely overlap that of the partially denervated muscles in rats which experienced normal caged activity. There was no significant difference in the mean MU twitch force between all the experimental groups for both partially denervated PL and SOL muscles (Fig. 3.5). There was no significant difference in the whole muscle twitch force as expected, but with one exception (Table 3.1). The whole muscle twitch force of partially denervated PL muscle with FES was significantly larger than that without FES treatment despite

the absence of significant difference in the mean MU twitch forces. This could be explained by the significantly higher number of MUs in partially denervated PL muscle as compared with normal caged activity. Muscle fiber CSA was not measured in PL and SOL muscles and therefore, the correction of MU and muscle twitch force for muscle fiber CSA was not made. Particularly for the few extensively denervated muscles which were examined histochemically in 100μm longitudinal sections (Fig. 3.9, C-F), atrophic denervated muscle fibers abound and innervated muscle fiber diameters might be smaller, as demonstrated for PL muscle (Pachter and Eberstein, 1992). Without direct measurement of muscle fiber diameter, the extent of MU enlargement is difficult to determine.

3.3.6. Axonal sprouting in partially denervated muscles

Using combined Ag/AChE histochemical staining, three different types of collateral sprouts were examined in this study: 1) intranodal (Fig. 3.6A); 2) preterminal (Fig. 3.6B) and ultraterminal sprout (Fig. 3.6C). There was usually only one type of sprout per axon in moderately denervated muscle, although the incidence of more than 1 type increases dramatically in extensively denervated muscle (Fig. 3.6E). A single axonal outgrowth, for example, originated from the node of Ranvier (Fig. 3.6A, intranodal sprout) or preterminal region (Fig. 3.6B, preterminal sprout). Another example is shown in Fig. 3.6C. A single axonal outgrowth originated from the endplate area (ultraterminal sprout). The different

sprouts observed in combination for the same axon is illustrated in the example in figure 3.6D. An ultraterminal sprout originated from the endplate region of the same axon from whence an intranodal sprout originated. We also observed that many intranodal sprouts originated from a single axon (Fig. 3.6E).

In normal PL muscle, the typical pattern of innervation is shown in figure 3.7A and B. The combined Ag/AChE histochemical staining on 100μm thick cryostat longitudinal sections revealed at high magnification (Fig. 3.7B) that an intramuscular nerve trunk composed of several axons branching down into single axons which innervated single motor endplates.

As compared to normal muscles (Fig. 3.7, A and B), we found that moderately denervated muscles (<75% partial denervation) contained considerable numbers of collateral sprouts (Fig. 3.7, C and D). Nevertheless, there was some free endplates at one month. These collateral sprouts appeared to be mainly intranodal sprouts. The number and size of intramuscular nerve trunks were decreased and the nerve branches became relatively longer consequent to the loss of axons and the extension of collateral sprouts to denervated endplates, respectively. For extensively denervated muscles (>75% partial denervation), the number and size of nerve trunks was dramatically reduced and collateral sprouts were more extensive (Fig. 3.7, E and F). Despite the more extensive axonal sprouting in highly denervated PL muscle, many free endplates were observed (Fig , 3.7G), consistent with incomplete recovery of

muscle force (Table 3.1) indicating that maximal axonal sprouting was insufficient to fully compensate the extensive loss of motoneurons.

3.3.7. Neural activity and axonal sprouting in partially denervated muscles

Increased neuromuscular activity, either by wheeling exercise (average 1757±310 m/day) or FES (20Hz, 8hrs daily) did not appear to affect the extent of axonal branching or sprouting in moderately denervated PL and Sol muscles (Fig. 3.8), consistent with the lack of effects on MU size (Fig. 3.5). We observed that the incidence of collateral sprouts and free endplates were comparable in both exercised muscles and muscles which experienced normal caged activity.

Occasionally, we did obtain extensive denervation in PL and SOL muscles. Histochemical staining revealed the dramatic effect of wheeling exercise and FES in reducing axonal sprouting and reinnervation of denervated endplates in extensively denervated PL muscle (Fig. 3.9). Both motor endplates and muscle fibers were obviously atrophic.

In order to obtain histochemical data to directly complement the electrophysiological evidence in extensive denervated muscles of the inhibitory effect of increased neuromuscular activity on axonal sprouting, we extensively denervated TA muscles by evulsion of L4 spinal roots and subjected the rats to either normal caged activity or wheeling exercise (8hrs daily), and removed and prepared TA muscle for Ag/AChE histochemical staining. Extensively denervated

TA muscle after 4 week-wheeling exercise (1569±278m/day) contained almost no sign of collateral sprouts and very high number of free endplates, as compared to those without exercise (Fig. 3.10). These results were consistent with the data obtained in the few extensively denervated PL and SOL muscles (Fig. 3.9 and 3.11). They demonstrate that the detrimental effect of increased neuromuscular activity is most obvious in extensively denervated muscles irrespective of the muscle. In addition, the effects of increased neuromuscular activity can be demonstrated both at the level of the nerve innervation of endplates and recording of MU forces.

3.3.8. Quantitation of the number of collateral sprouts

The relative proportion of different sprouts was comparable between fast PL and slow SOL muscles after moderate denervation (PD<75%), in contrast to the finding of the previous study of Brown et al. (1980a) in which they reported that the relative proportion of the sprout types was different between fast and slow muscles. Brown and his colleague showed that the fast extensor digitorum longus (EDL) muscle had more intranodal and less preterminal sprouts than the slow SOL muscle after partial denervation. Since 1) there was no significant differences in the relative proportion of different sprout types between PL and SOL muscles and, 2) we obtained similar range of the extent of partial denervation for both partially denervated PL and SOL muscles in each experimental group, we, therefore,

grouped and discussed the results from PL and SOL muscles together in each experimental group.

The number of collateral sprouts in 1 month partially denervated muscles increased as a function of the extent of partial denervation (Fig. 3.11, A and B). However, in view of the limit of axonal sprouting, the number of free endplates also increased as a function of the extent of partial denervation (Fig. 3.11, A and B). There was considerable variability in the number of collateral sprouts between the partially denervated muscles for similar extent of partial denervation, as previously reported in partially denervated SOL muscles (Brown et al., 1980a). For example, 3 muscles, which experienced normal caged activity, suffered a similar degree of partial denervation (40% to 50%), but contained different number of collateral sprouts. Two of them contained 15 or more collateral sprouts per 100 innervated endplates while the last one contained only about 2 per 100 innervated endplates for the same degree of partial denervation (Fig. 3.11A). The variability may arise as a result of: 1) variation in the orientation of muscles during sectioning and/or, 2) intra-animal variability.

The direct relationship between the number of collateral sprouts and free endplates and the percentage of partial denervation was also evident in the moderately denervated muscles which experienced increased neuromuscular activity, either by wheeling exercise (average 1757±310 m/day) or FES (20Hz, 8hrs daily). Increased neuromuscular activity did not appear to affect the number

of collateral sprouts and free endplates in moderately denervated muscles. It was only when partial denervation became extensive that a detrimental effect of increased neuromuscular activity of reducing the axonal sprouting was evident, as reported in extensively denervated TA muscle. While the extent of partial denervation of PL and SOL muscles averaged at about 50%, 6 of 12 partially denervated muscles which experienced wheeling exercise (Fig. 3.11A) and 2 of 8 partially denervated muscles which experienced FES (Fig. 3.11B) suffered extensive denervation (>75%). Of these, there was an abnormal reduction in axonal sprouting as compared to normal caged activity with the exception of 2 extensively denervated muscles which experienced wheeling exercise which had comparable number of collateral sprouts.

Number of collateral sprouts and free endplates in normally innervated and partially denervated muscles, and the effects of exercise and FES are shown in figure 3.12. The few collateral sprouts (~3%) present in normal muscles was also reported in the study of Pachter and Eberstein (1992). They are not, however, the collateral sprouts involved in compensatory sprouting to reinnervate denervated endplates in partially denervated muscles. They are simply axonal branches involved in normal innervation in normal muscles. However, under the examining criteria used in this study, this normal branching cannot be distinguished from the compensatory sprouting. The 10% free

endplates found in the normal muscles (Fig. 3.12B) were simply due to the artifactual effects of muscle sectioning.

The number of collateral sprouts increased about 2 fold after moderate denervation (<75% partial denervation). Nevertheless, these failed to compensate for the loss of motoneurons as shown by the significantly higher number of free endplates. Increased neuromuscular activity either by wheeling exercise or FES did not change this 2-fold increase of axonal sprouting. On the other hand, the number of collateral sprouts dramatically increased to more than 2 fold after extensive denervation (>75% partial denervation), and increased neuromuscular activity either by wheeling exercise or FES significantly reduced the axonal sprouting and, for FES, significantly increased the number of free endplates.

3.3.9. Three different types of collateral sprouts and effect of neural activity

Intranodal sprouts remained the predominant type of sprout in control and all experimental muscles (Fig. 3.13), as reported previously (Pachter and Eberstein, 1992). For moderate denervation, the relative proportion of the sprout types was not affected by the increased neuromuscular activity (Fig. 3.13A). For extensive denervation, on the other hand, intranodal sprouts were reduced by increased neuromuscular activity and FES eliminated preterminal and ultraterminal sprouts (Fig. 3.12B).

3.4. Discussion

There are two major findings in this study. (1) Effect of increased neuromuscular activity is dependent of the degree of partial denervation but is not muscle dependent. (2) Increased neuromuscular activity during the acute phase of axonal sprouting significantly compromises axonal sprouting and MU enlargement in extensively but not moderately denervated muscles.

3.4.1. MU enlargement by axonal sprouting in different muscles after partial denervation

Evulsion of L4 and L5 spinal roots axotomized more than 80% of TA and MG motoneurons and, on average 50% of PL and SOL motoneurons. The 20% or less remaining motoneurons in TA and MG musc-1Xles enlarged their MU size by axonal sprouting and reinnervation of denervated muscle fibers to compensate the loss of motor function. Force in all MUs increased in direct proportion to the extent of partial denervation, indicating that all remaining motoneurons participated in axonal sprouting, with the relative increase in unit size being the same (Fig. 3.3). This is consistent with the previous findings of proportional enlargement of MUs after partial denervation in study using cat MG muscle (Rafuse et al., 1992). It has been suggested that motoneuron size determines the final number of muscle fibers which the motoneuron supplies during reinnervation (Rafuse et al., 1992). In

the present study, the remaining MUs enlarged to a maximum of 3-fold which was not sufficient to fully compensate the loss of motoneurons, resulting in incomplete recovery of whole muscle force and large proportion of denervated endplates (Table 3.1; Fig. 3.11 and 3.12). The extent to which adult MU size can increase, however, remains controversial. The example of increase of MU size up to even 20 times in cats arises from the comparison of MU force of a few MUs with more than 100 fold range in MU force (Luff et al., 1988). Comparisons of representative force distributions of larger MU samples as performed in this study provides a more realistic measure of MU enlargement. Such comparisons made previously indicate maximum sprouting capacities of 3-5 times in mice (Thompson and Jansen, 1977) and rats (Brown and Ironton, 1978), 5-8 times in cats (Rafuse et al., 1992) and in human (Yang et al., 1990). The limit of MU enlargement after partial denervation has been attributed to an inability of remaining MUs to increase their territories by branching from the large proximal trunks (Gordon et al., 1991), and an obstruction of regenerating axons by structural barriers such as the perimysial sheets of connective tissue in the muscle (Kugelberg et al., 1970; Rafuse and Gordon, 1996a). Comparisons of MU twitch forces in normal and partially denervated muscles which experienced normal caged activity were made after correcting for changes in muscle fiber CSAs. This is because meaningful comparisons of MU size in terms of IRs, given that specific force of muscle fiber is a small contributing factor (Tötösy de Zepetnek et al., 1992a; Fu and Gordon,

1995a,b) could be made. However, since we used mean CSA of innervated muscle fibers to make this correction for reduced muscle fiber diameter in partially denervated muscles, the force of the smaller MUs might be underestimated and the larger MUs might be overestimated. This is due to the larger muscle fiber diameters in the fast fatiguable MUs as compared to the smaller muscle fiber diameters in slow, fatigue resistant MUs.

MU enlargement in moderately denervated PL and SOL muscles was much less. There was a trend seen for a shift to more forceful MUs after partial denervation (Fig. 3.3). These results are, nevertheless, consistent with previous studies of partially denervated muscles in not finding a significant increase in MU size until MU number was reduced below 50% of normal (Rafuse et al., 1992). Variability between animals in partial denervation may be a contributing factor, but more importantly, measurement of MU force without correcting for changes in muscle fiber diameters as for TA and MG muscles might have underestimated the MU size and IR (Fig. 3.3). A previous study by Pachter and Eberstein (1992) showed that there was a significant decrease in mean fiber size in partially denervated PL muscle at one month partial denervation. Hence, it is likely that MU force increases in partially denervated PL muscle. If it were corrected for reduced muscle fiber CSA, this would be more consistent with the significant increase in number of collateral sprouts after partial denervation (Fig. 3.12).

There was considerable variability in counting of collateral sprouts and free endplates due to variation in muscle orientation during cryostat sectioning. There was at least 10% loss of endplate innervation even in normal muscles resulting from multiple sectioning even when the sections were relatively thick (100μm; see Fig. 3.12B). The variability of number of collateral sprouts in response to similar degree of partial denervation (Fig. 3.10) was also reported in previous study of Brown et al. (1980).

3.4.2. Detrimental effect of increased neuromuscular activity after extensive denervation but not moderate denervation

Using both electrophysiological and histochemical techniques, we have demonstrated in the present study that increased neuromuscular activity, by either wheeling exercise (average 1757±310 m/day) or FES (20Hz, 8hrs daily), significantly reduced MU size in TA and MG muscles in which more than 80% of innervation was removed by section of L4 and L5 spinal root, respectively. This reduction of MU size by increased neuromuscular activity was also evident in relatively few extensively denervated PL and SOL muscles after L4 or L5 section. Reduction in MU size after increased neuromuscular activity was not evident in PL and SOL muscles in which average denervation was less than 50%. The detrimental effect of increased neuromuscular activity was, therefore, not dependent of muscle types, but dependent of the extent of partial

denervation. Increased neuromuscular activity significantly reduced the number of sprouts and increased the number of denervated endplates in PL and SOL muscle when partial denervation was extensive.

The striking finding of the present study that the detrimental effect of increased neuromuscular activity in reducing MU enlargement was evident only in extensively denervated muscles may account for the contradictory findings in previous studies (Gardiner et al., 1984; Gardiner and Faltus, 1986; Michel and Gardiner, 1989; Seburn and Gardiner, 1996). Many of these studies were done on PL and SOL muscles where on average, the extent of partial denervation is about 50% and might be below the limits of detection of inhibitory effects. The variability between animals in the extent of partial denervation makes it very difficult to detect any definitive effect of activity on mean MU size. particularly in experiments where the extent of partial denervation was not evaluated or quantitated. A 2-fold increase in MU size is difficult to detect electrophysiologically (Rafuse et al., 1992) and morphometrically (Brown et al., 1980). Although a trend was seen for a shift to more forceful MUs in partially denervated PL and SOL muscles (Fig. 3.3), many MUs had to be sampled to even detect these trends. This would be even more difficult to detect by average values where were used in most of those previous studies. Large sample of MUs is especially important when all MUs in partially denervated muscles are not affected similarly as described above. As seen in figure 3.4 and 3.5, it was evident in all

four types of partially denervated muscles that increased neuromuscular activity was more detrimental to the smaller and slower MUs, and there was an apparent sparing of the larger MUs. It is important to take into account the effect of denervation/reinnervation and neuromuscular activity on muscle fiber size which was ignored in many of those previous studies. In fact, by using the mean CSA, forces of the smaller MUs might be underestimated and the forces of the larger MUs might be overestimated, and the effects of increased neuromuscular activity might be even more pronounced. MU force is the product of innervation ratio, CSA and specific force of muscle fiber. MU force depends on both IR and CSA, and reflects innervation ratio and therefore axonal sprouting reasonably only if muscle fiber CSA is taken into account. Since specific force does not change after reinnervation (Tötösy de Zepetnek et al., 1992a; Fu and Gordon, 1995a,b), MU force corrected for muscle fiber CSA, therefore provides reasonable average measurement of MU enlargement, given that the relative underestimate of IRs of smaller MUs and overestimate of IRs of larger MUs are take into account.

3.4.3. Effects of increased neuromuscular activity are MU type or size dependent

Examination of the cumulative force distributions in Fig. 3.3 demonstrate that effects of increased neuromuscular activity did not equally affect different MUs. The smaller MUs were more detrimentally affected and the larger MUs were

relatively spared from the detrimental effect, evident in all four types of partially denervated muscles. In contrast to our finding that larger MUs were being spared from the detrimental effect of increased neuromuscular activity, there is evidence to support the notion that activity may promote MU enlargement in the larger MUs which are normally the fast-fatiguable and fast-intermediate units (Einsiedel and Luff, 1994). Einsiedel and Luff (1994), in their study of the effect of two-week treadmill walking on axonal sprouting in partially denervated MG muscle, suggested that there was a preferential effect of treadmill running exercise in promoting MU enlargement of fast-fatiguable and fast-intermediate units on the basis of comparisons of mean MU forces in different MU types which were distinguished by fatiguibility test. However, since activity alters MU type to more fatigue resistant MUs (for review, see Pette and Vrbová, 1992), analysis in terms of MU size, as in the present study, rather than type allows differences to be detected more readily.

One may argue that the larger MUs in partially denervated muscles might not be recruited during wheeling exercise and therefore that they were spared for the detrimental effect of activity. This argument may seem likely, especially when one considers the study of Walmsley et al. (1978) in which they have demonstrated that treadmill locomotion requires only 10-20% of the maximal force output of the MU pool in the cat MG muscle and that this relatively low force output is normally provided by slow and fatigue resistant units. In the present study,

however, denervation in TA and MG muscles was very extensive. Less than 20% of MUs remained in these muscles after extensive denervation and total muscle force of the extensively denervated muscles was only a fraction of normal muscles (Table 3.1). Most importantly, the sparing effect of larger MUs was also evident in all denervated muscles with FES which recruits all MUs. Low-threshold slow and fatigue resistant units are involved in postural maintenance and locomotion (Burke, 1981). In the present study, smaller MUs might very well be maximally active in the partially denervated muscles even during normal level activity (normal caged activity). Further involvement of these units in the increased level activity (wheeling exercise or FES) might have overloaded the sprouting units and therefore resulted in reduction of MU enlargement.

3.4.4. Effect of increased neuromuscular activity and different types of collateral sprouts

The possibility that activity differentially affects one type of collateral sprout and not another was excluded by examining different sprout types in both fast PL and slow SOL muscle. The relative proportion of the sprout types has been reported to be different between fast and slow muscles (Brown et al., 1978c, 1980a). Fast EDL muscle was shown to have more intranodal and less preterminal sprouts than the slow SOL muscle after partial denervation. The reason suggested was the possibility that fast muscles were less susceptible to changes associated

with muscle inactivity and nerve degeneration which have been suggested to be the two major sprouting stimuli for preterminal sprout (Brown et al., 1978c; for review, see Brown et al., 1981). However, we, in the present study, did not find significant differences in the proportion of different sprout types between fast PL and slow SOL muscles. The dominant form of collateral sprout was the intranodal and the least prevalent was the ultraterminal sprout in all muscles. Until more studies are done, the issue whether there is a muscle dependent effect on the types of collateral sprout occurred remains unclear.

3.4.5. Proposed mechanisms for the detrimental effect of increased neuromuscular activity on MU enlargement and axonal sprouting

Completion of sprout formation requires outgrowth of collateral sprouts from the intact axons, followed by successful synapse formation between the nerve terminal and the target muscle fiber. Reduction of MU enlargement in partially denervated muscles by activity could result from 3 possibilities: 1) withdrawal of terminals, 2) failure for synaptic contacts to mature, and 3) failure of axonal outgrowth.

3.4.5.1. Withdrawal of terminals

Withdrawal of terminals may be accounted for by a mechanism similar to one of the proposed mechanisms underlying synapse elimination in developing

skeletal muscles, suggested by Vrbová and her colleagues (Connold et al., 1986). Activity by sciatic stimulation at 8Hz promotes synapse elimination in neonatal SOL muscle while calcium reduction and protease inhibitors inhibit it (O'Brien et al., 1978, 1984; Connold et al., 1986). It was suggested that active muscles, in response to ACh, release calcium-activated protease which digests the membrane of the terminals. When a terminal is digested faster than replaced, it would be removed, resulting in withdrawal of that terminal. This is supported by the finding that indirect stimulation *in vivo* (stimulation of sciatic nerve) caused a remarkable increase in proteolytic enzyme activity at the neuromuscular junctions of adult rats (Poberai et al., 1972; Poberai and Sávay, 1976).

Our finding that increased neuromuscular activity either by wheeling exercise or FES was detrimental to MU enlargement in extensively denervated muscles conflicts with the finding that active nerve terminals had competitive advantage over the ones which were inactivated by tetradotoxin (TTX), for making synaptic connection with denervated muscle fibers in lumbrical muscles in rat foot (Ribchester and Taxt, 1983,1984; Ribchester, 1988). In those studies, however, no consideration was given to another possible alternative interpretation that TTX blockade might impair the membrane replacement process of inactive nerve terminals and thereby deprive them from an equal chance for competition with the active nerve terminals since TTX has been

shown to block transport of axonal proteins (Edwards and Grafstein, 1983; Antonian et al., 1987). This postulation, however, has yet to be confirmed.

3.4.5.2. Failure for synaptic contacts to mature

The second possibility to explain the detrimental effect of increased neuromuscular activity on axonal sprouting and MU enlargement is that chronic stimulation may exhaust quantal content of newly formed synaptic terminals. This is suggested by several findings. Gutmann and Jakoubek (1963) showed that fiber size of regenerating axons was significantly reduced and synapse maturation was retarded after nerve crush by increasing motor activity via a daily swimming regime of approximately 3-4 hours for 35 days. Soucy and his colleagues (1996) recently demonstrated that increased activity by 30-day treadmill running impaired reinnervation in LG muscles as indicated by the exacerbated "tetanic fade" phenomenon. This phenomenon has been explained as a gradual decrease in quantal content of neurotransmitters and indicates the failure of synapses to mature. After synapse is formed, the initial small subthreshold endplate potential matures into a potential sufficiently large to evoke a contractile response of nerve-muscle contact within 36 hrs and a further few days are required before the terminal can tolerate repeated stimulation (Camignoto et al., 1983). Therefore, the increased neuromuscular activity in the present study might impair the maturation process of newly formed synapses by exhausting the quantal content of terminals more rapidly because quantal

release was dramatically increased by chronic nerve stimulation (Pockett and Pygott, 1982).

3.4.5.3. Failure of axonal outgrowth

Another possible mechanism which could account for the detrimental effect of increased neuromuscular activity in reducing MU enlargement is blockade of axonal outgrowth from intact axons. Since partially denervated muscles were subjected to activity immediately after denervation surgery, activity might prevent collateral sprouts from growing out and/or continuing to grow before they could even reach the target muscle fibers.

A number of immunohistochemical studies have verified the original idea that collateral sprouts might be acompanied by Schwann cell processes (Duchen, 1971). Studies on partially denervated SOL muscle demonstrate that terminal Schwann cells induce and guide collateral sprouts by forming extended processes at both the innervated endplates and the denervated endplates (Reynolds and Woolf, 1992; Son and Thompson, 1995a,b; Son et al., 1996; Thompson and Kopp, 1996). In view of this close association of Schwann cells and axonal sprouting, candidates which either promote or inhibit axonal sprouting might be expected to perturb this association. The detrimental effects of increased neuromuscular activity in reducing axonal sprouting may arise indirectly from effects on the terminal Schwann cells. Striking evidence supporting this view comes from the recent studies of Love and his colleagues (Love et al., 1997) and our own

laboratory (Tam et al., 1998). Love and his colleagues (1997) found that direct muscle stimulation prohibited bridge formation of terminal Schwann cell processes and thereby reduced axonal sprouting in SOL muscle 7 days after partial denervation. From our own laboratory, studies of extensively denervated TA muscle 4 weeks after evulsion of L4 spinal root using triple immunofluorescence also showed that the increased neuromuscular activity associated with running on exercise wheel significantly reduced the number of collateral sprouts and Schwann cell bridge formation at early time points over the period of 4 weeks (Tam et al., 1998). Interestingly, we found that wheeling exercise actually increased the length of processes but complicated the orientation of these navigating processes. We suggested that this complication might account for the reduction in bridge formation. Another possibility is suggested by the findings that Schwann cells respond to neuromuscular activity by electrical or receptor-mediated elevations of intracellular calcium level (Jahromi et al., 1992; Reist and Smith, 1992; Reynolds and Woolf, 1993; Lev-Ram and Ellisman, 1995). The responses of terminal Schwann cells to acetylcholine released from nerve terminals may be essential for their normal function but high level of neuromuscular activity in partially denervated muscles may cause excessive Schwann cell activation which in turn may perturb the ability of Schwann cells to form processes and guide sprouting axons to denervated endplates.

In addition to the role of providing physical pathway for sprout growth, a role of Schwann cell for producing sprout-inducing substances was also suggested. Hoffman (1950) first sugested that collateral sprouts were guided to denervated endplates by some "elements" supplied by the denervated Schwann cells in the vacant nerve sheaths in partial denervated muscles. Since then, considerable amount of evidence supporting this view has become available from subsequent studies on the role of non-neural cells of the distal nerve stumps and terminal Schwann cells in producing sprout-inducing factors (David and Aguayo, 1985; Diaz and Peot-Dechassine, 1990; also see review Kuffler, 1987, 1994). Ciliary neurotrophic factor (Stöckli et al., 1989; Dobrea et al., 1992; Gurney et al., 1992; Sendtner et al., 1992; Smith et al., 1993; Kown and Gurney, 1994; Lee et al., 1995; Siegel and English, 1997) and growth factors analogous to nerve growth factor (Slack et al., 1983; Heumann et al., 1987) have been suggested to be prominent candidates of these sprout-inducing factors. Neuromuscular activity might somehow interfere with the ability of intact axons to respond to the sprouting stimuli.

Another source of sprouting stimuli is denervated or otherwise inactive muscle fibers (Brown et al., 1978a,b, 1980, 1981; Slack and Pockett, 1981; Pockett and Slack, 1982; Keynes, et al., 1983; Gurney, 1984; Gurney et al., 1986; Kuffler, 1989; Rassendren et al., 1992; Kuffler and Luethi, 1993). Suggested sprouting factors released from inactive muscle fibers include insulin-like growth

factors (Caroni and Grandes, 1990; Near et al., 1992; Caroni et al., 1994; Glazner et al., 1994,1995; Marsh et al., 1994; Pu et al., 1995; Thompson and Kopp, 1996), neural cell adhesion molecules (Gurney et al., 1986) and neurocrescin (Nishimune et al., 1997). Since restoration of muscle activity by direct muscle stimulation inhibits axonal sprouting in paralyzed (Brown et al., 1977, 1980) and partially denervated (Brown and Ironton, 1977; Brown and Holland, 1979) muscles, increased neuromuscular activity might reduce and/or inhibit production of sprout-inducing factors from inactive muscle fibers and thereby reduce axonal sprouting.

Adequate intracellular calcium concentration has been repeatedly shown, in *in-vitro* and *in-vivo* studies, to be critical for nerve outgrowth (Cohan et al., 1987; Mattson and Kater, 1987; Mattson et al., 1988; Kater et al., 1988, 1989; Connor et al., 1990; Collins et al., 1991; Kater and Mills, 1991; Rehder and Kater, 1992). Either excessive intracellular calcium level induced by electrical stimulation and calcium ionphores, and reduced intracellular calcium level caused by calcium channel blockers result in cessation of nerve outgrowth. Therefore, increased neuromuscular activity in the present study might overload sprout terminals with calcium, resulting in reduction of axonal sprouting.

The aforementioned mechanisms are mostly postulated and yet to be proven. Nevertheless, the consistent findings that the size relationships between motor axons, IR and MU force are restored after reinnervation argue strongly that the largest MUs are the least active: the orderly recruitment of MUs

according to size recruits the small slow MUs before the larger and faster MUs (Tötösy de Zepetnek et al., 1992b). Thus neuromuscular activity during reinnervation which is dictated by the size principle of recruitment appears to restore the normal size relationship between motoneurons and the number of muscle fibers that they reinnervate (Milner-Brown et al., 1973). This same size relationships are also restored after partial denervation which argues that the net increase in number of nerve terminals per MU is much greater in the large and infrequently recruited MUs than in the smaller and frequently recruited slow and fatigue resistable MUs (Rafuse et al. 1992). Thus the size of MUs increases as an inverse function of the amount of neuromuscular activity. The mechanism by which activity restricts MU size may simply be the same mechanism which operates during development to restrict the size of MUs according to activity.

3.5. Conclusion

Using 4 functionally different muscles and both electrophysiological and histochemical techniques, we, in the present studies, have resolved the controversial findings of previous studies on the effect of activity on axonal sprouting and been able to generalize the effect of increased neuromuscular activity on MU enlargement and axonal sprouting. The detrimental effect of increased neuromuscular activity in reducing MU enlargement and axonal sprouting depends critically on the extent of partial denervation of muscles.

Increased neuromuscular activity significantly reduced MU enlargement and axonal sprouting in extensively denervated muscles where only less than 20% of functional MUs remained.

We have demonstrated that normal physiological activity of sprouting motoneurons is conducive for MU enlargement during the acute phase of axonal sprouting while non-physiological activity can be detrimental. The findings of the present studies indicate that increased neuromuscular activity is not advised as rehabilitation immediately after motoneuron injury or in the early stages of motoneuron disease.

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Figure 3.1

Mean (±S.E.) percentage of ventral root L4 (open histograms) or L5 (filled histograms) innervation (A), mean muscle twitch forces (B), motor unit (MU) numbers (C) and MU twitch forces (D) of fast, flexor tibialis anterior (TA), extensor medial gastrocnemius (MG), plantaris (PL), and slow, extensor soleus (SOL) muscles. Evulsion of either L4 or L5 spinal roots resulted in more than 80% denervation in TA (average 89±3%) and MG (average 90±2%) muscles, respectively; but resulted in less extensive denervation in PL (average 45±4%) and Sol (average 47±4%) muscles. Muscle force varies more directly with mean MU number than mean MU force.

A
Ventral root innervation (%)
100
80
60
40
20
0
L4
L5
TA
MG
PL
SOL
B
Muscle twitch force (mN)
2000
1500
1000
500
0
TA
MG
PL
SOL
C
Motor unit number
160
140
120
100
80
60
40
20
0
TA
MG
PL
SOL
D
Motor unit twitch force (mN)
25
20
15
10
5
0
TA
MG
PL
SOL

Figure 3.2

Frequency and cumulative frequency histograms of MU twitch force distributions in control (A and B: open histograms; C: open squares) and partially denervated (PD) TA muscles (A and B: filled histograms; C: open circles). A) The arithmetic mean MU twitch force (vertical line) increased from 22.1±0.9mN for normal TA muscle to 67.4±7.0mN for partially denervated TA muscle. The logarithmic values of MU twitch force were more normally distributed. B) Geometric mean MU twitch force (vertical line), after normalized for muscle fiber cross sectional area (CSA), increased significantly from 19.1mN for normal TA muscle to 52.5mN for partially denervated TA muscle ($p<0.0001$). C) A parallel shift of cumulative frequency histograms on semi-logarithmic scales to the right after partial denervation indicated that all MUs in the population enlarged by the same factor.

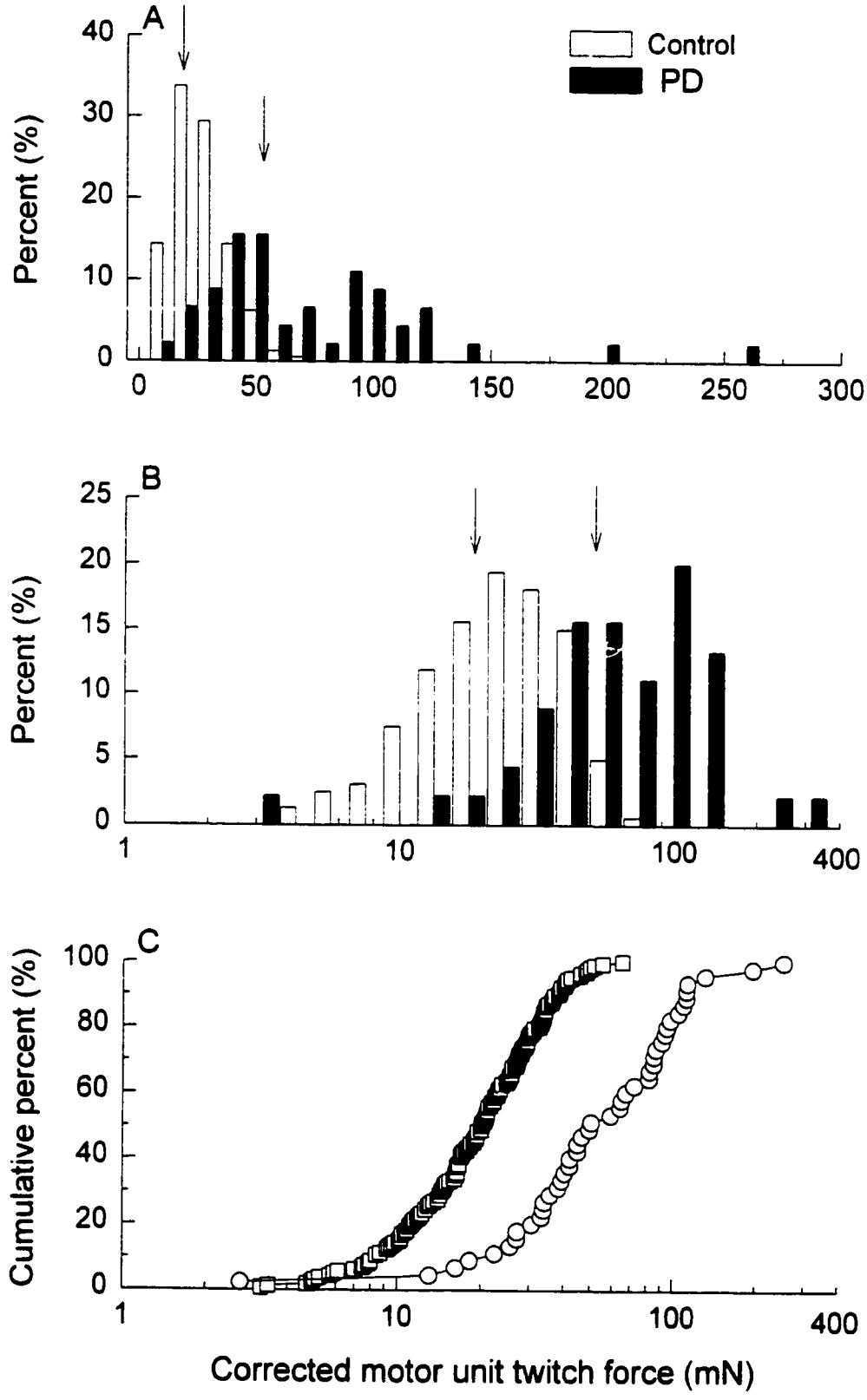

A
Control
PD
Percent (%)
40
30
20
10
0
0
50
100
150
200
250
300
B
Percent (%)
25
20
15
10
5
0
1
10
100
400
C
Cumulative percent (%)
100
80
60
40
20
0
1
10
100
400
Corrected motor unit twitch force (mN)

Figure 3.3

Cumulative frequency histograms of MU twitch force distributions in partially denervated TA (A), MG (B), PL (C) and SOL (D) muscles of rats experiencing normal caged activity (open circles) as compared to control muscles (open squares). In TA and MG muscles where partial denervation was greater than 80%, MU twitch forces, after normalized for muscle fiber CSA, were significantly larger than control as shown by the significant rightward shift of the MU twitch force distributions ($p<0.05$) with a significant increase in mean MU twitch forces (from 22.1±0.9mN to 67.4±7.0mN for TA; from 18.6±0.8mN to 60.2±4.5mN for MG; $p<0.0001$). The shift in the MU twitch force distributions for PL and SOL muscles was much less for moderate PD (PD<80%) with nevertheless a significant increase in mean MU twitch forces (from 23.9±1.9mN to 28.0±1.2mN for PL, $p<0.05$; from 11.4±0.7mN to 16.8±0.7mN for SOL, $p<0.0001$).

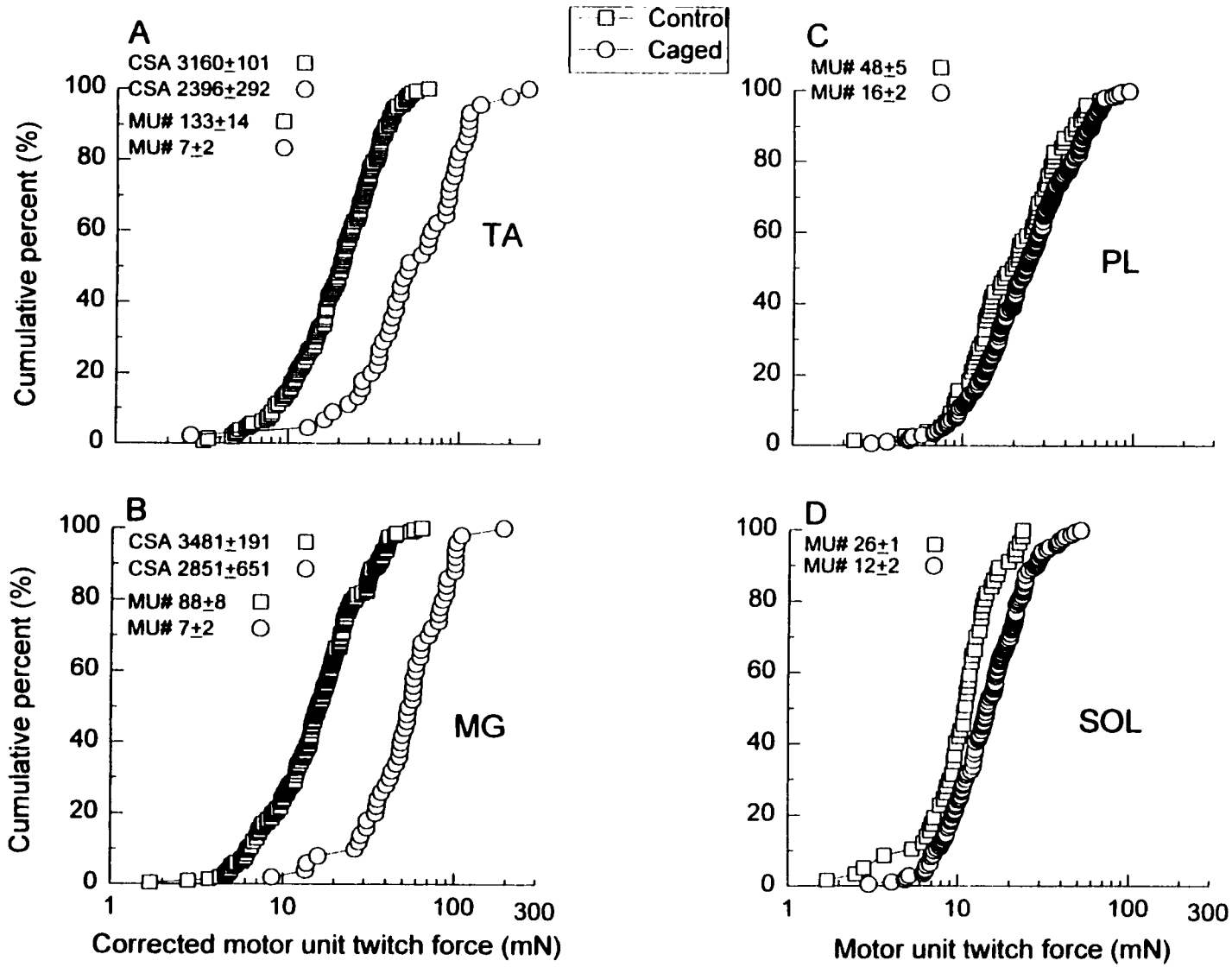

Control
Caged
A
CSA 3160±101
CSA 2396±292
MU# 133±14
MU# 7±2
TA
C
MU# 48±5
MU# 16±2
PL
B
CSA 3481±191
CSA 2851±651
MU# 88±8
MU# 7±2
MG
D
MU# 26±1
MU# 12±2
SOL
Cumulative percent (%)
Corrected motor unit twitch force (mN)
Motor unit twitch force (mN)
0
20
40
60
80
100
1
10
100
300

Figure 3.4

Cumulative frequency histograms of MU twitch force distributions after normalization for CSAs in partially denervated TA (A and C) and MG (B and D) muscles of rats experiencing normal caged activity (open circles) as compared to partially denervated muscles after running exercise (blue circles), and partially denervated muscles with functional electrical stimulation (FES, red circles). The dramatic effect of increased neuromuscular activity in reducing MU enlargement was seen as a shift in cumulative MU force distributions to the left of the extensively denervated TA and MG muscles in rats which experienced normal caged activity ($p<0.05$) with a significant decrease in mean MU twitch forces. Mean MU twitch forces were siginificantly reduced from 67.4±7mN to 26.8±4.6mN ($p<0.0001$) after exercise and to 50.3±12.5mN ($p<0.05$) FES in TA muscles, and from 60.2±4.5mN to 42.3±5.5mN ($p<0.0001$) after exercise and 49.6±6.3mN ($p<0.01$) after FES in MG muscles.

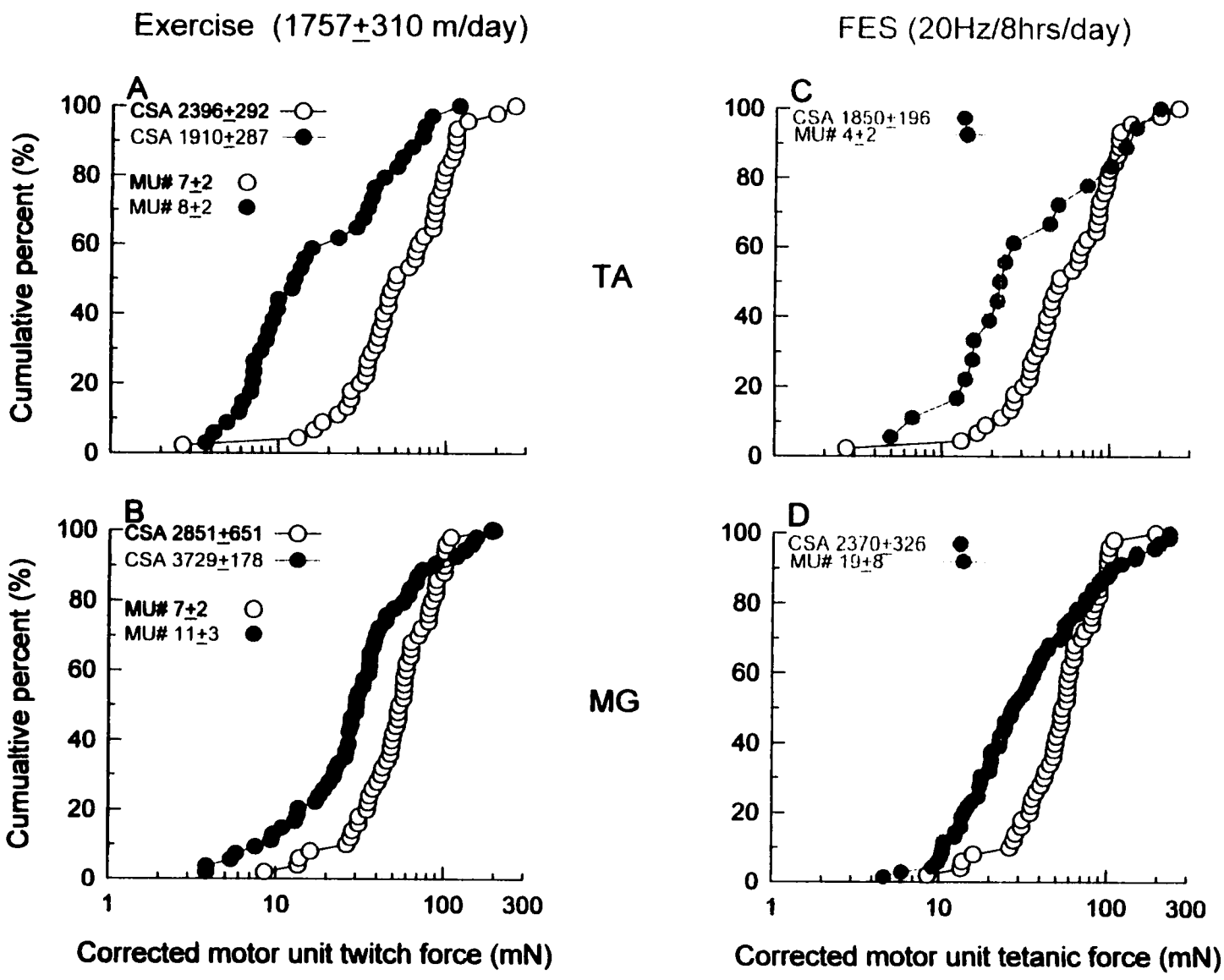

Exercise (1757±310 m/day)
FES (20Hz/8hrs/day)
A
CSA 2396±292
CSA 1910±287
MU# 7±2
MU# 8±2
C
CSA 1850±196
MU# 4±2
TA
B
CSA 2851±651
CSA 3729±178
MU# 7±2
MU# 11±3
D
CSA 2370±326
MU# 19±8
MG
Cumulative percent (%)
Cumulative percent (%)
0
20
40
60
80
100
1
10
100
300
Corrected motor unit twitch force (mN)
Corrected motor unit tetanic force (mN)

Figure 3.5

Cumulative frequency histograms of MU twitch force distributions in partially denervated PL (A and C) and SOL (B and D) muscles of rats experiencing normal caged activity (open circles) as compared to partially denervated muscles after running exercise (blue circles), and partially denervated muscles with FES (red circles). Increased neuromuscular activity did not significantly change the cumulative MU force distributions and the mean MU twitch forces in these moderately denervated muscles. Mean MU forces after exercise and FES were 33.4±22mN and 31.3±1.4mN respectively in PL muscle and were 19.8±1.7mN and 16.2±1.0mN respectively in SOL muscle.

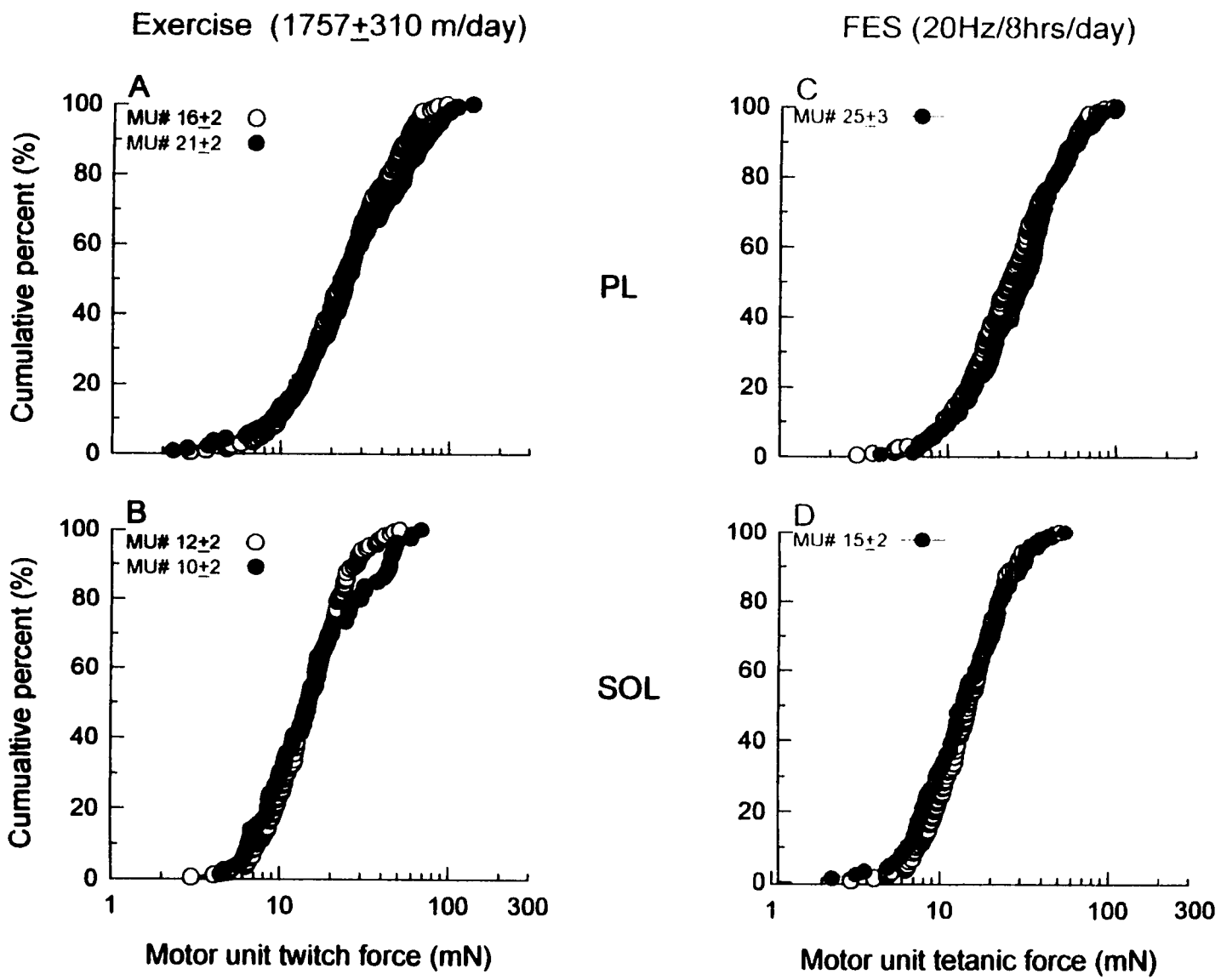
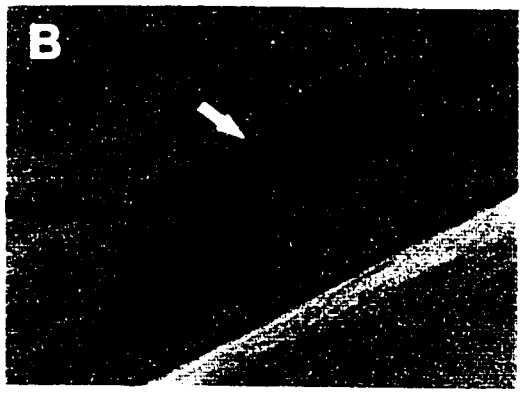
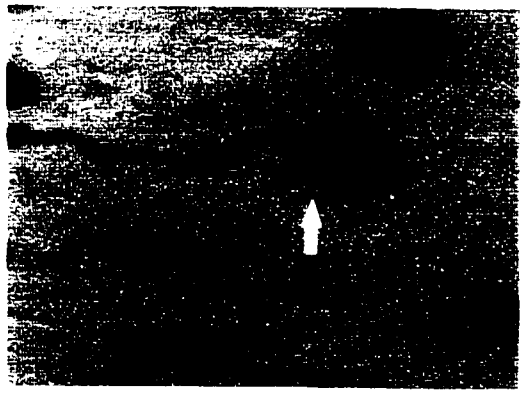

Exercise (1757±310 m/day)
FES (20Hz/8hrs/day)
A
MU# 16±2
MU# 21±2
B
MU# 12±2
MU# 10±2
C
MU# 25±3
D
MU# 15±2
PL
SOL
Cumulative percent (%)
Cumulative percent (%)
100
80
60
40
20
0
1
10
100
300
Motor unit twitch force (mN)
Motor unit tetanic force (mN)

Figure 3.6

Three different types of collateral sprouts: intranodal (A), preterminal (B) and ultraterminal sprouts (C), visualized with the combined silver/acetylcholinesterase (Ag/AChE) histochemical staining. In some cases, different collateral sprouts occurred in combination for the same axon (D and E). Arrows indicate collateral sprouts.


B



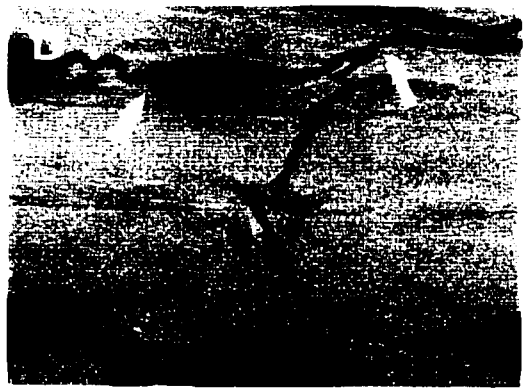


Figure 3.7

Low (A, C and E) and higher (B, D and F) power combined Ag/AChE histochemical photomicrographs of 100μm thick cryostat longitudinal sections of control (A and B), moderately denervated (C and D), and extensively denervated (E and F) PL muscles. In normal PL muscle, single endplates were innervated by single axons. After moderate (PD<75%) and extensive (PD>75%) denervation, denervated endplates were reinnervated by collateral sprouts (filled arrows). For PD>75%, many free endplates (open arrows) were seen.

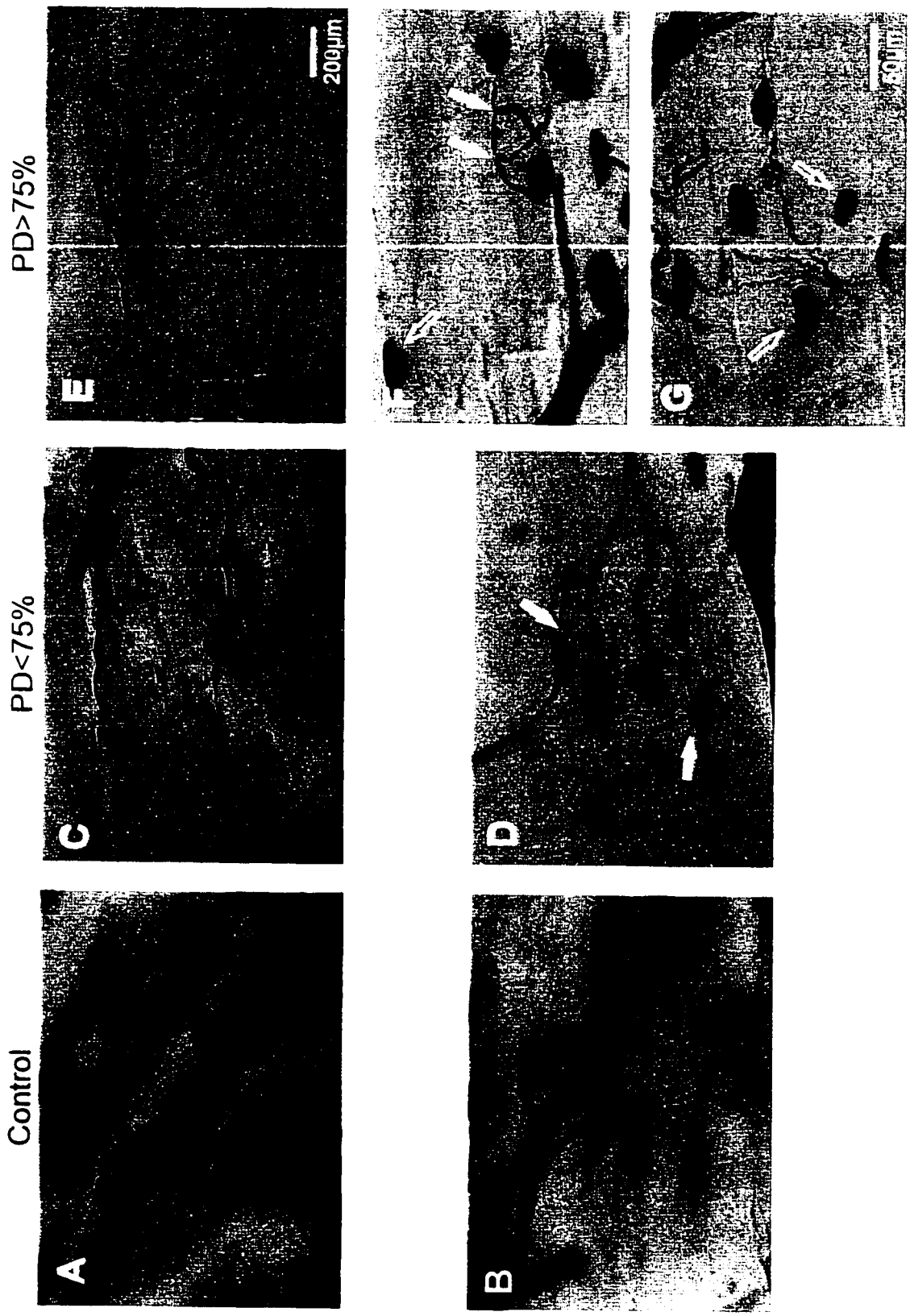

Control
PD<75%
PD>75%
A
B
C
D
E
F
G
200µm
200µm

Figure 3.8

Low (A, C and E) and higher (B, D and F) power combined Ag/AChE histochemical photomicrographs of 100μm thick cryostat longitudinal sections of moderately denervated PL muscles (PD<75%) after normal caged activity (A and B), wheeling exercise (C and D), and FES (E and F). There was no visual differences in branching and axonal sprouting all the experimental groups. Arrows indicate collateral sprouts.

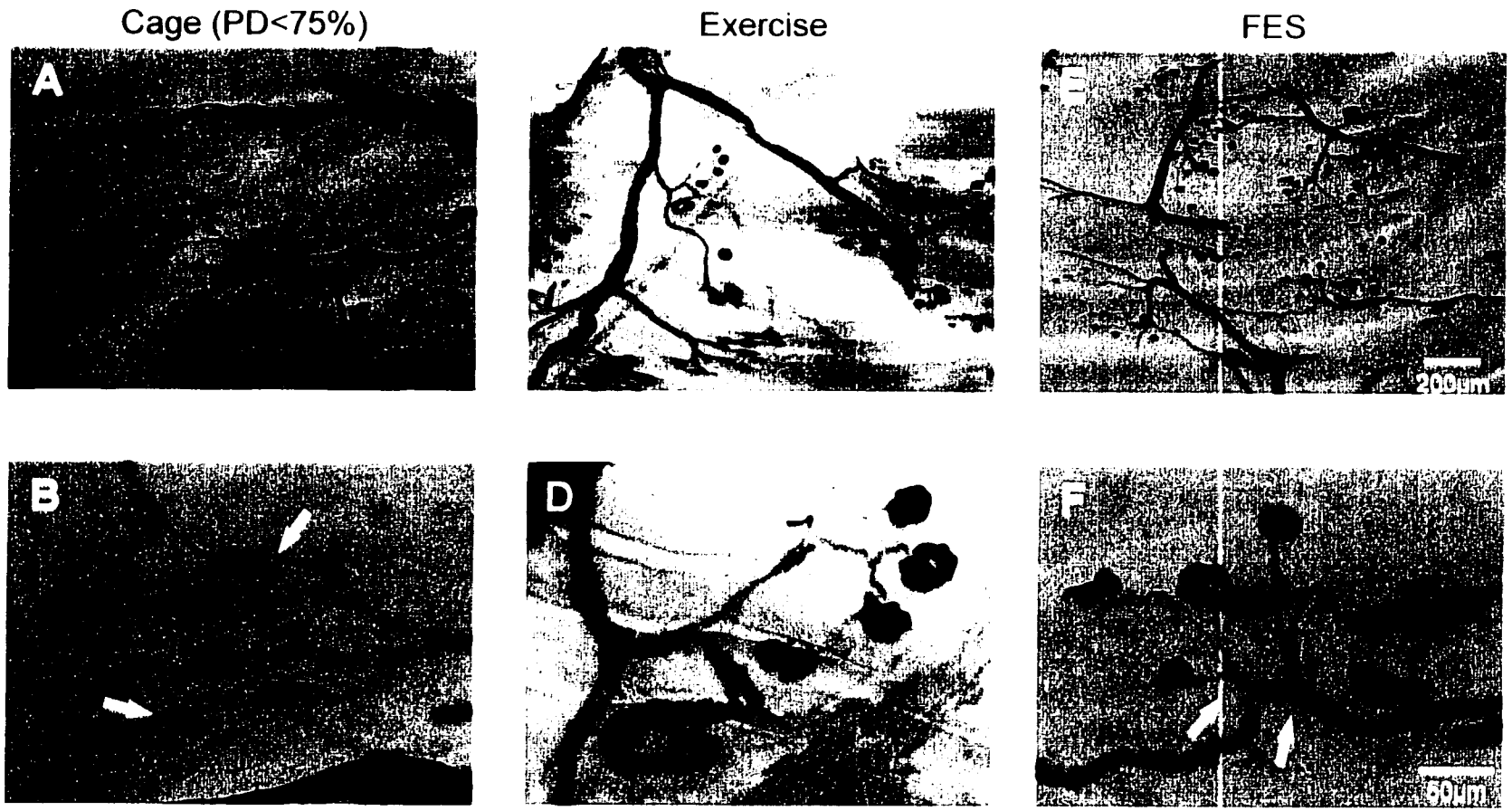

Cage (PD<75%)
Exercise
FES
A
B
D
F
200um
50um

Figure 3.9

Low (A, C and E) and higher (B, D and F) power combined Ag/AChE histochemical photomicrographs of 100μm thick cryostat longitudinal sections of extensively denervated PL muscles (PD>75%) after normal caged activity (A and B), wheeling exercise (C and D), and FES (E and F). Increased neuromuscular activity (wheeling exercise or FES) dramatically reduced amount of collateral sprouts and further increased amount of free endplates. Both motor endplates and muscle fibers became atrophic. Filled arrows indicate collateral sprouts, and open arrows indicate free endplates.

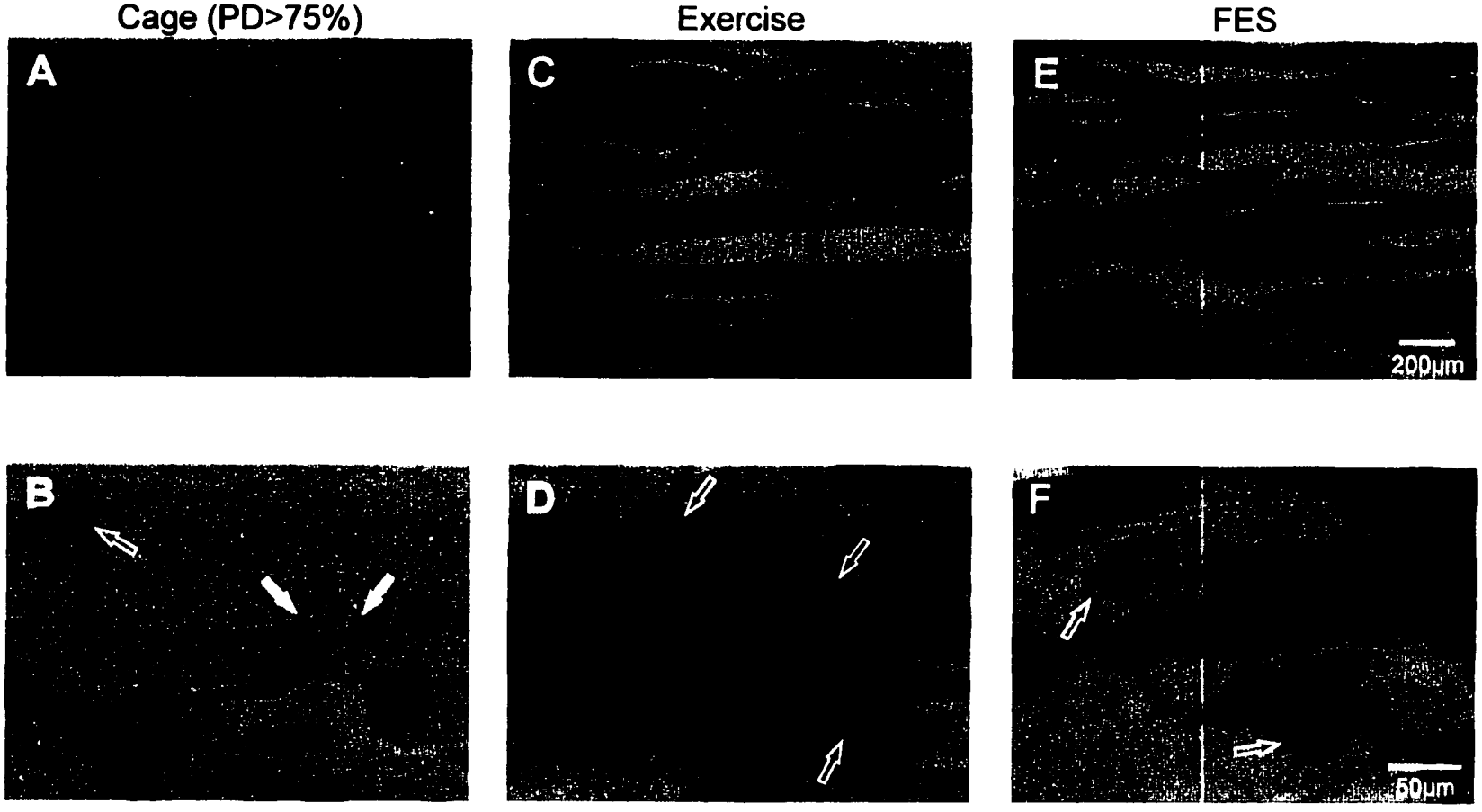

Cage (PD>75%)
Exercise
FES
A
C
E
200µm
B
D
F
50µm

Figure. 3.10

Low (A and C) and higher (B and D) power combined Ag/AChE histochemical photomicrographs of 100μm thick cryostat longitudinal sections of extensively denervated TA muscles with normal caged activity (A and B), and after wheeling exercise (C and D). Extensively denervated TA muscle after wheeling exercise contained almost no sign of collateral sprouts but contained very high number of free endplates, as compared to normal caged activity, and contained many vacant nerve sheaths (asterisks). Filled arrow indicates collateral sprout, and open arrows indicate free endplates.

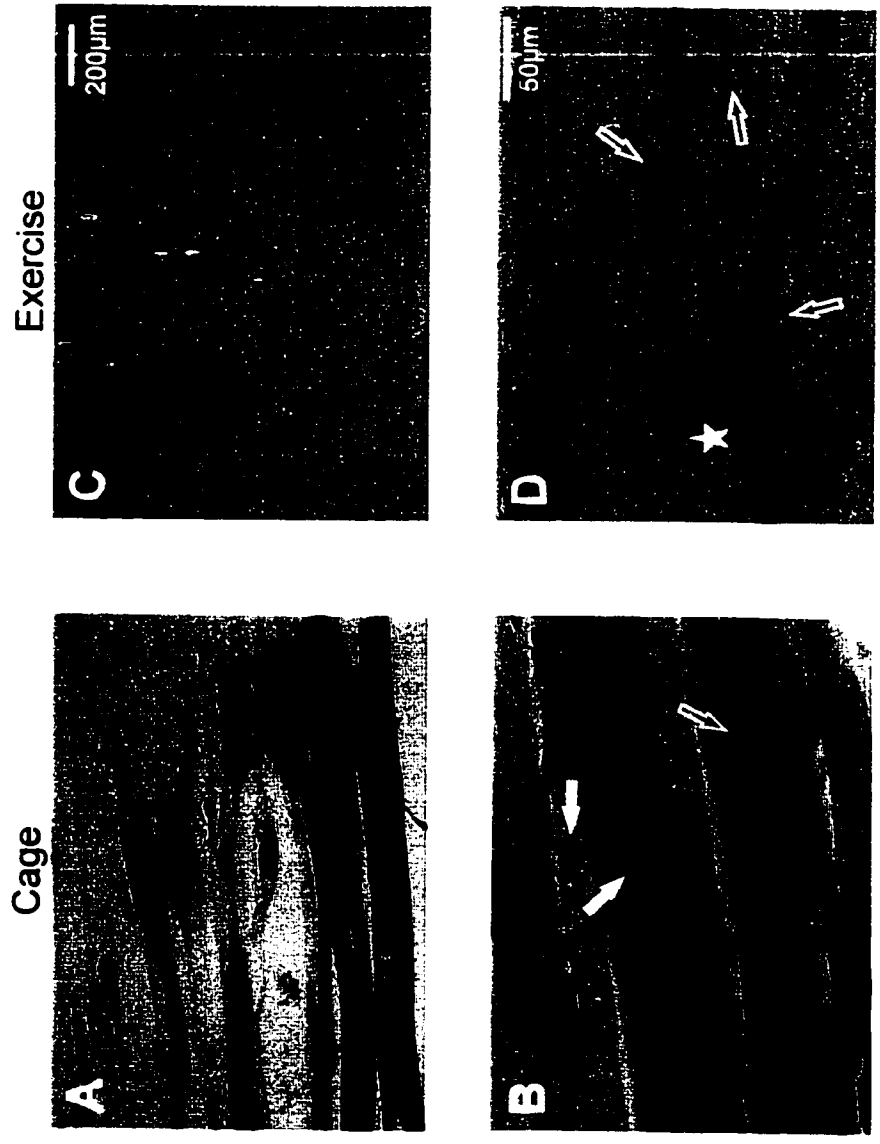

Cage
Exercise
A
B
C
D
200µm
50µm

Figure 3.11

Number of collateral sprouts per 100 innervated endplates (A and B), and number of free endplates per 100 endplates (C and D), of partially denervated PL and Sol muscles after normal caged activity (open circles), wheeling exercise (blue circles) and FES (red circles) plotted as a function of percentage of PD. For PD>75%, increased neuromuscular activity dramatically reduced the number of collateral sprouts with 2 exceptions (A), resulting in dramatic increase in number of denervated endplates, and in contrast, had no effect for PD<75%.

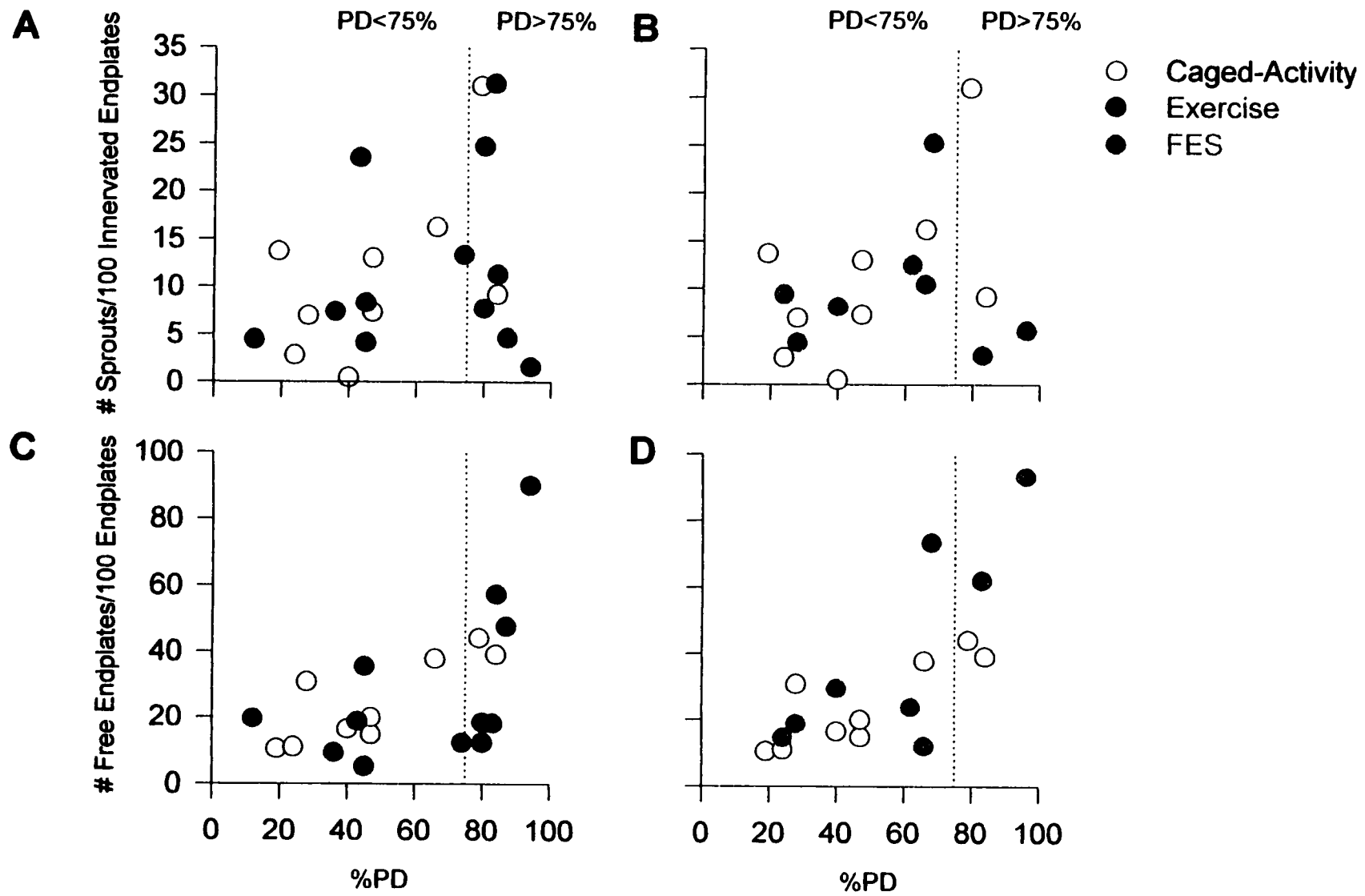

A
B
C
D
PD<75%
PD>75%
Sprouts/100 Innervated Endplates
Free Endplates/100 Endplates
%PD
Caged-Activity
Exercise
FES
0
5
10
15
20
25
30
35
40
60
80
100
0
20
40
60
80
100

Figure 3.12

Mean (±S.E.) of number of collateral sprouts per 100 innervated endplates (A), and 100 free endplates per endplates (B) of normal (open bars), moderately denervated (PD<75%; filled bars), and extensively denervated (PD>75%; hatched bars) PL and SOL muscles after normal caged activity (black), wheeling exercise (blue), and FES (red). For PD<75%, both the number of collateral sprouts and free endplates was significantly increased as compared to control (*$p<0.0001$). Wheeling exercise or FES did not have significant effect on axonal sprouting. For PD>75%, number of collateral sprouts and thus number of free endplates after caged activity were dramatically increased as compared to PD<75%. Increased neuromuscular activity reduced the number of sprouts and increased the number of free endplates.

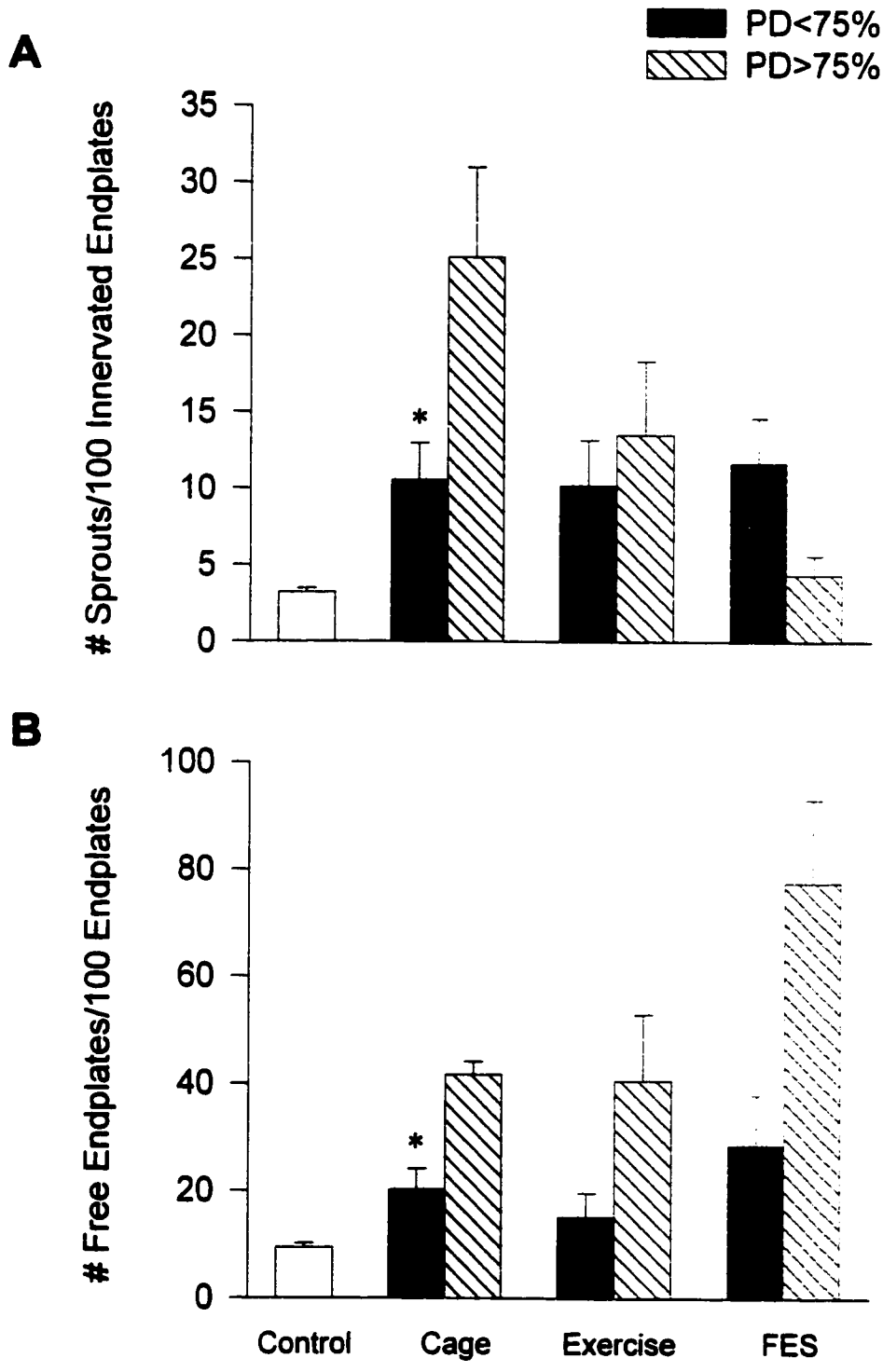

A
PD<75%
PD>75%
Sprouts/100 Innervated Endplates
35
30
25
20
15
10
5
0
*
B
Free Endplates/100 Endplates
100
80
60
40
20
0
*
Control
Cage
Exercise
FES

Figure 3.13

Mean (±S.E.) of number of intranodal, preterminal, and ultraterminal sprouts per number of innervated endplates in normal (open bars), moderately denervated (PD<75%; A), and extensively denervated (PD>75%; B) PL and SOL muscles after normal caged activity (black bars), wheeling exercise (blue bars), and FES (red bars). For PD<75%, the proportion of the sprout types was not affected by the increased neuromuscular activity. For PD>75%, all sprout types were reduced by increased neuromuscular activity. FES appeared having the most detrimental effect on preterminal and ultraterminal sprouts.

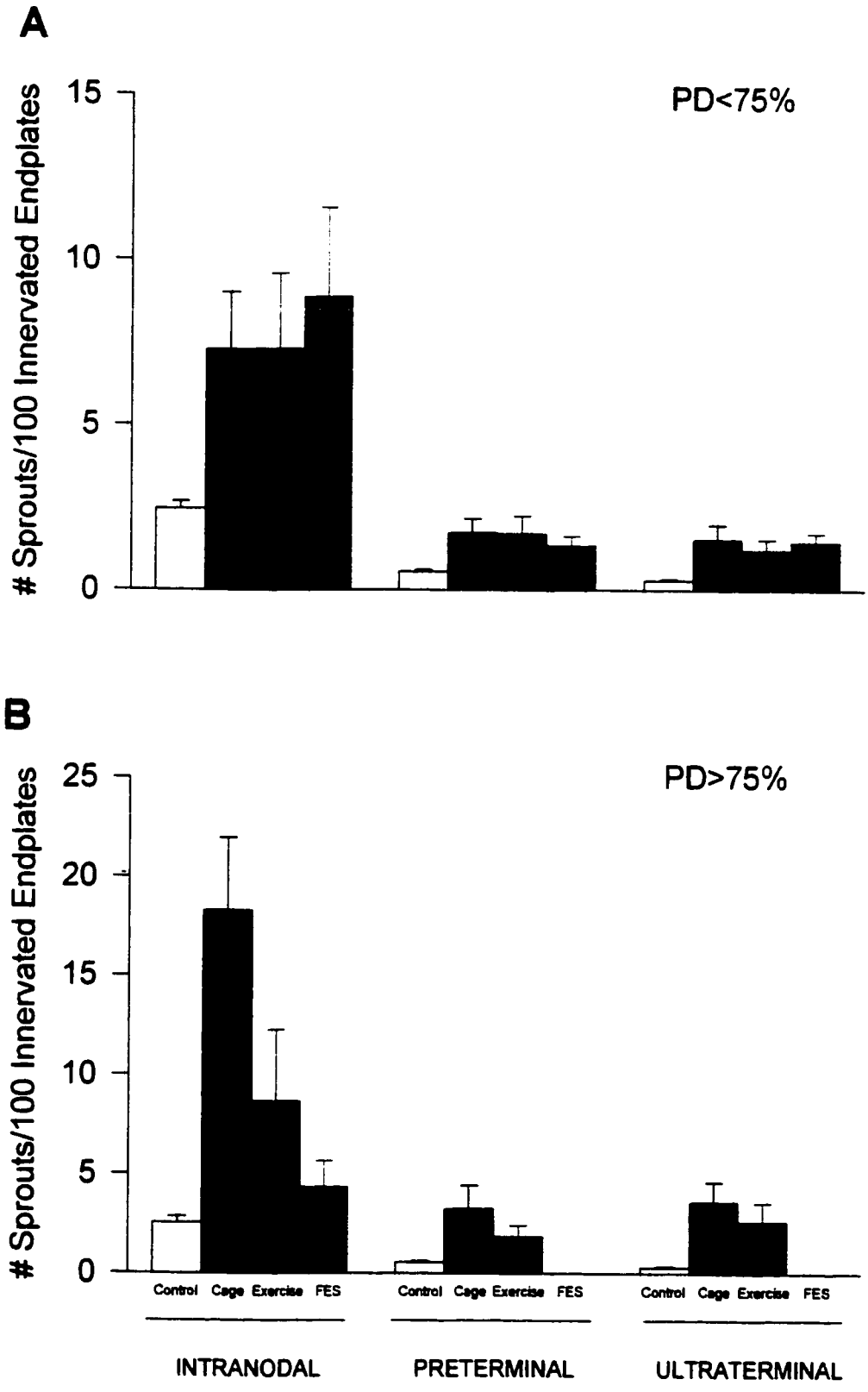

A
PD<75%
Sprouts/100 Innervated Endplates
15
10
5
0
B
PD>75%
Sprouts/100 Innervated Endplates
25
20
15
10
5
0
Control Cage Exercise FES
Control Cage Exercise FES
Control Cage Exercise FES
INTRANODAL
PRETERMINAL
ULTRATERMINAL

Table 3.1

Summary of mean muscle twitch forces in TA, MG, PL and SOL muscles in control, partially denervated muscles in rats experiencing normal caged activity (PD), partially denervated muscles after wheeling exercise (PD+Exercise) and partially denervated muscles with FES (PD+FES). (*) and (**) indicate statistical significance as compared to corresponding control and partially denervated muscles with normal caged activity, respectively.

Mean Muscle Twitch Forces (mN)				
Experimental Groups	TA	MG	PL	SOL
Control	1897±64	1744±78	747±38	256±13
PD	* 483±130 (p<0.0001)	* 401±71 (p<0.0001)	* 521±69 (p<0.0001)	* 194±22 (p<0.05)
PD+Exercise	** 115±29 (p<0.05)	356±97	598±105	195±37
PD+FES	349±246	632±183	** 778±66 (p<0.001)	232±36

CHAPTER 4

4. Effect of Neuromuscular Activity on Stability of Chronically Enlarged Motor Units

4.1. Introduction

Axonal sprouting from surviving or uninjured motor units (MUs) is a well recognized process which can, at least partially, compensate for loss of functional MUs following spinal cord injury or motoneuron disease (Wohlfart, 1957; McComas et al., 1971; reviewed by Miller, 1984; Dengler et al., 1989; Grimby et al., 1989; Trojan et al., 1991). In several cases, muscle function depends on the long-term functional integrity of the enlarged MUs. In some forms of spinal muscular atrophy, poliomyelitis and partial nerve injuries, enlarged MUs will subserve muscle function for life. However, many years after the acute phase of axonal sprouting, polio patients may suffer muscle weakness and fatigue (Halstead and Wiechers, 1987; Windebank et al., 1991; Halstead and Grimby, 1995). This so called post-polio syndrome was first reported 100 years ago (Halstead and Wiechers, 1987). Yet the basis for the muscle weakness is not understood and management of this syndrome is a dilemma.

The increased metabolic requirements of enlarged MUs established during the acute phase of the infection coupled with the further axonal sprouting to compensate for the normal motoneuron attrition of aging may lead to nerve terminal instability at neuromuscular junctions and thereby the withdrawal of unstable nerve terminals (Tuffery, 1971; Fahim and Robbins, 1982; Slack and Hopkins, 1982; Dalakas et al., 1986; Cashman et al., 1987; Halstead and Wiechers, 1987; Banker et al., 1983; Kelly and Robbins, 1983; Kelly and Robbins, 1986; Oertel, 1986; Hasizume et al., 1988; Rocel and Robbins, 1988; Lange et al., 1989; Emeryk et al., 1990; Jacob and Robbins, 1990a,b; Robbins et al., 1990; Agre et al., 1991; Trojan et al., 1991; Maselli et al., 1992; Dalakas, 1995). Exercise has been advocated as a treatment for the muscle weakness, and there is evidence of some improvement of muscle force output and endurance (Einarsson and Grimby, 1987; Feldman and Soskolne, 1987; Milner-Brown and Miller, 1988; Einarsson, 1991). However, exercise is an added metabolic stress and its benefits may be outweighed by detrimental effects on the stability of the enlarged MUs. In discussion of short-term benefits of a 6 week isokinetic exercise program, Dr. Wiechers addressed his concern that "we may have just cost these patients 2 additional years of ambulation by hyperfunctioning their residual motoneurons to gain this strength" and asked the question "Will exercise have a negative effects in 5 years?" (Einarsson and Grimby, 1987). Therefore, it is essential to evaluate any rehabilitative

approaches carefully in animal models before extensive use in patients whose neuromuscular system is already significantly compromised.

From animal studies, there is evidence for (Hoffman, 1952; Herbison et al., 1986; Einsiedel and Luff, 1994) and against (Brown and Holland, 1979; Gardiner et al., 1984; Gardiner and Faltus, 1986; Michel and Gardiner, 1989) activity promoting MU enlargement. However, careful evaluation of natural (exercise) and artificial (functional electrical stimulation, FES) neuromuscular activity on a number of different partially denervated rat hindlimb muscles indicate that high level of neuromuscular activity actually constrains MU enlargement during the acute phase of axonal sprouting, especially for extensive denervation (Tam et al. 1995, 1996; Archibald et al., 1996).

There is good evidence in humans and animals for age-related motoneuron attrition (Tuffery, 1971; Oertel, 1986; Robbins et al., 1990). The normal capacity for axonal sprouting and MU enlargement compensates well for the decline. As a result, muscle weakness is not perceived unless compounding motoneuron disease removes more than 80% of the normal complement of MUs. Surprisingly there has been few studies on the stability of established enlarged MUs in partially denervated muscles with time and/or age which pertains to the syndrome of post-polio syndrome as well as the long-term functional fate of enlarged MUs some time after the acute phase of axonal sprouting.

Change in MU number and size with age and capacity to sprout have been evaluated in animals (Jacob and Robbins, 1990a,b; Rosenheimer, 1990). These studies demonstrated both a decrement in sprouting capacity and a loss of the large fast fatigable MUs, respectively. However these studies do not address the issue of the stability of enlarged MUs many months or years after axonal sprouting and MU enlargement. The increased jitters of electromyographic (EMG) signals in muscles of patients with post-polio syndrome and the decreased safety factor of neuromuscular transmission *in vitro* support the hypothesis that enlarged MUs become progressively more unstable and may lose terminals (Dalakas et al., 1986; Cashman et al., 1987; Lange et al., 1989; Maselli et al., 1992).

We have undertaken the present study to evaluate 1) the long-term stability of enlarged MUs in partially denervated muscles and 2) effects of short period natural (running on exercise wheels) and artificial (functional electrical stimulation, FES) neuromuscular activity on this stability. We chose to study 4 different muscles in rat hindlimb with different physiological functions (physiological extensors: medial gastrocnemius (MG), plantaris (PL), and soleus (SOL) and flexor: tibialis anterior (TA), fast-twitch (TA, MG and PL) and slow-twitch (SOL) and large (TA, MG) and small (PL, SOL) muscles). In the present study, we demonstrated that MU enlargement after normal caged activity, measured electrophysiologically and morphologically was significantly reduced at

12 month in extensively but not moderately denervated muscles as compared to 1 month (i.e. during the acute phase of sprouting, from chapter 3). One month period of increased neuromuscular activity further compromised the time-related reduction in MU size in the extensively but not moderately denervated muscles.

The present results have been presented and published in abstract form (Tam et al. 1997).

4.2. Experimental design

In 44 female Sprague Dawley rats, either the L4 (n=21) or L5 (n=23) spinal root was evulsed under surgical anesthesia (sodium pentobarbital administered intraperitoneally as 0.07ml/g body weight) and using sterile procedure (see section 2.1.). Cutting L4 or L5 resulted in extensive denervation of TA and MG muscles, respectively and a range of partial denervation in the SOL and PL muscles. All animals were housed individually in cages for a period of 11 months before initiating an 4-week exercise program in 44 rats (body weight 382±7gm). The rats were divided into 3 groups: 1) normal caged activity (n=20), 2) wheeling exercise (n=14): daily 8hrs running on an exercise wheel (588±127m/day), and 3) FES (n=10): daily 8hrs continuous maximal stimulation of sciatic nerve at 20Hz (see section 2.2.). The unoperated muscles of the left side hindlimb of the experimental rats was used to serve as contralateral control and 6 unoperated rats were used as normal control.

Twelve months after partial denervation and 1 month after exercise, muscle twitch and tetanic forces were recorded *in vivo* in isolated TA, MG, SOL and PL muscles in response to stimulation of the sciatic nerve and MU forces were recorded in response to stimulation of teased filaments of the exposed L4 and L5 ventral roots (see section 2.3). Twelve micrometer thick cryostat cross sections of fresh TA and MG muscles were stained for acid or alkaline-myosin ATPase (see section 2.4.) and muscle fiber cross-sectional area (CSA) was measured from these sections (see section 2.5.). PL and SOL muscles were fixed in 4% formalin and cut into 100μm thick cryostat longitudinal sections on which combined silver/acetylcholinesterase (Ag/AChE) histochemical staining was done (see section 2.4.). Number of collateral sprouts and free endplates were counted from the sections.

4.3. Results

Throughout this study, no significant difference was found between the contralateral control and normal control for all parameters examined. Thus, the results from both groups were collated and used as the overall control.

4.3.1. Partial denervation

Because of the bilateral symmetry of the contribution of each spinal root to the motor innervation of the muscles on the contralateral unoperated side (Buller

and Pope, 1977; Gordon et al., 1986), the extent of partial denervation of the muscles was estimated by using the ratio of muscle tetanic force elicited by the stimulation of each spinal root to that elicited by the stimulation of sciatic nerve on the contralateral unoperated control side. As shown in figure 4.1A, evulsion of either L4 or L5 spinal roots resulted in more than 80% denervation in TA (average 86±4%) and MG (average 88%±2%) muscles, respectively. Partial denervation of PL and SOL muscles, on the other hand, ranged from 4% to 99% (average 54±4%) and 8% to 96% (average 54±4%), respectively. Also shown in figure 4.1 are the differences in the muscles with respect to the number of MU's derived from division of muscle twitch force by mean MU twitch force in each muscle. Muscle twitch force varies more directly with MU number than mean MU force which is very similar in fast-twitch muscles and significantly smaller in the slow twitch SOL muscle which contains predominantly slow MUs.

4.3.2. Evidence for chronically partial denervation as an animal model of post-polio syndrome: reduced MU enlargement with time in normal caged activity

For TA and MG muscles where denervation was more than 80%, the mean MU number was significantly reduced from 129±14 to 22±6 and 107±6 to 18±4 respectively (Fig. 4.2). As shown in figure 4.2, mean MU twitch forces of chronically denervated muscles as compared to the contralateral control were

increased from 18.3±0.4mN to 51.3±3.8mN for TA muscle and 21.5±0.5mN to 45.3±3.5mN for MG muscle. This is illustrated in the figure 4.2 when the MU twitch forces, corrected for muscle fiber CSA, were plotted as a cumulative frequency histogram on a semi-logarithmic scale. As shown for the acute phase of axonal sprouting (data from chapter 3; Fig. 4.2 A and B), the enlargement of MU size was also evident from the rightward shift of the MU twitch forces to the larger force values for both TA and MG muscles at 12 months (Fig. 4.2, C and D).

In chronically denervated muscles, the number of MUs was significantly reduced, by section of either L4 or L5 spinal roots, from 55±4 to 22±3 for PL muscles and 23±2 to 15±2 for SOL muscles (Fig. 4.3). As in the acute phase of axonal sprouting (data from chapter 3; Fig. 4.3, A and B), MU twitch force in PL and SOL muscles after 12 month moderate denervation increased less than 2 fold and the rightward shift of distribution was much less, as compared to TA and MG muscles (Fig. 4.3, C and D). Nevertheless, the mean of MU twitch forces for both partially denervated PL and SOL was statistically higher as compared to normal. The mean MU twitch forces increased from 20.1±0.5mN to 27.7±1.4mN for PL muscle and from 13.3±0.3mN to 18.3±1.3mN for SOL muscle (Fig. 4.3).

As shown in figure 4.2 and 4.3, the shift to the right 12 months after partial denervation was less than that 1 month after partial denervation, indicating that there might have been a decline in MU size with time after partial denervation,

consistent with the view of retraction of axonal sprouting in chronically denervated MUs and evidence for reduced synaptic efficacy as a function of age.

In comparison, cumulative MU force distributions for 12 month partial denervation (chronic) and 1 month (acute) are compared directly in figure 4.4C and D together with comparisons of normal MUs in the contralateral limbs (Fig. 4.4, A and B). In contrast to the contralateral limbs where the distribution histograms overlapped in the 2 graphs (Fig. 4.4, A and B), there was a significant shift of the cumulative histograms to the left for the chronically partially denervated TA and MG muscles, except for the largest MUs (Fig. 4.4, C and D). The MU enlargement was significantly lower at the chronic phase as compared to acute phase with mean MU twitch forces significantly reduced from 60.2±4.5mN (acute phase; data from chapter 3) to 45.3±3.5mN (chronic phase; fig. 4.4A) for MG muscle and from 67.4±7.0mN (acute phase; data from chapter 3) to 51.3±3.8mN (chronic phase; Fig. 4.4B). Thus, evidence for age-related reduction in size of enlarged MUs after chronic denervation is consistent with time-related post-polio evidence of muscle weakness.

For PL and SOL muscles where increase in MU size was considerably less after acute sprouting, time-related reductions in MU size were not detected. There was no significant difference in the mean MU twitch forces between the acute phase (PL: 28.0±1.2mN, SOL: 16.8±0.7mN; data from chapter 3) and the chronic phase (PL: 27.7±1.4mN, SOL: 18.3±1.3mN; see Fig. 4.5). The

cumulative distribution histograms of MU twitch forces of the acute phase and the chronic phase almost completely overlapped.

4.3.3. Effect of neural activity on chronically enlarged MUs

When chronically denervated muscles were subjected to 1 month increased neuromuscular activity either by wheeling exercise or FES, there was relatively little effect (Fig. 4.6 and 4.7). With the exception of a small but significant reduction in MG MU force (from 45.3±3.5mN to 35.3±3.7mN), the increased neuromuscular activity was without measurable effects on the chronically enlarged MUs. These findings indicate that time-related reductions in MU size after partial denervation are not greatly exacerbated by increased neuromuscular activity. Interestingly the significant effects of wheeling exercise were mediated on the smaller and not larger MUs, consistent with the findings of the inhibitory effects of wheeling exercise and FES on acute MU enlargement and axonal sprouting.

4.3.4. Comparison of acutely and chronically denervated intramuscular branching an sprouting

As shown in figure 4.8, there are few differences in the branching of intramuscular nerves and innervation of motor endplates in normal muscles. Predominant thick intramuscular nerve trunks with several axons branch into

single axons which innervate single motor endplates, and each motor endplate exhibited typical and simple oval shape. However, there are occasional axonal sprout in the older muscles (Fig. 4.8E), possibly associated with terminal withdrawal and subsequent reinnervation by axonal sprouting (Tuffery, 1971; Oertel, 1986; Robbins et al., 1990).

Comparisons of collateral sprouts and endplate morphology in moderately denervated muscles 1 month and 12 months later in figure 4.9 show several features which are time-related and are similar to findings of age-related changes in normal junctions. As for the acute partial denervation, there are three different types of collateral sprouts identified in chronically enlarged units in partially denervated muscles: 1) intranodal (Fig. 4.9D); 2) preterminal (Fig. 4.9E) and ultraterminal sprout (Fig. 4.9F). Most of them occurred individually as shown in figure 4.9D, E and F. In some cases, the different sprout types occurred in combination for the same axon (Fig. 4.9, G, H and I). Two intranodal sprouts grew out of the same axon (Fig. 4.9G). A preterminal sprout came out from the preterminal region of an endplate which was in turn innervated by another preterminal sprout which had grown out from the preterminal region of another endplate (Fig. 4.9H). We also observed that two preterminal sprouts originated from the same preterminal region of an endplate (Fig. 4.9I). Hence there is an increase in endplate size with associated dispersion of terminals over the

endplate site and increased complexity of the sprouting terminals (see also Fig. 4.11, C, D and E).

The overall nerve branching pattern in moderately denervated (<75% partial denervation) muscles appeared to be comparable to normal muscle except that the nerve branches are longer (Fig. 4.10). They contained many collateral sprouts which reinnervated denervated endplates. One collateral sprout per axon was the predominant pattern of axonal sprouting in these muscles. Likewise in the extensively denervated muscles (>75% partial denervation), there were few differences in the pattern of intramuscular branching and sprouting between the acute and chronic phase (Fig. 4.11). The terminal branches became predominant as collateral sprouts became more extensive with reasonably complex patterns of axonal sprouting. Most notably the endplates became more diffused with separated synaptic gutters. These changes in motor endplate morphology are consistent with previous observations. The greater complexity of terminal arborization at older junctions is associated with dispersion of terminals into more synaptic regions, indicating the decrease in ability of aged motoneurons to maintain motor innervation (Tuffery, 1971; Fahim and Robbins, 1982; Banker et al., 1983; Kelly and Robbins, 1983; Kelly and Robbins, 1986; Oertel, 1986; Robbins et al., 1990).

4.3.5. Effect of neural activity on endplate morphology and on number of collateral sprouts in moderately denervated muscles

Comparisons of contralateral control and chronically partially denervated muscles which were subjected to FES showed remarkably complex branching of intramuscular nerves in the unoperated contralateral muscles (Fig. 4.12, A and B) and increased complexity of the endplate regions in the partially denervated muscles (Fig. 4.12, C-F). However, little detectable effects of increased neuromuscular activity were noted with the number of collateral sprouts in moderately denervated muscles (Fig. 4.12, C-F). In exercised muscles, on the other hand, little change was seen in contralateral controls (data not shown) or between non-exercised and exercised muscles (Fig. 4.12, E and F).

Numbers of collateral sprouts were counted from the longitudinal muscle sections. Since 1) there was no significant differences in the number of collateral sprouts between PL and SOL muscles and, 2) we obtained similar range of the extent of partial denervation for both partially denervated PL and SOL muscles in each experimental group, we, therefore, grouped and discussed the results from PL and SOL muscles together in each experimental group.

Similar to 1 month partial denervation (acute phase, data from chapter 3), the number of collateral sprouts increased as a function of the extent of partial denervation at 12 month partial denervation (chronic phase). There appeared to be no difference in the number of collateral sprouts in partially denervated

muscles in rats which experienced normal caged activity between the acute and chronic phase (Fig. 4.13A), indicating time-related reduction in MU size was not evident in moderately denervated muscles. These results are consistent with the electrophysiological data. One month period of increased neuromuscular activity, either by wheeling exercise or FES did not affect the number of collateral sprouts in moderately denervated muscles in which the time-related reduction in MU enlargement was not evident (Fig. 4.13, B and C).

4.4. Discussion

There are three major findings in this study. (1) We have established an animal model of post-polio syndrome. Prolonged and extensive partial denervation of rat muscles results in destabilisation of chronically enlarged MUs and reduction of their MU size. Therefore, MU destabilisation is one of the key factors responsible for the muscle weakness of post-polio syndrome. (2) The destabilisation of chronically enlarged MUs results in loss of nerve terminals which leads to reduction in MU size. (3) Increased neuromuscular activity further exacerbates the time-dependent reduction in MU size in extensively denervated muscles.

4.4.1. MU enlargement by axonal sprouting in different muscles after prolonged partial denervation

Evulsion of L4 and L5 spinal roots removed more than 80% MUs from TA and MG muscles, respectively, and about 50% MUs on average from PL and SOL muscles. The remaining MUs in the partially denervated muscles enlarged their MU size by forming collateral sprouts to compensate the loss of motor function. The less than 20% remaining MUs in partially denervated TA and MG muscles enlarged to only 2-fold. In contrast to the previous findings of proportional enlargement of MUs after partial denervation in study using cat MG muscle (Refuse et al., 1992), there appeared to be a larger inhibitory effect of the chronic partial denervation on the smaller active MUs (Fig. 4.2, C and D). This is consistent with the effects of activity on the smaller MUs seen 1 month after increased neuromuscular activity and axonal sprouting. One of the objectives of this study was to determine whether there was time-dependent reduction in MU size of chronically enlarged MUs which would contribute an animal model of post-polio syndrome. Therefore, MU size was measured in rat muscles 1 year after the initial partial denervation. As a result, time-dependent reduction in MU enlargement, if it exists, would mask the actual degree of enlargement of MU size, especially when enlarged MUs are affected differently in accordance to their types.

Enlargement of MUs in moderately denervated PL and SOL muscles was much less than the extensively denervated TA and MG muscles. MU force enlarged by only about 1-fold. These results are, nevertheless, consistent with

previous studies of partially denervated muscles in not finding a significant increase in MU size until MU number was reduced below 50% of normal (Rafuse et al., 1992). A 2-fold increase in unit size is relatively difficult to detect electrophysiologically in large samples. It may simply be related to the wide range of MU force, variability between animals in partial denervation and difficulties in obtaining an adequate sample of MU number. On the other hand, MU force measurement might have underestimated the MU size or IR since muscle fiber CSA in partially denervated PL and SOL muscles were not taken into account. A previous study by Pachter and Eberstein (1992) showed that the mean fiber size in partially denervated PL muscle was significantly reduced as compared to control 1 year following partial denervation. In the present study, there may also have been reduction in muscle fiber size. Therefore, not having taken fiber CSA in PL and SOL might have underestimated the increase of MU force. But, this is relatively unlikely because we did not see differences in muscle fiber CSA in MG and TA muscles.

4.4.2. Time-dependent reduction in size of chronically enlarged MUs after prolonged and extensive partial denervation: animal model of post-polio syndrome

In order to determine whether there was a reduction in MU size with time as in animal model of post-polio syndrome, the extent of MU enlargement 12

months after partial denervation (chronic) was compared to that 1 month after partial denervation (acute; data from chapter 3). Comparison of MU size in control muscles between acute phase and chronic phase rats should disclose if any MU enlargement due to normal motoneuron attrition of aging occurs.

Using both electrophysiological and histochemical techniques, we found no significant differences in MU twitch forces, sprout counts and morphology in the control muscles between acute and chronic phase. For TA and MG muscles where more than 80% innervation was removed by section of L4 and L5 spinal roots, respectively, MU enlargement was significantly reduced at 12 months as compared to 1 month, indicating that MU size had declined as suggested for the establishment of post-polio syndrome in these muscles. The time-related reduction in MU enlargement was, however, less pronounced in TA muscle, suggesting that post-polio syndrome was less severe in this muscle.

For PL and SOL muscles where denervation was only moderate, time-dependent reduction in MU enlargement was not evident, suggesting that post-polio syndrome was less well established in these moderately denervated muscles. Morphometric analysis of extensively denervated PL muscle which we occasionally obtained, showed that motor endplates exhibited features such as greater complexity of terminal arborization and dispersion of terminals (Fig. 4.11, C, D and E), indicating age-related sign of unstability (Tuffery, 1971; Fahim and

Robbins, 1982; Banker et al., 1983; Kelly and Robbins, 1983, 1986; Oertel, 1986; Robbins et al., 1990).

Although histochemical analysis revealed the presence of occasional axonal sprouting in control muscles (Fig. 4.8E), comparison of cumulative MU force distributions which included much larger sample size supports that MU enlargement due to attrition of aging was only minor in this study. There is, however, good evidence from human and animal studies for age-related motoneuron attrition (Tuffery, 1971; Oertel, 1986; Robbins et al., 1990). There is also evidence showing that aged motoneurons undergo synaptic remodeling which have been generally believed to be the results of adaptation or compensation for synaptic deficits associated with aging (Fahim and Robbins, 1982; Banker et al., 1983; Fahim et al., 1983; Kelly and Robbins, 1983, 1986; Wernig et al., 1984; Elkerdany and Fahim, 1993). Although age changes at neuromuscular junction appear to be rather successful compensations for cellular deficits associated with age, the increasing extent of adaptation entails progressively more fragility. Aged motoneurons have also been found to lose the full ability to sprout (Jacob and Robbins, 1990a,b; Rosenheimer, 1990). Therefore, under normal conditions, the normal capacity for axonal sprouting and MU enlargement compensates well for the decline attributed by the age-related motoneuron attrition. However, when compounding motoneuron disease removes more than 80% of the normal complement of MUs, muscle weakness

becomes obvious. It may explain the results of the present study that time-related reduction in MU enlargement was only evident in extensively denervated but not in moderately denervated muscles. In control muscles. MU enlargement due to attrition of aging was only minor, and therefore, was not reflected in cumulative MU force distributions as a significant change. However, when more than 80% of the normal complement of MUs was removed, the already enlarged and aged motoneurons remaining simply cannot maintain the undue functional metabolic demands and therefore lose their terminals. Nevertheless, the interesting findings of the present study that the time-dependent reduction in MU enlargement was only evident in extensively denervated but not in moderately denervated muscles, are consistent with the clinical observation that symptomatic post-polio syndrome is experienced primarily in patients who suffered severe motoneuron loss in the prior poliomyelitis (Cashman et al., 1987; Maselli et al., 1992).

4.4.3. Loss of unstable nerve terminals after prolonged and extensive partial denervation

It has been suggested that the over-exhaustion and failure of chronically enlarged MUs to maintain their enlarged peripheral innervation field results in withdrawal of unstable nerve terminals and therefore, accounts for the progressive muscle weakness associated with chronic denervation syndrome

such as post-polio syndrome. Supporting evidence coming from muscle biopsy and quantitative EMG studies of patients with prior poliomyelitis has suggested a link between this progressive muscle weakness and fatigue and increased 'jitters' indicative of synaptic instability and impaired neuromuscular transmission (Dalakas et al., 1986; Cashman et al., 1987; Lange et al., 1989; Maselli et al., 1992). In the present study, MU twitch force of enlarged MUs was significantly reduced at 12 months after partial denervation as compared to 1 month in extensively denervated MG muscle, and to a less extent in extensively denervated TA muscle. Since MU force is the product of IR, CSA and specific force of muscle fiber, and specific force does not change after reinnervation (Tötösy de Zepetnek et al., 1992; Fu and Gordon, 1995a,b), we normalized MU twitch force values by CSA in order for MU force to reflect IR. All muscle fiber CSAs were measured and corrected for in TA and MG muscles to reveal that IR declined with time after partial denervation. Therefore, the time-dependent reduction in MU enlargement in extensively denervated muscles was not accounted for by the changes in muscle fiber size if any, but by the loss of functional nerve terminals.

In view of the very small number of extensively denervated PL and SOL muscles, representative sprout counts were not available to confirm our electrophysiological results. Nevertheless, our electrophysiological results suggesting that loss of functional nerve terminals accounts for the time-

dependent reduction in MU enlargement in extensively denervated muscles are consistent with the previous histological findings (Pachter and Eberstein, 1992). They examined partially denervated PL muscle histologically over a period of 12 months, and found that the number of collateral sprouts was significantly reduced at 12 months, as compared to the earlier periods after partial denervation, and became comparable to the level of control muscle. More evidence comes from the immunohistochemical detection of myofibers expressing NCAM from muscle biopsies of patients experiencing post-polio syndrome, indicating ongoing denervation in the muscles of these patients (Cashman et al., 1987). These findings provide direct histological evidence to support the electrophysiological evidence obtained in the present study in suggesting that withdrawal of unstable nerve terminals accounts for, at least in part, the progressive muscle weakness and fatigue associated with chronic denervation syndrome.

4.4.5. Detrimental effect of increased neuromuscular activity in extensively denervated muscles where time-dependent reduction in MU enlargement was most evident

We have demonstrated that 1 month period of wheeling exercise (average 588±127m/day) further exacerbated the time-related reduction in MU enlargement in extensively denervated (MG and TA) but not moderately denervated (PL and SOL) muscles. The exacerbation in time-related reduction in

MU enlargement was most pronounced in MG muscle in which phenomenon of post-polio syndrome was most evident, and was less pronounced in TA muscle. Unexpectedly, a one month period of FES (20Hz, 8hrs daily) had almost no effect on MU enlargement in both extensively denervated MG and TA muscles (Fig. 4.6, C and D). For moderately denervated PL and Sol muscles where the time-related reduction in MU enlargement was not evident, both electrophysiological and histochemical analysis showed that 1 month period of increased neuromuscular activity by either wheeling exercise or FES did not reduce their MU size.

Exercise regimes have been advocated and some positive effects on muscle strength and endurance have been reported in patients with post-polio syndrome (Einarsson and Grimby, 1987; Feldman and Soskolne. 1987; Milner-Brown and Miller, 1988; Einarsson, 1991). Positive effects may, however, arise from several possible adaptations including increased central drive, MU and muscle recruitment, MU firing and/or muscle fiber hypertrophy. Other problems with most of these studies include the lack of a clear definition of post-polio syndrome, and the lack of well defined and quantifiable measures of muscle strength and endurance. It is also important to know the degree of involvement of initial polio and the severity of post-polio syndrome before the effect of muscle exercise can be accurately assessed. In addition, exercise is also an added metabolic stress and therefore, its benefits may be outweighed by the detrimental

effects on the stability of the enlarged MUs. The very same concern was also raised by Dr. Wiechers. He questioned whether regaining of the muscle strength might just be the result of hyperfunctioning of the residual motoneurons which in turn might cause the patients even more harm in the future (Einarsson and Grimby, 1987).

Surprisingly, there has been only few studies on the effect of neural activation on the stability of established enlarged MUs in partially denervated muscles with time and/or age which pertains to the syndrome of post-polio syndrome, despite of the popularity of using exercise as a clinical treatment for muscle weakness of patients suffering chronic denervation syndromes. From animal studies, there has been conflicting evidence regarding the effects of activity on MU enlargement during the acute phase of axonal sprouting, with some reports indicating it was beneficial for sprouting (Hoffman, 1952; Herbison et al., 1986; Einsiedel and Luff, 1994) while others reported that it was detrimental (Brown and Holland, 1979; Gardiner et al., 1984; Gardiner and Faltus, 1986; Michel and Gardiner, 1989). However, careful evaluation of natural (wheeling exercise) and artificial (FES) neuromuscular activity on a number of different partially denervated rat hindlimb muscles indicate that high level of neuromuscular activity actually constrains MU enlargement during the acute phase of axonal sprouting, especially for extensive denervation (Tam et al. 1995, 1996; Archibald et al., 1996). Consistent with these studies, the results of the

present study also showed that high levels of neuromuscular activity further exacerbated the time-related reduction in MU enlargement only in extensively denervated but not moderately denervated muscles. The results of the present study suggest that after prolonged and extensive denervation, chronically enlarged MUs become over-exhausted and nerve terminals become unstable, resulting in terminal withdrawal. Since exercise is an added metabolic stress, increased neuromuscular activity may simply impose extra amount of stress to the already exhausted and unstable nerve terminals, resulting in more nerve terminal withdrawal.

4.5. Conclusion

Using 4 functionally different muscles and both electrophysiological and histochemical techniques, we, in the present studies, have successfully established a rat model of post-polio syndrome of extensively denervated muscles. We have shown that prolonged and extensive partial denervation of rat muscles results in destabilization of nerve terminals and reduced MU size. We have further provided evidence showing that destabilization of chronically enlarged MU results in loss of nerve terminals. The detrimental effect of increased neuromuscular activity in further exacerbating time-related reduction in MU enlargement depends on the extent of partial denervation of muscles. Increased neuromuscular activity has a small effect in exacerbating the time-

related reduction in MU size in extensively denervated muscles. This suggests that the overloaded intact MUs by axonal sprouting for compensating for the extensive motoneuron loss may have been further stressed by increased neuromuscular activity, resulting in more nerve terminal loss.

Our findings suggest that increased levels of activity may not be advised as rehabilitation for progressive muscle weakness and fatigue of post-polio syndrome, especially when muscle weakness and fatigue is pronounced.

4.6. Bibliography

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Figure 4.1

Mean (±S.E.) percentage of ventral root L4 (open histograms) or L5 (filled histograms) innervation (A), motor unit (MU) numbers (B), muscle twitch forces (C) and motor unit twitch forces (D) of fast, flexor tibialis anterior (TA), extensor medial gastrocnemius (MG), plantaris (PL), and slow, extensor soleus (SOL) muscles. Evulsion of either L4 or L5 spinal roots resulted in more than 80% denervation in TA (average 86±4%) and MG (average 88%±2%) muscles, respectively; but resulted in less extensive denervation of PL (average 54±4%) and Sol (average 54±4%) muscles. Muscle force varies more directly with mean MU number than mean MU force.

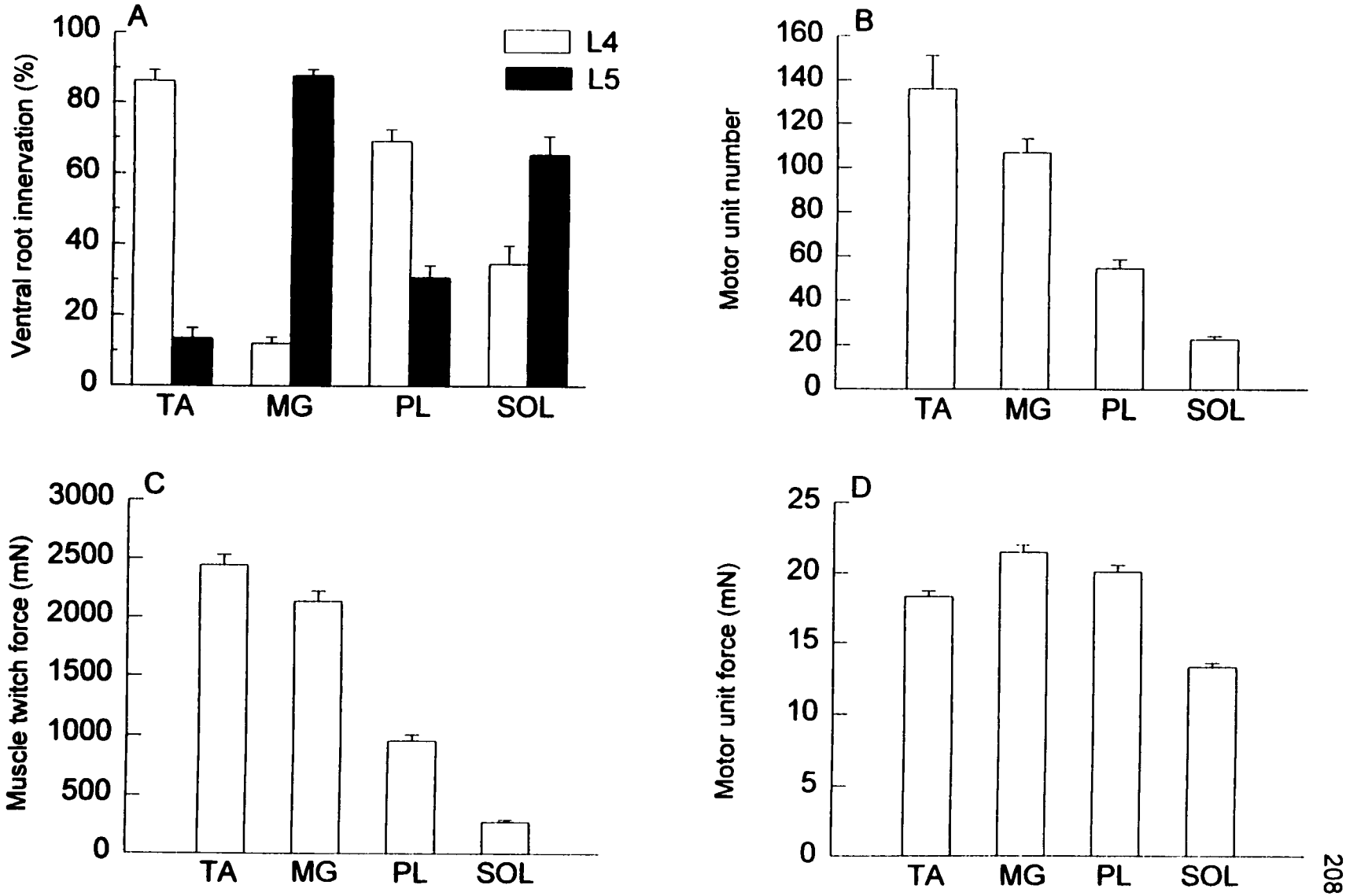

A
Ventral root innervation (%)
100
80
60
40
20
0
L4
L5
TA
MG
PL
SOL
B
Motor unit number
160
140
120
100
80
60
40
20
0
TA
MG
PL
SOL
C
Muscle twitch force (mN)
3000
2500
2000
1500
1000
500
0
TA
MG
PL
SOL
D
Motor unit force (mN)
25
20
15
10
5
0
TA
MG
PL
SOL

Figure 4.2

Cumulative frequency histograms of MU twitch force distributions in control (Acute phase: open circles; Chronic phase: open triangles) and partially denervated TA (A and C) and MG (B and D) muscles of rats experiencing normal caged activity (Acute phase: blue circles; Chronic phase: red triangles). As in the acute phase, the MU twitch force distributions, after normalized for muscle fiber cross-sectional area (CSA), significantly shifted to the right from the controls ($p<0.05$) with a significant increase in mean MU twitch forces from 18.3±0.4mN to 51.3±3.8 mN for TA muscle ($p<0.0001$) and from 21.5±0.5mN to 45.3±3.5mN for MG muscle ($p<0.0001$).

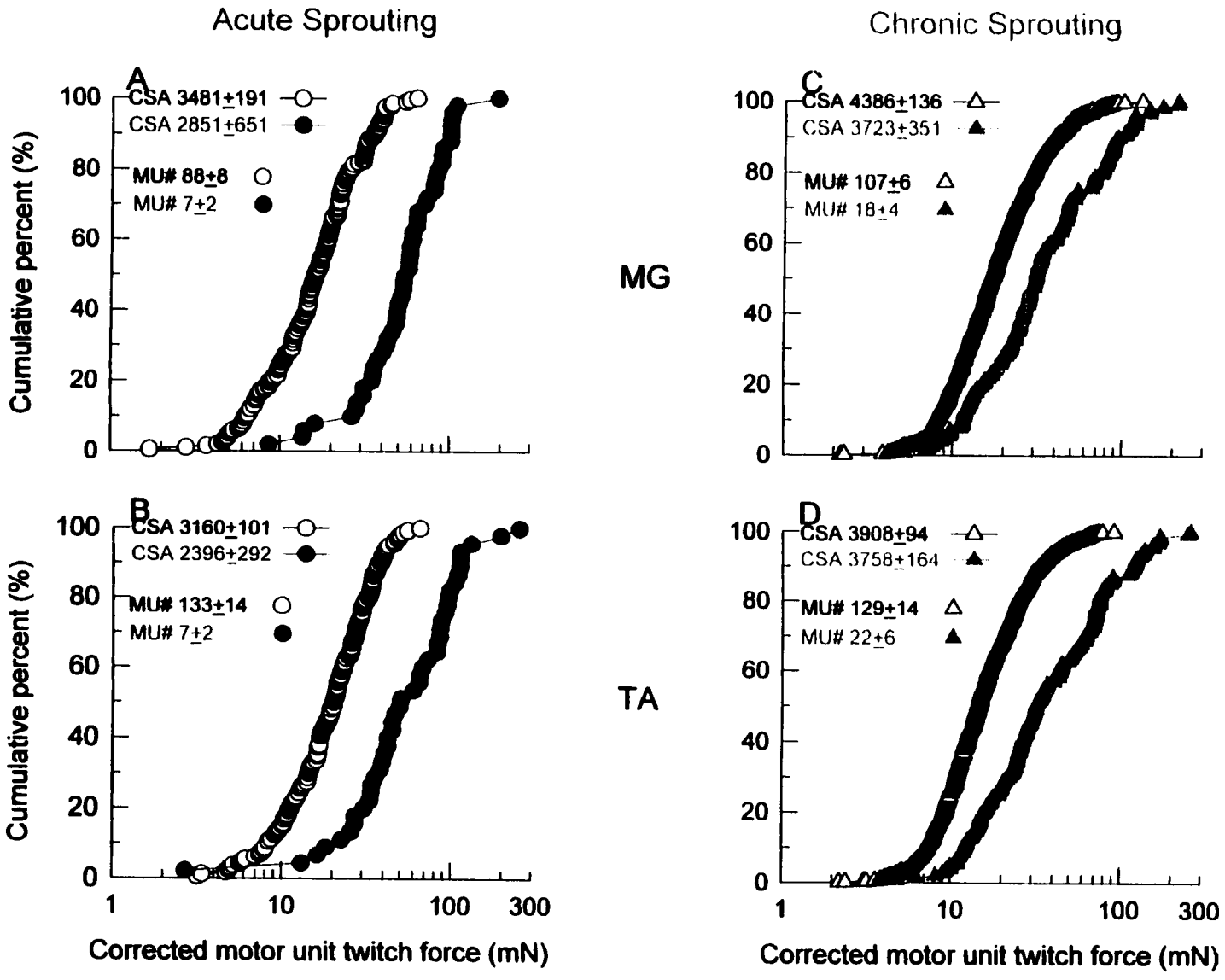

Acute Sprouting
Chronic Sprouting
A
CSA 3481±191
CSA 2851±651
MU# 88±8
MU# 7±2
C
CSA 4386±136
CSA 3723±351
MU# 107±6
MU# 18±4
MG
B
CSA 3160±101
CSA 2396±292
MU# 133±14
MU# 7±2
D
CSA 3908±94
CSA 3758±164
MU# 129±14
MU# 22±6
TA
Cumulative percent (%)
Corrected motor unit twitch force (mN)
0
20
40
60
80
100
1
10
100
300

Figure 4.3

Cumulative frequency histograms of MU twitch force distributions in control (Acute phase: open circles; Chronic phase: open triangles), and partially denervated PL (A and C) and SOL (B and D) muscles of rats experiencing normal caged activity (Acute phase: blue circles; Chronic phase: red triangles). As in the acute phase, the rightward shift in the MU twitch force distributions for PL and Sol muscles (PD<80%) was much less than TA and MG muscles with a significant increase in mean MU twitch forces from 20.1±0.5mN to 27.7±1.4mN for PL muscle ($p<0.0001$) and from 13.3±0.3mN to 18.3±1.3mN for SOL muscle ($p<0.0001$).

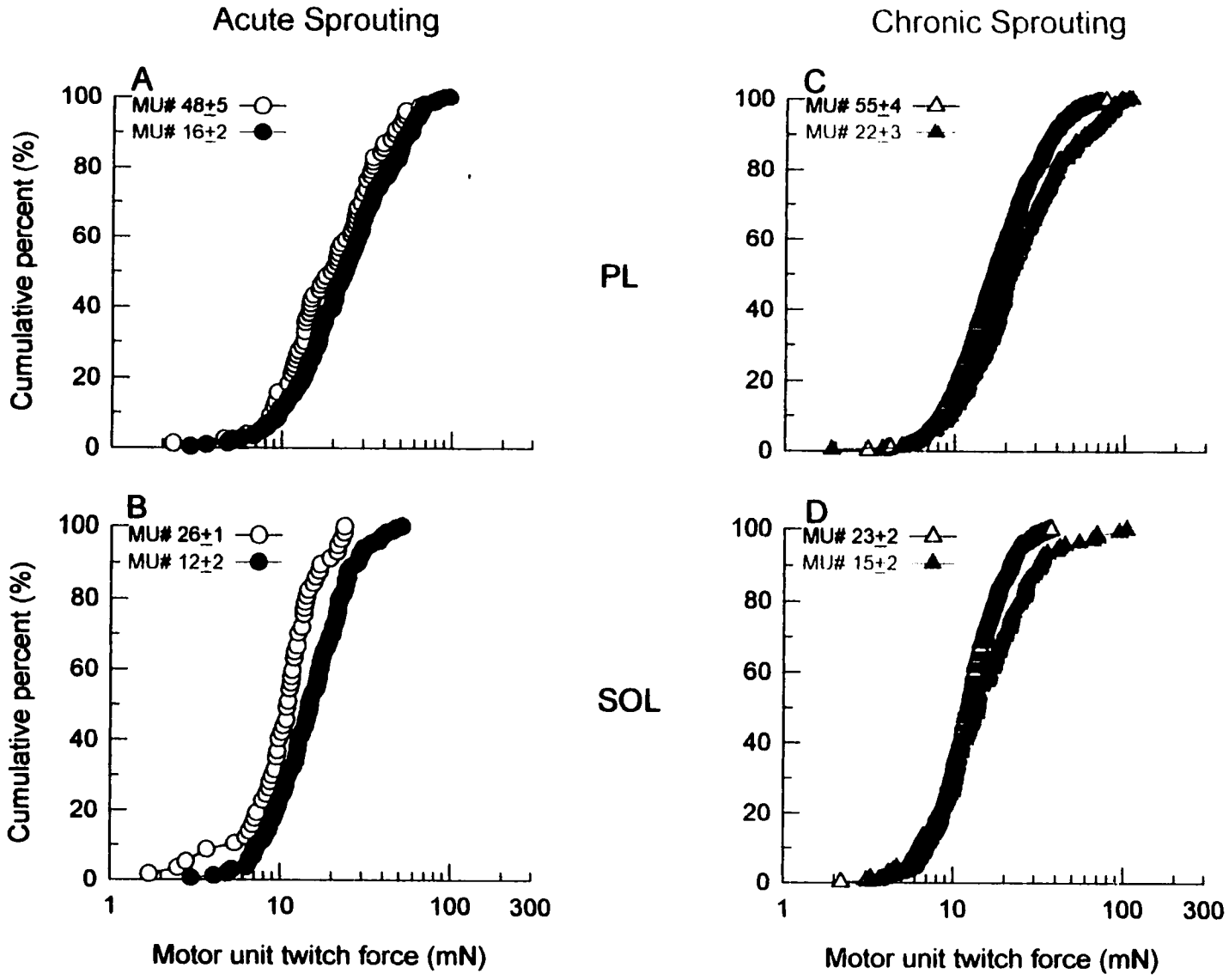

Acute Sprouting
Chronic Sprouting
A
MU# 48±5
MU# 16±2
B
MU# 26±1
MU# 12±2
C
MU# 55±4
MU# 22±3
D
MU# 23±2
MU# 15±2
PL
SOL
Cumulative percent (%)
Motor unit twitch force (mN)
0
20
40
60
80
100
1
10
100
300

Figure 4.4

Cumulative frequency histograms of MU twitch force distributions, after normalization for CSAs, in control (Acute phase: open circles; Chronic phase: open triangles), and partially denervated TA (A and C) and MG (B and D) muscles of rats experiencing normal caged activity (Acute phase: blue circles; Chronic phase: red triangles). There was no significant differences between the acute and chronic phase in the MU twitch forces in the controls. MU enlargement was significantly reduced at chronic phase in extensively denervated MG muscle as compared to acute phase (from 60.2±4.5mN to 45.3±3.5mN, $p<0.05$), indicating a time-related reduction in MU enlargement which was less pronounced in TA muscle (from 67.4±7.0mN to 51.3±3.8 mN, $p<0.05$).

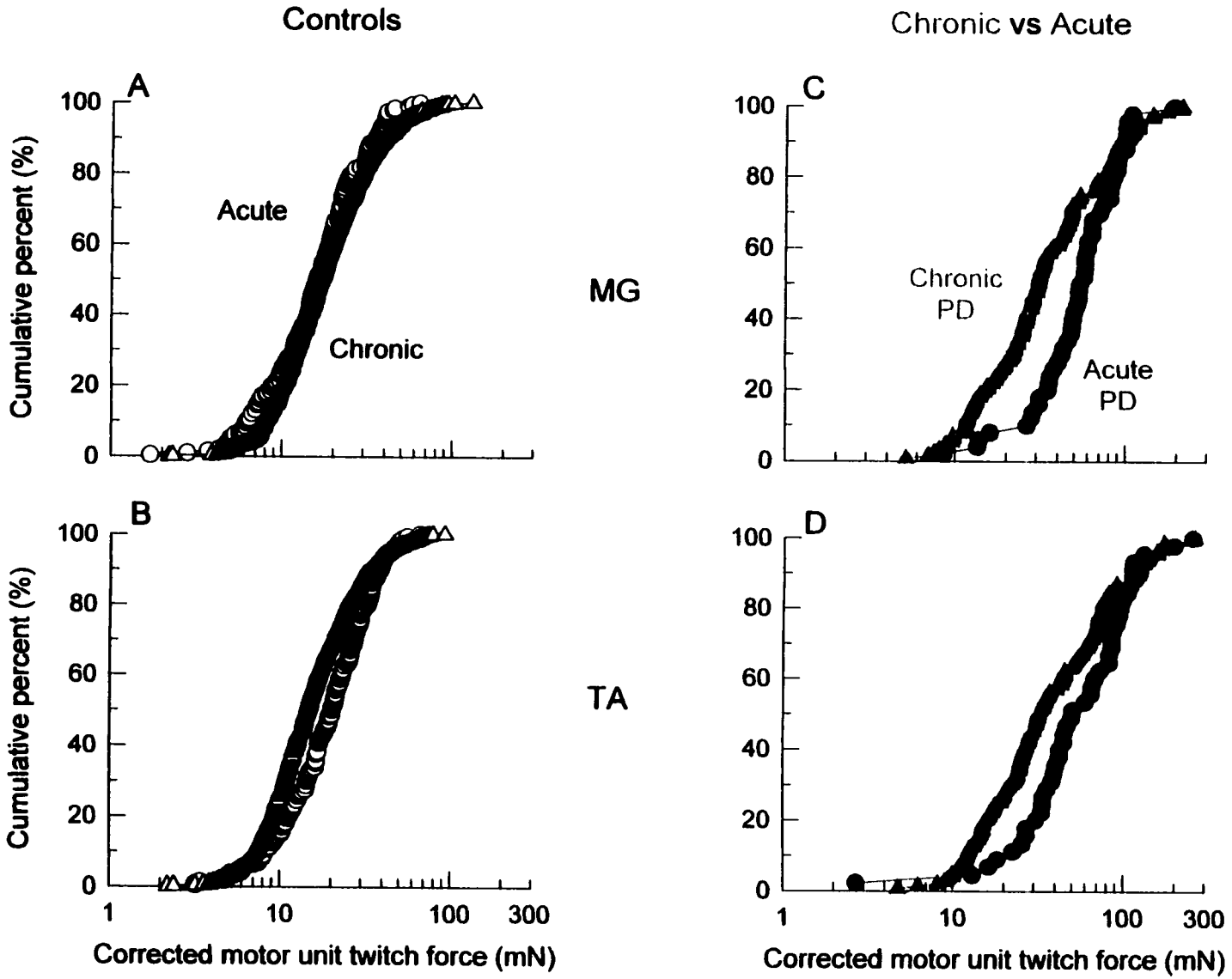

Controls
Chronic vs Acute
A
B
C
D
Acute
Chronic
Chronic PD
Acute PD
MG
TA
Cumulative percent (%)
Corrected motor unit twitch force (mN)
0
20
40
60
80
100
1
10
100
300

Figure 4.5

Cumulative frequency histograms of MU twitch force distributions in control (Acute phase: open circles; Chronic phase: open triangles), and partially denervated PL (A and C) and SOL (B and D) muscles of rats experiencing normal caged activity (Acute phase: blue circles; Chronic phase: red triangles). There was no significant differences in the MU twitch forces between the acute and chronic phase in control and moderately denervated muscles, indicating no evidence of time-related reduction in MU enlargement.

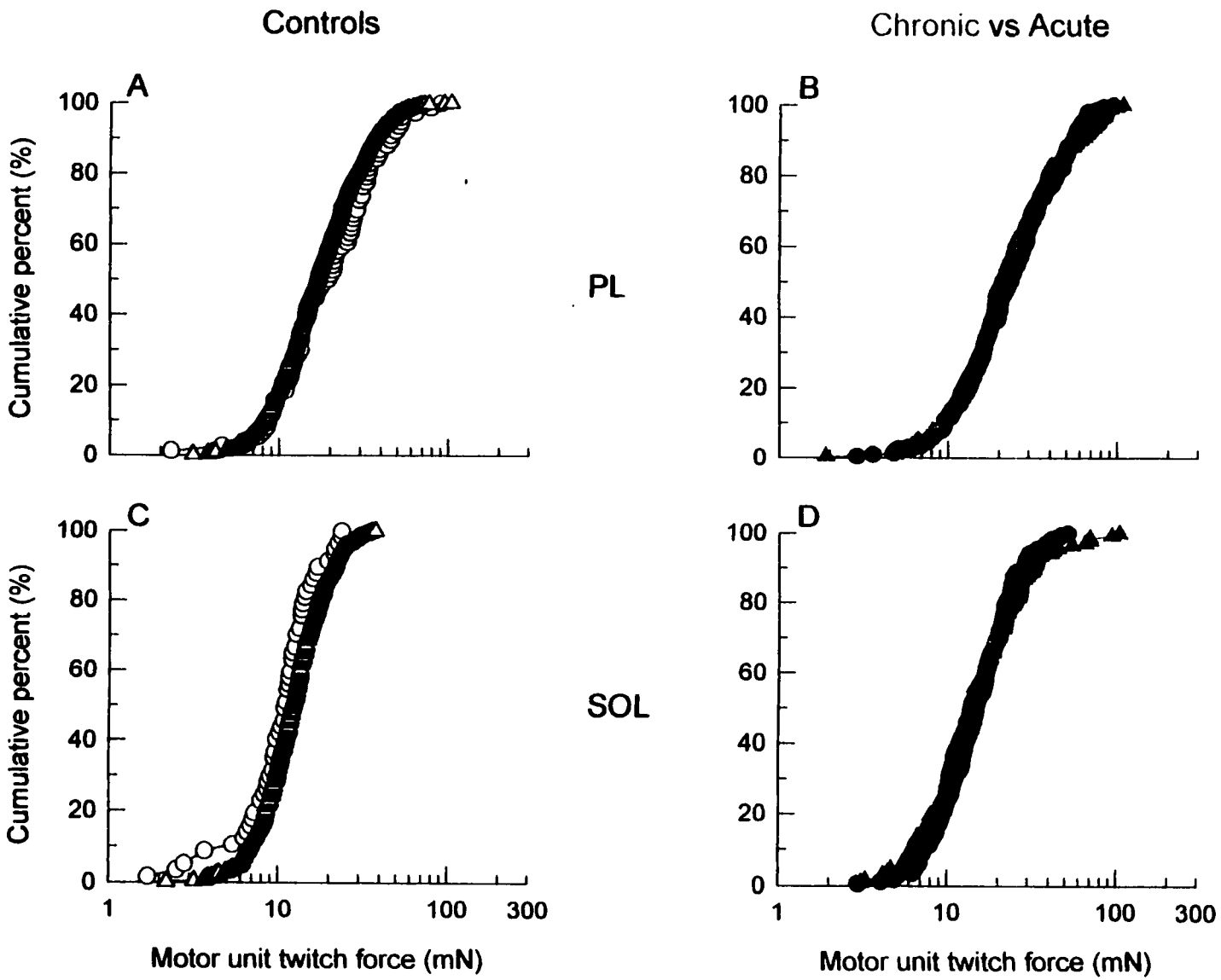

Controls
Chronic vs Acute
A
B
C
D
PL
SOL
Cumulative percent (%)
Cumulative percent (%)
100
80
60
40
20
0
1
10
100
300
Motor unit twitch force (mN)
Motor unit twitch force (mN)

Figure 4.6

Cumulative frequency histograms of MU twitch force distributions in chronically denervated TA (A and C) and MG (B and D) muscles of rats experiencing normal caged activity (red triangles) as compared to wheeling exercise (blue squares), and functional electrical stimulation (FES, purple squares). With the exception of MG muscle after wheeling exercise, increased neuromuscular activity did not affect the time related-reduction in MU size in chronically and extensively denervated muscles. One month period of wheeling exercise further exacerbated the time-dependent reduction in MU size in MG muscle, indicated as a leftward shift in cumulative MU force distributions ($p<0.05$) with a significant decrease in mean MU forces from 45.3±3.5mN to 35.3±3.7mN ($p<0.0001$).

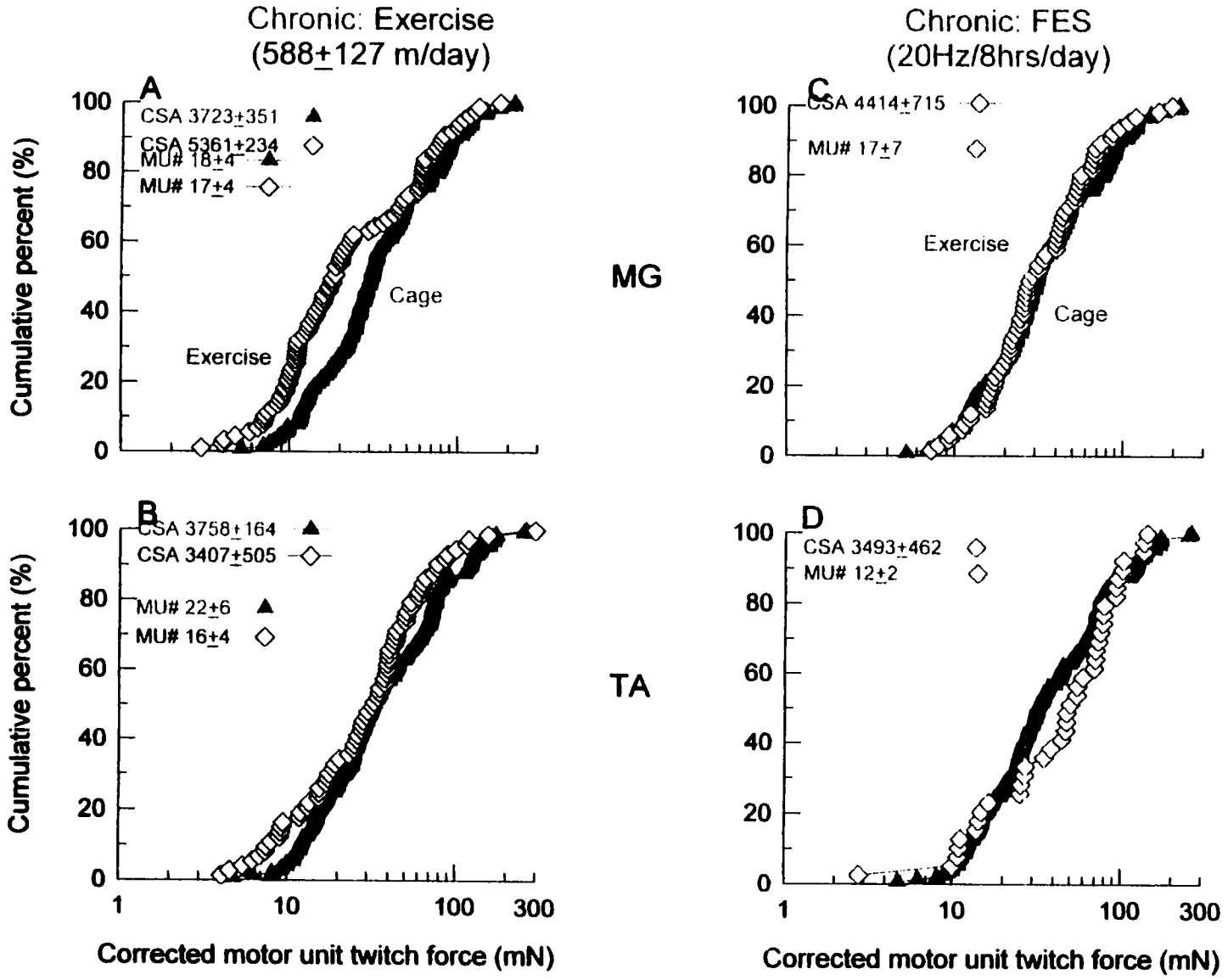

Chronic: Exercise
(588±127 m/day)
Chronic: FES
(20Hz/8hrs/day)
A
B
C
D
CSA 3723±351
CSA 5361±234
MU# 18±4
MU# 17±4
CSA 3758±164
CSA 3407±505
MU# 22±6
MU# 16±4
CSA 4414±715
MU# 17±7
CSA 3493±462
MU# 12±2
Exercise
Cage
MG
TA
Cumulative percent (%)
Corrected motor unit twitch force (mN)
0
20
40
60
80
100
1
10
100
300

Figure 4.7

Cumulative frequency histograms of MU twitch force distributions in chronically denervated PL (A and C) and Sol (B and D) muscles of rats experiencing normal caged activity (red triangles) as compared to wheeling exercise (blue squares), and FES (purple squares). One month period of increased neuromuscular activity did not affect the size of MUs in the moderately denervated PL and SOL muscle.

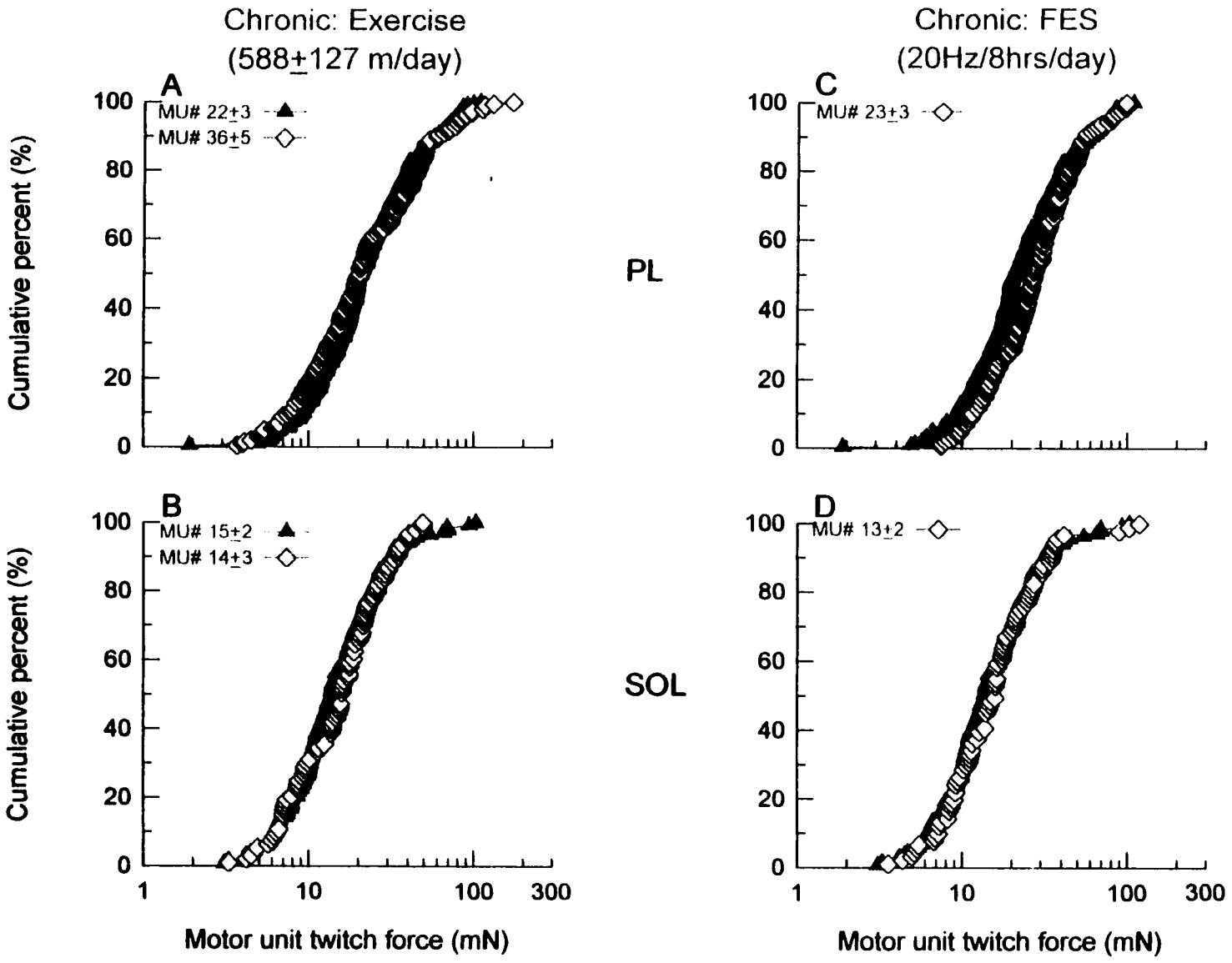

Chronic: Exercise
(588±127 m/day)
Chronic: FES
(20Hz/8hrs/day)
A
MU# 22±3
MU# 36±5
C
MU# 23±3
PL
B
MU# 15±2
MU# 14±3
D
MU# 13±2
SOL
Cumulative percent (%)
Cumulative percent (%)
100
80
60
40
20
0
1
10
100
300
Motor unit twitch force (mN)
Motor unit twitch force (mN)

Figure 4.8

Low (A and C) and higher (B, D and E) power combined Ag/AChE histochemical photomicrographs of 100μm thick cryostat longitudinal sections of control PL muscles at 1 month (Acute; A and B) and 12 months (Chronic; C-E).

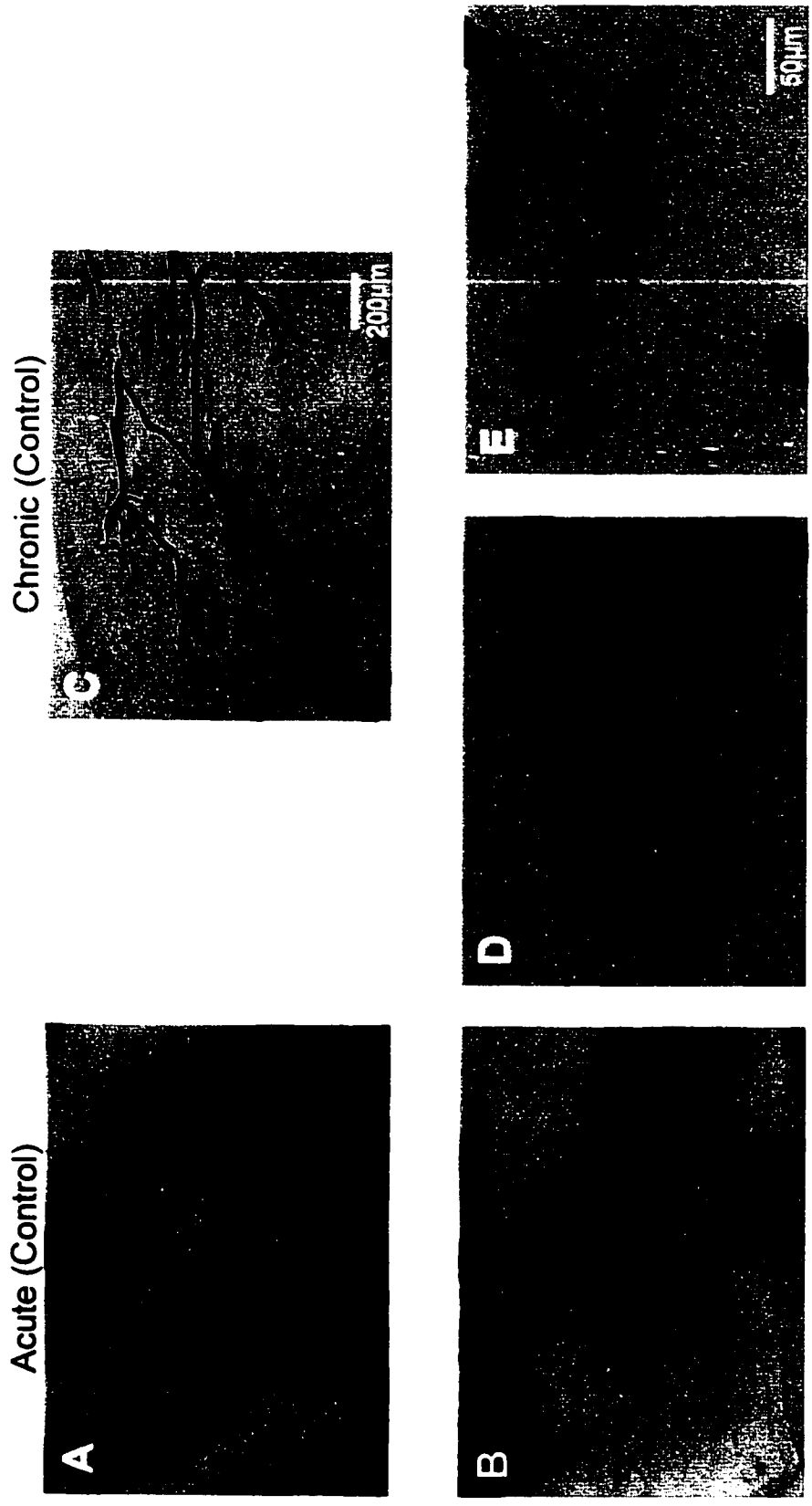

Acute (Control)
Chronic (Control)
A
B
C
D
E
200µm
50µm

Figure 4.9

Three different types of collateral sprouts: intranodal (A and D), preterminal (B and E) and ultraterminal sprouts (C and F), visualized with combined Ag/AChE histochemical staining. Motor endplates were bigger at 12 months (chronic; D-I) than 1 month (acute; A-C). As shown for acute phase (A-C), sprouts occurred either singly for the same axon (D-F) or in combination (G-I). Filled arrows inidcate collateral sprouts, and asterisks indicate motor endplates.

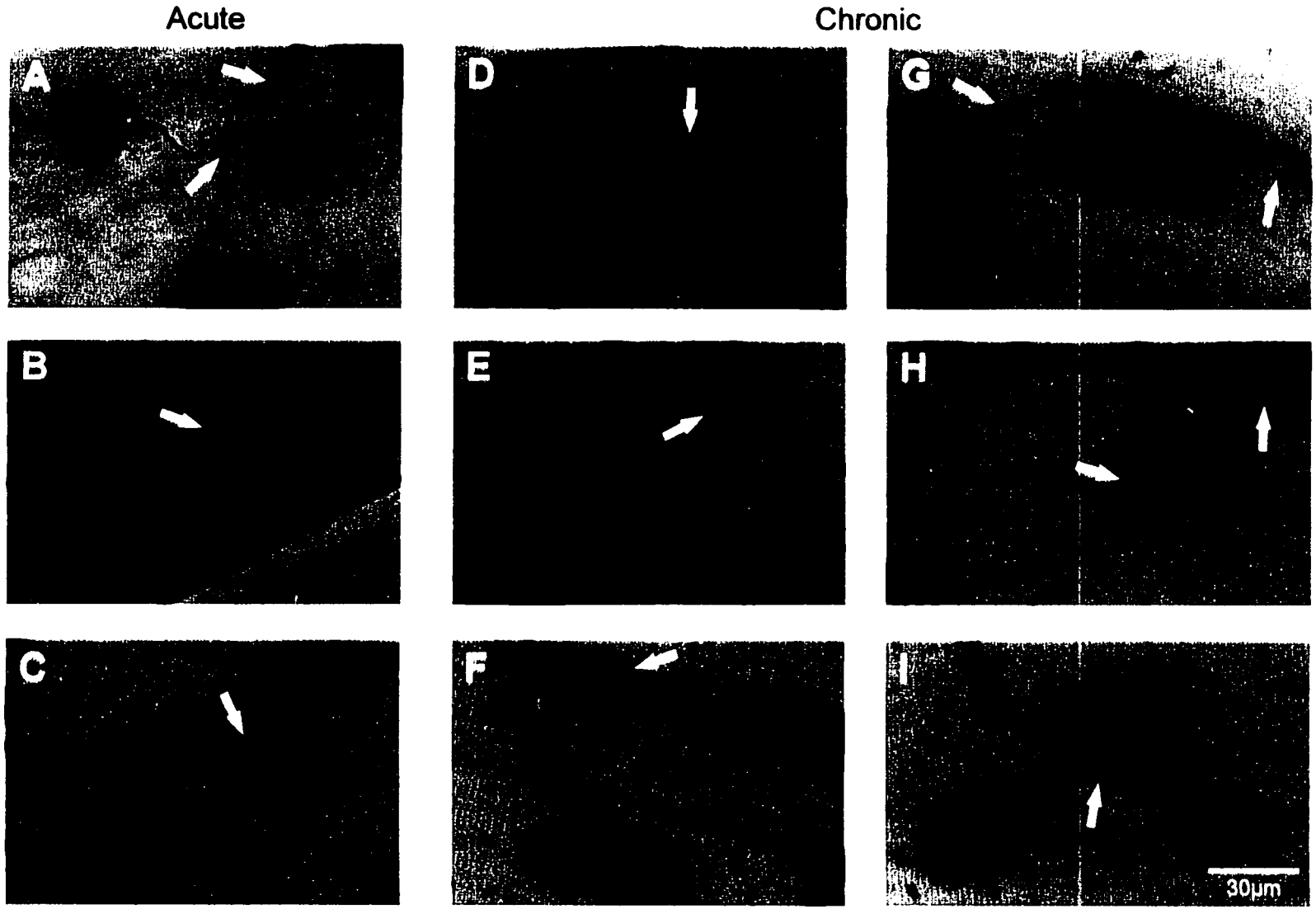

Acute
Chronic
A
B
C
D
E
F
G
H
I
30µm

Figure 4.10

Low (A and C) and higher (B and D) power combined Ag/AChE histochemical photomicrographs of 100μm thick cryostat longitudinal sections of moderately denervated PL muscles (PD<75%) at 1 month (Acute; A and B) and 12 months (Chronic; C and D). The overall nerve branching pattern in moderately denervated muscles appeared similar betwen acute and chronic phase (A and C), and the nerve branches were longer than in normal muscles Fig.4.8). Collateral sprouts (arrows) reinnervated denervated endplates.

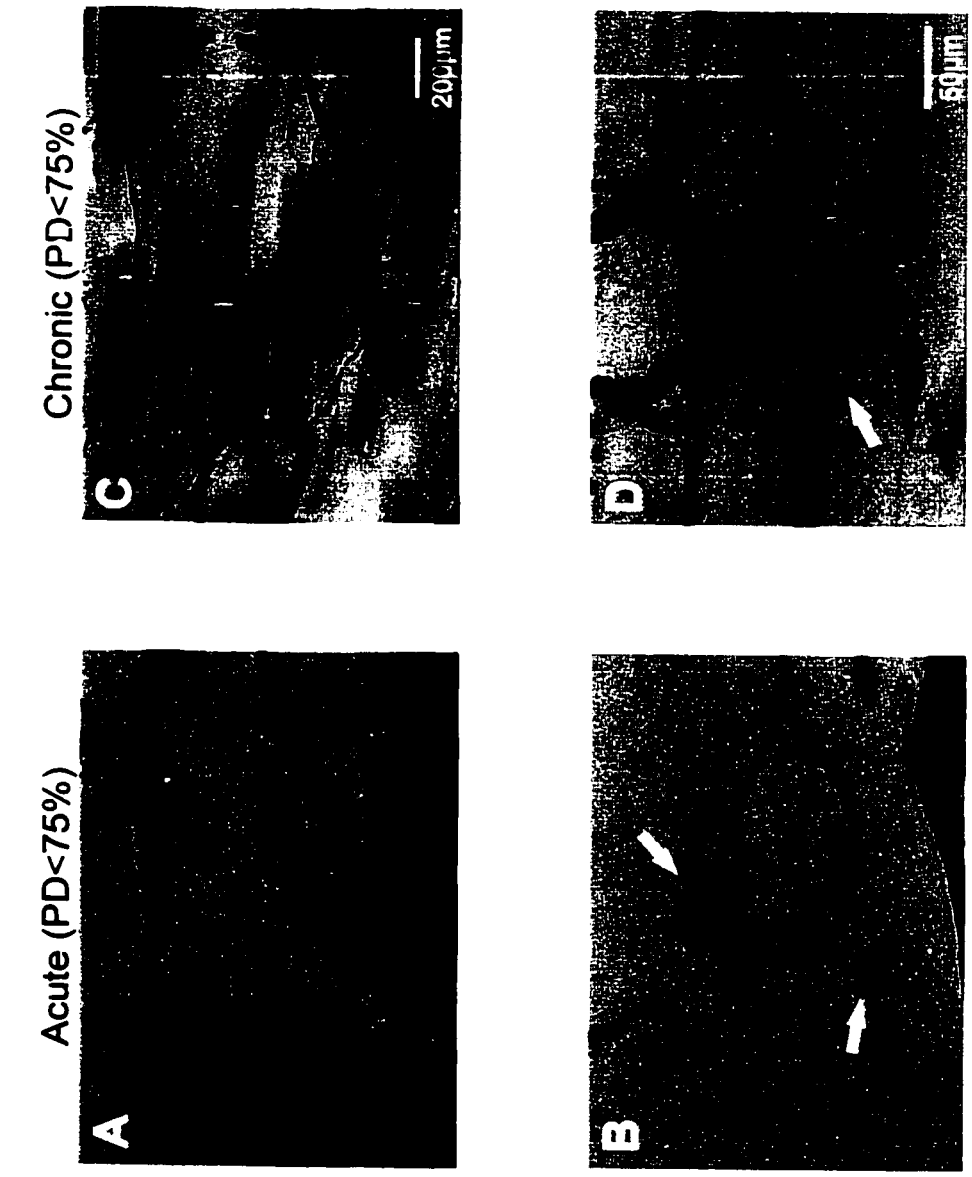

Acute (PD<75%)
Chronic (PD<75%)
A
B
C
D
200μm
50μm

Figure 4.11

Low (A and C) and higher (B, D and E) power combined Ag/AChE histochemical photomicrographs of 100μm thick cryostat longitudinal sections of extensively denervated PL muscles (PD>75%) at 1 month (Acute; A and B) and 12 months (Chronic; C-E). At both acute and chronic phase, terminal branches became predominant as collateral sprouts became more extensive (A and C) with complex pattern of axonal sprouting (B, D and E). At chronic phase (D and E), some endplates became more diffused (asterisks). Arrows indicate collateral sprouts.

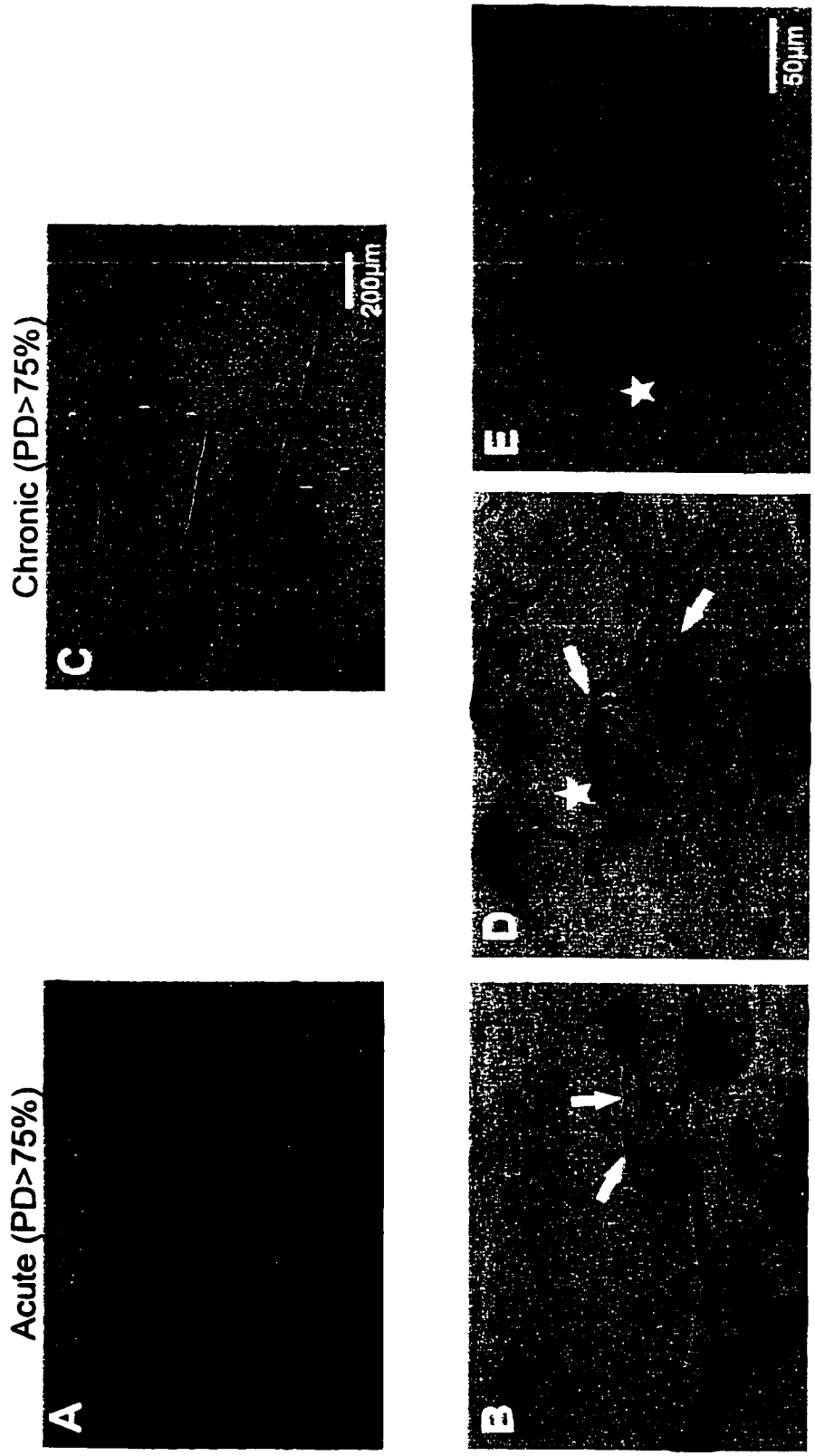

Acute (PD>75%)
Chronic (PD>75%)
A
B
C
D
E
200µm
50µm

Figure 4.12

Low (A, C and E) and higher (B, D and F) power combined Ag/AChE histochemical photomicrographs of 100μm thick cryostat longitudinal sections of contralateral control (A and B) and moderately denervated PL muscles (PD<75%) after FES (C and D) and moderately denerrvated PL muscle after wheeling exercise (E and F). There was no visually obvious differences in axonal branching pattern in moderately denervated PL muscle among all the experimental groups, as compared to normal caged activity (Fig. 4.10, C and D). Denervated endplates were reinnervated by sprouts (filled arrows) from remaining intact MUs. But motor endplates became more diffused (asterisks).

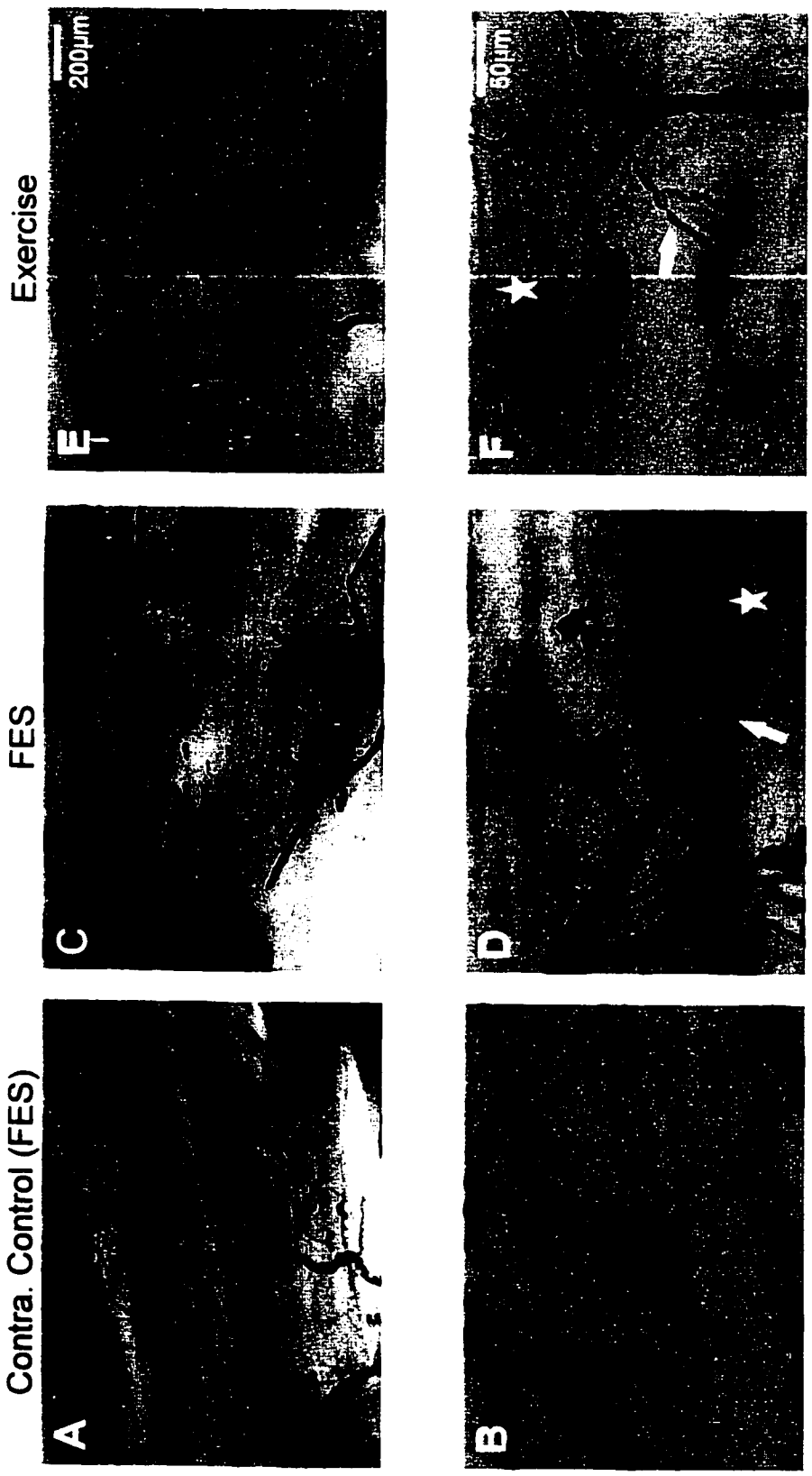
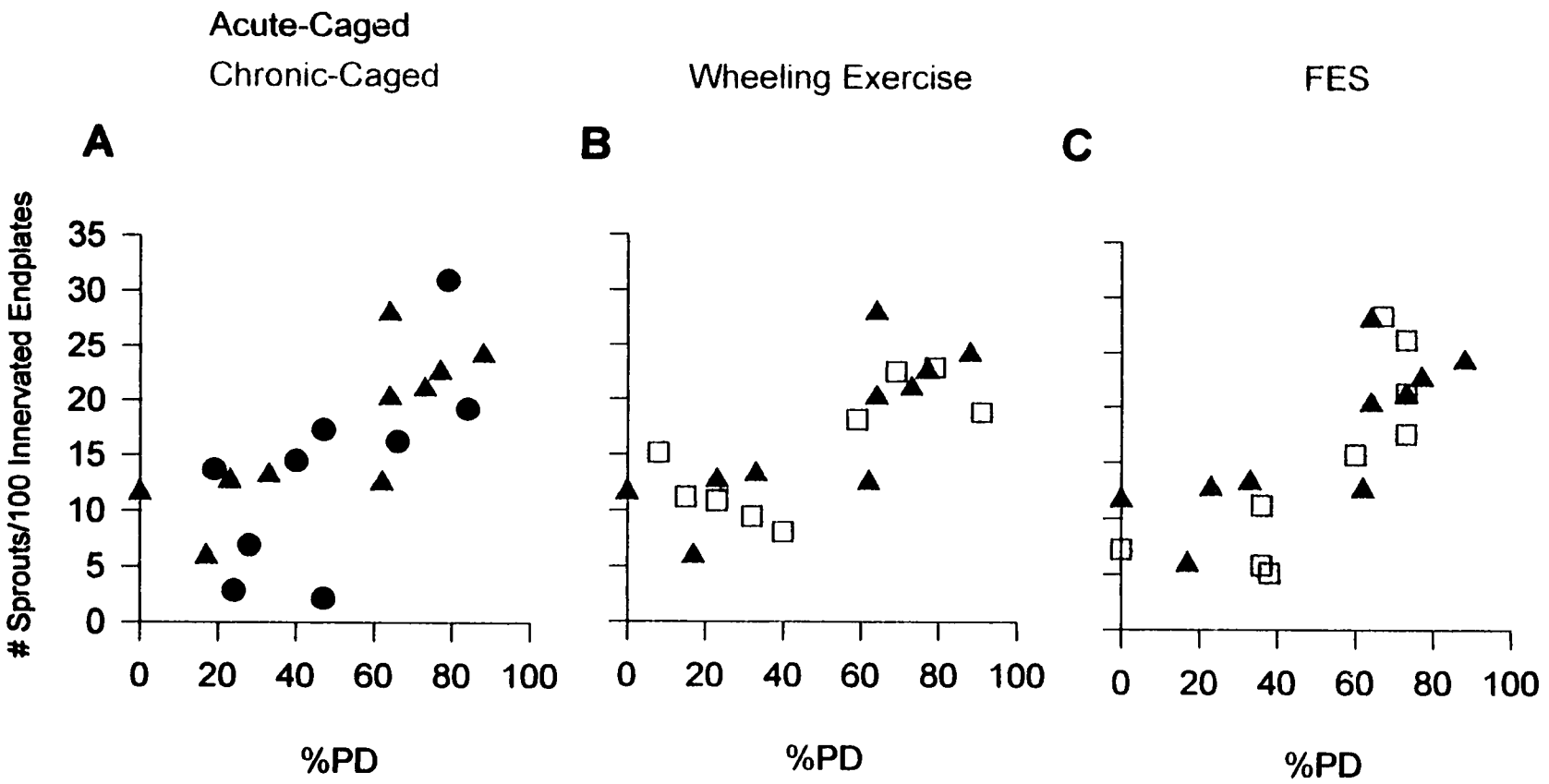

Contra. Control (FES)
FES
Exercise
A
B
C
D
E
F
200μm
50μm

Figure 4.13

Number of collateral sprouts per 100 innervated endplates of partially denervated PL and Sol muscles of rats experiencing normal caged activity during the acute phase (blue circles), as compared to the chronic phase (red triangles), chronically denervated PL and SOL muscles after wheeling exercise (blue squares) and FES (purple squares) plotted as a function of percentage of PD. There was no significant differences in the number of collateral sprouts in moderately denervated muscles of rats experiencing normal caged activity between the acute phase and the chronic phase. Increased neuromuscular activity did not affect axonal sprouting in moderately denervated muscles.


Acute-Caged
Chronic-Caged
Wheeling Exercise
FES
A
B
C
Sprouts/100 Innervated Endplates
35
30
25
20
15
10
5
0
0
20
40
60
80
100
%PD

CHAPTER 5

5. Effect of Muscle Paralysis on Motor Unit Enlargement

5.1. Introduction

The results from the first two studies (chapter 3 and 4) demonstrated that increased neuromuscular activity significantly reduced axonal sprouting within 1 month of partial denervation and stability of chronically enlarged motor units (MUs) in the rat hindlimb muscle model of partial denervation. One may then suspect that neuromuscular inactivity, on the other hand, might promote axonal sprouting and stability of chronically enlarged MUs. This is supported by previous findings of Brown and Ironton (1977) that short-term muscle paralysis induced either by tetrodotoxin (TTX) or α-bungarotoxin (α-BTX) always induces axonal sprouting in normally innervated muscles (Holland and Brown, 1980). Later experiments of Connold and Vrbová (1991), however, showed that α-BTX blockade of neuromuscular activity significantly reduced MU enlargement in 2-10 month partially denervated soleus muscles. Neuromuscular activity blockade by α-BTX also failed to maintain the large MU size in neonatal muscles in the presence of partial denervation (Connold and Vrbová, 1990). They suggested that neuromuscular inactivity reduced MU enlargement by reducing the ability of

collateral sprouts to maintain the synaptic contacts with muscle fibers. Another possibility is that long term neuromuscular inactivity may have prevented nerve outgrowth simply due to lack of calcium influx at the nerve terminals. It has been demonstrated in numerous studies that neurite outgrowth depends on a narrow range of intracellular calcium concentration (Cohan et al., 1987; Mattson and Kater, 1987; Mattson et al., 1988; Kater et al., 1988, 1989; Connor et al., 1990; Collins et al., 1991; Kater and Mills, 1991; Rehder and Kater, 1992). Too low an intracellular calcium level resulted in neurite outgrowth arrest.

To address this issue, we have undertaken the present study to test the hypothesis that muscle paralysis by TTX blockade of sciatic nerve reduces MU enlargement during both the acute and chronic phase of axonal sprouting. We 1) chose to examine the effect of TTX on axonal sprouting and stability of chronically enlarged MUs on 4 functionally different muscles: tibialis anterior (TA), medial gastrocnemius (MG), plantaris (PL) and soleus (SOL) muscles, 2) used complementary force measurement and histochemical methods to quantitate MU enlargement and axonal sprouting, respectively, and 3) compared the effect of TTX on MU enlargement during the acute and chronic phase of axonal sprouting.

5.2. Experimental design

In 77 female Sprague Dawley rats, either the L4 (n=34) or L5 (n=31) spinal root was evulsed under surgical anesthesia (sodium pentobarbital administered intraperitoneally as 0.07ml/g body weight) and using sterile procedure (see section 2.1.). Cutting L4 or L5 resulted in extensive denervation of TA and MG muscles, respectively and a range of partial denervation in the SOL and PL muscles. Either immediately (ACUTE) or 11 months later (CHRONIC), the partially denervated muscles were subjected to 4 week program of 1) normal caged activity (ACUTE: n=20, from chapter 3; CHRONIC: n=20, from chapter 4), or 2) muscle paralysis by chronic TTX (80μg per ml) administration via an implanted miniosmotic pump to sciatic nerve at the rate of 2.5μl per hour (ACUTE: n=13; CHRONIC: n=12). The unoperated muscles of the left side hindlimb of the experimental rats was used to serve as contralateral control and 12 unoperated rats were used as normal control (ACUTE: n=6, from chapter 3; CHRONIC: n=6, from chapter 4).

Within 1 month, muscle twitch and tetanic forces were recorded *in vivo* in isolated TA, MG, SOL and PL muscles in response to stimulation of the sciatic nerve and MU forces were recorded in response to stimulation of teased filaments of the exposed L4 and L5 ventral roots (see section 2.3). Twelve micrometer thick cryostat cross sections of fresh TA and MG muscles were stained for acid or alkaline-myosin ATPase (see section 2.4.) and muscle fiber cross-sectional area was measured from these sections. PL and SOL muscles

were fixed in 4% formalin and cut into 100μm thick cryostat longitudinal sections on which combined silver/cholinesterase (Ag/AChE) histochemical staining was done (see section 2.4.). The number of collateral sprouts and free endplates were counted from the sections.

5.3. Results

No significant difference was found between the contralateral control and normal control for all parameters examined. Thus, the results from both groups were collated and used as the overall control.

5.3.1. Effect of muscle paralysis on MU enlargement in partially denervated muscles during the acute phase of sprouting

As shown in chapter 3 for TA and MG muscles where section of spinal roots L4 or L5 removed more than 80% of their innervation, mean MU twitch force in the extensively denervated muscles increased about 2 to 3 fold on average with a significant increase in mean MU twitch force after normalization for muscle fiber CSA (Fig. 5.1). In moderately denervated PL and SOL muscles where section of spinal roots L4 or L5 removed more than an average of 50% innervation, mean MU twitch force increased significantly by about 1.5 fold (Fig. 5.2.). Effects of TTX blockade of neuromuscular activity on axonal sprouting were examined in the extensively denervated TA and MG muscles where mean

numbers of remaining MUs were 8±1 in TA muscle and 9±1 in MG muscle, and in the moderately denervated PL and SOL muscles where mean numbers of remaining MUs were 22±2 in PL muscle and 12±2 in SOL muscle. The numbers of MUs were not significantly different from that in the partially denervated muscles in rats which experienced normal caged activity.

TTX blockade of neuromuscular activity severely reduced MU enlargement in both extensively denervated TA and MG muscles and moderately denervated PL and SOL muscles. The cumulative distribution histograms were shifted far to the left of the partially denervated muscles in rats which experienced normal caged activity with a significant reduction in mean MU twitch forces (Fig. 5.1 and 5.2). There was a relative sparing of the fastest and largest MUs to the detrimental effects of TTX blockade of neuromuscular activity in extensively denervated TA and MG muscles. More detailed analysis of the cumulative frequency histograms which compare MUs from active and normal activity showed that the detrimental effect was most pronounced for the smaller MUs and less so for the MUs which developed larger forces.

Because whole muscle force is the product of MU number and MU force, whole muscle twitch force was expected to be reduced with the reduction in MU twitch forces by increased neuromuscular activity. With the exception of partially denervated SOL muscle, the muscle twitch forces of the partially denervated muscles after TTX blockade of neuromuscular activity, however, were not found

to be significantly reduced as compared to normal caged activity (Table 5.1). It could be explained by the fact that the larger and more forceful MUs were relatively spared in the extensively denervated TA and MG muscles, and relatively less detrimental effects on PL muscle. Therefore, the detrimental effect of TTX blockade of neuromuscular activity on axonal sprouting in these 3 muscles was not always detected by measuring the whole muscle force.

Since MU force is the product of IR, CSA and specific force of muscle fiber, and specific force does not change after reinnervation (Tötösy de Zepetnek et al., 1992; Fu and Gordon, 1995a,b), we normalized MU twitch force values for CSA in order for MU force to reflect IR. All muscle fiber CSAs were measured and the MU twitch forces corrected for in TA and MG muscles. The detrimental effect of TTX blockade of neuromuscular activity on MU enlargement in extensively denervated TA and MG muscles plotted in figure 5.1 reflects a reduction in IR directly and is not accounted for by the reduced muscle fiber size.

Because muscle fiber CSAs were not measured in PL and SOL muscles, the correction of MU and muscle twitch force for muscle fiber CSA was not made. Particularly for the few extensively denervated muscles which were examined histochemically in 100μm longitudinal sections (Fig. 5.5., C and D), atrophic denervated muscle fibers abounded and innervated muscle fiber diameters were obviously smaller. It is also known that muscle paralysis or disuse promotes atrophy in normally innervated muscles, consistent with the

atrophy in TTX-treated TA and MG muscles in the present study (Fig. 5.1). Thus, not having taken direct measurement of muscle fiber CSA might have exaggerated the detrimental effects of TTX blockade of neuromuscular activity on moderately denervated PL and SOL muscles.

5.3.2. Effect of muscle paralysis and quantitation of axonal sprouting in partially denervated muscles

Since 1) there was no significant differences in the relative proportion of different sprout types between PL and SOL muscles and, 2) we obtained similar range of the extent of partial denervation for both partially denervated PL and SOL muscles in each experimental group, we, therefore, grouped and considered the results from PL and SOL muscles together in each experimental group.

As shown for the partially denervated muscles in rats which experienced normal caged activity (data from chapter 3; Fig. 5.3), the number of collateral sprouts in 1 month moderately denervated (<75% partial denervation) muscles, which were inactivated by TTX, increased as a function of the extent of partial denervation (Fig. 5.3A). However, because of the limit of axonal sprouting, the number of free endplates also increased as a function of the extent of partial denervation (Fig. 5.3B). TTX blockade of neuromuscular activity did not appear to affect the number of collateral sprouts and free endplates in moderately

denervated muscles. It was only when partial denervation became extensive (>75% partial denervation) that detrimental effect of TTX blockade of reducing the axonal sprouting was evident.

Mean number of collateral sprouts and free endplates in normally innervated and partially denervated muscles (data from chapter 3), and the effects of TTX blockade are shown in figure 5.4. The number of collateral sprouts dramatically increased to more than 2 fold after extensive denervation (>75% partial denervation), and TTX blockade of neuromuscular activity dramatically reduced the number of collateral sprouts, indicating that axonal sprouting was significantly blocked by TTX.

Variability between animals in partial denervation and, more importantly, measurement of MU forces in PL and SOL muscles without correcting for changes in muscle fiber diameters as for TA and MG muscles might have over-exaggerated the detrimental effects of TTX blockade of reducing MU size and IR (Fig. 3.3). If MU sizes were corrected for the likely reduced muscle fiber CSA, this would be more consistent with the quantitation of collateral sprouts which showed that TTX blockade did not change the number of collateral sprouts in 1 month moderately denervated PL and SOL muscles (Fig. 5.3).

5.3.3. Effect of muscle paralysis and morphometric analysis of axonal sprouting in partially denervated muscles

Histochemical staining revealed the dramatic effect of TTX blockade of neuromuscular activity in reducing axonal sprouting and reinnervation of denervated endplates in extensively denervated PL muscle (Fig. 5.5). Both motor endplates and muscle fibers were obviously atrophic.

5.3.4. Effect of muscle paralysis on chronically enlarged MUs

As shown in chapter 4 and figures 5.6 and 5.7, for TA and MG muscles where more than 80% innervation was removed by section of L4 and L5 spinal roots, respectively, both electrophysiological and histochemical evidence showed that MU enlargement was significantly reduced at 12 months as compared to 1 month. These findings indicated that MU size declined with time, akin to post-polio syndrome. The time-related reduction in MU enlargement was, however, less pronounced in TA muscle, suggesting that post-polio syndrome was less severe in this muscle. For PL and SOL muscles where denervation was only moderate, time-dependent reduction in MU enlargement was not detectable (Fig. 5.2), suggesting that the post-polio like syndrome was less well established in these moderately denervated muscles.

Effects of 1 month TTX blockade of neuromuscular activity on chronically enlarged MUs were examined in the extensively denervated TA and MG muscles where mean numbers of remaining MUs were 7±2 in TA muscle and 14±3 in MG muscle, and in the moderately denervated PL and SOL muscles where numbers

of remaining MUs were 19±4 in PL muscle and 13±1 in SOL muscle. With the exception of partially denervated TA muscle in which the number of MUs was significantly smaller, the numbers of MUs were not significantly different from that in the partially denervated muscles in rats which experienced normal caged activity. When chronically denervated muscles were subjected to 1 month TTX blockade of neuromuscular activity at 11 months after partial denervation, there was relatively little effect (Fig. 5.6 and 5.7).

The whole muscle twitch force of the partially denervated muscles after TTX blockade of neuromuscular activity was also not significantly different as compared to normal caged activity (Table 5.1), as expected, with the exception of partially denervated TA muscle where the significantly lower muscle twitch force of partially denervated TA muscle could be explained by the significantly lower MU number.

5.3.5. Effect of muscle paralysis on number of collateral sprouts in chronically denervated muscles

Numbers of collateral sprouts were counted in longitudinal muscle sections. Since 1) there was no significant differences between PL and SOL muscles in the number of collateral sprouts and, 2) the extent of partial denervation was the same in both partially denervated PL and SOL muscles in

each experimental group, we grouped and considered the results from PL and SOL muscles together in each experimental group.

There appeared to be no difference in the number of collateral sprouts between the acute and chronic phase of sprouting in partially denervated muscles in rats which experienced normal caged activity (data from chapter 4; Fig. 5.8A). This indicated that the time-related reduction in MU size seen for the extensively denervated TA and MG muscles was not evident in moderately denervated PL and SOL muscles. Even 1 month period of TTX blockade of neuromuscular activity did not affect the number of collateral sprouts in these moderately denervated muscles (Fig. 5.8B).

5.4. Discussion

There are two major findings in this study. (1) TTX blockade of neuromuscular activity during the acute phase of axonal sprouting significantly compromises axonal sprouting and MU enlargement in extensively but apparently not in moderately denervated muscles. (2) TTX blockade of neuromuscular activity does not appear to have detrimental effects on chronically enlarged MUs.

5.4.1. Detrimental effect of TTX blockade of neuromuscular activity on axonal sprouting after extensive denervation but not moderate denervation

Using both electrophysiological and histochemical techniques, we have demonstrated in the present study that TTX blockade of neuromuscular activity significantly reduced MU size in extensively denervated muscles. We have also found that the detrimental effect was not dependent on muscle types, but dependent on the extent of partial denervation. TTX blockade of neuromuscular activity significantly reduced MU size in extensively denervated TA and MG muscles, and the number of sprouts and increased the number of denervated endplates in PL and SOL muscle when partial denervation was extensive. The detrimental effects was, however, not evident in PL and SOL muscles in which average denervation was less than 75%.

The striking finding of the present study that the detrimental effect of muscle paralysis induced by TTX in reducing MU enlargement and axonal sprouting was evident in extensively denervated muscles conflicts with findings of few previous studies. Short-term muscle paralysis induced either by TTX (Brown and Ironton, 1977) or α-BTX always promotes axonal sprouting in normally innervated muscles (Holland and Brown, 1980). However, in those experiments, the muscles were studied for only a short period of time (up to 1 week) after axonal sprouting was induced. Since it is the final expansion of MU territory in partially denervated muscles which determines the functional recovery of the muscles, the ultimate effect of neuromuscular inactivity on axonal sprouting could not be assessed accurately in the paralyzed muscle in these studies. Although TTX

promotes axonal sprouting in normally innervated muscles, our findings are quite consistent with those of Connold and Vrbová (1991) who demonstrated that axonal sprouting was significantly reduced in the partially denervated SOL muscles after a 2-10 month period of post-synaptic blockade of neuromuscular activity using α-BTX. Thus, TTX blockade is very effective in destabilizing formation of neuromuscular junctions by collateral sprouts. Connold and Vrbová (1990) showed that partial denervation failed to maintain the large MU size in neonates in the presence of α-BTX. This suggested that neuromuscular inactivity reduced MU enlargement by reducing the ability of newly formed sprouts to maintain the synaptic contacts with muscle fibers. Hence, TTX-induced paralysis reduced MU enlargement possibly by destabilizing synaptic contacts on muscle fibers.

5.4.2. No measurable effect of TTX blockade of neuromuscular activity on chronically enlarged MUs

We have demonstrated that a 1 month period of muscle paralysis induced by TTX, did not exacerbate the time-related reduction in MU enlargement in extensively and chronically denervated TA and MG muscles. MU size in moderately denervated PL and SOL muscles in which time-related reduction in MU enlargement was not detectable, was also not affected by TTX blockade. Thus, these results indicate that 1 month period of muscle paralysis induced by

TTX did not affect MU size of chronically enlarged MUs, irrespective of extent of partial denervation or the effect with time in reduction of MU size.

Surprisingly, there has not been any studies on the functional fate of enlarged MUs with muscle inactivity, particularly with reference to post-polio syndrome where exercise programs have been advocated. However, our earlier studies of the effects of increased neuromuscular activity by wheeling exercise and functional electrical stimulation on axonal sprouting in a number of partially denervated rat hindlimb muscles indicate that increased levels of neuromuscular activity further exacerbated the destabilization of chronically enlarged MUs in extensively denervated muscles, resulting in loss of nerve terminals and reduction in MU size (Tam et al. 1995, 1996; Archibald et al., 1996; also see chapter 4). We have suggested that, since exercise is an added metabolic stress, increased neuromuscular activity may simply impose more stress to the already exhausted and unstable nerve terminals, resulting in more nerve terminal withdrawal (chapter 4). In line with this rationale, neuromuscular inactivity could possibly prevent the over-exhaustion of already stressed and unstable nerve terminals. Thus may account for the lack of a time-related reduction in MU size. Nevertheless, our finding that TTX did not affect the MU size of chronically enlarged MUs is consistent with the findings of numerous developmental studies that diminished activity results in a reduction or cessation of synapse elimination

(Benoit and Changeux, 1975; Thompson et al., 1979; Brown et al., 1982; Greensmith and Vrbová, 1991).

5.4.3. Possible mechanisms accounting for the effects of TTX on axonal sprouting and chronically enlarged MUs

Successful sprouting requires 2 steps: 1) the availability of sprout-inducing stimuli and 2) subsequent nerve outgrowth. Both steps are affected by neuromuscular activity or inactivity. Evidence that 1) axonal sprouting is stimulated in normally innervated muscle fibers which are inactivated by BuTX (Brown et al., 1982a), and 2) direct electrical stimulation of muscle fibers which are inactivated by TTX inhibits axonal sprouting (Brown and Holland, 1979) have suggested that muscle inactivity increases the availability of sprout-inducing factors derived from inactive muscle fibers. In partially denervated muscles in contrast, TTX blockade of nerve activity and in turns muscle activity reduced MU enlargement. The difference here is that with TTX both nerve and muscle are inactive not just muscle. So, inactive muscle promotes axonal sprouting (Brown and Ironton, 1977; Holland and Brown, 1980) but inactive nerve either inhibits process of axonal sprouting and/or the stability of the neuromuscular junctions formed by the collateral sprouts as suggested by Connold and Vrbová (1990, 1991). We cannot distinguish between these 2 possibilities. However, the first possibility must be considered in the context of the calcium influxes that normally

occur in the growing collateral sprouts, the terminal Schwann cells and the collateral sprout-innervated endplates.

Muscle inactivity induced by TTX in the present study would reduce the influx of calcium into nerve terminals, Schwann cells and muscles. Adequate intracellular calcium concentration has been repeatedly shown, in *in-vitro* and *in-vivo* studies, to be critical for nerve outgrowth (Cohan et al., 1987; Mattson and Kater, 1987; Mattson et al., 1988; Kater et al., 1988, 1989; Connor et al., 1990; Collins et al., 1991; Kater and Mills, 1991; Rehder and Kater, 1992). Reduced intracellular calcium level caused by calcium channel blockers has been shown to stop nerve outgrowth. Recent findings that Schwann cells respond to neuromuscular activity by electrical or receptor-mediated elevations of intracellular calcium level (Jahromi et al., 1992; Reist and Smith, 1992; Reynolds and Woolf, 1993; Lev-Ram and Ellisman, 1995). The responses of terminal Schwann cells to ACh released from nerve terminals are essential for their normal function. Reduced level of neuromuscular activity in partially denervated muscles by TTX may therefore perturb the ability of Schwann cells to migrate, form processes and guide sprouting axons to denervated endplates.

If TTX blocks calcium influx completely, it would have reduced MU enlargement and axonal sprouting in all partially denervated muscles during the acute phase of sprouting, regardless of the extent of partial denervation. We, however, found that TTX blockade of neuromuscular activity detrimentally reduced

MU enlargement and axonal sprouting only in extensively denervated muscles. Thus, the extent of partial denervation and the distance of growth stimuli from innervated to denervated muscle fibers may be contributing factors to the relative degree of availability of sprout-inducing stimuli to nerve outgrowth. The idea may perhaps be that the higher number of denervated muscle fibers in extensively denervated muscles would require more extensive sprouting and require collateral sprouts to extend further away in order to reinnervate denervated muscle fibers. Topological analysis of the positions of collateral sprouts upon partial denervation have shown that they occur in a very close range, up to 200μm from denervated muscle fibers, suggesting that sprout-inducing stimuli are possibly available within a short range (Brown et al., 1978, 1980; Slack and Pockett, 1981; Pockett and Slack, 1982). In other words, extensive denervation requires more extensive sprouting and increases the distance between the short-range diffusible sprout-inducing stimuli and intact axons. Thereby remaining axons in intact MUs will become progressively remote from sprout-inducing stimuli from distant denervated muscle fibers. As a result, extensive denervation may exacerbate the effects of TTX of inhibiting nerve outgrowth by progressively distancing the availability of sprout-inducing stimuli from denervated muscle fibers. Thus, the final outcome would be a progressive reduction of MU enlargement and axonal sprouting in extensively denervated muscles.

Findings that TTX had no effect on chronically enlarged MUs indicate that the stabilization of long-term nerve-muscle contacts in contrast to short-term was not affected by TTX blockade.

5.5. Conclusion

Using 4 functionally different muscles and both electrophysiological and histochemical techniques, we, in the present study, have shown that the detrimental effect of TTX blockade of neuromuscular activity in reducing MU enlargement depends on the extent of partial denervation of muscles. the detrimental effect of TTX blockade of neuromuscular activity in reducing MU enlargement during the acute phase of sprouting was only evident in extensively denervated, but not moderately denervated muscles. We have also shown that TTX blockade of neuromuscular activity did not have measurable effect on chronically enlarged MUs.

Our findings that TTX-induced paralysis counteracts the axonal sprouting which compensates for partial denervation, demonstrates that neuromuscular inactivity is as detrimental as increased levels of neuromuscular activity to MU enlargement.

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Figure 5.1

Cumulative frequency histograms of MU twitch force distributions in partially denervated TA (A and C) and MG (B and D) muscles of rats experiencing normal caged activity (open circles) as compared to control muscles (open squares), and partially denervated muscles with tetrodotoxin (TTX) treatment (green circles). With TTX treatment, MU twitch forces after normalized for muscle fiber CSA were significatnly reduced as shown by the leftward shift of the MU force distributions ($p<0.05$) with a significant reduction in mean MU twitch forces in both extensively denervated TA (from 67.4±7mN to 41.5±6.2mN, $p<0.001$) and MG muscles (from 60.2±4.5mN to 32.0±3.8mN, $p<0.0001$).

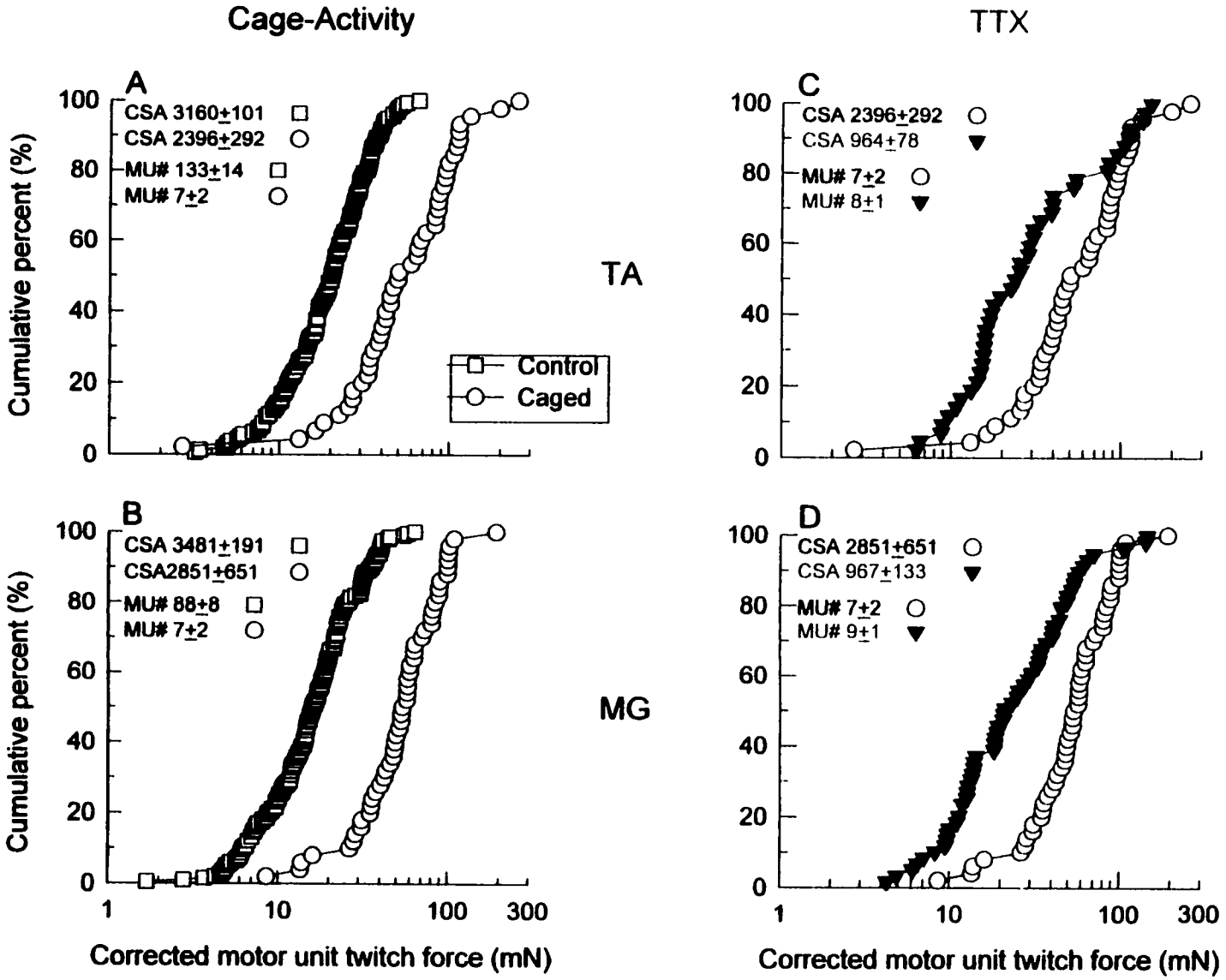
Cage-Activity
TTX
A
B
C
D
CSA 3160±101
CSA 2396±292
MU# 133±14
MU# 7±2
CSA 3481±191
CSA2851±651
MU# 88±8
MU# 7±2
CSA 2396±292
CSA 964±78
MU# 7±2
MU# 8±1
CSA 2851±651
CSA 967±133
MU# 7±2
MU# 9±1
Control
Caged
TA
MG
Cumulative percent (%)
Corrected motor unit twitch force (mN)
0
20
40
60
80
100
1
10
100
300

Figure 5.2

Cumulative frequency histograms of MU twitch force distributions in partially denervated PL (A and C) and SOL (B and D) muscles of rats experiencing normal caged activity (open circles) as compared to control muscles (open squares), and partially denervated muscles with tetrodotoxin (TTX) treatment (green circles). The detrimental effect of TTX treatment was seen as a leftward shift of the MU force ($p<0.05$) with a significant reduction in mean MU twitch forces from 28.0±1.2mN to 20.2±1.0mN in moderately denervated PL muscle ($p<0.0001$), and from 16.8±0.7mN to 9.3±0.6mN in moderately denervated SOL muscle ($p<0.0001$).

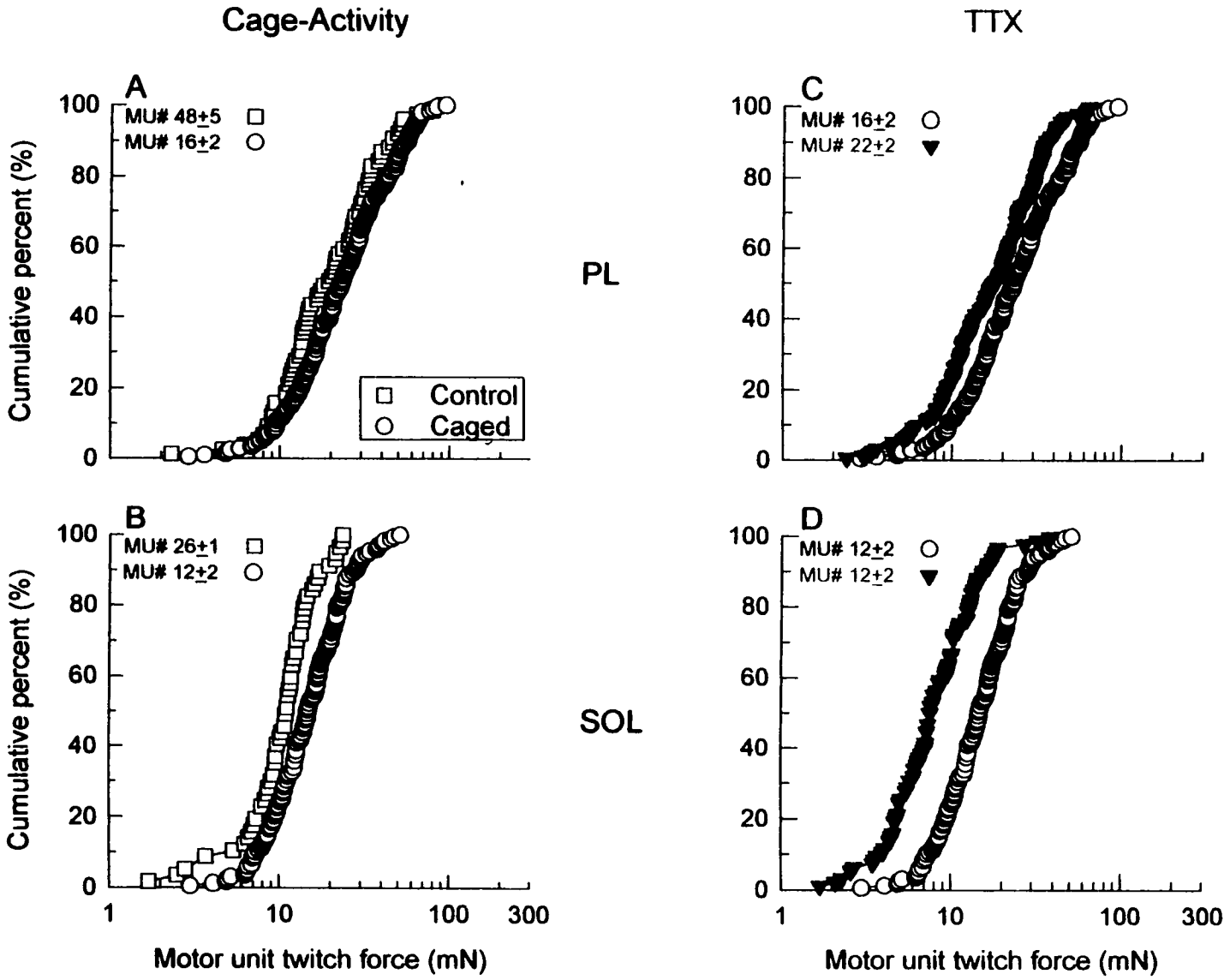
Cage-Activity
TTX
A
MU# 48±5
MU# 16±2
Control
Caged
C
MU# 16±2
MU# 22±2
PL
B
MU# 26±1
MU# 12±2
D
MU# 12±2
MU# 12±2
SOL
Cumulative percent (%)
Motor unit twitch force (mN)
0
20
40
60
80
100
1
10
100
300

Figure 5.3

Number of collateral sprouts per 100 innervated endplates (A and B), and number of free endplates per 100 endplates (C and D), of partially denervated PL and SOL muscles after normal caged activity (open circles) and TTX treatment (green circles) plotted as a function of percentage of partial denervation (PD). TTX treatment dramatically reduced the number of collateral sprouts and increased the number of free endplates for PD>75%.

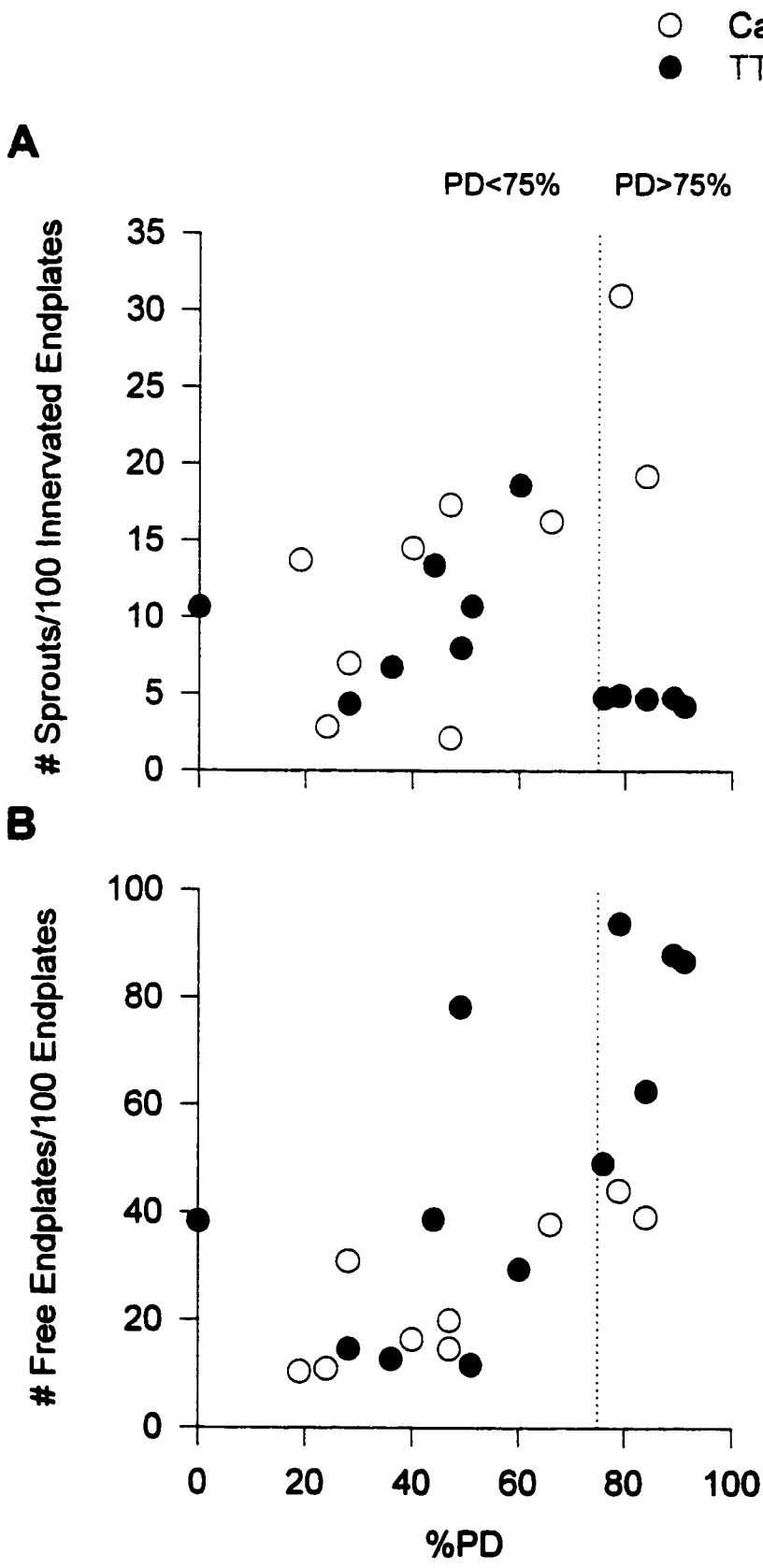
Caged-Activity
TTX
A
PD<75%
PD>75%
Sprouts/100 Innervated Endplates
35
30
25
20
15
10
5
0
B
Free Endplates/100 Endplates
100
80
60
40
20
0
0
20
40
60
80
100
%PD

Figure 5.4

Mean (±S.E.) number of collateral sprouts per 100 innervated endplates (A), and 100 free endplates per endplates (B) of control (open bars), moderately denervated (PD<75%; filled bars), and extensively denervated (PD<75%; hatched bars) PL and SOL muscles after normal caged activity (black) and TTX treatment (green). TTX treatment did not have significant effect on axonal sprouting for PD<75%, but dramatically reduced the number of collateral sprouts and hence increased the number of free endplates for PD>75%.

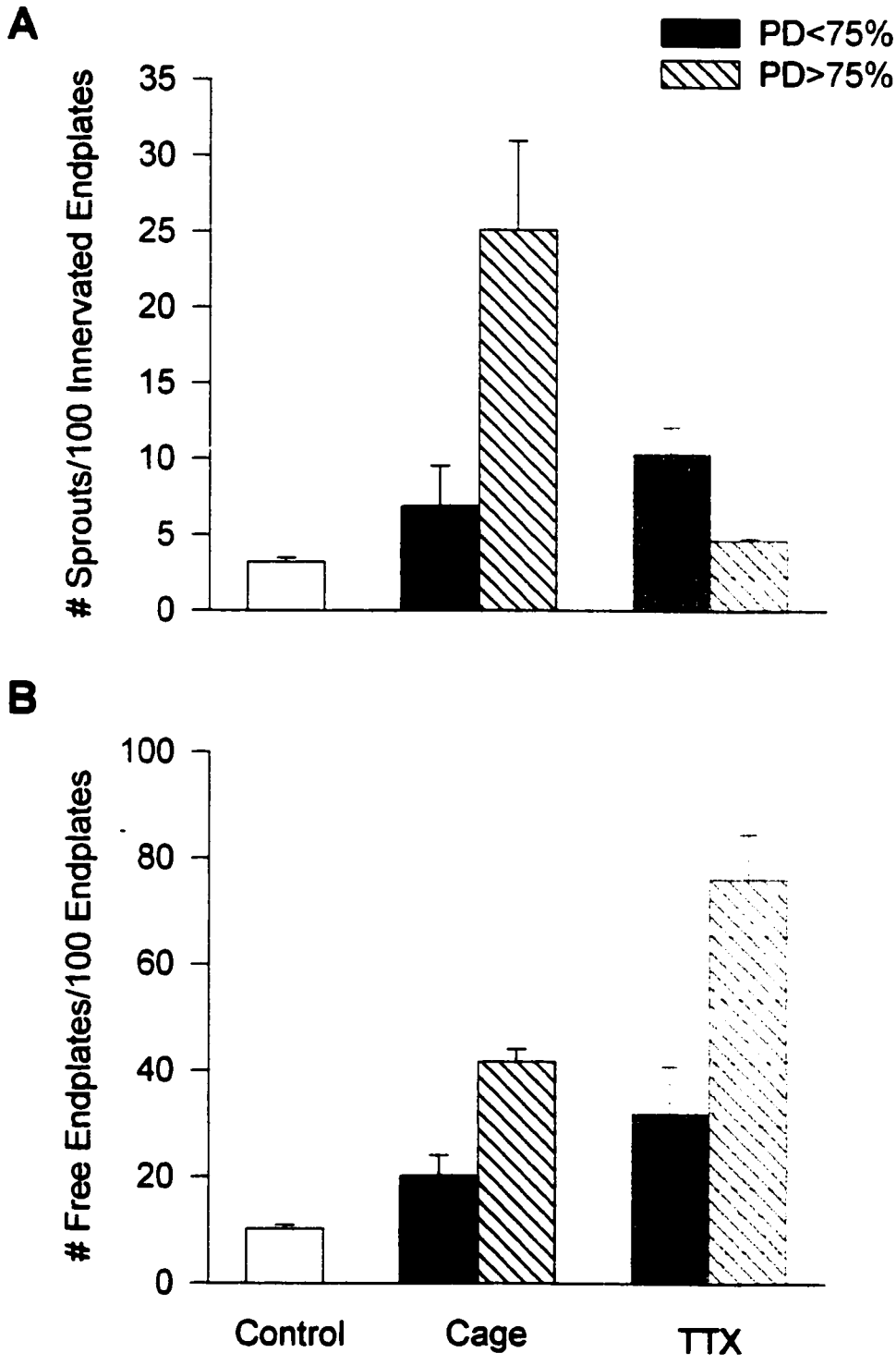
A
PD<75%
PD>75%
Sprouts/100 Innervated Endplates
35
30
25
20
15
10
5
0
B
Free Endplates/100 Endplates
100
80
60
40
20
0
Control
Cage
TTX

Figure 5.5

Low (A and C) and higher (B and D) power combined Ag/AChE histochemical photomicrographs of 100μm thick cryostat longitudinal sections of extensively denervated PL muscles (>75% PD) after normal caged activity (A and B), and TTX treatment (C and D). TTX blockade of neuromuscular activity dramatically reduced incidence of collateral sprouts and further increased the amount of free endplates. Both motor endplates and muscle fibers became atrophic. Filled arrows indicate collateral sprouts, and open arrows indicate free endplates.

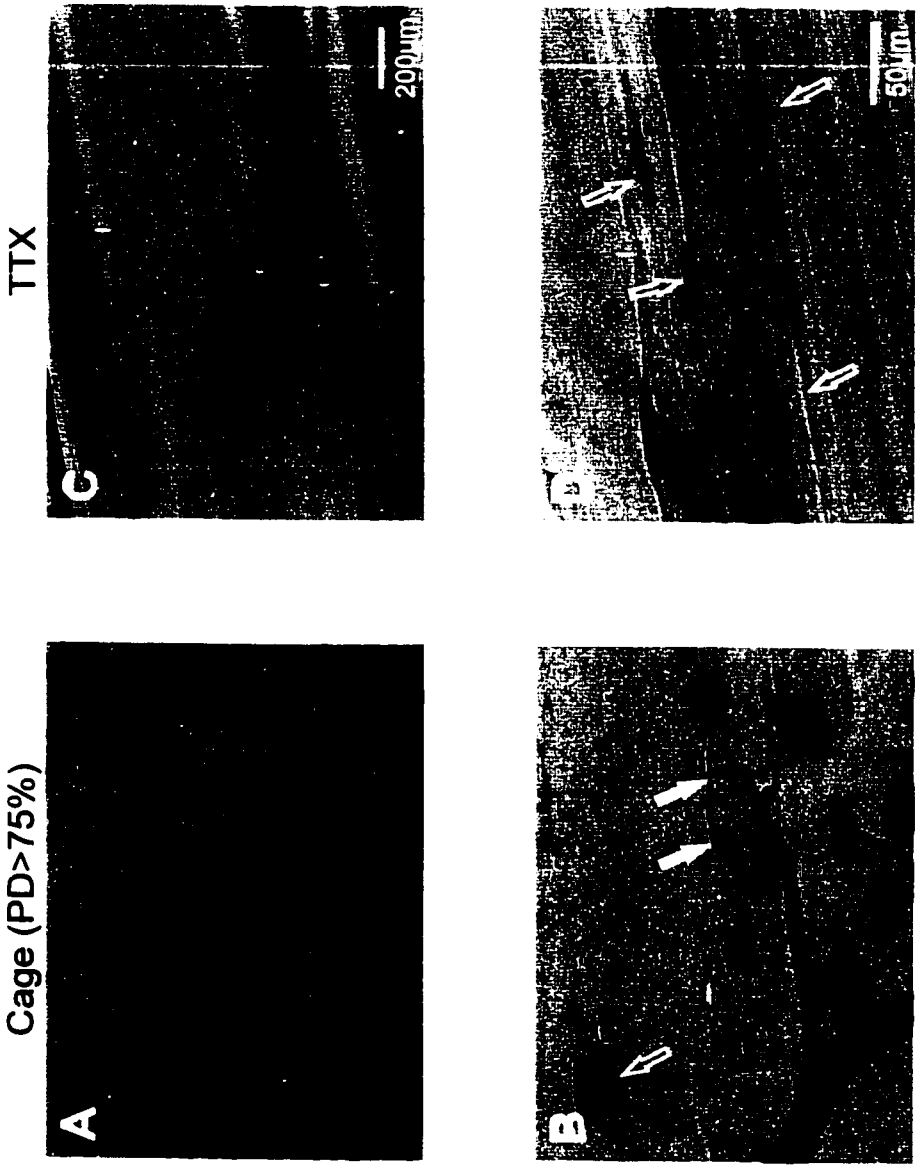
Cage (PD>75%)
TTX
A
B
C
200 µm
50 µm

Figure 5.6

Cumulative frequency histograms of MU twitch force distributions after normalization for CSAs in partially denervated TA (A and C) and MG (B and D) muscles of rats experiencing normal caged activity at 12 month PD (chronic, red triangles) as compared to 1 month PD (acute, blue circles) and chronically denervated muscles after TTX treatment (green squares). One month period of TTX treatment did not affect the time-related reduction in MU size in extensively denervated TA and MG muscles. Mean MU twitch forces of extensively denervated TA and MG muscles were 51.3±3.8mN and 45.3±3.5mN respectively during the chronic phase, and were 42.5±4.3mN and 52.6±5.6mN respectively after TTX treatment.

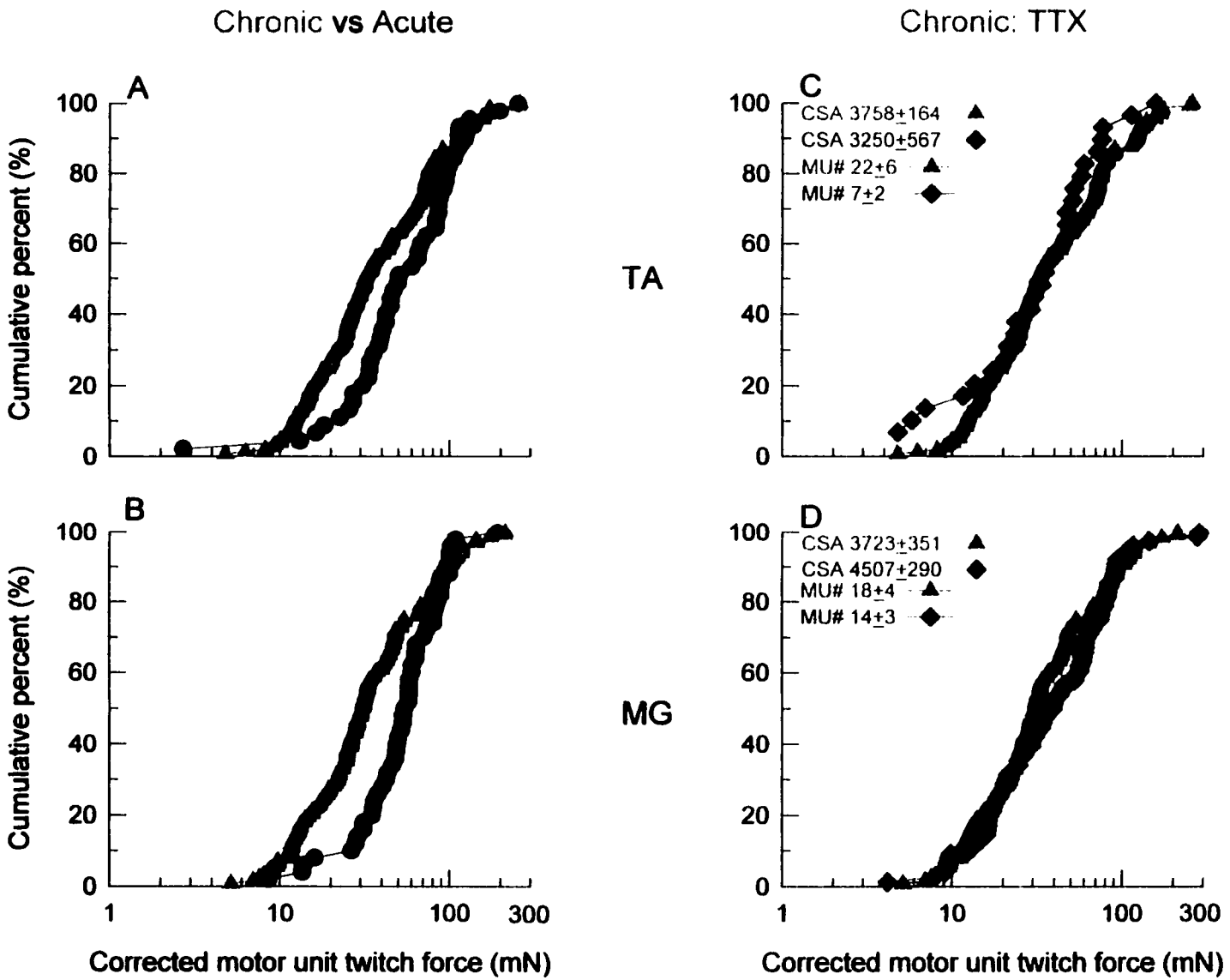
Chronic vs Acute
Chronic: TTX
A
B
C
D
TA
MG
Cumulative percent (%)
Corrected motor unit twitch force (mN)
CSA 3758±164
CSA 3250±567
MU# 22±6
MU# 7±2
CSA 3723±351
CSA 4507±290
MU# 18±4
MU# 14±3
0
20
40
60
80
100
1
10
100
300

Figure 5.7

Cumulative frequency histograms of MU twitch force distributions in partially denervated PL (A and C) and SOL (B and D) muscles of rats experiencing normal caged activity during the chronic phase (red triangles), as compared to acute phase (blue circles) and chronically denervated muscles after TTX treatment (green squares). One month period of TTX treatment did not affect size of MUs in moderately denervated PL and SOL muscles. Mean MU twitch forces of moderately denervated PL and SOL muscles were 27.7±1.4mN and 18.3±1.3mN respectively during the chronic phase, and were 26.7±2.2mN and 17.6±0.9mN respectively after TTX treatment.

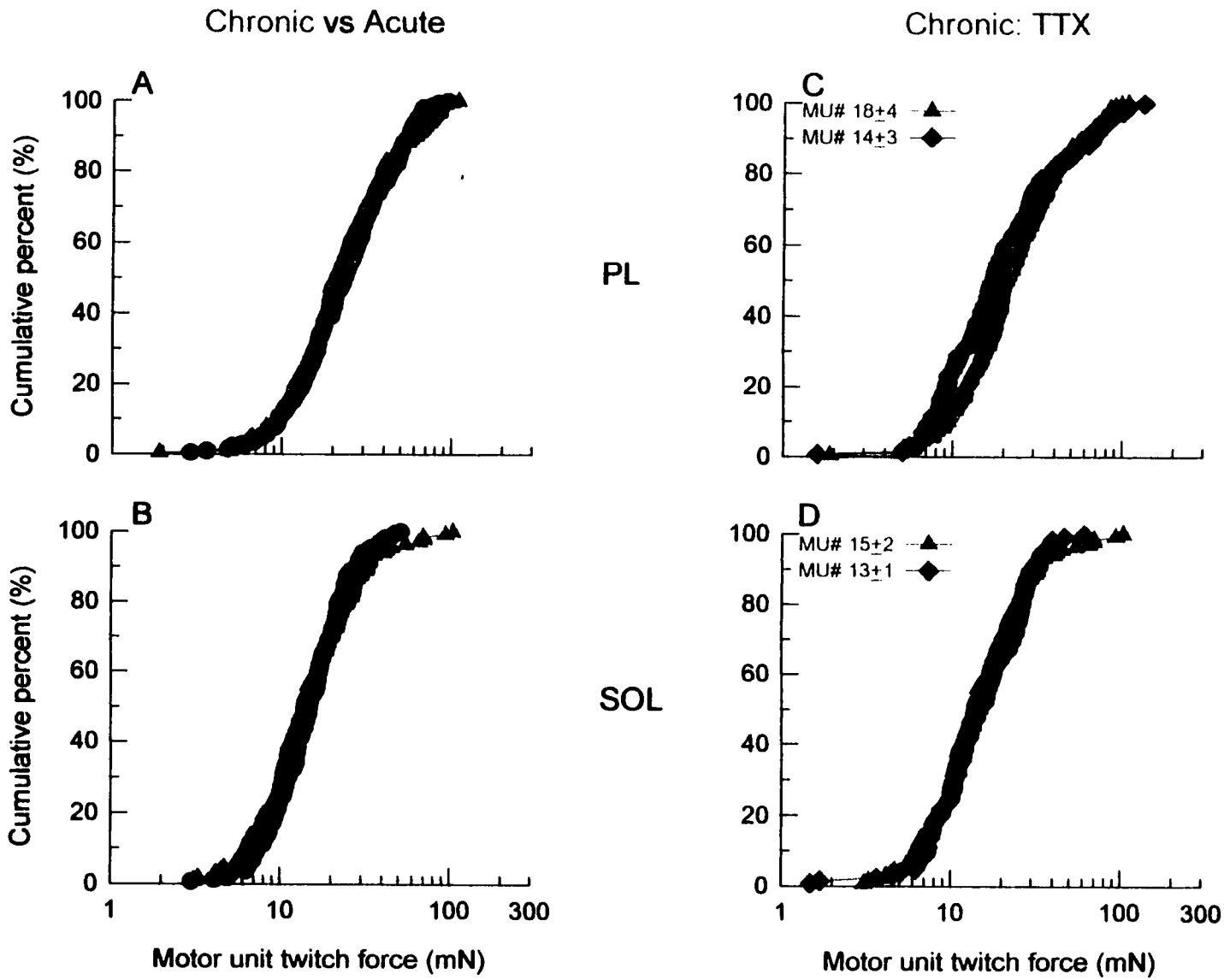
Chronic vs Acute
Chronic: TTX
A
B
C
D
PL
SOL
Cumulative percent (%)
Motor unit twitch force (mN)
MU# 18±4
MU# 14±3
MU# 15±2
MU# 13±1
0
20
40
60
80
100
1
10
100
300

Figure 5.8

Number of collateral sprouts per 100 innervated endplates of partially denervated PL and Sol muscles of rats experiencing normal caged activity during the acute phase (blue circles), as compared to chronic phase (red triangles) and chronically denervated PL and SOL muscles after TTX treatment (green squares) plotted as a function of percentage of PD. One month period of TTX treatment had no significant effect on the number of collateral sprouts in moderately and chronically denervated muscles.

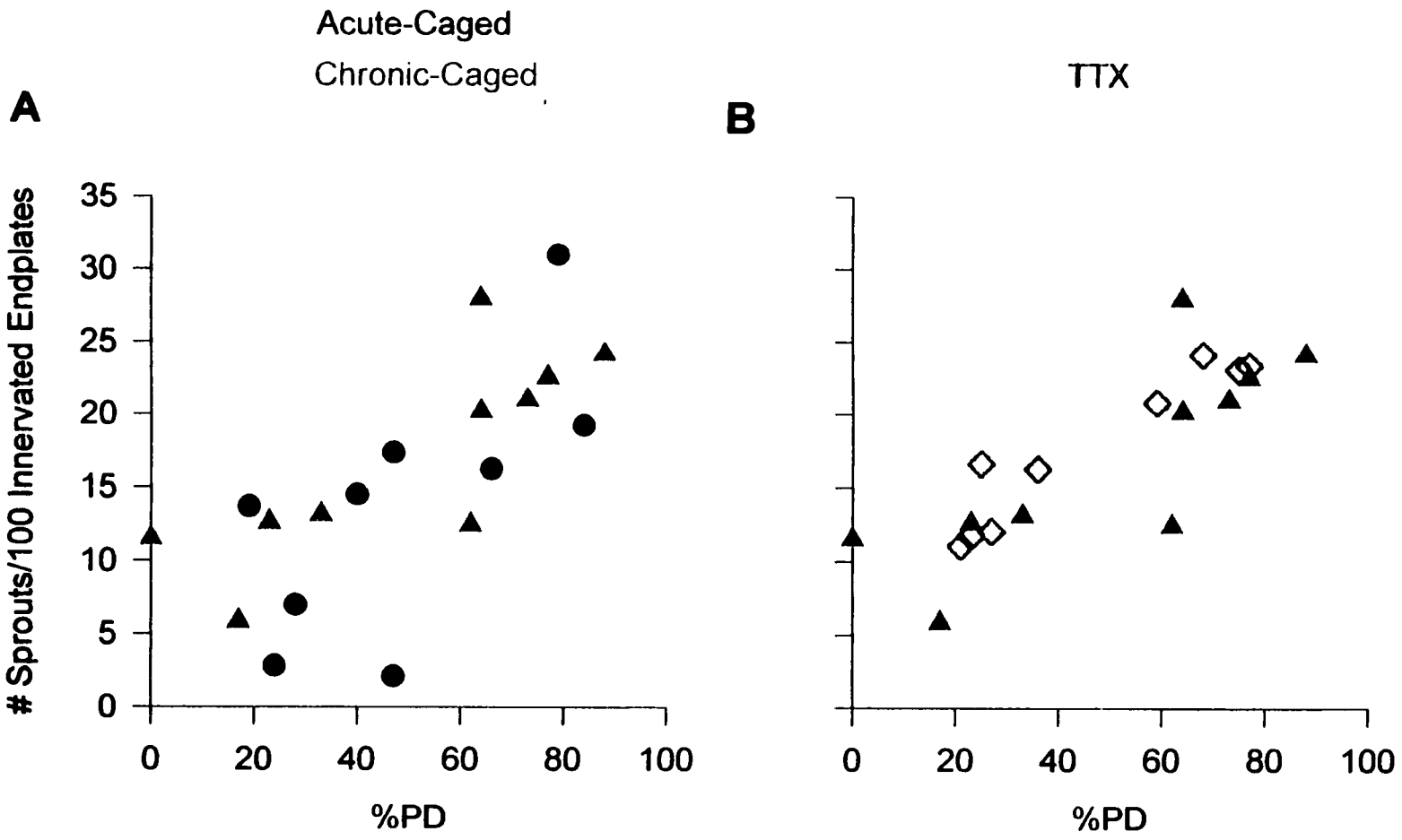
Acute-Caged
Chronic-Caged
A
B
TTX
Sprouts/100 Innervated Endplates
0
5
10
15
20
25
30
35
0
20
40
60
80
100
%PD
0
20
40
60
80
100
%PD

Table 5.1

Summary of mean muscle twitch forces in control muscles, and partially denervated muscles of rats experiencing 4 weeks of normal caged activity (PD) and with TTX treatment (TTX+PD) immediately (acute) and 11 months (chronic) after PD. (*) and (**) indicate statistical significance as compared to corresponding control and partially denervated muscles with normal caged activity, respectively.

Mean Muscle Twitch Forces (mN)				
Acute				
Experimental Groups	TA	MG	PL	SOL
Control	1897±64	1744±78	747±38	256±13
PD	* 483±130 (p<0.0001)	* 401±71 (p<0.0001)	* 521±69 (p<0.0001)	* 194±22 (p<0.05)
PD+TTX	310±56	367±59	418±71	**113±19 (p<0.05)
Chronic				
Experimental Groups	TA	MG	PL	SOL
Control	2447±89	2135±87	960±50	274±17
PD	* 1026±227 (p<0.0001)	* 776±188 (p<0.0001)	608±65	260±22
PD+TTX	** 271±71 (0.0001)	678±132	453±98	229±28

CHAPTER 6

6. General Conclusions

6.1. Summary

Motor axonal sprouting takes place under conditions of loss of motoneurons for diseases such as poliomyelitis and amyotrophic lateral sclerosis (ALS) and partial nerve injuries to compensate for the functional loss (Wohlfart, 1957; McComas et al., 1971; reviewed by Miller, 1984; Halstead and Wiechers, 1987; Dengler et al., 1989; Grimby et al., 1989; Trojan et al., 1991). Despite the compensatory process, the prognosis of these conditions is rather disappointing. In cases such as ALS, the outcome is even deadly. In some cases such as post-polio syndrome, even the etiology is unclear. Management of patients with these conditions is also unclear.

Exercise regimen has been advocated because of the strong association of muscle exercise with strength and endurance (Einarsson and Grimby, 1987; Feldman and Soskolne, 1987; Milner-Brown and Miller, 1988; Einarsson, 1991). Although some positive effects have been reported, the issue of whether exercise regimes are beneficial remains contentious. A number of studies using animal models of partial denervation has been carried out in the past to examine

whether neuromuscular activity is good or bad for axonal sprouting (Hoffman, 1952; Brown and Holland, 1979; Gardiner et al., 1984; Gardiner and Faltus, 1986; Herbison et al., 1986; Michel and Gardiner, 1989; Connold and Vrbová, 1990, 1991; Einsiedel and Luff, 1994). To date, however, the evidence for any beneficial effect is controversial.

The purpose of this thesis project was to systematically and thoroughly re-examine whether neuromuscular activity is good or bad for motor unit (MU) enlargement and stability of chronically enlarged MUs, using animal models of partial denervation and both electrophysiological and histochemical techniques,.

We drew 3 main conclusions from our animal studies of partial denervation. 1) Increased neuromuscular activity by daily exercise or functional electrical stimulation reduces axonal sprouting within 1 month of partial denervation. 2) Chronically enlarged MUs become destabilized with time. The destabilization is exacerbated by increased neuromuscular activity after 11 month partial denervation. 3) Tetratodoxin (TTX) blockade of neuromuscular activity profoundly reduces axonal sprouting and establishment of enlarged MUs, but has little effect on chronically enlarged MUs.

The detrimental effects of increased neuromuscular activity in reducing axonal sprouting may arise from deleterious effects on 1) prohibiting the bridge formation of terminal Schwann cells (Love et al., 1997; Tam et al., 1998), 2) downregulating sprout-inducing stimuli from Schwann cells, 3) interferring with the

ability of intact axons to respond to denervated muscle-derived sprouting stimuli, and 4) directly inhibiting nerve growth due to excessive calcium influx. Since Schwann cells respond to neuromuscular activity by electrical or receptor-mediated elevations of intracellular calcium level (Jahromi et al., 1992; Reist and Smith, 1992; Reynolds and Woolf, 1993; Lev-Ram and Ellisman, 1995), increased neuromuscualr activity may cause excessive Schwann cell activation which in turns 1) prohibits bridge formation of terminal Schwann cell processes, 2) downregulate sprout-inducing stimuli from Schwann cells, and 3) interferring with the ability of intact axons to respond to denervated muscle-derived sprouting stimuli. Finally, since excessive intracellular calcium level has been shown to cause collapse of nerve growth cones and stop neurite growth, high levels of neuromuscular activity may simply overload sprout terminals with calcium, resulting in reduction of axonal sprouting (Cohan et al., 1987; Mattson and Kater, 1987; Mattson et al., 1988; Kater et al., 1988, 1989; Connor et al., 1990; Collins et al., 1991; Kater and Mills, 1991; Rehder and Kater, 1992).

With age, the instability of motor endplates and reduction in MU size became evident. Thus, the small but significant effect of increased neuromuscular activity of further exacerbating the time-related reduction in MU size demonstrated a role of increased level of neuromuscular activity in the progressive destabilization of chronically enlarged MUs, particularly with reference to the fact that exercise is an added metabolic stress.

TTX-induced muscle paralysis did not have the contrasting effects of preventing axonal sprouting but rather decreased it. Since too low a level of intracellular calcium has been shown to cause collapse of nerve growth cones and stop neurite growth, TTX blockade of neuromuscular activity may block access of sprout terminals to calcium which is critical for nerve outgrowth, resulting in reduction of axonal sprouting (Cohan et al., 1987; Mattson and Kater, 1987; Mattson et al., 1988; Kater et al., 1988, 1989; Connor et al., 1990; Collins et al., 1991; Kater and Mills, 1991; Rehder and Kater, 1992).

In summary, our findings suggest that too much or too little neuromuscular activity does not appear to be safe for patients suffering motoneuron disease and/or partial nerve injury which results in extensive motor unit (MU) loss. Our results indicate that only moderate levels of neuromuscular activity is appropriate particular with reference to its effects on axonal sprouting and stability of chronically enlarged MUs.

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