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The Effect of Partial Denervation on the Anterior
Tibialis Motoneuron Pool of Normal (C57BL/6J) and
Dystrophic (dy2j/dy2j) Mice.

by

Heidi A. Tissenbaum

A thesis submitted to the School of Graduate Studies of the
University of Ottawa in partial fulfillment of the requirements
for the degree, Master of Science in the Department of
Physiology, Faculty of Health Sciences.

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LIST OF ABBREVIATIONS

C57-	normal strain of black mouse (C57BL/6J)
dy2j-	dystrophic strain of the black mouse (C57BL/6J dy2j/dy2j)
dy/dy-	dystrophic strain of the ReJ 129 mouse
ReJ 129-	a normal strain of mouse
BDNF-	brain derived neurotrophic factor
CG-	ciliary ganglion
CN-	central nucleation
CT-	connective tissue
CTA-	control (normal) tibialis anterior muscle
DMD-	Duchenne muscular dystrophy
EDL-	extensor digitorum longus
FCU-	flexor carpi ulnaris
FDL-	flexor digitorum longus
FIR-	functional innervation ratio
ION-	isthmo-optic nucleus
HRP-	horseradish peroxidase
LG-	Limb-girdle muscular dystrophy
LMN-	lumbar motoneurons
MD-	Muscular dystrophy
MHC-	myosin heavy chain
NCAM-	neural cell adhesion molecule
NGF-	Nerve Growth Factor
PD-	partial denervation
PD/PE-	partial denervation with partial extirpation of TA
SMH-	Sick Motorneurone Hypothesis
TA-	tibialis anterior

ABSTRACT

Reinnervation of the tibialis anterior (TA) muscle was examined in both dystrophic (dy2j) and control (C57) mice following partial denervation. Partial denervation allows the possibility of examining the ability of axons to sprout collaterals and capture denervated muscle fibers, resulting in an increase in motor unit size. This has been previously documented in the normal animal but has yet to be reported in the dystrophic animal.

Attempts were made to determine motor unit sizes both physiologically and morphologically following partial denervation. There was little success with the physiological recording measurement of motor unit size. Morphologically, mean motor unit size can be quantified by the total number of innervated muscle fibers in TA divided by the number of *alpha* motoneurons supplying the TA. However, problems arose when determining this calculation. A unimodal distribution of the motoneuron sizes limited an accurate determination of the number of *alpha* motoneurons since there was no clear separation of the small (presumed *gamma*) and large (presumed *alpha*) motoneurons. The number of innervated muscle fibers could not be accurately calculated as well since there was no clear differentiation of the denervated and innervated muscle fibers. These two factors limited any accurate assessment of the sprouting ability of the motor nerves in dystrophic mice.

Experiments were performed to determine if selective reinnervation occurs following partial denervation using immunohistochemistry with antibodies to myosin heavy chains (i.e. different muscle fiber types).

There was little evidence of 'type-grouping' present in the TA following recovery. This may indicate that the partial denervation were not extensive enough to produce clear type-grouping, as seen following nerve section.

Using intramuscular injection of horseradish peroxidase, the number and sizes of motoneurons supplying the tibialis anterior were examined in both normal (C57) and dystrophic (dy2j) mice. After partial denervation, there was a significant decrease ($p \leq 0.05$) in the number of motoneurons supplying the tibialis anterior (TA). This was accompanied by a significant ($p \leq 0.05$) increase in the mean cross-sectional area of these motoneurons when compared to the control (unoperated) side. In 12 C57 mice, there was a mean of 140 ± 6 TA motoneurons with a mean cross-sectional soma area of $245 \pm 19 \mu\text{m}^2$. With partial denervation, the TA motoneuron supply was significantly reduced ($p \leq 0.05$) to a mean of 82 ± 10 motoneurons with a significant increase ($p \leq 0.05$) in the mean soma area to $299 \pm 16 \mu\text{m}^2$. In 12 dy2j mice, the control TA had a mean of 112 ± 3 motoneurons with a mean soma area of $235 \pm 18 \mu\text{m}^2$. With partial denervation, this was significantly reduced to 60 ± 5 motoneurons with a mean soma area of $292 \pm 18 \mu\text{m}^2$ on the operated side. It is possible that the increased cross-sectional area may be associated with the increased motor unit size known to occur with partial denervation. In an attempt to prevent this increase, experiments were performed involving partial denervation accompanied by partial extirpation (PD/PE) of TA. Following recovery, in 12 C57 mice, there was a mean of 142 ± 4 TA motoneurons with a mean soma area of $251 \pm 15 \mu\text{m}^2$ on the control side. With PD/PE, this number was significantly

reduced ($p \leq 0.05$) to 66 ± 5 TA motoneurons. However there was no significant change ($p \geq 0.05$) in the mean soma area on the operated side. With partial denervation accompanied by partial extirpation, the mean soma cross-sectional area was $256 \pm 19 \mu\text{m}^2$. In 12 dy2j mice, there was a mean of 114 ± 2 TA motoneurons with a mean soma area of $257 \pm 16 \mu\text{m}^2$ on the control side. With PD/PE, the TA motoneuron pool was significantly reduced ($p \leq 0.05$) to 55 ± 4 motoneurons with no significant change ($p \geq 0.05$) in mean soma area ($247 \pm 11 \mu\text{m}^2$) on the operated side.

The results suggest that there is a direct relationship between motoneuron area and the size of the peripheral field.

INTRODUCTION

1) RATIONALE FOR THE STUDY

a) Historical Perspective- Human Muscular Dystrophy

The etiology of the muscular dystrophies has been under extensive investigation in the past regarding a myogenic or neurogenic origin of the disease. The muscular dystrophies are a group of genetically inherited disease characterized by the progressive degeneration of muscle fibers. In the early stages of the disease, spontaneous degeneration of the muscle fibers occurs accompanied by the regeneration of new muscle fibers. However, as the afflicted individual ages and the disease progresses, the capability of the muscle to regenerate is greatly reduced. Dystrophic muscles will slowly deteriorate and the so-called symptoms of "muscle wasting" occur. This has been observed in muscle biopsies of patients afflicted with Duchenne muscular dystrophy (Mastaglia and Kakulas 1969) and in dystrophic mice of both the dy^{2j} strain (Montgomery and Swenarchuk 1978; Parry and Parslow 1981) and the dy/dy strain (Michelson et al. 1955; Rowe and Goldspink 1969).

When muscular dystrophy was first described by Meryon (1852), it was described as a purely myopathic disease. Meryon, a physician, reported his clinical observations of his brothers afflicted with muscular dystrophy. The disease was described as being hereditary, affecting predominantly males with no signs of neural abnormality upon post-mortem evaluation. Several years later, Duchenne (1861) described the symptoms of the disease that bears his name.

The histopathological description of dystrophy by Erb in 1883, was so clear that it is still relevant to date. Although the disease was still described as a myopathy, there was evidence for some sort of neural involvement since there was a selectivity of the muscles afflicted by the disease.

It was many years later that the pure myopathic basis of this disease came into question. McComas et al. (1971c) developed the Sick Motorneurone Hypothesis which described several peripheral nervous system disorders as primary neuropathies as opposed to primary myopathies. They stated that muscular dystrophy and other related diseases typically characterized by muscle wasting or weakness were not a primary disorder of the muscle but rather a problem of a dysfunctional, or 'sick' motoneuron, resulting in a loss of motor units.

A definition of the motor unit would be appropriate at this time. Sherrington (1925) first defined the motor unit as an *alpha* motoneuron along with its motor axon and all the muscle fibers that this axon innervates. In the Sick Motorneurone Hypothesis, the *alpha* motoneuron could potentially exist in three distinct states. These are 'healthy', 'dead' and 'sick'. In the 'healthy' state, the motoneuron has normal functioning connections with the muscle. In states such as partial denervation, where the nervous input supplying a muscle is reduced, the remaining normal motoneurons can respond by sprouting collaterals from their motor axons. The collateral branches can then establish successful synaptic connections with the now denervated muscle fibers resulting in an increase in motor unit size. Motoneurons in the 'dead' state no longer

have any influence on the muscle fibers they originally innervated. The third state in which a motoneuron could potentially exist was called the 'sick' state. When motoneurons are 'sick', they are not capable of transmitting a signal across the neuromuscular junction. Although they maintain some degree of trophic control of the muscle fibers within their own motor unit, the 'sick' motoneurons lack the ability to increase their motor unit size through axonal sprouting. In later studies (McComas et al. 1975), it was suggested that muscle fibers innervated by 'sick' motoneurons would not be eligible for innervation by any surviving healthy motoneurons. This would continue for as long as some axoplasmic transport occurred from the 'sick' motoneuron.

McComas et al. reasoned that if these diseases were of myopathic origin and affected the muscle fibers in a random fashion, then one would predict that all motor units in the muscle pool would show signs of the disease. In the early stages of the disease, there should still be a normal number of motor units in diseased muscle but their mean size would be reduced. As the disease progressed, since more muscle fibers would degenerate, motor unit sizes would decrease until eventually some motor units would lose all their muscle fibers or become so small they could not be detected. The number of functioning motor units would now be decreased with the mean size of the remaining motor units decreasing as well.

However, if these diseases were peripheral nervous system disorders, motor axons would degenerate. As the disease progressed, the number of motor units would decrease in accordance with the loss of motor axons. The decrease in the number of motor axons should be accompanied by

an increase in motor unit size of the remaining motor axons through collateral sprouting (as seen in various Motoneuron Diseases).

The evidence that McComas et al. (1971c) obtained from patients afflicted with muscular dystrophy, did not reflect either of the two above situations. Using electrophysiological recordings, they were able to estimate the numbers and sizes of functioning motor units in a muscle. With this method, McComas et al. reported losses of functioning motor units in muscles from the dystrophic patients when compared with the normal subjects. The sizes of the remaining functioning motor units were identical or slightly lower than the sizes from normal muscle. Consequently, McComas and co-workers proposed the first neural hypothesis of muscular dystrophy whereby three states of the motoneuron existed as described above and accounted for the disease manifestations. With this hypothesis, the observations of reduced numbers of motor units could be explained by dead motoneurons. The remaining units would then exist in either the healthy or sick state. Since there were losses of units with no increase in motor unit size, the results indicated that the surviving axons could not sprout and capture the denervated muscle fibers suggesting that these motoneurons belonged to the 'sick' category.

In later studies on patients with muscular dystrophy, McComas (1977) found additional evidence of neural abnormalities associated with the disease. When a stimulus was applied to the muscle, a contraction was recorded. However, when the stimulus was applied indirectly via the nerve, the muscle fibers failed to respond. McComas referred to this state as "functional denervation". Muscle fibers in this state, still obtain

sufficient trophic influence from their axon (as opposed to completely denervated muscle fibers) but they are unable to maintain an electrophysiologically efficient synapse. Thus, the presence of functionally denervated muscle fibers adds to a neural basis or at least a neural abnormality in human muscular dystrophy.

b) Murine Muscular Dystrophy

Research efforts concerning the etiology of muscular dystrophy were at a clear disadvantage before the discovery of an experimental animal model. One of the laboratory animals that has proven to be a good model of a genetic type of dystrophy is the mouse. In 1955, Michelson et al. observed a genetic form of dystrophy in the ReJ 129 strain of mouse. This dy/dy form of dystrophy affected the mice so severely that they had a very short life span and could not reproduce thus providing some disadvantages when trying to maintain an active colony. This problem was alleviated in 1970 by Meir and Southard when they found a genetic type of dystrophy in the C57BL strain of mouse known as the dy2j/dy2j dystrophic mouse. These genetically dystrophic mice which are less severely affected and are able to breed, have been used extensively to provide information necessary to try and solve the problems of human dystrophy.

The first report of neural abnormalities in murine dystrophy was by McComas and Mrozek (1967). With intracellular electrodes, recordings were made from the tibialis anterior and gastrocnemius muscles of the dy/dy dystrophic mouse. When stimulation was applied indirectly, via the nerve, some of the muscle fibers failed to produce an action potential.

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However, when stimulation was applied directly to these muscle fibers, action potentials were produced. They concluded that these muscle fibers were denervated since neuromuscular transmission was absent in these muscle fibers. McComas and Mrozek (1967) suggested that the presence of the 'functionally denervated' muscle fibers could be a major factor contributing to the muscle weakness typically seen in muscle of the dystrophic mouse. This finding was later confirmed by Law and Atwood (1972).

A neural component to the etiology of murine muscular dystrophy was also reported when functional denervation of dystrophic muscle fibers was shown by Law et al. (1976) and Parry (1977). In the latter study, Parry determined the Functional Innervation Ratio (FIR) of both the slow-twitch soleus and the fast-twitch extensor digitorum longus (EDL) muscles of normal (C57) and dystrophic (dy2j) mice of various ages. The FIR was measured as follows:

$$\frac{\text{maximum twitch tension from nerve stimulation (indirect)}}{\text{maximum twitch tension from muscle stimulation (direct)}} \times 100$$

A decrease in the FIR was found in the EDL and soleus muscles of the dy2j mice older than ten weeks. However, clear signs of the dystrophy are seen in these mice several weeks earlier. Parry concluded that the study supported the presence of a neural abnormality associated with the disease but that this may be secondary to a primary myopathy.

With the suggestion that functional denervation was important in

the pathogenesis of murine muscular dystrophy, Harris and Ribchester (1978, 1979) examined the EDL muscle of normal (ReJ 129) and dystrophic (dy/dy) mice. Physiological recordings were performed to determine if neuromuscular transmission was present in these muscles. This was accompanied by intracellular staining of the muscle fibers being stimulated. They found that of 24 muscle fibers of the dystrophic mouse in which neuromuscular transmission occurred, 19 displayed morphological abnormalities upon microscopic visualization. They concluded that a peripheral block of neuromuscular transmission, as in 'functional denervation', could not be the cause of the pathological state of the muscle fibers in murine dystrophy.

Although these discrepancies in the results may be partly attributable to strain differences in the mice (dy/dy versus dy2j/dy2j), the main reason for these differences has yet to be answered.

Parry and Melenchuk (1981) evaluated the reinnervation process in both fast twitch (EDL) and slow twitch (soleus) muscles of control (C57) and dystrophic (dy2j) mice at various periods following nerve section. In the C57 mice, full recovery was observed within six weeks, whereas, in the dystrophic mice, recovery was somewhat impaired. Rates of reinnervation were reduced in both young and old dystrophic mice when compared to age-matched control mice. In the older dystrophic mice, recovery from nerve section was still incomplete even twelve weeks after nerve section while in the young dy2j mice, recovery seemed to be almost complete at six weeks. Although from these results one cannot show direct proof of a neurogenic origin of murine dystrophy, Parry and Melenchuk

(1981) concluded that the reinnervation process is impaired in dy2j mice. The problem in interpreting these results is that the study did not contain any measurement of motor unit sizes. Thus, any similarities between murine dystrophy and the situation described by the Sick Motorneurone Hypothesis (McComas et al. 1971C) in human muscular dystrophy could not be conclusive.

Desypris (1986) performed a similar series of experiments and measured motor unit numbers and sizes. Following soleus nerve section at six months of age, motor units were measured physiologically, by ventral root splitting in both dy2j and C57 mice. In C57 mice, there was complete reinnervation of the soleus one month after surgery with the number of motor units restored to control levels. However, in the dy2j mice this was not so; a reduced number of motor units was observed in the operated soleus muscle. Thus, at least a partial explanation for the impaired reinnervation reported previously by Parry and Melenchuk (1981) may be found in the reduced number of axons that are able to grow back and reinnervate the muscle.

Interpretation of these results requires some attention. The impaired reinnervation observed in the dy2j mouse may be simply due to factors such as the formation of a neuroma in the path of the axon that blocked further successful nerve regeneration (Scott 1986) and may not reflect an impaired relationship between the nerve and the muscle fibers. Therefore, to further examine the impaired reinnervation associated with muscular dystrophy, a technique such as partial denervation might be more informative. Partial denervation experiments, in which part of the nerve

supply is interrupted, allows the possibility of examining the ability of nerve axons to sprout and capture denervated muscle fibers thereby increasing their own motor unit size. An increase in motor unit size under these conditions has been shown to occur in the normal mouse (Brown and Irons 1978) but has yet to be examined in the dystrophic mouse. This technique provides some advantages for regeneration studies over nerve crush or nerve section since it eliminates the additional obstacles faced by the axon in trying to regain its peripheral field connections. With partial denervation, redirection of axon growth across a lesion does not create a problem since the intact motor axons are already "on site".

2) PARTIAL DENERVATION

a) Collateral Sprouting

Since it was first observed and recorded by Van Harreveld (1945), collateral sprouting in partially denervated muscle has been extensively studied. Collateral sprouting has been also noted in many other tissues such as brain (Raisman 1969, Lynch et al. 1977, and Morse et al. 1986), spinal cord (Goldberger 1972, Murray and Goldberger 1974), sympathetic ganglia (Murray and Thompson 1957) and skin (Weddell et al. 1941). Following partial denervation, a definite pattern has been described by Edds (1953) and later by Brown (1984). Immediately after partial denervation, the isometric tension produced by the affected muscle decreases which is directly due to the loss of motor axons. Within a few days, fine outgrowths emerge from both the nodes of Ranvier (nodal sprouting) and the motor nerve terminals (terminal sprouting) of the

remaining intact motor axons. Growth of these sprouts is directed towards the denervated endplates. The sprouts that form proper neuromuscular junctions with the denervated muscle fibers will become myelinated and the rest of the sprouts will disappear. When complete reinnervation has occurred, since one motoneuron innervates an increased number of muscle fibers, motor unit size increased. Complete reinnervation by collateral sprouting has been noted to occur within two weeks of partial denervation (Guth et al. 1980).

The response of motor axons to partial denervation was first documented in the early 1950s by Hoffman (1950) and Edds (1953). Motor axons can sprout in response to several conditions. Terminal sprouts arise from the intact nerve terminal and occur primarily when transmission of the neural signal is blocked or reduced such as in paralysis, botulinum toxin and partial denervation (Guth et al. 1980, Brown 1984). Terminal sprouts are further classified into preterminal and ultraterminal sprouts the former arising from the unmyelinated axons proximal to the nerve terminal and the latter from a part of an existing terminal (Barker and Ip 1966). Often, as in this thesis, the ultraterminal and preterminal sprouts are grouped together and called terminal sprouts.

As previously stated, sprouts may arise from the intramuscular nodes of Ranvier of the intact motor axons and are termed nodal sprouts. Nodal sprouts occur only in response to partial denervation (Guth et al. 1980, Hopkins and Slack 1980, Brown 1984).

✓
b) Stimulus for Sprouting

Over the past ten years, many studies have examined the source of the stimulus for nodal and terminal sprouting. Pockett and Slack (1982) were the first to suggest that the stimulus for terminal sprouting was a factor produced by the muscle. Gurney et al. (1986) provided the most convincing evidence for such a muscle-derived stimulus for terminal sprouting. They isolated a 56000 dalton muscle-derived protein and produced monoclonal antibodies against it. When the monoclonal antibodies against this muscle-derived protein were applied to the denervated neuromuscular junction, terminal sprouting was suppressed. Gurney et al. (1986) concluded that following either partial denervation or paralysis, at least part of the stimulus for terminal sprouting must come from the muscle. Terminal sprouting has also been shown to be suppressed by direct electrical stimulation of the denervated or paralysed muscle (Brown and Holland 1979, Brown et al. 1981b).

Nodal sprouting is known only to occur under conditions of partial denervation. It is not suppressed by direct electrical stimulation of the muscle (Brown et al. 1981b). Therefore, it seems that the molecular requirements for these two types of sprouting are different. Hoffman (1950) and later Hopkins and Slack (1981) suggest degeneration of the axon is necessary to produce a pathway for the nodal sprout to grow and therefore may be the stimulus for nodal sprouting. Degeneration of a nerve seems necessary since nodal sprouts are present following PD, but absent in response to paralysis and botulinum toxin poisoning.

c) Motor Unit Sizes Following Partial Denervation

Past studies suggest that following partial denervation, motor unit territory can increase up to five times. Van Harreveld (1945) performed partial denervation of the medial sartorius and the quadriceps, in both hindlimb muscles of the rabbit, by removal of the sixth lumbar segment (L₆), which supplies the majority of the innervation for both these muscles. After a two month recovery, the motor units of the fifth lumbar segment (L₅) had expanded their peripheral field to include the muscle fibers originally innervated by L₆. The sizes of the motor units in L₅ were shown to have increased on average to 4.5 times their normal size. Similar results have been found with partial denervation of the cat flexor digitorum longus (FDL) (Westerman 1978, Hatcher 1985), rat extensor digitorum longus (EDL) (Guth et al. 1980) and soleus (Thompson and Jansen 1977, Gorio et al. 1983) as well as mouse peroneus tertius (Brown and Ironton 1978) where it was also suggested that motor units can increase their size up to five times.

In the study of Hatcher et al. (1985), the relationship between the extent of denervation and the size of the sprouted units was examined. Cat flexor digitorum longus (FDL) muscles were partially denervated by sectioning one of the two ventral roots responsible for its innervation. The contribution of each root to the total motor innervation of the FDL was determined prior to the partial denervation so that the exact percentage of denervation was known in each animal. Following a recovery period of 100 days, motor unit sizes in the partially denervated FDL were

determined using the glycogen depletion technique (to be explained later). They found a positive correlation between the size of the remaining sprouted motor units and the percent of denervation of the muscle. This indicates that the increase previously shown in the motor unit sizes following partial denervation may not represent the absolute limit to the extent that a motor unit can increase. It seems that the number of motor units removed with partial denervation is important when the sizes of the motor units are examined after sprouting has occurred.

A functional limit to the expansion of an individual motor unit after partial denervation has been recently shown in a slow twitch muscle. Luff et al. (1988) examined the cat flexor digitorum longus (FDL), a fast-twitch muscle, and the soleus, a slow-twitch muscle, 100 days after partial denervation. In the soleus muscle, with denervations of more than 85%, force production from the muscle began to decline. This decrease became more apparent as the extent of the original denervation increased above this 85% value. This decrease in force production was accompanied by an increased number of small-diameter muscle fibers seen in histological examination of the soleus muscle. These small muscle fibers were presumed to be denervated. Luff et al. (1988) suggested that there is a limit to the amount a soleus motor unit can sprout. Nonetheless, if only 15% of the original innervation remains after partial denervation, the motor unit will still expand its peripheral territory without any apparent atrophic fibers. Thus according to Luff et al. (1988), the limit that the slow-twitch motor unit can increase in size is between six and seven times. In the FDL muscle, although partial denervations above 85% existed, there was

no decline in the force production in these units. Thus, conclusions as to a functional limit of motor unit expansion cannot be made with the FDL motor units.

It is worth noting that the increased tension observed in partially denervated motor units could be due to either an increase in the number of muscle fibers supplied by an individual motoneuron or hypertrophy of the muscle fibers. Westerman et al. (1979) found that the increase in tension of such motor units was in part attributable to muscle fiber hypertrophy. Following partial denervation by ventral root section, the FDL and the soleus muscles of cats were examined both physiologically and histochemically. In histological sections, there was a significant increase in the mean diameter of the fibers in the partially denervated muscle. However, along with the hypertrophy of some of the muscle fibers, atrophic fibers were also seen. These results provide some evidence for the possibility of muscle hypertrophy contributing partly to the recovery of the muscle following partial denervation.

In contrast, both Hatcher et al. (1985) and Luff et al. (1988) measured the cross-sectional areas of the muscle fibers within the enlarged motor units. Both studies indicated that no difference existed between the cross-sectional areas of the control muscle fiber pool and the muscle fibers of the partially denervated muscle. This was true for both the fast-twitch FDL and slow-twitch soleus muscles. Thus, the motor units must have increased in size by increasing the number of muscle fibers innervated by the motor axon and not by hypertrophy of the muscle fibers. Both groups also found that there was no change in either axonal diameter

or conduction velocity with partial denervation. They concluded that motoneurons can expand their peripheral field and subsequently generate increased tensions without any change in axonal diameter, conduction velocity or hypertrophy of the muscle fibers.

d) Alterations in Muscle Fiber Type Grouping

The definition of a motor unit has been previously noted in the above sections. Edström and Kugelberg (1968), using the technique of glycogen depletion, to be described in detail later in the introduction, mapped the area occupied by the individual motor units within the tibialis anterior (TA) of the rat. They found that the muscle fibers in an individual motor unit are randomly distributed over a large area of the muscle. The technique of glycogen depletion provides an advantage over others since the physiological properties of the motor axon can be correlated with the histochemical profile of the muscle fibers within an individual motor unit.

i) acute denervation

Since the discovery of the random dispersion of muscle fibers in a motor unit, several studies have examined how this compares with the motor units of reinnervated muscle. The studies by Karpati and Engel (1968) and Kugelberg et al. (1970) were the first to show changes in muscle fiber patterns following reinnervation. Motor units in the rat hindlimb were examined by glycogen depletion following recovery from acute denervation.

Although the muscle fibers of a given motor unit are normally scattered over a large area of the muscle, they are histochemically homogeneous. However, in a self-reinnervated muscle section, both Karpati and Engel (1968) and Kugelberg et al. (1970) found the muscle fibers within a motor unit were grouped into small compact units which they referred to as type-grouping. With type-grouping, each small group of muscle fibers will be homogeneous, representing an individual motor unit.

ii) cross-innervation

Cross-innervation experiments involve removal of the original innervation supplying a muscle and implanting a nerve from a different muscle. This has been shown to alter the contractile properties of the muscle (Buller et al. 1960, Robbins et al. 1969, Prewitt and Salafsky 1970). The presence of type-grouping might be expected since there is complete denervation of the muscle followed by reinnervation. This is very similar to the situation of acute denervation except that innervation now involves a different nerve supply. Type-grouping has been shown with cross-innervation by Romanul and Van Der Meuluen (1967), while the reports of Prewitt and Salafsky (1970) and Robbins et al. (1969) do not show the presence of type-grouping as clearly.

iii) partial denervation

Previously, two studies have examined the effect of partial denervation on the muscle fiber distribution. Kugelberg et al. (1970) examined the TA after partial denervation by ventral root section of

either the fourth ventral root, L₄, (responsible for 80% of the innervation) or the fifth ventral root, L₅, (responsible for 20% of the innervation). Following a five to six month recovery period, glycogen depletion was used to examine motor unit groupings in the TA muscle sections. When partial denervation was performed by sectioning L₅, moderate denervation was produced and resulted in little change in the muscle fiber type pattern. However, when L₄ was severed, only 20% of the axons remained, and this was associated with the presence of type grouping throughout most of the muscle.

Narusawa (1985) examined the rat soleus muscle following recovery from partial denervation. Partial denervation was performed by severing one of the nerve branches supplying the soleus muscle very close to its entry point into the muscle. The surgery attempted to produce a denervation in excess of 30% since with partial denervation of 30% or less, little change was noted in the number of muscle fibers and the nature of the remaining nerve (Narusawa 1985). Although, the results showed that the number of type II muscle fibers was significantly reduced after an eight week recovery period, there did not seem to be extensive evidence of type-grouping in the reinnervated soleus muscle.

The reason for discrepancy between the results of Kugelberg et al. (1970) and the results of Narusawa (1985) has yet to be determined. One can suggest that it may simply be the extent of denervation that is responsible for the discrete groups since the type grouping observed by Kugelberg et al. (1970) was only seen when partial denervation was performed by sectioning the fourth lumbar root (L₄). L₄ was the dominant

root and when sectioned, only 20% of the motor units remained. In a later review, Kugelberg (1981) reported that moderate partial denervations, (e.g.: sectioning of a non-dominant root), resulted in a few very small groups of closely packed fibers with reinnervation. Fiber aggregates will become larger when the extent of denervation is increased. The results indicate that in order to obtain type-grouping, a critical number of axons must be removed with partial denervation. Large distinct fiber groups may only be shown with large denervations and these may be particularly difficult to achieve when partial denervation is performed by transection of the peripheral nerve as in this thesis and the work by Narusawa (1985). This latter author concluded that with moderate levels of denervation, as in sectioning of a non-dominant ventral root, when reinnervation has occurred, the fiber type distribution will show small groups of closely packed fibers and not the large type groups seen with more severe transections. With increased denervation, it was stated that the fiber aggregates become larger.

e) Axonal Sprouting in Murine Dystrophy

Previous studies on the reinnervation of dystrophic muscle have used the techniques of complete denervation by either nerve section or nerve crush. A technique of partial denervation can provide some added insight into the problems associated with muscular dystrophy. As previously discussed, Gurney et al. (1986) isolated a muscle-derived protein that influences terminal sprouting. One might speculate that if there is a biochemical alteration in muscle of the dy2j mice, this factor

may not be present and subsequent to partial denervation, reinnervation could be impaired since terminal sprouting would not occur. The technique of partial denervation will determine whether, given every opportunity, motor axons of the dystrophic mouse can sprout collaterals and increase their motor unit size. The existence of collateral sprouting by the axons of the dystrophic mouse has been previously examined by Law and Atwood (1973). When a morphological estimate of average motor unit size was calculated (as described later in the introduction), they found that soleus motor units of dy/dy mice were slightly but not significantly larger than those of the normal mice. Since functionally denervated muscle fibers are known to be present in dystrophic mice (McComas and Mrozek 1967; Law and Atwood 1973), these results may suggest that motor units in dystrophic mice cannot increase their size through collateral sprouts.

Examining the ability of the nerves of the dy2j mice to sprout collaterals requires some added attention. Dystrophic mice are known to be considerably less active than their normal counterparts. This could potentially influence the interpretation of any results on the effect of the partial denervation since several studies have shown that exercise may play an important role in recovery from partial denervation. Gardiner et al. (1984) found that rats subjected to daily exercise training 10 weeks prior to partial denervation of both the plantaris and the soleus muscles, showed enhanced sprouting in the plantaris muscle but reduced sprouting in the soleus. They concluded that exercise training enhances short-term sprouting in the motoneurons supplying fast muscles. Different sprouting stimuli and the degree of overload of the muscle prior to partial

denervation were suggested by Gardiner et al. (1984) as possibilities for the difference in the results from the two muscles.

In a later study, Gardiner and Faltus (1986) examined the contractile responses of the rat plantaris muscles ten days after partial denervation. During these ten post-operative days, the rats were subject to a daily program of intense exercise. When the functional indices of motoneuron sprouting were assessed, no changes were observed in the exercised rats when compared to the control rats. These results seem to indicate that their training program was not intense enough to produce any change in activity.

This is in contrast to the increased sprouting seen with exercise training following partial denervation by Hoffman (1952) and Maehlen and Nja (1982). These studies involved delivering short periods of electrical stimulation to the muscle immediately after partial denervation. These brief durations of increased neural activity enhanced the sprouting observed in the remaining intact motoneurons seven days later.

Herbison et al. (1986) electrically stimulated the sciatic nerve of rats following partial denervation of the soleus muscle. Electrodes had been implanted unilaterally adjacent to the sciatic nerve following bilateral partial denervation of the soleus muscle. Stimulation was applied to the sciatic nerve for any of 2, 4, or 8 hours per day, 5 days of the week for a total of six weeks. Following recovery, muscle weight, tension, contraction time and fiber areas of type I and type II were examined. Contraction times were unaffected by either partial denervation or electrical stimulation. Muscle weight, twitch and tetanic tensions and

fiber type areas were reduced with partial denervation alone. With electrical stimulation, recovery from partial denervation significantly increased the muscle weight and tension production but did not produce any change in the fiber type areas when compared to the partially denervated unstimulated animals. The experiments seem to provide evidence that stimulation of intact axons of partially denervated muscle enhances recovery possibly by increasing the sprouting response.

Although these studies indicate some contradictory results, it seems that the intensity of the exercise and the muscle type play an important role in determining the effect of exercise on recovery following partial denervation. Since the dy2j mice are considerably less active than the age-matched C57 mice, the influence of exercise on recovery should be considered.

3) Techniques for Measuring Motor Unit Size

This section will serve to introduce the reader to some of the methods used for determining the number and sizes of motor units in a muscle.

a) Physiological Recordings

Within physiological recording techniques, there are many parameters that can be measured in the assessment of an individual motor unit. The method that is often used in physiological assessment of the motor units is ventral root splitting. The main reason for the frequent use is that it seems to limit any sample bias on the basis of motor unit

size or physiological properties since the ventral roots are split at random (Parry et al. 1982).

The ventral root splitting technique will be described briefly in this section as it is fully explained in the methods section. In this procedure, following a laminectomy, the animal is secured in a clamp device and then the experimental muscle is isolated and prepared for isometric tension recording. The ventral roots supplying the given muscle are then exposed and split sequentially until a filament which contains only one *alpha* motor axon innervating the muscle under investigation is isolated. The size of the individual motor unit is determined by recording the tension elicited when the ventral root filament is stimulated. This tension is then expressed as a percentage of the total tension recorded from the muscle. The size is then a fraction of the total ~~force~~ produced by the muscle. The number of motor units is determined using the average size of the motor units divided into the total force produced by the muscle.

The concept of the motor unit was first suggested by Sherrington (1906). Since this time, there have been many studies attempting to correlate the structural and histochemical characteristics of the muscle fibers with the physiological properties of their motor axons.

Techniques for this correlation were not developed until the works of Edström and Kugelberg (1968, 1973) and Burke et al. (1968; 1973a, 1973b). These authors utilized the method of glycogen depletion which allows a direct study of the interrelationship between the physiology and the histochemistry of an individual motor unit. This method is essentially

ventral root splitting with one additional procedure. The artery supplying blood to the given experimental muscle is clamped to produce ischemia in the muscle. The single motor nerve is repetitively stimulated with low frequency fatiguing impulses to deplete the muscle fibers in this unit of glycogen. After a given time, the glycogen stores will be exhausted. When the muscle is examined histologically, the depleted muscle fibers stain pale with PAS technique for glycogen. These pale muscle fibers will belong to the motor unit isolated and characterized by ventral root splitting.

Burke et al. (1971; 1973; 1974a; 1974b) used this technique and also examined some physiological measurements of the motor unit. Twitch and tetanus tensions and fatigue index of the motor units were measured in an attempt to achieve a classification of motor units. Their classification was originally applied to cat muscles, and in later years, has proven to be useful in the accumulation of new data.

Burke and co-workers (1974) divided the motor unit populations into three categories:

- 1) FF- fast-twitch, fatiguable
- 2) FR- fast-twitch, fatigue-resistant
- 3) S- slow-twitch, fatigue-resistant

In later studies, Burke (1978) included a fourth type of motor unit in the cat muscle unit classification. This is an FI unit referring to a motor unit with intermediate fatigue resistance, and a fast-twitch. Thus far, this so-called 'tetrapartite' classification has been applied to many hindlimb muscles in the cat including the tibialis anterior and extensor digitorum longus (Dum and Kennedy 1980), the tibialis posterior (McDonaugh

et al. 1980), the flexor hallicus longus (Dum et al. 1979; Westerman et al. 1979), and the soleus and gastrocnemius (Burke et al. 1982).

Motor unit studies using the glycogen depletion technique have yet to be performed with success on the mouse. However, motor unit studies using the conventional ventral root splitting technique have been performed on the mouse extensor digitorum longus (Bateson and Parry 1983) and soleus (Lewis et al. 1982) muscles.

b) Morphological Techniques

Law and Atwood (1974) examined the extent of axonal sprouting in soleus muscle of ReJ129 and dy/dy mice using a morphological estimation of motor unit size. The study required determining the number of motor end-plates, the number of myelinated axons, and the estimated number of motor axons. The number of motor end-plates were compared in the normal and dystrophic mice by staining the muscles for acetylcholinesterase, while the number of axons was determined histologically by sectioning the soleus nerve and then estimating the number of motor axons. An estimate of motor unit size was then made by dividing the number of motor end-plates by the number of motor axons. This technique uses the assumption that each of the motor end-plates corresponds to formation of a neuromuscular junction and therefore can correspond to the number of muscle fibers supplied by the motor axons. This technique has not been used extensively perhaps because of this large assumption since dystrophy may affect the formation of proper neuromuscular junctions.

The most common morphological technique for counting motor units is horseradish peroxidase (HRP) labelling. The definitive demonstration for the use of HRP in tracing motoneuronal connections did not occur until the work of Kristensson and Olsson (1971). These authors stated that following intramuscular administration, HRP could be readily demonstrated in the motoneurons of fixed spinal cord. Since this time, HRP has been used to examine the motoneuron population in many muscles of both the mouse and the rat (Parry et al. 1982; Peyronnard and Charron 1983; Krishnan et al. 1985; Parry and Falconer 1986; Desypris 1986; Lowrie et al. 1987).

A measure of motor unit size could be obtained morphologically by determining the total number of innervated muscle fibers and dividing by the number of *alpha* motoneurons supplying the muscle. This raises the problem of identifying specifically alpha motoneurons as well as the number of innervated muscle fibers. Following intramuscular injection of HRP, the number and sizes of motoneurons can be determined. If the areas of the cells are plotted as a frequency distribution, one would expect to observe a bimodal distribution of the motoneurons. This bimodality would be expected with a clear separation of the large (presumed *alpha*) and the small (presumed *gamma*) motoneurons. Similarly, the total number of innervated muscle fibers can be counted from a histological section of the muscle. NCAM, or neural cell adhesion molecule has been shown to be present on the surface of denervated muscle fibers (Rutihauser et al. 1983; Covault and Sanes 1985; Moore and Walsh 1986; Walsh and Moore 1986). In muscle sections stained with a monoclonal antibody directed against

NCAM, the denervated muscle fibers stain positively, while the innervated muscle fibers did not stain. This technique should enable the number of innervated muscle fibers to be counted.

d) Past Work on Muscular Dystrophy

Since the first neural hypothesis was developed by McComas et al. (1971), many studies have looked into the neuropathology of muscular dystrophy. The Sick Motoneurone Hypothesis of McComas et al. (1971C) describes the etiology of human muscular dystrophy as a problem of neural origin based in the spinal motoneuron. However, only very limited research had been performed on examining the actual morphology of dystrophic motoneurons in both humans and in animal models.

Spinal motoneurons have been examined in our lab in dy2j mice to determine if the dystrophic process manifests itself in the murine model as a change in the motoneuron itself as suggested by McComas et al. (1971; 1977; 1988) in human dystrophy. These studies include that of Parry et al. (1982) where the number and sizes of motoneurons in the soleus muscle were examined in both C57 and dy2j mice. Using the HRP technique, the dy2j mice had a 20% reduction in the number of motoneurons supplying the soleus muscle when compared with age-matched C57 mice. Associated with this decreased number in the dy2j mice, was an increase in the mean soma area of the motoneurons when compared to the C57 mice. This phenomenon was also observed by Parry and Falconer (1986) in a forelimb motornucleus.

These results can lead to some questions regarding the increased soma area in the dystrophic motoneurons. A review of the factors governing the size of spinal motoneurons will now be given.

4) Controlling Factors of Motoneuron Size

As mentioned above, results from our lab have shown (Parry et al. 1982; Parry and Falconer 1986) that in the dy2j mouse, there is an increased soma size associated with a decreased number of motoneurons in the soleus and forelimb motornucleus respectively. This can lead to some interesting questions. Does the dystrophic process manifest itself by changing the size of the motoneuron? Can the increased soma area be a compensatory response to the reduced number of motoneurons supplying the muscle? The answers to these questions can be explored by examining the relationship between motoneuron size and motor unit size. In order to do this, circumstantial evidence for the existence of this relationship should be considered. By relating the motoneuron size to both axonal diameter and conduction velocity, then relating conduction velocity to motor unit size and type, a relationship between motoneuron size and motor unit size and type can be inferred. This section will review the past literature on the known related parameters.

a) Cell Size and Axonal Diameter/Conduction Velocity

Cajal, in 1909, related the size of a cell to the diameter of its axis cylinder and secondly to the number and thickness of its collaterals and terminals. In this review paper, Cajal based this relationship on examples of large cells with large axons and numerous collateral branches such as: spinal motoneurons, giant corpuscles of the torpedo, Golgi cells of the cerebellum and horizontal cells of the retina. Small cells with thin axons which give rise to few collaterals were mentioned with examples of the granular cells of the cerebellum, bipolar cells of the retina and

the granular cells of the fascia dentata. Cells in the dorsal root ganglia were also presented as evidence since the ganglion cells with large volumes had thick axons while those with small volumes had thin axons.

Henneman et al. (1965) reviewed the work of Cajal in their research examining the functional significance of cell size in spinal motoneurons. They stated that in the mammalian nervous system the idea of Cajal, that cell size, axon diameter and collateral number are inter-related, seems to be true without exception. Consequently, Henneman and associates concluded that the diameter of an axon was a reliable index of the size of its cell.

In the early 1970's, Barrett and Grill (1971) examined motoneurons histologically. A positive correlation was shown to exist between the size of the *alpha* motoneurons and their axonal conduction velocity. Once this was established, they relied on the close relationship between conduction velocity and axonal diameter. Since axonal diameter is one of the main determinants of conduction velocity, they stated that cell body size was proportionally related to axonal diameter. With this indirect method, axonal diameter correlated closely with both cell body size and the number of dendrites from the cell body. Although this study did provide more direct evidence, a better method was still needed to elucidate the relationship between motoneuron size and axonal conduction velocity.

The relationship between conduction velocity and axonal diameter has been known for many years. In a review by Erlanger and Gasser (reprinted 1968) past studies were examined to determine how this relationship came into existence. The first suggestion of a relationship

between these two parameters was made by Gøthlin in 1907 when he developed his cable hypothesis of conduction velocity of nerve fibers and this was later confirmed by Lapique and Legendre (1913). Erlanger and Gasser (1927) derived a positive relationship between these two parameters from mathematical models that they created based on physiological tracings recorded from the bullfrog nerve. Later, Douglass et al. (1934) and Hursh (1939) provided more experimental evidence that the conduction rate varied as a function of the size of the axonal diameter.

More direct confirmation of the relations among cell body size, axon diameter and axon conduction velocity of *alpha* motoneurons came with the work of Cullheim (1978) and Cullheim and Kellerth (1978A,B and C) using the technique of intracellular injection of HRP. This was performed as follows: After a laminectomy, intracellular electrodes were placed directly into the spinal cord. *Alpha* motoneurons were then identified by the action potentials following antidromic stimulation of the nerve and by their conduction velocities, time to peak tension, and fatigue indices were measured for characterization of the motor unit type being stimulated. Once the physiological recordings were finished, horseradish peroxidase was injected directly into the *alpha* motoneuron by passing a positive current through a micro-electrode filled with the horseradish peroxidase solution. This staining technique of intracellular injection of horseradish peroxidase allows the possibility of examining a physiologically identified motoneuron morphologically under both light and electron microscopy. Therefore, the relationship between morphology and physiology in the motoneuron can be examined.

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The first results using this technique were reported by Cullheim (1978) in adult cat sciatic *alpha* motoneurons. Significant positive correlations were found between cell body size and axon diameter and cell body size and axon conduction velocity. This report was the first direct morphological evidence for a close correlation between cell body size and axon diameter in spinal *alpha* motoneurons. As mentioned, there was a positive correlation between axonal diameter and conduction velocity. Kernell and Zwaagstra (1981) confirmed the positive correlation existed in cat hindlimb motoneurons between the axonal conduction velocity and motoneuron size with the same technique as Cullheim and Kellerth (1978A,B, and C).

In other studies from the same lab, Cullheim and Kellerth (1978A, 1978B, 1978C) used this technique to study *alpha* motoneurons with their axons and their axon collaterals supplying different hindlimb muscles in the cat hindlimb. The five different muscles that were examined were the quadriceps (knee extensor), posterior biceps (knee flexor), gastrocnemius-soleus (ankle extensor), tibialis anterior (ankle flexor) and the plantar (toe extensor) muscles. No differences were observed in the mean axon diameters among all five muscle groups. Recalling that Cullheim (1978) related the axon diameter positively with cell body size, this study suggest that the mean motoneuron size is similar in all the hindlimb muscles of the cat even though each muscle is responsible for different motor function.

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b) Conduction Velocity and Motor Unit Size/Type

The relationship between motor unit size and axonal conduction velocity has been the subject of many research studies. In these studies, the size of the motor unit is determined by the tension elicited or peak tetanic tension. Close correlations have been shown between the axonal conduction velocity and the size of the motor units in the smallest muscles of the cat's hindlimb, the lumbrical muscles (Appelberg et al. 1967; Bessou et al. 1963;1965; Emonet-Denand et al. 1971), and in the larger cat hindlimb muscles (McPhedran et al. 1965; Wuerker et al. 1965; Bagust et al. 1974; 1979;1981; Jami and Petit 1975; Proske and Waite 1976; Binder et al. 1978; McDonagh et al. 1980B). However, it seems that in the larger muscles, the correlations are not as close as in the smaller muscle units. No clear relation has been established between conduction velocity and motor unit size in the cat flexor digitorum brevis muscle (Gerlach et al. 1976), in the mouse extensor digitorum longus muscle of the hindlimb (Bateson and Parry 1983), and in all but one of the animals in mouse soleus muscle (Lewis et al. 1982).

c) Motoneuron Size and Motor Unit Size/Type

Henneman and his associates examined several physiological parameters in relation to motoneuron size. Using ventral root splitting, axonal conduction velocity, the amplitude and time course of single motor unit twitches were measured in the cat soleus and gastrocnemius muscles. The first experiments (Henneman et al. 1965; McPhedran et al. 1965; Wuerker et al. 1965) correlated the diameter of a motor nerve fiber with

the number of muscle fibers it innervates indirectly using axonal conduction velocity as the indicator of axon diameter and tension production for the number of muscle fibers innervated (motor unit size). From this positive correlation, this group has stated that the largest units are innervated by the thickest axons. This is very important since it suggests that the diameter of the axon is directly related to the size of the motor unit. Henneman et al. (1965) had already stated that the diameter of an axon is a reliable index of cell size. If so, then one can relate the size of the motor unit to the size of the cell body in the spinal cord. This seems quite plausible since a large motoneuron must have sufficient axonal substance to give off a large number of terminals (Henneman et al. 1965). When the soleus muscle was examined in cross-section, the muscle fibers were very similar in area. This indicates that the increased force production could be explained by the fact that the motor units were larger due to an increased number of muscle fibers rather than hypertrophy of the same number of fibers.

Henneman et al. (1965) examined the recruitment of the motoneurons in activity in what is now referred to as the Size Principle. The Size Principle dictates the relationship between the size of the *alpha* motoneuron and its electrophysiological properties. It was found that the threshold voltage of a motoneuron was dependent upon its soma size. The size of the motoneuron was based on extracellular recordings of axonal action potential amplitude from the axon. They assumed that the large amplitude action potentials arose from large diameter motor axons. Translating axonal diameter into cell size, the size of a motoneuron will

dictate the threshold response of the motor unit. Therefore, the smaller motoneurons or motor units have lower thresholds while the larger motoneurons or motor units fire only when a large stimulus is applied. Henneman and colleagues stated that the results indicated that the motoneuron pool is graded according to size with the small motoneurons (slow contracting, small tensions) recruited first and the large motoneurons (fast contracting, large tensions) later. However, since axonal diameter was used as an indication of motoneuron size and not motoneuron size itself, direct evidence for controlling factors of motoneuron size was still not evident.

These results by Henneman should not be confused with the results by Petrofsky and Phillips (1979) who showed that when examining recruitment of motoneurons by electrical stimulation of peripheral nerves, the larger axons are recruited first. With stepwise increase in stimulation voltage to the motor nerve, smaller axons are subsequently recruited. The order of recruitment is thus from large (fast) to small (slow) axons. This discrepancy can be attributed to the fact that the experiments in Hennemans' work observed recruitment in the spinal cord, the central nervous system order. Petrofsky and Phillips (1979) examines recruitment by the peripheral nerve. In the large motoneurons, the membrane area is increased and requires a greater stimulus for excitation. However, in the peripheral nerves, the large axons have a decreased membrane resistance and should in theory be recruited first if recruitment is performed by stimulation of the peripheral nerve.

Using intracellular injection of HRP into the *alpha* motoneurons of

the cat triceps surae muscle, Cullheim and Kellerth (1978C) examined the morphological and physiological properties of the different motor unit types. Ten alpha motoneurons of each of four motor unit types including the soleus S, gastrocnemius S, FR and FF were examined. The definition of each of these four groups was taken from Burke et al. (1973) as previously described. Mean axonal diameters of the alpha motoneurons were compared among the different motor unit types in the triceps surae. The soleus S type motor units had a significantly smaller mean axonal diameter when compared with all the other three types. Among the gastrocnemius units, a significant reduction was observed in the mean axonal diameter in the S type when compared with the fast motor units while significant differences among the fast motor unit axonal diameters did not occur.

Since Cullheim (1978) has already confirmed the relationship between axonal diameter and conduction velocity, then the results can give further information. Since conduction velocity is directly related to contraction time, then one would expect that the axonal diameters of the fast twitch units be the same as seen in these experiments. One would also expect to see significant differences in the axonal diameters as such among the slow and fast contracting motor units. This is in agreement with the physiological studies on the triceps surae muscle where the soleus units exhibited the slowest conducting motor axons while the FF and FR motor units were the fastest. The gastrocnemius S unit type had intermediate conduction velocity as would be expected.

Since the relationship between axonal diameter and motoneuron size was also confirmed by Cullheim (1978), the results by Cullheim and

Kellerth (1978A,B,C) can be used to estimate cell body size. Since differences were observed in the axonal diameters among the different motor unit types, this may also reflect differences in motoneuron size in these groups. Motor units of the soleus S type would have the smallest cell body size, and in order of increasing size of motor unit type, gastrocnemius S type, FF and FR.

The first direct evidence that alpha motoneurons innervating fast-twitch muscle units are larger than those innervating slow-twitch units was by Burke et al. (1982) studying the cat triceps surae and plantaris muscles. Following identification of the motor unit innervated by a given alpha motoneuron, the cells were labeled with HRP by intracellular injection so that the dimensions of the soma and the stem dendrites could be made. The results showed that the alpha motoneurons that were physiologically identified to innervate the fast-twitch motor units had larger motoneurons than those innervating the slow motor units. Burke et al. (1982) suggested a correlation between motoneuron size and motor unit type exists and helps to add to data on recruitment of the motoneurons by Henneman et al. (1965) with the Size Principle. Similarly, Luff et al. (1988) provided evidence that fast motor units are larger than slow motor units in the cat soleus and FDL muscles.

With all the data together, there is now a link between functional excitability, motor unit type and motoneuron size. Secondly, they suggest that motoneuron size may be directly related to the size of the peripheral territory (i.e. the number of innervated muscle fibers) as suggested by the work of Henneman et al. (1965).

STATEMENT OF THE PROBLEM

The aim of this research study is to examine some of the motor unit properties of both control (C57BL/6J) and dystrophic (C57BL/6J dy2j/dy2j) mice, following partial denervation.

The technique of partial denervation effectively removes part of the nerve supplying the muscle. In the normal animal, this is known to cause the remaining intact axons to sprout collaterals and capture the denervated muscle fibers. This results in an increased motor unit size. This has never been examined in the dystrophic animal.

The following questions will be asked:

(1) Can dystrophic mice increase their motor unit size in response to partial denervation?

This will be approached by two methods both examining motor unit size. The first method is a morphological estimate of motor unit size. Using horseradish peroxidase (HRP) retrograde labeling, the number of motoneurons in the anterior tibialis (TA) muscle pool can be determined. From the same animal, muscle sections will be taken and photographed. One can then obtain a morphological estimate of mean motor unit size by dividing the total number of innervated muscle fibers in TA by the total number of *alpha* motoneurons determined by HRP. Secondly, by the technique of ventral root splitting, a physiological quantification of motor unit size can be obtained, both in absolute (force produced) and relative (motor unit force/whole muscle force) terms.

2) Is there any selective reinnervation in the anterior tibialis muscle following partial denervation?

Cryosections of the anterior tibialis muscle from each leg of the animal will be taken. Using immunohistochemical staining techniques, muscle sections can be classified into different muscle fiber types based on their myosin heavy chain composition. Since the anterior tibialis muscle consists mainly of type IIA and IIB with a few type I muscle fibers, antibodies directed against these three muscle fiber types will be used to stain the TA sections. Using sections from each leg of the animal, one can then obtain any changes in the proportions of these muscle fiber types following recovery from the surgery.

Problems arose during the course of the data collection with the determination of the morphological estimate of motor unit size. When the motoneuron pool was quantified and the sizes were measured, the distribution of motoneuron sizes revealed a unimodal distribution. In a motor pool supplying a muscle; the motoneurons should reveal a bimodal distribution with a clear separation of the small (presumed gamma) and the large (presumed alpha) motoneurons. However, the data in this study revealed no such separation. This causes a problem since in order to estimate motor unit size, the number of alpha motoneurons innervating the mouse anterior tibialis muscle is required.

Secondly, when examining cross-sections of the muscle, the total number of innervated muscle fibers was very difficult to determine in the dystrophic partially denervated muscle. Many fibers showed signs of

degeneration and deterioration. This made it very difficult to determine if indeed the muscle fibers were innervated, in the process of being reinnervated, regenerating or degenerating. The problem was thought to be alleviated by staining the muscle fibers with anti-NCAM. These sections revealed problems as well. As a result of these problems, a morphological estimate of motor unit size was not able to be determined.

The other method for measuring motor unit size is by the ventral root splitting technique. However, after three months of constant endeavors, a lack of results made it rather important to examine another area of study.

These problems will be dealt with further in the results section of the thesis but it was clear when examining the HRP labelled motoneurons that motoneuron size increased with partial denervation. This then gave rise to another related question.

3) Is the increase in the motoneuron soma area a reflection of a compensatory response to an increased motor unit size as a result of the expected decreased number of motoneurons induced by the partial denervation surgery?

Experiments will be performed whereby both partial denervation as well as partial extirpation of the anterior tibialis (TA) are performed. Following a two month recovery, motoneuron and motor unit sizes will be measured in order to examine the influence of the size of the peripheral field on motoneuron soma size.

MATERIALS AND METHODS

1) Animals

The strains of mice used in this research study were the control (C57BL/6J) and dystrophic (C57BL/6J dy2j/dy2j) mutant.

These mice were originally obtained from Jackson Laboratories, Bar Harbour, Maine but have since been bred at our own facilities. The animals were given mice chow and water *ad libidum*. The animals were maintained on a twelve hour day and night cycle.

2) Partial Denervations

All the animals in the study, both control (C57) and dystrophic (dy2j), were initially operated on at three months of age and a period of eight weeks was allowed for reinnervation to occur. One group however, was allowed to reinnervate for a period of 18 months after partial denervation surgery.

The first series of experiments involved performing a partial denervation on the animals while the second series required a partial denervation accompanied by a partial extirpation of the tibialis anterior muscle.

The protocol for the partial denervation surgery was as follows. Animals were anesthetized with Somnotol (sodium pentobarbital), 80 $\mu\text{g/g}$ by body weight, administered intraperitoneally. The left lateral popliteal nerve which is the sole innervator of the anterior tibialis muscle was isolated in the area adjacent to the patella. This was performed by

cutting through a layer of fascia and connective tissue. Care was taken not to damage any of the surrounding muscle and the blood and nerve supply. The lateral popliteal nerve, once exposed and isolated, was split in half with the aid of a corneal dissecting knife. The partial transection was performed over a distance of approximately 1 centimeter (from the patella to the hip) and half of the nerve was removed. This was in an attempt to remove approximately 50% of the axons innervating TA. After removal of the nerve, the hindlimb of the animal was sutured in layers with 6/0 silk, the wound was cleaned with distilled water, and the animal was left to recover for a period of eight weeks. Following surgery, the animals were checked daily until their wounds were completely healed and void of infection. If the wound was opened, as seen in several animals, the animals were reanesthetised as above, and the wound was resutured with 6/0 silk.

3) Partial Extirpation

The second series of experiments involved both partial denervation and a partial extirpation where part of the TA was removed. Partial denervation as described above, was performed on both C57 and dy2j mice. Once this was completed, a skin incision was made on the lower left hindlimb allowing the anterior tibialis muscle to be exposed from the ankle to knee. With aid of fine forceps and surgical scissors, approximately half of TA was then removed and weighed. All precautions were taken to minimize any damage to the vascular supply. Once this was completed, the leg was then sutured and the wound was cleaned with

distilled water. The animal was left to recover for a period of eight weeks. In every animal, the same part of the muscle was removed which included both superficial and deep portions of the muscle.

4) Complete Denervation

In four animals, two dy2j and two C57, following administration of anesthesia, as above, the left lateral popliteal nerve was exposed from the patella to the hip. The nerve was isolated, sectioned and removed from this entire area which was approximately one centimeter of nerve. The leg was then sutured in layers and the animal was left to recover for a period of eight weeks. After the two month period, the muscles were removed from each leg of the animals and frozen for histological use.

5) Horseradish Peroxidase Injection

Horseradish peroxidase (HRP) can be injected into a given muscle and will then be taken up by the motor axons of the muscle. The axons will carry the HRP by rapid retrograde axoplasmic flow to the cell bodies in the spinal cord. The HRP after appropriate staining with a chromagen, will then appear as colored intracytoplasmic granules in the motoneurons of the given muscle pool (Mesulam 1978).

The number and sizes of motoneurons supplying the tibialis anterior muscle were quantified with this method. The tibialis anterior was chosen because it is a large hindlimb muscle. Therefore, when labelling the motoneurons with intramuscular injection of HRP, any spread of the label to the adjacent EDL muscle, would probably not have made

significant differences in the motoneuron counts. However, if the EDL muscle was used and the HRP spread into the TA, since the EDL is a much smaller muscle, then it would have affected the results significantly.

After a two month recovery from surgery, animals were anesthetized and the tibial nerve was exposed in the region from the hip to the knee. The tibial nerve was then cut below its separation from the lateral popliteal nerve. The leg was now completely denervated except for the lateral popliteal nerve. This was an added precaution to prevent any chances of uptake of the HRP label into other motoneuron pools in the spinal cord. Once denervated, the incision was closed with 6/0 silk. Denervation was performed on both the right and the left leg.

100 μ l of a 20% solution of horseradish peroxidase (Sigma Type VI) in saline containing 2% dimethyl sulfoxide (DMSO) was mixed in a microcentrifuge tube and the solution was drawn up into a 100 μ l Hamilton microsyringe. A drop of Paraffin oil was drawn up above and below the HRP solution. This helped to prevent the HRP solution from drying out in the microsyringe and clogging the fine needle tip. A capillary tube was placed on an electrode puller apparatus and pulled to make a very fine tipped micropipette. This pipette was then placed on the Hamilton microsyringe and sealed with wax. The tibialis anterior muscle of each leg was exposed and 25 μ l of the HRP solution was slowly injected into the muscle. It should be noted that some muscles including the dy2j TA and the PD/PE C57 muscles, required less than 25 μ l of HRP to obtain complete staining. HRP was injected into the TA of both legs so that each animal had its own control in the right motoneuron pool. Injections were performed at several

points along the longitudinal axis of the muscle. Once all the HRP was injected, the electrode was removed and the muscle was gently blotted. The legs were then sutured with 6/0 silk and the wound was cleaned with distilled water.

The animals were allowed to recover for a period of 18 to 24 hours. At this time, the animals were reanesthetized and the TA from each leg was removed and frozen in isopentane frozen in liquid nitrogen and stored at -80° until required for histology.

After the leg muscles were removed, the chest area was exposed by reflecting back the skin and the sternum with the help of a clamp. An incision was made in the right atrium and the left ventricle. The animals were then transcardially perfused through the left ventricle with (1) 100 ml of 0.9 % saline; (2) 125 ml of 4% glutaraldehyde (JBS Chem) in 0.1M potassium phosphate buffer (pH 7.4); and (3) 100 ml of 10% sucrose in 0.1M potassium phosphate buffer (pH 7.4) chilled to 4° via a cannula consisting of polyethylene tubing attached to an 18 gage needle on a 50 ml syringe.

Once the perfusion was completed, an incision was made along the back of the animal and muscle was cleared to expose the vertebral column. A laminectomy was performed and the spinal cord was completely exposed from the sacral region to the insertion of the first rib. A diagonal cut was made at the top of the thoracic level of the spinal cord. This permitted easy identification of each side of the spinal cord once the spinal cord was stained. The spinal cord was then cut at the sacral region and removed. The block of cord was then placed in a solution of chilled 10% sucrose in 0.1M potassium phosphate (pH 7.4) and stored in the

refrigerator for one hour (Rosene and Mesulam 1978).

After one hour, the spinal cord was removed from the refrigerator and placed in a small petri dish filled with the sucrose solution and the dura, dorsal and ventral roots were removed. The spinal cord was then placed on a precooled chuck and embedded in OCT compound (Miles Scientific) on a freezing plate of an IEC Minotome Cryostat. The cryostat was routinely set at -10° for this procedure. $40\ \mu\text{m}$ serial longitudinal sections were taken and put on gelatin subbed slides. The spinal cord sections were then covered with a drop of 0.1 M phosphate buffer (pH 7.4). If it was required, sections were straightened out with a fine haired paintbrush. The mounted sections were then air dried for approximately one hour at room temperature and processed according to the technique of Mesulam (1978) using tetramethylbenzidine as the chromagen. The sections were processed through an alcohol dilution series, cleared with Xylene and mounted with Permount.

Using a phase contrast microscope fitted with a drawing tube attachment, motoneurons were visualized and traced. This permitted accurate tracing around the periphery of labelled motoneurons. Composite images of the entire motoneuron pool were made by drawing every stained motoneuron. However, only motoneurons with a visible nucleolus were counted. Figure 1 in the results section shows motoneurons that would have been counted. An image analyzer (IBAS) was then used to calculate the areas and maximum diameters of the labelled cell bodies from the tracings. In this thesis, cross-sectional areas were used to indicate soma size.

When the motoneuron pool was quantified in the soleus muscle,

the protocol was identical to that of the TA except that the denervation required sectioning of all the nerves except the soleus nerve. The only other difference was that intramuscular injection only required 10 μ l to stain the entire soleus motornucleus.

6) Ventral Root Splitting

The technique of ventral root splitting takes several hours and once the animal was set up in the apparatus, it was very difficult to move it in any direction. Therefore, for these two reasons, the first step in this procedure was to make a small incision in the peritoneum of the animal. Once this was done, a small cannula attached to a 1.0 ml syringe filled with Somnotol anesthetic was placed into the peritoneum. The cannula was then secured in place with several sutures and a small drop of Krazy glue. This allowed supplemental doses of anesthetic to be administered whenever it was necessary throughout the entire experiment. Extreme care was required throughout the ventral root splitting procedure to minimize the blood loss to the animal.

Once the cannula was secured, an incision was made around the patella area on the left leg of the animal. The lateral popliteal nerve was isolated very carefully as close to the muscle as possible. The left TA or EDL muscle was isolated next with care to minimize damage to both the nerve and vascular supply. When EDL motor units were to be isolated, it was necessary to denervated all other muscles in the lower leg to avoid interference with the recording of motor unit tensions by contractions elicited from the other muscles. With the aid of a pair of small

dissecting scissors, an incision was made along the side of the muscle to loosen the muscle from the surrounding tissue so the muscle could contract freely upon stimulation. The distal tendon was then loosened from the surrounding connective tissue and muscle by cutting through the ligaments in the ankle. The tendon was freed and then severed. Once freed, the tendon was attached to a small piece of stainless steel wire with looped ends and secured with the help of some 3/0 silk.

The second part of the surgery first required a skin incision from the neck to the sacrum. The skin was loosened to allow exposure of the rib cage, spine and sacrum. The sacrum was exposed next and cleared of muscle so it could be attached to a spinal clamp device that secured the animal in one position throughout the recording measurements.

It should be noted that the two protocols described above, preparing the spinal column and the hindlimb preparation of the animal for the recording measurements were done alternatively. This seemed to help to reduce the blood loss and trauma to the animal.

After this, the spine was cleared of muscle from the neck region down to the sacrum. A laminectomy was performed removing the lumbar vertebrae from L₁ to L₅. Cotton wads and Gelfoam were used to gently blot the spine whenever bleeding occurred. Once the spinal cord was cleared, the animal was moved to the tension measuring apparatus. The spinal clamp was first attached as follows. Two pairs of opposing metal pins were positioned at the sacrum and at the mid-portion of the spine. Once the animal was secured, the skin of the back was tied to the bent part of the metal pins to form a pool that could be filled with solution. This was

first filled with Mouse Ringer (see composition in appendix).

The leg was then secured with two separate clamps each consisting of a pair of Dumont #5 stainless steel forceps. One clamp was placed on the foot of the animal and the second one was attached to the femur. This prevented any movement of the lower limb that could interfere with the results. Once the hindlimb was secured, the leg was sealed in a bath with cotton that was previously soaked in a 5% aqueous Agar solution. When the leg was completely sealed, the bath was filled with Mouse Ringer solution and checked for leaks. If any occurred, the bath was resealed with the agar-soaked cotton. The bath was bubbled with a gas mixture consisting of 95% oxygen and 5% carbon dioxide. Temperature of the bath was maintained at 37 °c throughout the experiment by a heating coil apparatus at the base of the bath.

The severed distal tendon of the muscle was attached to a force transducer by means of the stainless steel wire. The signal from the transducer was amplified and then the tension was recorded and visualized on an oscilloscope. Once the tendon was attached, a wire stimulating electrode (distal electrode) was placed on the lateral popliteal nerve just above its entry into the muscle. The optimal muscle length was then established by indirect stimulation of the muscle via the distal electrode. Muscle length was adjusted until the twitch tension recording was maximal for three consecutive length adjustments.

With the back pool filled with Mouse Ringer, the dura of the spinal cord was removed next with the aid of a pair of very fine forceps and a pair of fine dissecting scissors. Once this was completed, the bath

was emptied with a vacuum and the bath was refilled with paraffin oil to decrease any possible spread of current during ventral root stimulation.

The dorsal roots on the left side were isolated, cut and then reflected to the side. Ventral roots L₄ and L₅ were cut and mounted on a pair of stimulating electrodes (central electrode) consisting of a pair of platinum wires. The twitch resulting from supramaximal stimulation of ventral roots L₄ and L₅ was recorded on the oscilloscope. The tension obtained from stimulation of the ventral roots should be equal to that produced by stimulation of the muscle-nerve.

One ventral root was then placed on a stainless steel dissecting stage that was immersed in the paraffin pool. The root was then split with a pair of very fine forceps. The split root was then mounted on the central electrode and a supramaximal stimulus was applied. The voltage was then lowered until the threshold voltage was reached. The threshold voltage was defined as the voltage at which an all or none twitch response was recorded 50% of the time when a series of ten pulses were given. If the tension that was recorded at threshold was less than the supramaximal tension then the root was split further. The root was repeatedly split until the tension recorded from a threshold voltage stimulus was equal to that of a supramaximal voltage stimulus. If this occurred, a single unit was considered to have been isolated.

Once the single motor unit was isolated, several parameters were measured following stimulation of the ventral root filament. These include: time to peak tension, maximum twitch tension, maximum tetanic tension and half relaxation time. The sizes of the individual motor units

are determined as a percentage of the total tension recorded from the muscle following stimulation to the nerve. When these parameters are measure and recorded, the ventral root is then dissected again until another motor unit is isolated.

7) Histological Processing of the Tibialis Anterior Muscle

a) Sectioning

Both the left (operated) leg and the right (unoperated) leg were removed from the animal prior to the transcardiac perfusion with glutaraldehyde. The muscles were bathed in a solution of 0.9 % saline, blotted dry, and the wet weights were recorded. The muscles were then frozen in isopentane precooled in liquid nitrogen. Once frozen, the muscles were placed in small plastic vials and stored in a freezer set at -80° until further processing occurred. Muscles were sectioned at various times later. Sectioning was done on a IEC Minotome Cryostat set in the range of -20 to -24 °c. Muscles were mounted on precooled chucks with OCT compound (Miles Scientific). 10 µm sections were taken and placed either on coverslips or gelatin subbed slides. The sections were either stained immediately or stored in a freezer set at -20°. Since sections were made of both legs, the right leg was used as the control for each animal.

To assess the specificity of the NCAM antibody, whole hindlimbs were removed from 17 day gestation fetuses (mice) and mounted together with adult hindlimb mouse muscle.

Many of the muscles, including the muscle from the mice embryos were wrapped in adult diaphragm and then frozen. The diaphragm muscle both

limited loss of muscle fibers at the periphery and served as a control for the myosin antibody staining.

b) Staining

Three types of staining were performed on the TA sections. The first type was a hematoxylin and eosin stain. These sections show a general morphological portrait of the muscle. Some of the sections were photographed to make composites of the stained muscle sections for muscle fiber counts.

The second type of staining that was done was immunohistochemical using monoclonal antibodies directed against the different myosin heavy chain isoforms. The TA sections were stained with monoclonal antibodies directed against type I, type IIA and type IIB myosin since this muscle is known to consist of these three fiber types. The muscles were examined for evidence of selective reinnervation following partial denervation as typically seen with reinnervated muscle after acute denervation (Karpati and Engel 1968).

Sections were incubated with the first antibody in small incubation chambers at room temperature for 24 hours. The sections were then rinsed in 0.1M PBS (phosphate buffered saline-pH 7.4) for thirty minutes changing the PBS every ten minutes. Excess liquid was removed from the sections and the sections were incubated with the second antibody, goat anti-mouse IgG (HRP conjugated) solution diluted 1:75 with 0.5% PBS-albumin solution. The slides were incubated with the second antibody for three hours. Slides were rinsed again in PBS as above and then processed

with diaminobenzidine (DAB) as follows. The sections were put in a solution of the following dilution: 10 ml PBS:10 mg DAB: 10 μ l 30% aqueous hydrogen peroxide. These were processed in the dark and checked occasionally until the staining reached the proper intensity. The sections were rinsed in distilled water and processed through the alcohol series and then mounted with Permount.

The last type of staining that was performed on the sections was immunohistochemistry for the demonstration of neural cell adhesion molecule (NCAM). NCAM is known to be present in denervated muscle (Covault and Sanes 1985, Walsh and Moore 1986). This stain was performed with a polyclonal antibody directed against NCAM. Anti-NCAM was used at various dilutions from 1:250-1:1000 with 0.5 % bovine serum albumin in 0.1M phosphate-buffered saline (pH 7.4) (PBS). The sections were stained with this first antibody for 24 hours at room temperature. After a thirty minute rinse with 0.1M PBS changing the PBS three times as above, excess water was once again removed. The second antibody was FITC labelled goat anti-rabbit IgG. This was incubated for two hours and then the slides were rinsed once with PBS-Nonidet and mounted with glycerol and stored in the dark at -20°. Sections were then observed using an FITC filter with a Zeiss Axiophot microscope.

8) Composition of Mouse Ringer

Mouse Ringer was made and used routinely in the ventral root splitting procedure as described above and consisted of the following:

NaCl: 7.071 g/L

KCl: 0.354 g/L

NaHCO₃: 2.10 g/L

KH₂PO₄: 0.068 g/L (add last)

CaCl₂: 0.1666 g/L

MgCl₂-6H₂O: 0.047 g/L

Glucose: 2.00 g/L

9) Statistical Analysis

All values represent means \pm standard error of the mean. Analysis for statistical significance used both the Students T test (for comparing different animal strains) and Analysis of Variance (for comparing effects of the surgery within a mouse strain).

RESULTS

1) TA MOTONEURON POOL CHARACTERISTICS

A) HRP Labeling

i) control muscle

The numbers and sizes of motoneurons supplying the (TA) muscle of control and dystrophic mice are presented in Table 1. In the dystrophic mice, there was a significant reduction of approximately 20% in the number of motoneurons supplying the TA muscle when compared to the C57 mice. No significant difference was observed between the motoneuron areas of the two groups.

Table 1 shows that the sample size is 24 animals in both the C57 and dy2j groups. These refer to the unoperated sides of the twelve animals from the partial denervation experiments and the twelve animals from the partial denervation/partial extirpation (PD/PE) experiments. The groups of twelve are further divided into 6 males and 6 females. Although the data from the sexes were separated at first, no difference was found in any of the measured parameters and the results from mice of both sexes were combined.

In a given motoneuron pool, there exists both *alpha* and *gamma* motoneurons. Therefore, if the individual cell soma areas are plotted on a frequency distribution, a bimodal distribution with a clear separation of the large (presumed *alpha*) and small (presumed *gamma*) motoneurons might be expected. When this was done, the raw data for both the C57 and the dy2j TA motoneuron pools lacked any sign of bimodality (Figure 2 and 3).

FIGURE 1 - Motoneurons supplying the tibialis anterior muscle labelled with horseradish peroxidase. Arrows point to motoneurons with nucleoli, indicating a motoneuron that would be counted (220X).



TABLE 1*

THE NUMBER AND SIZES OF MOTONEURONS SUPPLYING THE ANTERIOR
TIBIALIS MUSCLE*

	NUMBER OF MOTONEURONS	MOTONEURON AREA (μm^2)
C57 MICE (N=24)	141.9 \pm 3.3	264.8 \pm 12.9
dy2j MICE (N=24)	109.3 \pm 5.2 ^a	247.8 \pm 11.5

* data are expressed as means \pm standard error of mean

^a- significant decrease ($p < 0.05$) when compared with control side

FIGURE 2 - Frequency distribution of soma areas in the TA motoneuron pool of C57 mice. Areas are plotted as raw data (N=1610).

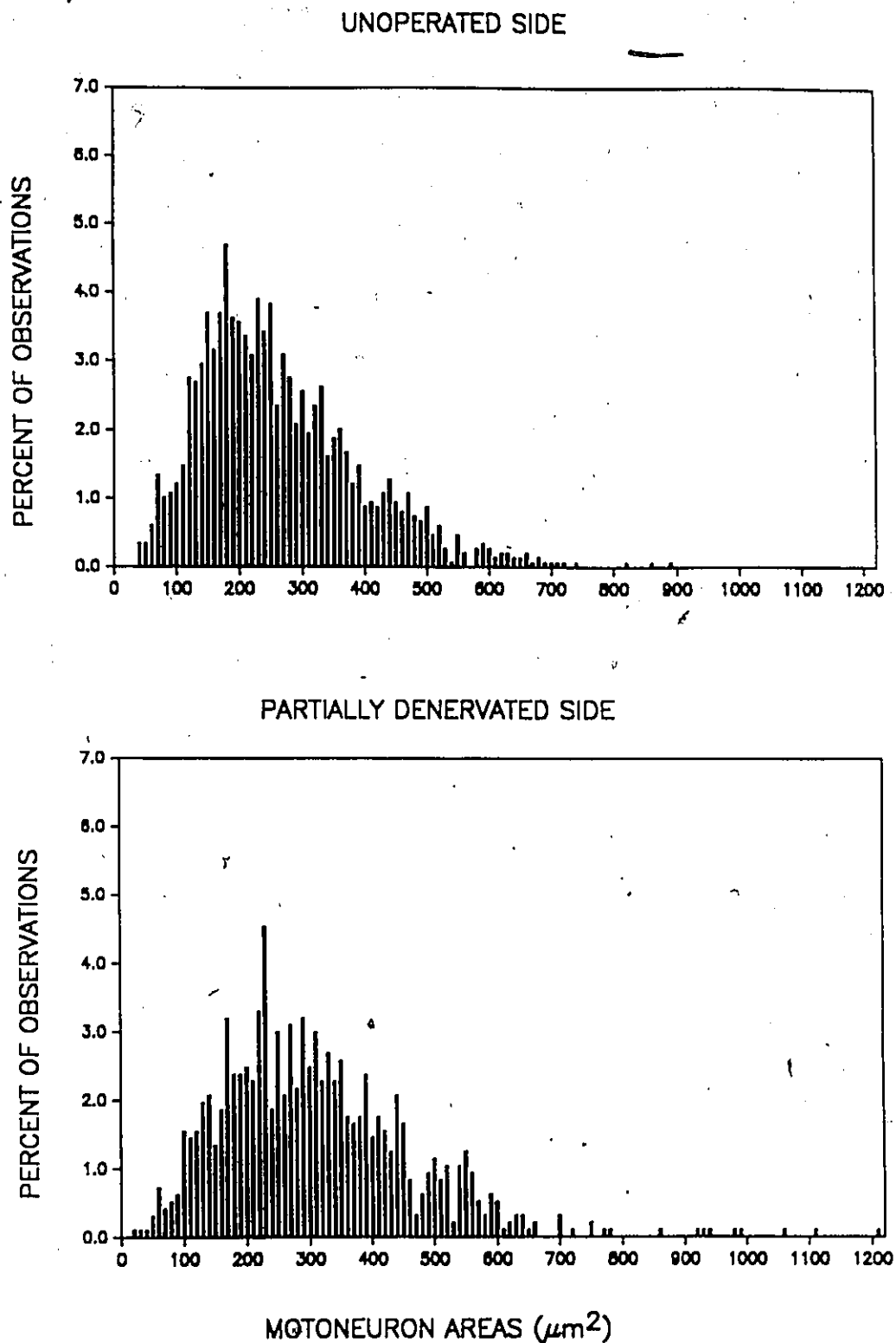
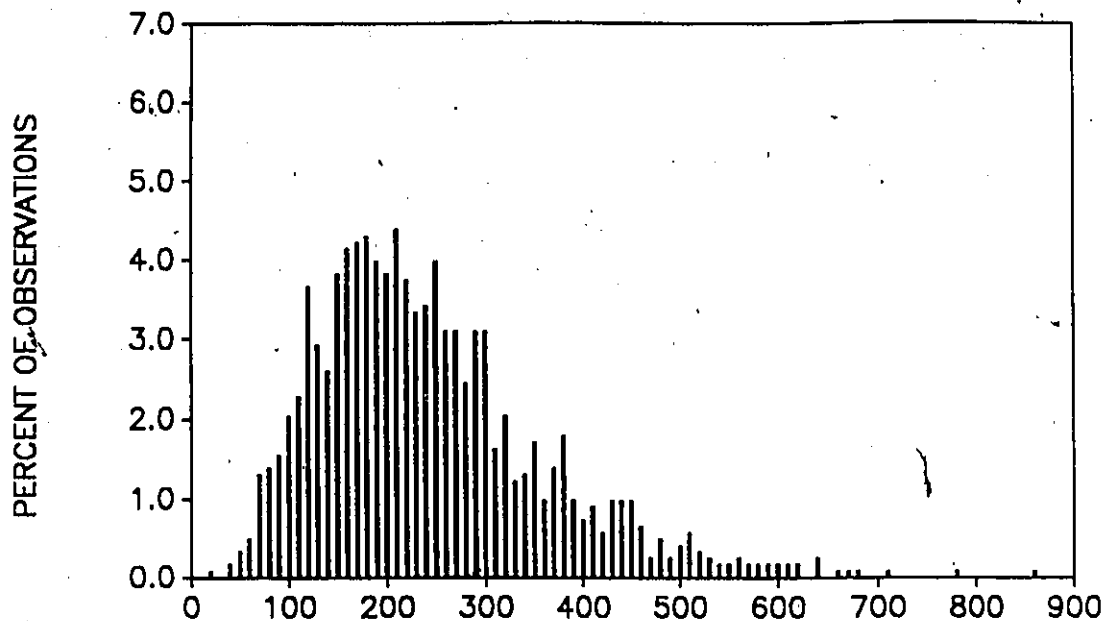
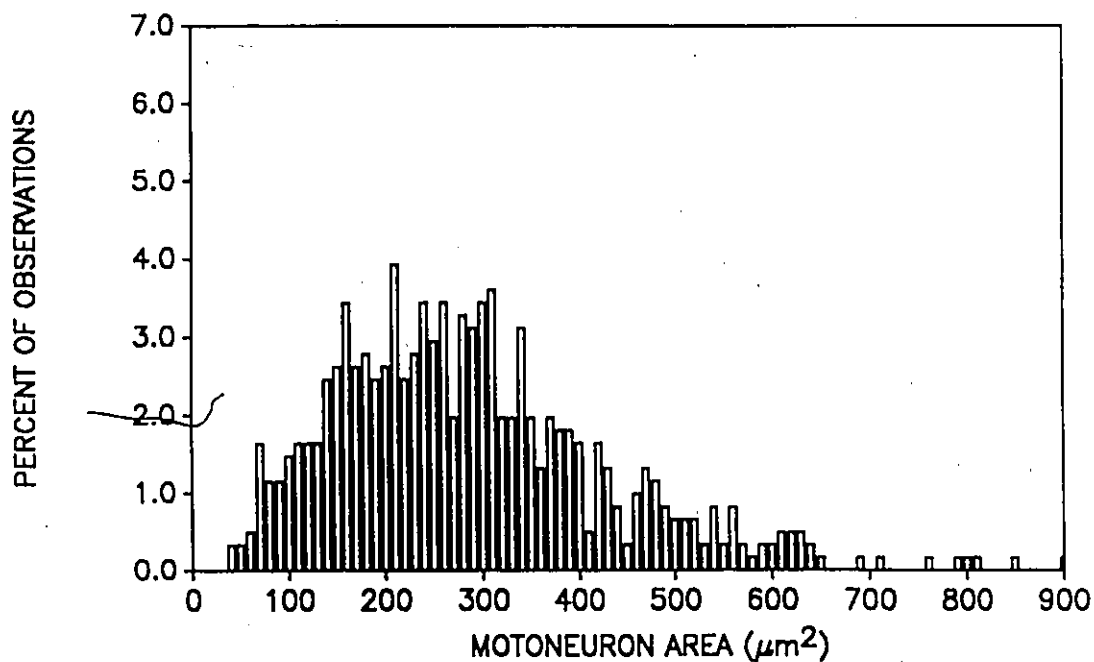


FIGURE 3 - Frequency distribution of soma areas in the TA motoneuron pool of dy2j mice. Areas are plotted as raw data (N=1234).

UNOPERATED SIDE



PARTIALLY DENERVATED SIDE



However, plotting the raw data of all the individual animals' motoneuron areas together could create some problems since the mean soma areas of the individual animals varied. For example, in C57 mouse #1 the mean soma area could be $325 \mu\text{m}^2$ while C57 mouse #2 had a mean area of $195 \mu\text{m}^2$. In mouse #1, the *gamma* motoneurons could then be in the range of $195 \mu\text{m}^2$ whereas in mouse #2 this area would include the *alpha* motoneurons. This could tend to mask any sort of bimodality in the distribution if the raw data were plotted in a frequency distribution. A technique was then sought to give a clearer description of the distribution. The raw data from each individual animal was converted to a percent value of that individual animal's mean soma area so that the soma areas from all animals could be grouped together. Figure 4 illustrates the frequency distribution of the C57 animals' mean soma areas plotted in this way. However, even when this was done, there was no indication of a bimodal distribution. Since there was a lack of bimodality, a precise estimate of the percentage of *alpha* and *gamma* motoneurons could not be determined. All the tables reporting the TA motoneuron pools therefore, are total numbers including both *alpha* and *gamma* motoneurons.

The dy2j motoneuron areas were converted to the percent value of the individual means as above. However, once again, there was no signs of a bimodal distribution as illustrated in figure 5. When one compares the mean soma area of the TA motoneuron pool of C57 mice with that of the dy2j mice, there was no difference ($p \geq 0.05$) between the two groups.

FIGURE 4 - Frequency distribution of soma areas in the TA motoneuron pool of C57 mice. Areas are plotted as a percent of the individual animal's mean soma area (N=1610).

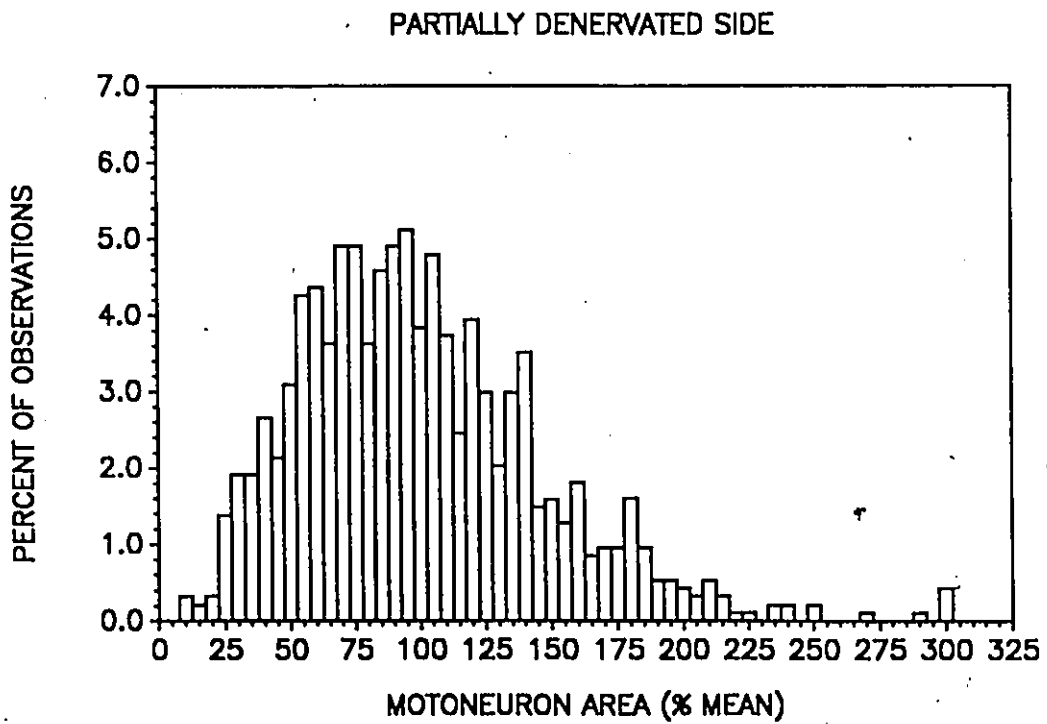
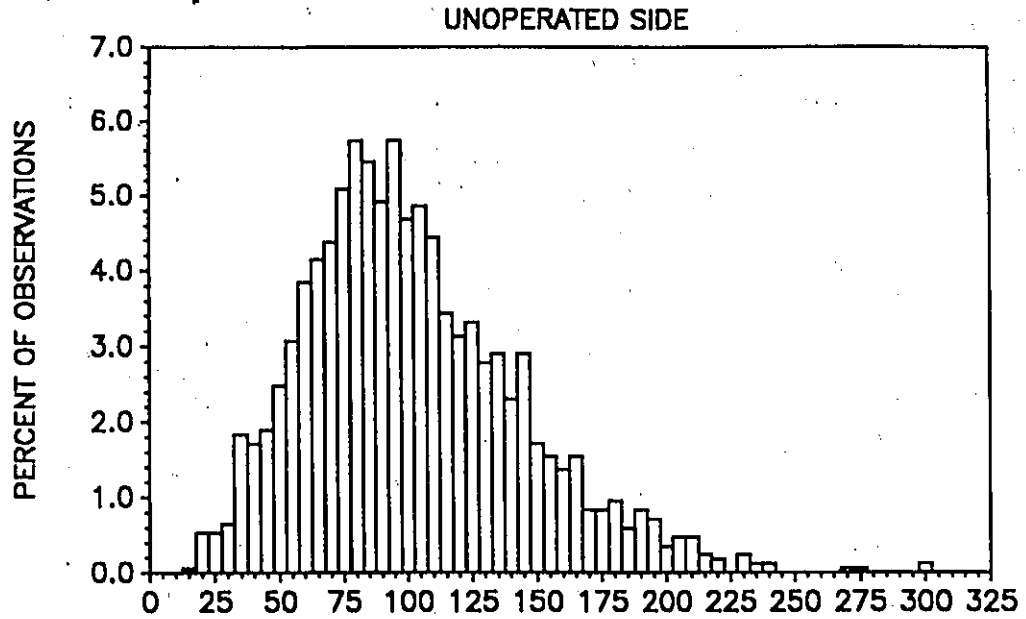
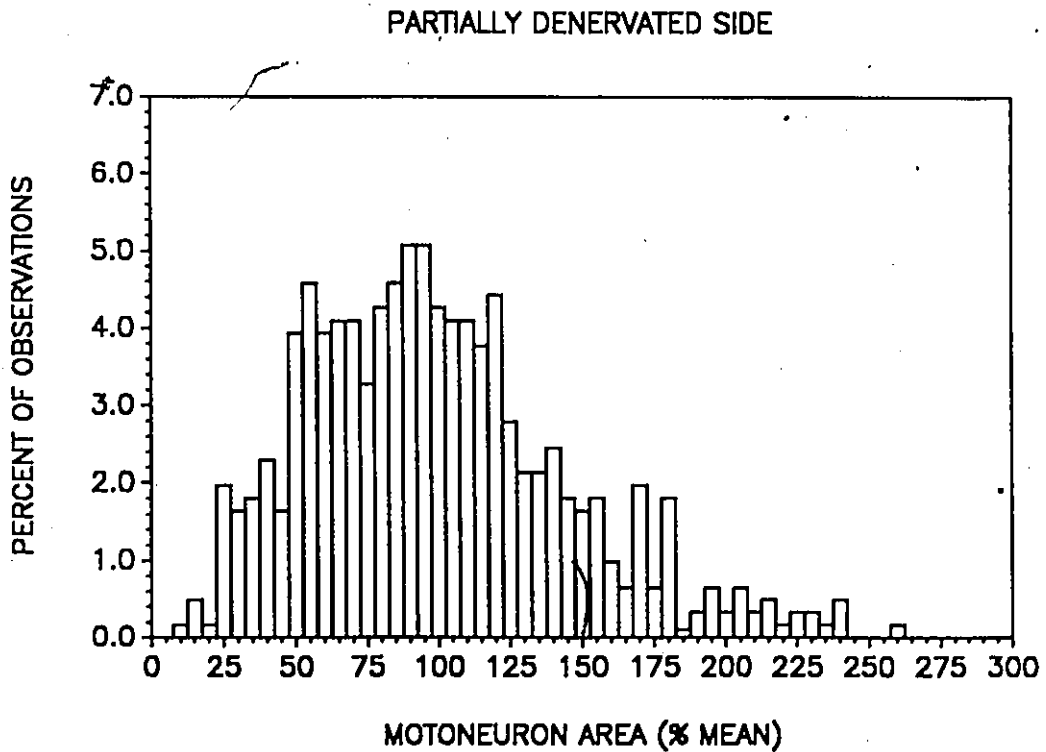
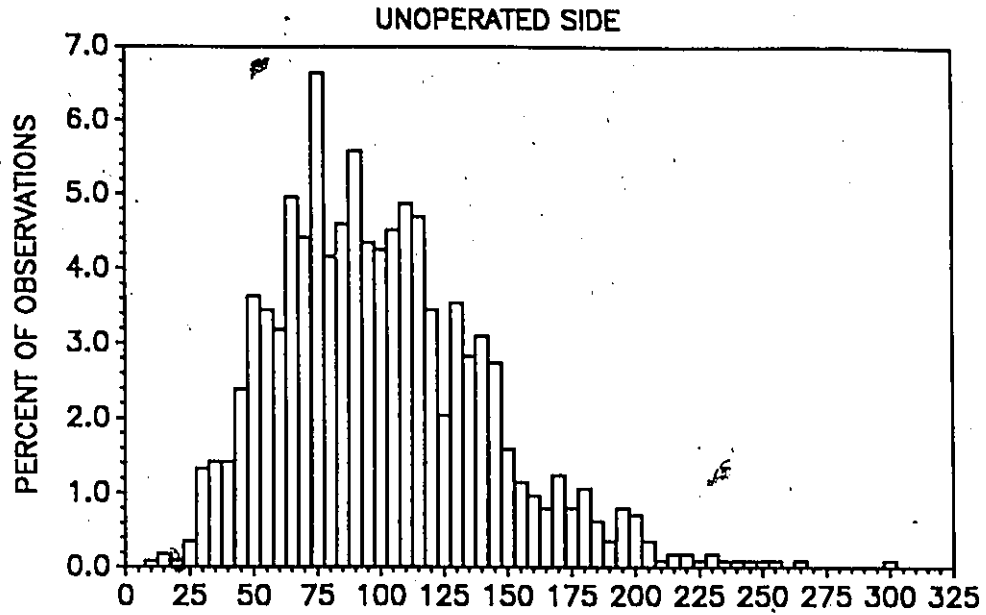


FIGURE 5 - Frequency distribution of soma areas in the TA motoneuron pool of dy2j mice. Areas are plotted as a percent of the individual animal's mean soma area (N=1234).



ii) Partial Denervation

Table 2 displays the effect of partial denervation on the numbers and sizes of motoneurons supplying the tibialis anterior muscle in both C57 and dy2j mice. In 12 C57 mice, the mean number of motoneurons in the TA muscle was significantly reduced ($p \leq 0.05$) from 140 ± 6 motoneurons to 82 ± 5 motoneurons following partial denervation.

The dy2j motoneuron pool was also significantly reduced as a result of the partial denervation surgery. In the dystrophic motoneuron pool, (N=12), the mean of 112 ± 7 motoneurons observed on the unoperated side was significantly reduced ($p \leq 0.05$) to 60 ± 5 on the partially denervated side. The experimental procedure achieved an almost identical degree of reduction in the motoneurons in both C57 and dy2j.

Table 2 also shows the mean soma areas on the unoperated and partially denervated sides of the C57 and dy2j mice. In the C57 mice, there was a significant increase ($p \leq 0.05$) in the motoneuron soma area. The overall mean soma area on the unoperated side was $245 \pm 19 \mu\text{m}^2$ and this value increased significantly to a value of $299 \pm 16 \mu\text{m}^2$ with partial denervation. In the dy2j mice, the mean soma area was $235 \pm 18 \mu\text{m}^2$ on the unoperated side and this value increased significantly to $292 \pm 18 \mu\text{m}^2$ with partial denervation. Therefore, following partial denervation, there is a decreased motoneuron supply to the TA muscle accompanied by an increase in the soma area of these neurons in both the C57 and dy2j mice.

TABLE 2

THE EFFECT OF PARTIAL DENERVATION ON THE NUMBER AND SIZES OF MOTONEURONS SUPPLYING THE ANTERIOR TIBIALIS MUSCLE

		NUMBER OF MOTONEURONS	MOTONEURON AREAS (μm^2)
C57 MICE (N=12)	CONTROL SIDE	140.3±6.0	245.1±18.5
	PARTIALLY DENERVATED SIDE	81.5±9.6 ^a	298.5±15.9 ^b
	PARTIAL DENERVATION AS % OF CONTROL SIDE	57.6±6.1‡	126.3†
dy2j MICE (N=12)	CONTROL SIDE	111.8±2.7 ^c	235.4±17.6
	PARTIALLY DENERVATED SIDE	60.4±5.4 ^a	291.8±17.6 ^b
	PARTIAL DENERVATION AS % OF CONTROL SIDE	49.1±5.1‡	127.3†

♦ data are expressed as means ± standard error of mean.

a - significant decrease ($p \leq 0.05$) when compared with control side

b - significant increase ($p \leq 0.05$) when compared with control side

c - significant decrease ($p \leq 0.05$) when compared with C57 mice

‡ - $\frac{\text{number of motoneurons on PD side} \times 100}{\text{number of motoneurons on control side}}$

† - $\frac{\text{individual motoneuron sizes with PD} \times 100}{\text{mean of control side for that individual animal}}$

iii) Older Animals with Partial Denervation

With partial denervation, there was an increase in the mean soma area when compared to the unoperated side in both C57 and dy2j mice. To ensure that this was not a transient response to nerve damage or reinnervation, the number and sizes of motoneurons in the TA muscle pool was determined for animals that had been partially denervated and left to recover for eighteen months. Table 3 indicates that even after an 18 month recovery from partial denervation, in both the C57 and dy2j mice, there is a significant reduction in the motoneuron supply to the TA. This is accompanied by an increase in the soma area of these remaining neurons.

iv) Partial denervation/Partial extirpation (PD/PE)

Table 4 shows the effect of the partial denervation accompanied by a partial extirpation of the tibialis anterior muscle. The number and the sizes of the motoneurons on the unoperated side of both C57 and dy2j mice are similar to those that were observed in the first set of experiments and are combined in Table 1.

The effect of the PD/PE surgery on the number of motoneurons supplying the TA muscle is very similar to that of the partial denervation surgery alone. On the unoperated side the mean number of motoneurons in the C57 mice went from 142 ± 4 to a significantly reduced (≤ 0.05) 66 ± 0 motoneurons. The same degree of reduction in motoneuron pool numbers was observed in both the dy2j mice and the C57. There were 114 ± 2 motoneurons on the unoperated side and only 55 ± 4 motoneurons on the PD/PE side of the dy2j mice.

TABLE 3

THE EFFECT OF PARTIAL DENERVATION ON THE NUMBER AND SIZES
OF MOTONEURONS SUPPLYING THE ANTERIOR TIBIALIS MUSCLE
OF OLDER MICE♦

		NUMBER OF MOTONEURONS	MOTONEURON AREAS (μm^2)
C57 MICE (N=3)	CONTROL SIDE	145.7±13.6	218.9±6.1
	PARTIALLY DENERVATED SIDE	76.7±20.7 ^a	312.7±7.2 ^b
	PARTIAL DENERVATION AS % OF CONTROL SIDE	51.5±9.1‡	144.7†
dy2j MICE (N=5)	CONTROL SIDE	113.0±4.9 ^c	260.3±10.8
	PARTIALLY DENERVATED SIDE	71.0±11.5 ^a	339.7±12.4 ^b
	PARTIAL DENERVATION AS % OF CONTROL SIDE	63.4±10.9‡	132.5†

♦ data are expressed as means ± standard error of mean

a - significant decrease ($p \leq 0.05$) when compared with control side

b - significant increase ($p \leq 0.05$) when compared with control side

c - significant decrease ($p \leq 0.05$) when compared with C57 mice

‡ - $\frac{\text{number of motoneurons on PD side} \times 100}{\text{number of motoneurons on control side}}$

† - $\frac{\text{individual motoneuron sizes with PD} \times 100}{\text{mean of control side for that individual animal}}$

When one examines the effect of the PD/PE surgery on the motoneuron soma areas, it is evident that there is no significant change ($p \geq 0.05$) in the mean soma area when comparing the operated side with the unoperated side in both the C57 and the dy2j mice as shown in Table 4.

B) Physiological Recordings

The first attempt at measuring the number and size of motor units in the TA following partial denervation was using the method of ventral root splitting described in the Methods section. A major problem occurred with this procedure since it was impossible to isolate a filament which contained a single unit. With muscles having fewer motor units, this difficulty should not arise as indicated by the successful reports of ventral root splitting on the mouse EDL (Bateson and Parry 1983) and on the mouse soleus (Lewis et al. 1982, Desypris 1986). However, soon after the length-tension relationship for EDL was established with the muscle set at optimal length, the tension following indirect stimulation dropped. This occurred over a period of ten to twenty minutes. The tension decreased to less than 50% of the maximal tension initially and then slowly dropped. Consequently, these experiments were discontinued and determination of the number and sizes of motor units by a morphological technique was attempted.

TABLE 4

THE EFFECT OF PARTIAL DENERVATION WITH PARTIAL EXTIRPATION
(PD/PE) ON THE NUMBER AND SIZES OF MOTONEURONS SUPPLYING
THE ANTERIOR TIBIALIS MUSCLE

		NUMBER OF MOTONEURONS	MOTONEURON AREAS (μm^2)
C57 MICE (N=12)	CONTROL SIDE	141.8 \pm 4.2	250.7 \pm 14.5
	PD/PE SIDE	66.3 \pm 4.9 ^a	255.7 \pm 19.1
	PD/PE AS % OF CONTROL SIDE	47.8 \pm 3.4 \ddagger	102.8
dy2j MICE (N=12)	CONTROL SIDE	113.8 \pm 2.4 ^b	257.3 \pm 16.3
	PD/PE SIDE	54.8 \pm 3.7 ^a	247.9 \pm 20.5
	PD/PE AS % OF CONTROL SIDE	48.2 \pm 2.9 \ddagger	98.9

♦ data are expressed as \pm standard error of mean

a - significant decrease ($p \leq 0.05$) when compared with control side

b - significant decrease ($p \leq 0.05$) when compared with C57 mice

\ddagger - $\frac{\text{individual motoneuron number with PD/PE} \times 100}{\text{motoneuron number on control side}}$

\ddagger - $\frac{\text{individual motoneuron sizes with PD/PE} \times 100}{\text{mean of control side for each individual animal}}$

FIGURE 6- Frequency distribution of soma areas in the TA motoneuron pool of C57 mice. Motoneurons are plotted as a percent value of the mean soma area of the control side as in Table 2 and 3. Arrows indicate mean value (PD = 126% and PD/PE = 103%).

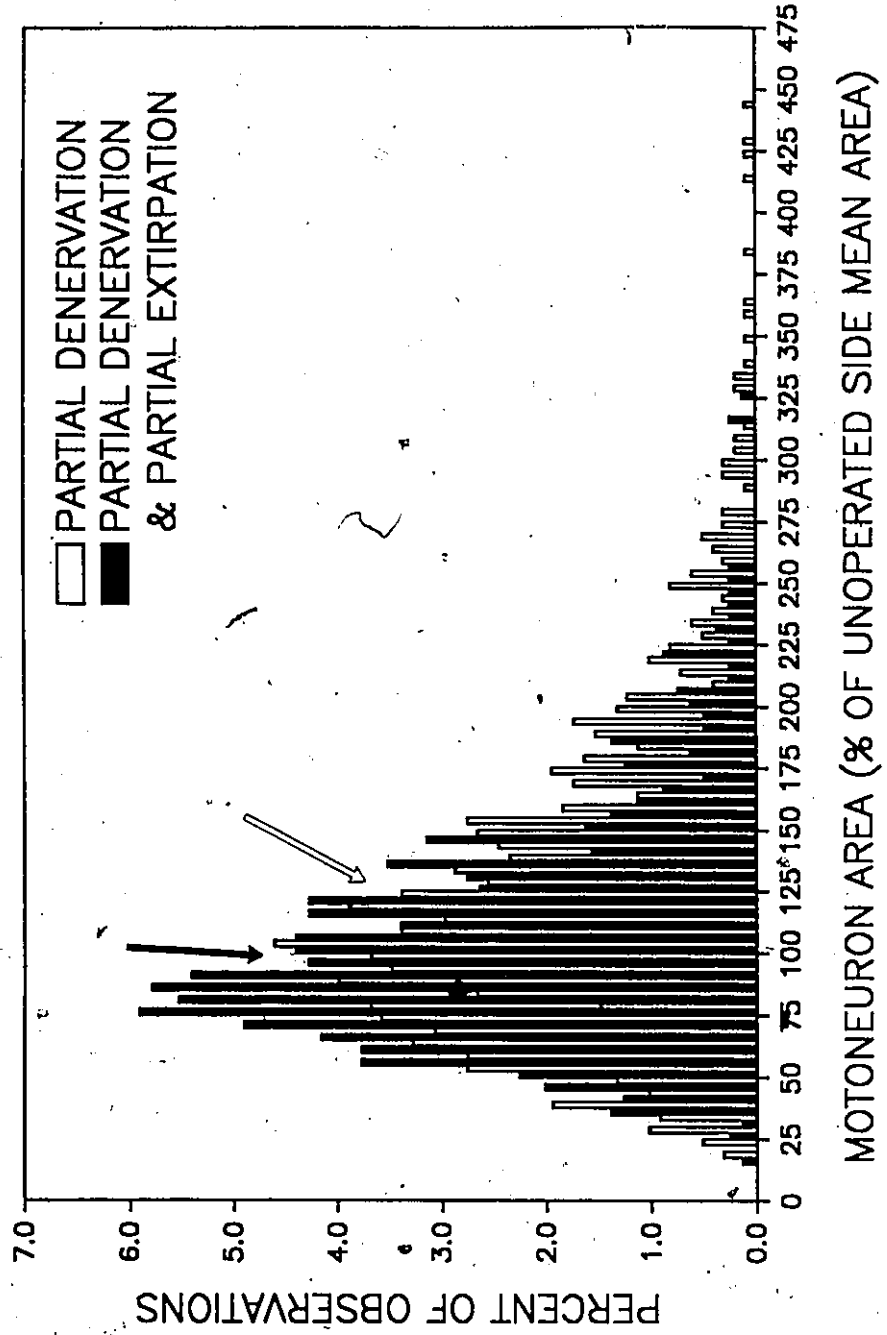
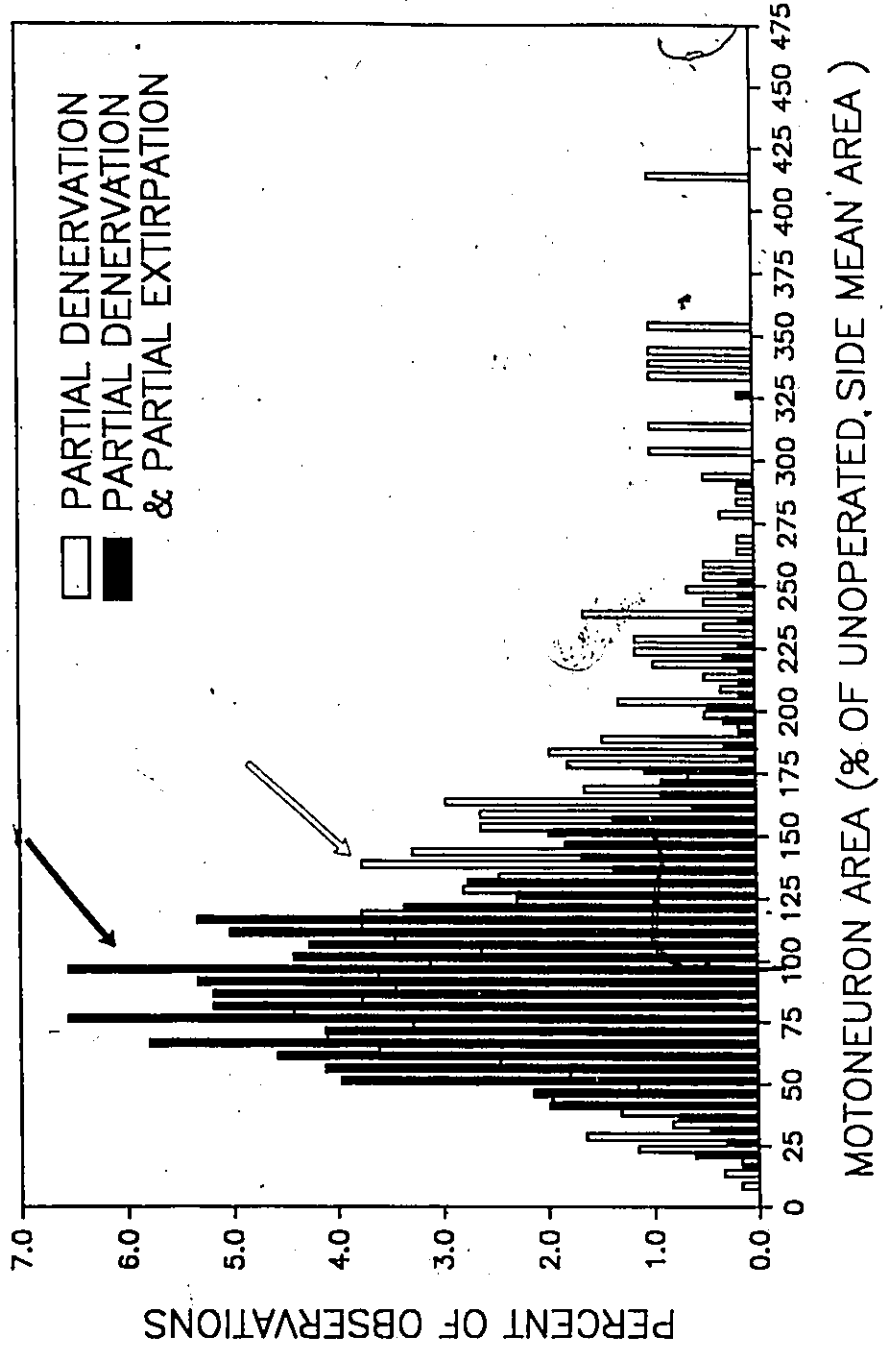


FIGURE 7- Frequency distribution of soma areas in the TA motoneuron pool of dy2j mice. Motoneurons are plotted as a percent value of the mean soma area of the control side as in Table 2 and 3. Arrows indicate mean value (PD = 127% and PD/PE = 99%).



2) Soleus Muscle Injections

To examine the possibility of a mechanical or technical error accounting for the lack of bimodality in the TA motoneuron area distributions, HRP injections were performed on the soleus motoneuron pool where a bimodal distribution has already been cited (Parry et al. 1982; Lewis et al. 1982; Desypris 1986). Three animals were subject to intramuscular injection of HRP with the same protocol as described for the TA. A total of 87 motoneurons were counted and sized indicating a mean of 29 motoneurons supplying the soleus muscle. The frequency distribution of soma areas are in Figure 8. The plot suggests that a bimodal distribution of the soma areas is present in the soleus motoneuron nucleus.

3) Histology

a) Muscle Fiber Counts

Figures 9-10 show the normal and partially denervated tibialis anterior muscles in both C57 and dy2j mice stained with H and E. All the photos except figure 10d (which was 440X) were taken at a magnification of 220X. In the C57 mice, there was a relatively small range of fiber sizes and nuclei are at the periphery of the muscle fibers. However, with partial denervation, the fibers were more variable in size. Occasionally, areas as indicated in Figure 9C, were seen where muscle fibers became necrotic during the two months following partial denervation surgery. The situation in the dystrophic muscle was more complex. Figure 10 shows the TA from a dy2j mouse which normally exhibits variability amongst the fiber diameters

Figure 8- Frequency distribution of soma areas in the soleus motoneuron pool. Areas are plotted as a percent of the individual animal's mean soma area (N=87).

SOLEUS MOTONEURON POOL

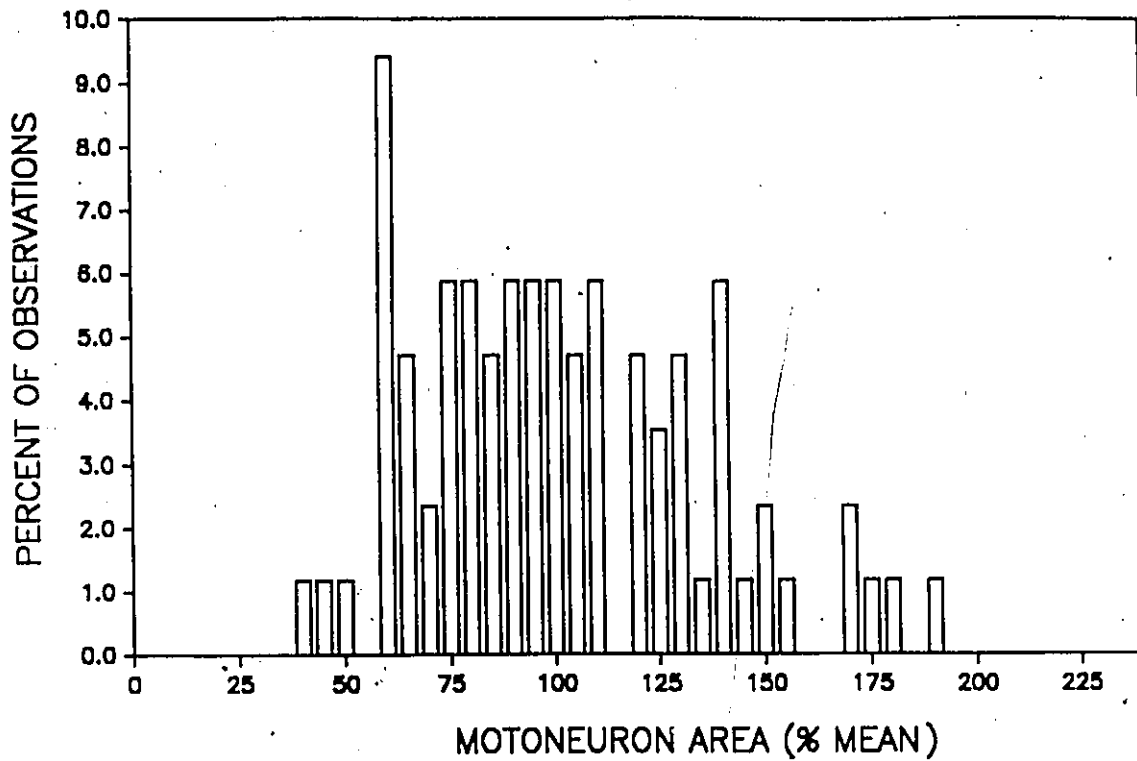
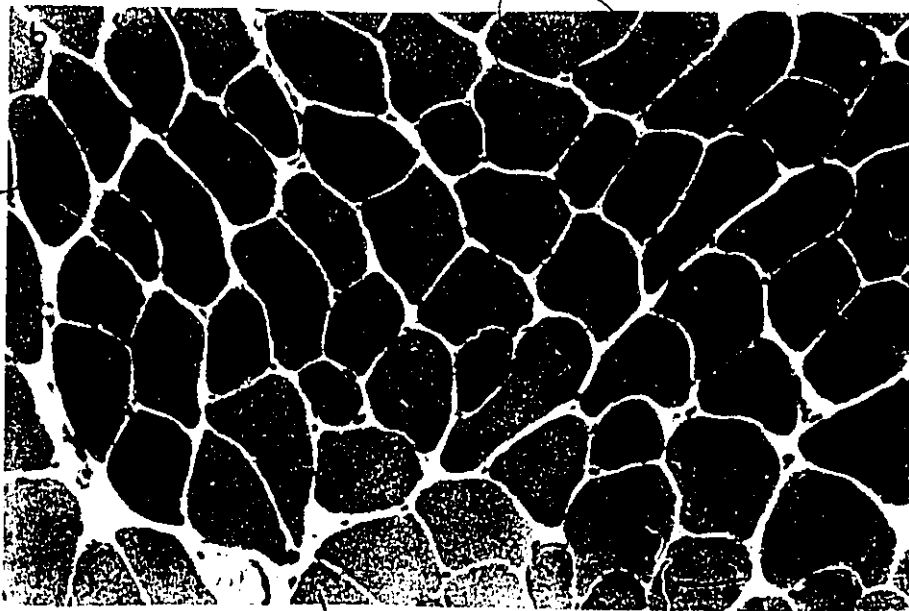
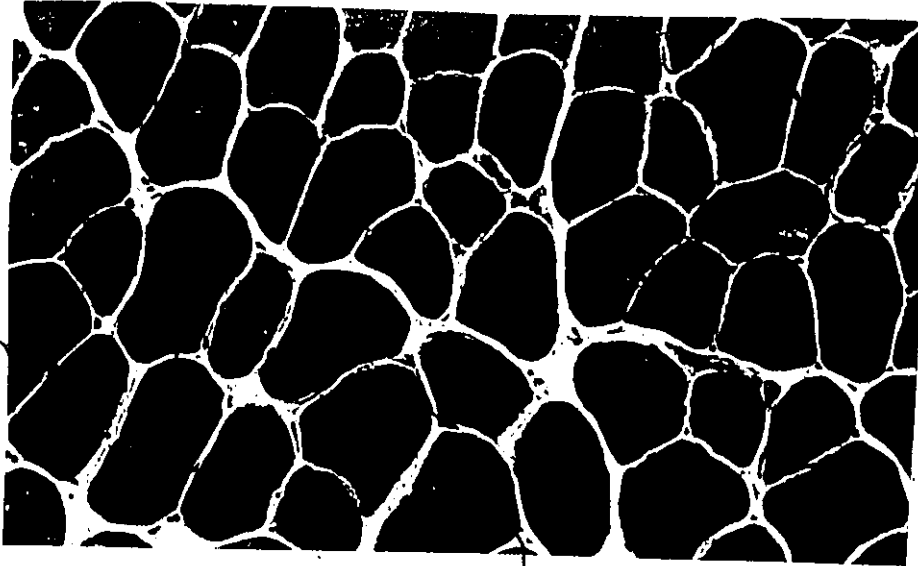


Figure 9- Muscle section from the TA muscle of a C57 mouse stained with Hematoxylin and Eosin. Figure 9a- unoperated, control muscle. Figure 9b and 9c- partially denervated muscle following a two month recovery. Stars indicate necrotic fibers. Note the small range of fiber sizes in the control C57 muscle and the position of the nuclei (indicated by the arrows). Figure 9d- after a two month recovery from complete denervation. Note the 'normal' appearance of many of the fibers indicated with the circles. All photos are 220X magnification.



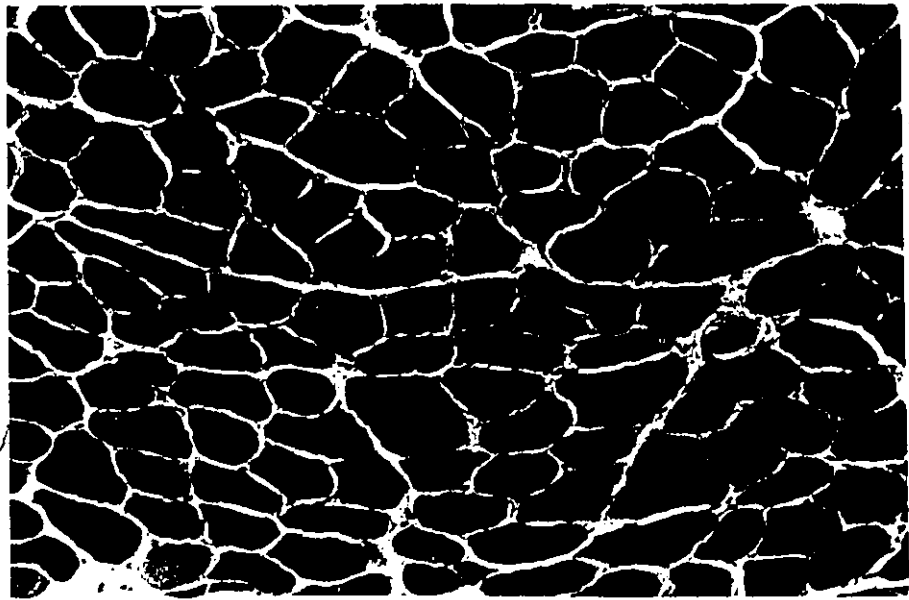
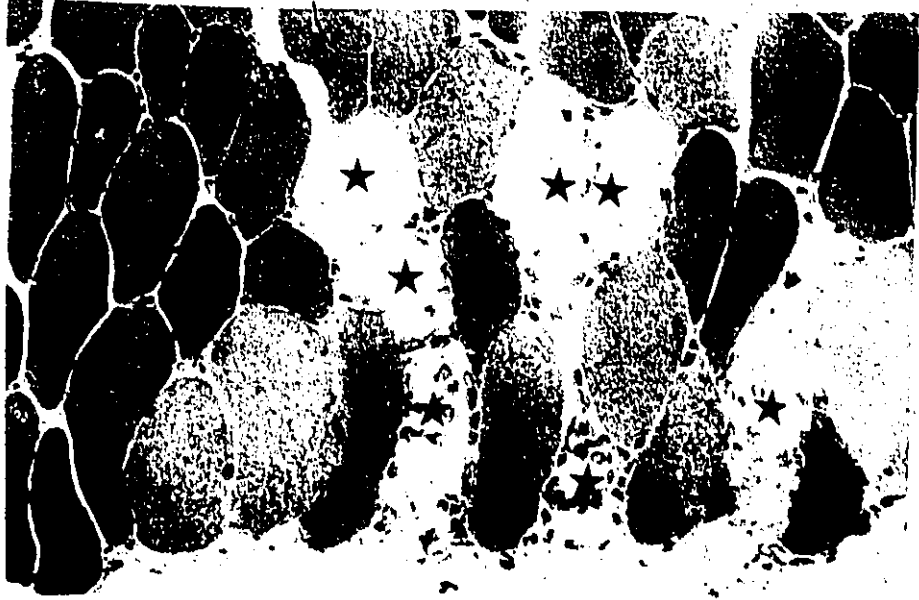
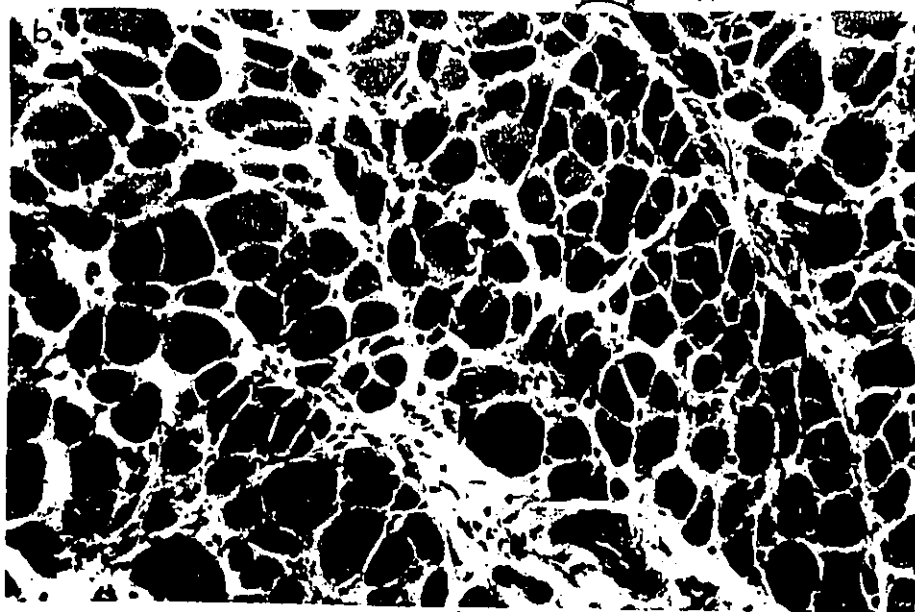
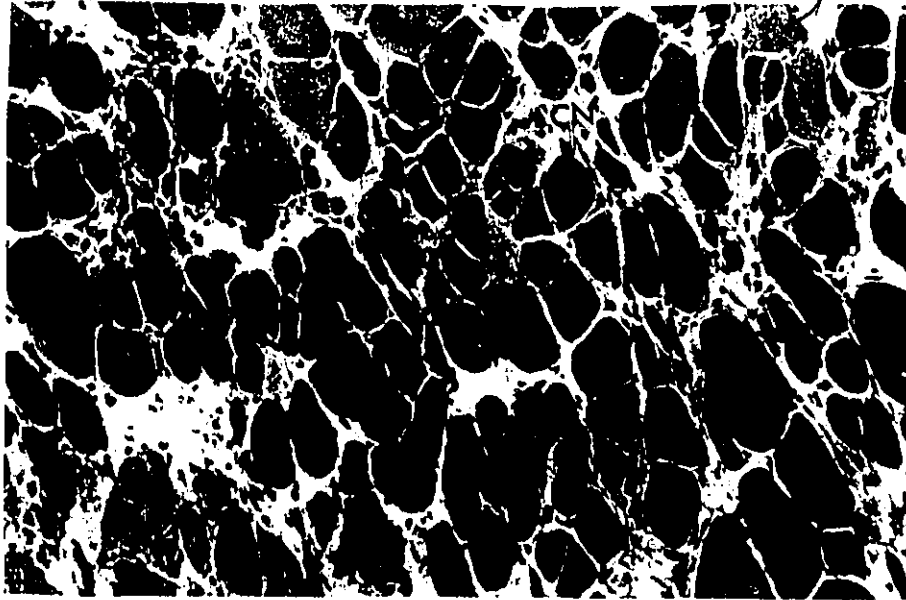
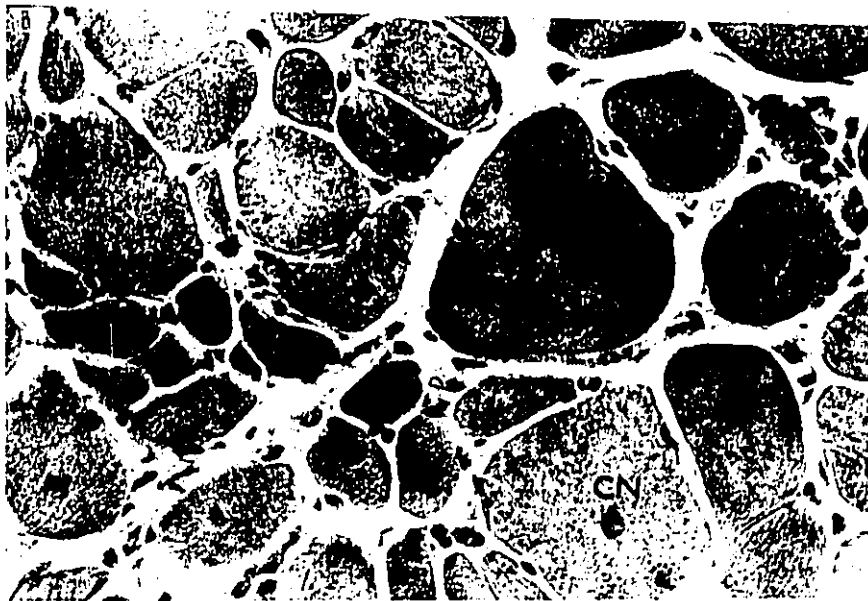


Figure 10- Muscle section from the TA muscle of a dy2j mouse stained with Hematoxylin and Eosin. Figure 10a- unoperated muscle. Figure 10b,c and d- partially denervated muscle following a two month recovery. Note the large variation in muscle fiber sizes, the presence of central nucleation (CN) and the connective tissue infiltration (CT) and the massive degeneration of the muscle tissue when partially denervated. Figure 10 a,b, and c are 220X magnification. Figure 10d is 440X magnification.





as well as central nucleation (regenerating muscle fibers) and the presence of connective tissue. However, with partial denervation the situation in the muscle from the dy2j mice was even more complex. Many of the muscle fibers were so small that it was very difficult to assess whether they were denervated, innervated, degenerating or regenerating.

When the muscle fiber composites were made, the fiber counts did not reveal any significant differences in the C57 mice when the unoperated TA was compared with the partially denervated TA (Table 5). In the dy2j mice, no changes were seen between the two muscles (Table 5). However, some caution is required when interpreting these results. The dy2j muscle fiber counts included all the very small fibers which might not be innervated muscle fibers (see for example figure 10b,c). It seems likely then that there was an overestimation of the number of muscle fibers in the dy2j partially denervated muscle (Figure 10b,c).

b) Immunohistochemistry- NCAM

As briefly mentioned, NCAM has been observed to be present on the surface of denervated muscle fibers (Rutishauser et al. 1983; Covault and Sanes 1985; Moore and Walsh 1986; Walsh and Moore 1986). Staining for the presence of denervated muscle fibers uses an immunohistochemical technique with an antibody to NCAM. A number of experiments were performed with this antibody. These include staining of the following tissues: embryonic muscle, partially denervated muscle (after four days and two months), completely denervated muscle (after four days and two months), normal C57 TA and unoperated dy2j TA wrapped in diaphragm. The figures of

TABLE 5

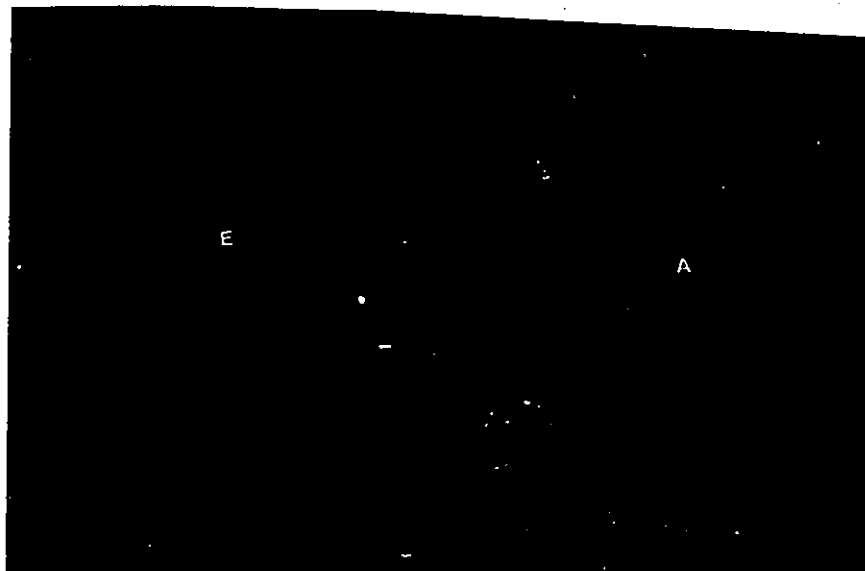
**MUSCLE FIBER COUNTS IN THE TIBIALIS ANTERIOR MUSCLE
OF NORMAL AND DYSTROPHIC MICE**

	C57 MICE			dy2j MICE _y		
	CONTROL TA	PD. TA	<u>PDx100</u> CTA	CONTROL TA	PD TA	<u>PDx100</u> CTA
ANIMAL #1	2242	2132	95.1	1261	1272	100.9
ANIMAL #2	2136	2020	94.6	1773	2142	120.8
ANIMAL #3	2333	2221	95.2	1645	1710	104.0
MEAN	2237	2125	95.0	1557	1708	109.7

each of these sections are shown in Figures 11-16. The histological sections stained with the anti-NCAM as the first antibody were then labelled with a secondary FITC-labelled (fluorescent) antibody. A control stain was performed on the sections in which the primary antibody was omitted, to determine the specificity for the NCAM labeling. This helps to indicate that the intense staining in the muscle fibers itself and surrounding the muscle fibers with the anti-NCAM primary antibody, is a positive reaction with the NCAM itself.

As previously mentioned, embryonic muscle has been shown to stain positively with antibodies to NCAM since the muscle fibers have not yet innervated at the early stages of neural development (Rutishauser et al. 1983; Covault and Sanes 1985; Moore and Walsh 1986; Walsh and Moore 1986). Staining of embryonic muscle was performed to examine the specificity of the NCAM antibody since this has never been used in this laboratory. Figure 11 contains a muscle section composed of both embryonic (day 14) hindlimb and adult soleus muscle wrapped in diaphragm stained with anti-NCAM. The section reveals the intense positive staining around the entire muscle fibers in the embryonic tissue indicating that they not innervated as expected (Rutishauser et al. 1983). In the adult soleus muscle, none of the muscle fibers reveal the intense positive staining around the muscle fibers as expected.

Figure 11- Section of embryonic hindlimb (E) and adult soleus muscle (A) wrapped in diaphragm from a C57 mouse (220X). Figure 11a stained with rabbit anti-rat NCAM and goat anti-rabbit IgG FITC. Figure 11b- with goat anti-rabbit IgG FITC alone. Positive NCAM fibers indicate embryonic muscle fibers have not been innervated. Positive staining is observed around the entire muscle fiber.



The partial denervation surgery removes part of the nervous supply to the TA. To examine which muscle fibers are affected as a result of the partial denervation, two C57 animals were examined four days after surgery. These animals were subjected to partial denervation of the left lateral popliteal nerve, and a complete denervation of the right lateral popliteal nerve both performed as in the methods section. After a four day recovery period, the TA muscles were removed, wrapped in diaphragm, frozen and sectioned. The soleus muscles were removed for control tissue. The muscles were stained with anti-NCAM to determine the randomness of the muscle fibers denervated from partial transection. Figure 12 shows this technique of partial denervation seems to remove motor axons that innervate mainly the superficial portion of the tibialis anterior muscle. This is important in examining the results since one area of the muscle is more affected than the other and therefore, the partial denervation is truly not a random surgery. If any type-grouping occurs with reinnervation, it will be seen in this area when the muscle is stained with monoclonal antibodies. This will be addressed in the next section. Figure 13 shows the TA stained with anti-NCAM four days after complete denervation. This experiment was performed to show the level of staining in completely denervated fibers. When one compares the four day PD with the four day complete denervation, it seems that there are no clear distinctions between the two groups, suggesting that not all of the denervated muscle fibers were expressing NCAM.

Figure 12- Section of adult TA muscle from a C57 mouse four days after partial denervation (220X). Figure 12a- stained with rabbit anti-rat NCAM and goat anti-rabbit IgG FITC. Figure 12b- with goat anti-rabbit IgG FITC alone. Positive fibers are stained around the periphery and throughout the muscle fiber.

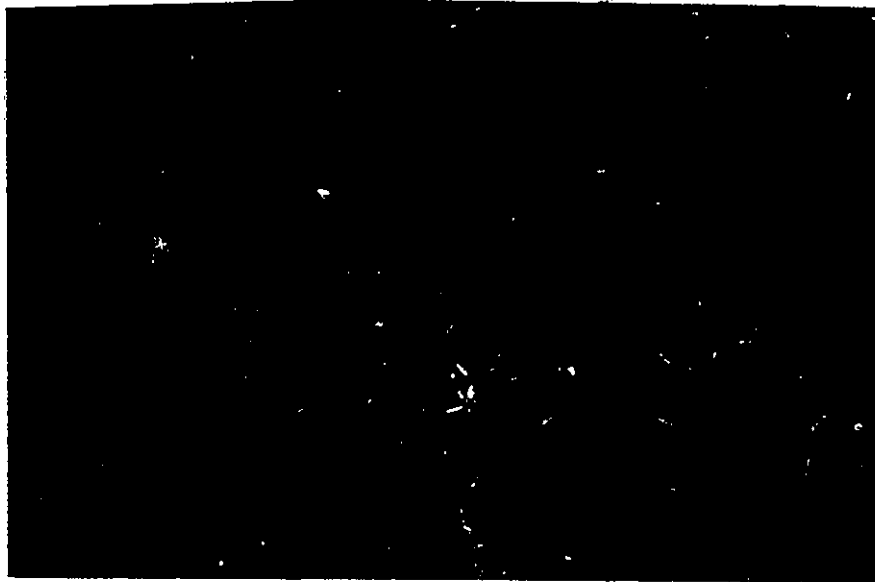
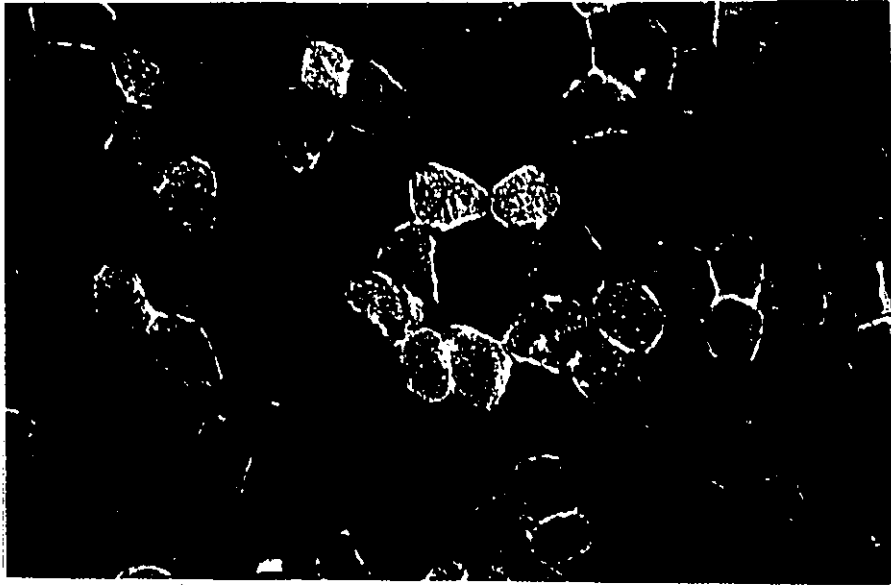


Figure 13- Section of adult TA muscle four days after complete denervation from a C57 mouse (220X). Figure 13a- stained with rabbit anti-rat NCAM and goat anti-rabbit IgG FITC. Figure 13b-with goat anti-rabbit IgG FITC alone. Positive (denervated) fibers are stained around the periphery and throughout the muscle fiber.

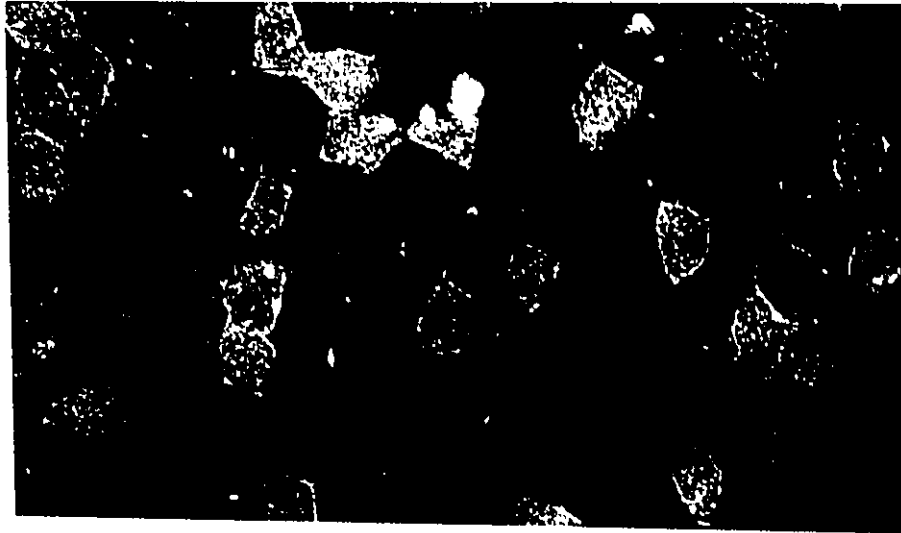


Figure 14 shows the TA stained with anti-NCAM two months after partial denervation. Very few NCAM-positive fibers were found in these muscles, the very small, bright fibers representing denervated muscle fibers.

Even after eight weeks of denervation (Figure 15), 'normal' size fibers were still present that did not stain with anti-NCAM. This clearly makes assessment of the total number of innervated fibers after partial denervation more difficult since some of the 'normal' (NCAM negative) fibers in the partially denervated TA section could be counted as innervated fibers but in fact could be denervated. Therefore, any morphological estimate of motor unit size would be clearly overestimated. At this time, the morphological assessment of motor unit size was abandoned and efforts were concentrated on trying to make assessments of the sprouting ability of the motor axons in the C57 mouse and on the factors controlling motoneuron size.

Figure 16 demonstrates that there seems to be positive staining of some of the normal adult tibialis anterior muscle fibers and as well many fibers in the diaphragm. This adds to the problem of determining an accurate assessment of the number of innervated muscle fibers.

Figure 14- Section of adult TA muscle from a C57 mouse two months after partial denervation (220X). Figure 14a- stained with rabbit anti-rat NCAM and goat anti-rabbit IgG FITC. Figure 14b- with goat anti-rabbit IgG FITC alone. Positive (denervated) fibers are stained around the periphery and throughout the muscle fiber itself. These appear to be the small, very bright fibers. Arrow indicates the same muscle fiber which stains positive for NCAM (denervated fiber).

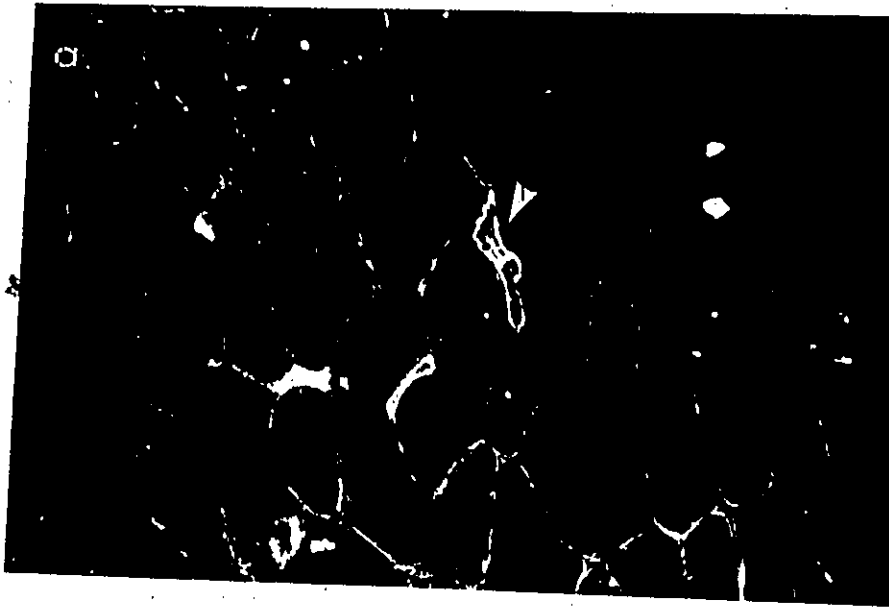


Figure 15- Section of adult TA muscle two months after complete denervation from a C57 mouse (220X). Figure 15a- stained with rabbit anti-rat NCAM and goat anti-rabbit IgG FITC. Figure 15b- with goat anti-rabbit IgG FITC alone. Note: most of the fibers appear NCAM positive (denervated) but still even after two months, many of the muscle fibers look 'normal' (NCAM negative) as indicated by arrows.

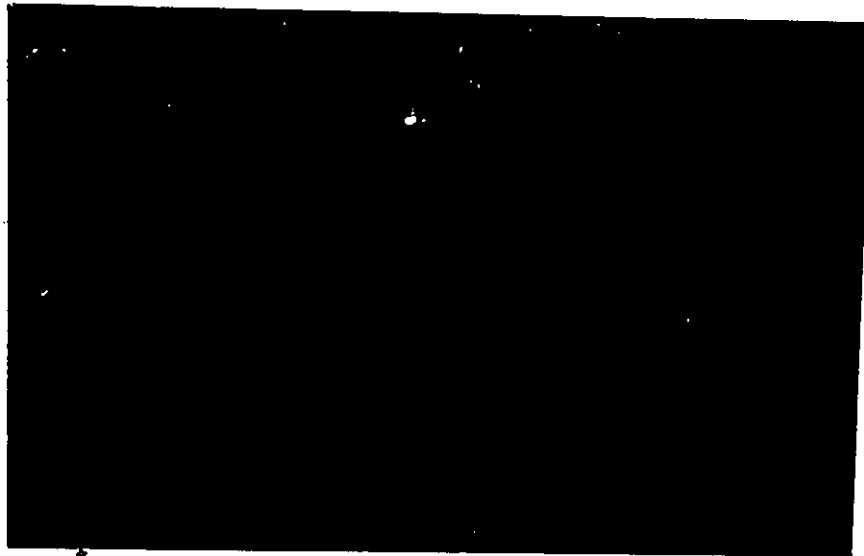
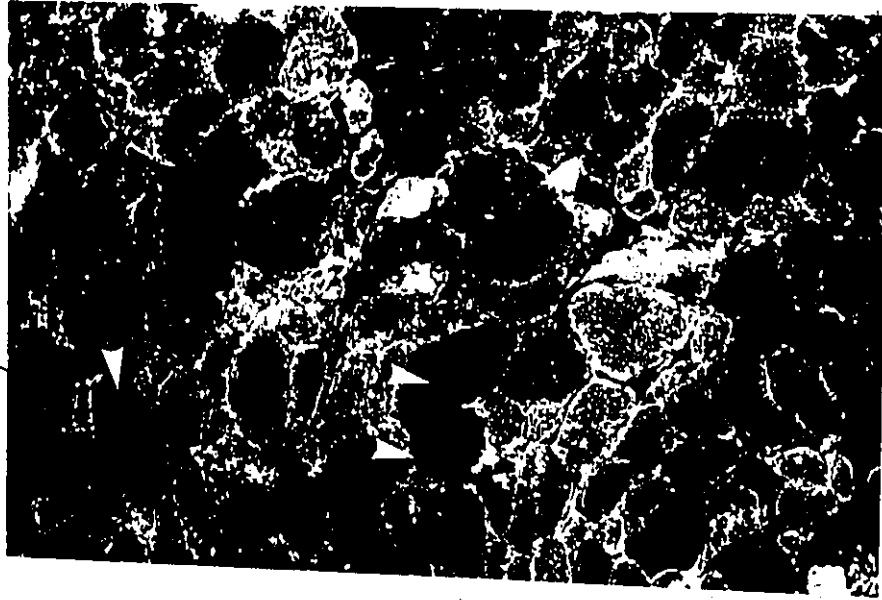
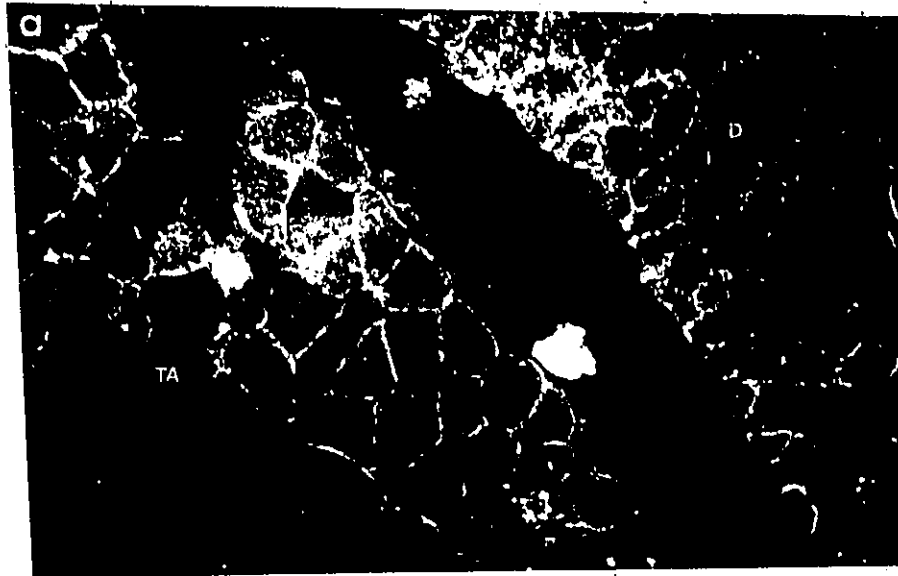


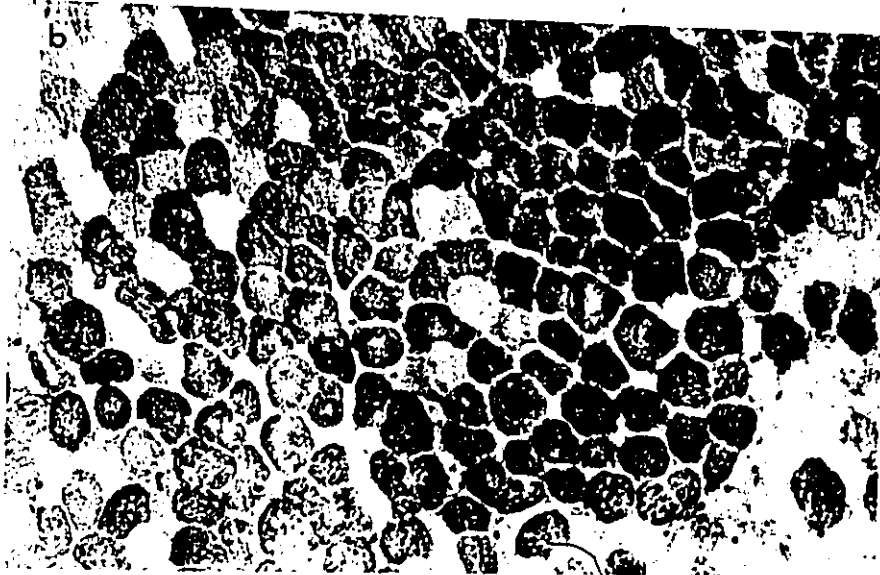
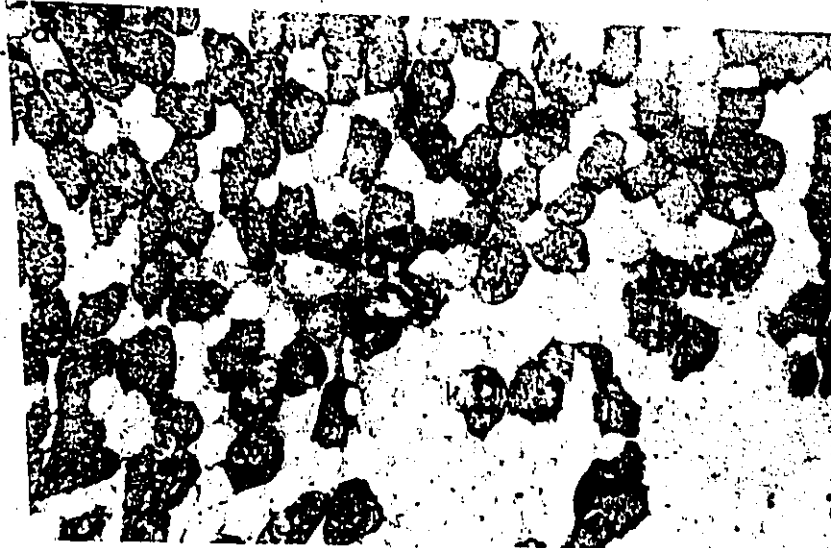
Figure 16- Section of adult TA muscle wrapped in diaphragm from a C57 mouse (220X). Figure 16a-stained with rabbit anti-rat NCAM and goat anti-rabbit IgG FITC. Figure 16b- with goat anti-rabbit IgG FITC alone. Note: the prescense of staining in the control tissue making it difficult to assess denervated muscle fibers.



c) Immunohistochemistry- Myosin Heavy Chains

Figures 17 reveal that when the TA sections were stained with the F₃ antibody which detects the IIB muscle fiber type, small areas of type groups occur. When stained with the other antibodies detecting type I and IIA fiber types, no clear changes in the muscle fiber composition were observed with partial denervation when compared to the normal TA.

Figure 17- Muscle section of adult TA from a C57 mouse stained with myosin heavy chain antibody F₃ which labels the type IIb muscle fibers (220X). Figure 17a- control TA muscle. Figure 17b- partially denervated muscle with two month recovery. Note: these are the same areas of the muscle. The random dispersion is not as evident with partial denervation, however, clear type-grouping is not present.



DISCUSSION

1) Characteristics of the TA motoneuron pool

a) Motoneuron Numbers

1) C57 Mice

The number of motoneurons supplying the normal C57 tibialis anterior muscle is of interest since this number has yet to be quantified in this strain of mouse. Using retrograde transport of HRP, the TA motoneuron pool in the C57 mouse contains approximately 142 neurons including both *alpha* and *gamma* motoneurons. Only one previous study, similar in both species and methods, has been reported. Wasserschaff and Kleinebel (1988), using intramuscular injection of HRP, found a mean of 128 ± 28 motoneurons supplying the TA of an experimental group of 36 mice. The authors do not mention whether this includes both the *alpha* and *gamma* motoneurons or simply the *alpha* motoneurons. The strain of mouse was not indicated but the numbers are very similar to those in this thesis.

Using HRP retrograde labeling, Peyronnard and Charron (1983) examined the number of motoneurons supplying the TA of the rat. With a sample size of five, they found a mean of 159 ± 18 labelled motoneurons following exposure of the motor nerve to HRP. Similarly, in the rat TA motoneuron pool, using retrograde axonal transport of HRP, Krishnan et al. (1985) found values ranging from 140 to 185 motoneurons, while Lowrie et al. (1987) found 149 motoneurons. These numbers are comparable with those presented in this thesis, especially when one considers that the size of the rat motoneuron pool has also been shown to be larger in other hindlimb

muscles in the rat when compared to the mouse (e.g. the soleus muscle (Thompson and Jansen 1977), the EDL muscle (Gorio et al. 1980; Guth et al. 1983)).

Harris and Wilson (1971) used the physiological recording technique of incremental stimulation of the sciatic nerve to estimate the number of motor units in the tibialis anterior muscle of the ReJ 129 strain of mouse. This technique involved first finding the threshold stimulation voltage applied through an electrode placed on the sciatic nerve. Once the threshold was recorded, the stimulus voltage was gradually increased until an increment in the twitch tension was recorded, i.e. a single motor unit. This was performed until the first four incremental tensions were recorded and then the stimulus was increased and a series of summated motor units was recorded. Following this, a maximum stimulus was applied to the nerve and the response was recorded. By dividing the maximum twitch tension of the whole muscle by the mean value of the first four increments, it was estimated that the TA contained 214 motor units. This number is much greater than the estimate in this thesis of the same motoneuron pool especially when considering that the HRP method includes both the *alpha* and the *gamma* motoneurons, and the physiological procedure only yields the number of *alpha* motoneurons. This large discrepancy may be attributed to several factors. First of all, different strains of mice were used. However, since there was such an enormous difference between the two populations, the results of the study of Harris and Wilson (1971) may indicate sciatic nerve incremental stimulation leads to an overestimation of motor unit numbers at least in the larger muscles. In

these experiments, the number of motor units were based on estimates from a sample of only four units (the first four increments recorded). In a large muscle such as the tibialis anterior, this would represent a very small proportion of the total number of units. This may account for the great overestimation in the number of motor units in the TA estimated by Harris and Wilson (1971). Perhaps in small muscles, where a sample of four motor units represent a larger proportion of the total motor unit population, the method of incremental stimulation of the sciatic nerve can be used in order to determine the number of motor units.

ii) Dystrophic (dy2j) Mice

The number of motoneurons in the tibialis anterior muscle of dystrophic mice has only been examined in one other study. This was the study of Harris and Wilson (1971) discussed above with incremental stimulation of the sciatic nerve. These authors found that the dy/dy TA motoneuron pool contained a mean of 63 motor units. This is much smaller than the estimate in this thesis with the dy2j mice of approximately 111 motoneurons. There are several important factors to consider when comparing these two studies. As previously stated, the method of incremental stimulation only estimates the number of *alpha* motoneurons while the results in this thesis include both *alpha* and *gamma* motoneurons. The strain difference between the two experiments may play a more significant role when comparing the TA motoneuron pools from the dy2j pool with the dy/dy (Harris and Wilson 1971) pool. The dy/dy strain of dystrophic mouse is known to be more severely affected by the dystrophic

process than the dy2j strain, thus motoneuron populations may be even more reduced than in the dy2j mice. This could potentially contribute to the significantly reduced number of motoneurons recorded by Harris and Wilson in the dy/dy mouse. As in the C57 mice, the large discrepancy may still rest in the fact that incremental stimulation of the sciatic nerve is not an accurate method for determining the number of motor units in large muscles.

iii) C57 vs. dy2j Motoneuron Pool Numbers

When comparing the motoneuron pool numbers in the C57 and the dy2j TA, there is approximately a 20% reduction in the number of motoneurons in the dy2j mice. This is in agreement with other studies using HRP to quantify other motoneuron pools of both C57 and dy2j mice.

Parry et al. (1982) observed a 20% reduction in the number of motoneurons supplying the soleus muscle (a slow-twitch hindlimb muscle) of the dy2j mouse when compared with the C57 mouse. Using the HRP technique, 21 motoneurons were found in the C57 soleus motoneuron pool, while there were only 17 motoneurons in the dy2j motoneuron pool. These numbers include only the *alpha* motoneurons and therefore, reflect the number of motor units in the soleus muscle. These results are similar to those of Law and Caccia (1975) where physiological recording parameters were used to examine the number of motor units in the soleus muscle of both the ReJ 129 and the dy/dy mice. Physiological recordings indicated a 27% decrease in the number of motor units supplying the soleus muscle of the dy/dy mice when compared with the normal mice. The mean number of motor units in the

soleus of control mice was 22 while in the dy/dy soleus, 17 motor units were observed. Evidence from both physiological (Law and Caccia 1975) and morphological studies (Parry et al. 1982), indicate a significant reduction in the number of motor units in the soleus muscle of dystrophic mice when compared to age-matched normal mice.

Bateson and Parry (1983) examined the number of motor units in both the C57 and the dy2j extensor digitorum longus (EDL), a fast-twitch muscle in the hindlimb, using the physiological recording method of ventral root splitting. Most of the motor units in the dy2j mice developed smaller than normal tensions but only two of the dy2j animals showed a reduced number of motor units in the EDL. These two mice had the most advanced cases of dystrophy. Bateson and Parry (1983) suggest that the apparent reduction in the number of motor units in these two mice might have resulted from some of motor units becoming so small that they could not be measured.

Parry and Falconer (1986) examined the motoneuron pools of the flexor carpi ulnaris (FCU), a forelimb muscle, in both C57 and dy2j mice, using the method of retrograde axonal transport of HRP. C57 mice had a mean of 35 neurons in the forelimb motoneuron pool, while the dy2j mice had a mean of only 26.5 motoneurons. This was the first study on a forelimb muscle from a dystrophic animal. The results show that the dystrophic process manifests itself in the same manner in both the hindlimb and the forelimb muscles. Unlike the forelimbs, the hindlimbs of the dy2j mouse display spontaneous activation which is observed as constant twitching of the hindlimbs. This continuous action potential

generation is thought to be of neural origin arising from amyelination of the spinal lumbar roots (Rasminsky 1978). The amyelination causes spread of the signal current and a so-called state of "cross-talk" arises. The final result is the continuous activation of the hind limb muscles (Rasminsky 1978). Since the reduction of the motoneuron supply is evident in both the forelimb and the hindlimb muscles, Parry and Falconer (1986) suggested that the spontaneous activity of the hindlimbs does not seem to be the primary reason for the changes in the dy2j motoneuron pool. The reduced motoneuron supply may be more a reflection of the dystrophic process itself and not the activity or use of the muscle.

This reduction in motoneuron numbers in the dystrophic mice, is not unique to the laboratory animal. Past studies on patients afflicted with muscular dystrophy have also indicated a reduced motoneuron supply to the muscle. It would seem likely that this reduction is more a reflection of a neural abnormality associated with the disease rather than simply being confined to the dystrophic laboratory animal. For example, in patients affected with Duchenne muscular dystrophy (DMD), limb-girdle (LG) and facioscapulohumeral (FSH) dystrophy, McComas et al. (1971A,B,C, and 1971D, 1975, 1977) have found reduced number of motor units in the thenar, a hand muscle, and two muscles in the hind limb, the extensor digitorum brevis (EDB) and the soleus, when compared with age-matched normal human subjects. However, they reported normal values for the number of motor units in the hypothenar (a muscle in the hand) from patients with DMD, LG, and FSH. This is of particular interest since in patients afflicted with axonal peripheral neuropathies, the hypothenar is the only muscle that is

not affected as well. In other studies on patients with ~~DMD~~, LG, and FSH dystrophy, both Ballantyne and Hansen (1974); Panayiotopoulos et al. (1974) and Panayiotopoulos and Scarpalezos (1976) found normal numbers of motor units in the EDB muscle both using physiological recording techniques. McComas and associates (1975;1977;1988) criticized these studies since the number of patients that were examined was much lower in both these studies. The discrepancies may be caused by the disease affecting each patient differently. Some may be severely affected and others may not. Patients afflicted with myotonic dystrophy consistently showed losses of motor units in the EDB (McComas et al. 1971c; Ballantyne and Hansen 1974; Panayiotopoulos and Scarpalezos 1976), soleus (McComas et al. 1974), thenar and hypothenar (McComas et al. 1978) dystrophic muscle.

The results of motor unit counting experiments have provided evidence for similar occurrences among both the dystrophic laboratory animal and humans afflicted with the disease. Both the patients and animal afflicted with dystrophy have shown evidence for a reduced number of motoneurons supplying the muscle. These results suggest that there is a degenerative neuropathy associated with muscular dystrophy. Whether this occurs as a primary neuropathy or as a primary myopathy still remains unanswered in both human and murine dystrophy.

Some suggestion of a neuropathic origin of murine muscular dystrophy has come from several studies where reduced axon counts have been observed innervating the hindlimb muscles of the dystrophic mouse. This is a partial explanation of the reduced number of motor units that have been shown in the dystrophic animal (Harris and Wilson 1971; Bateson

and Parry 1983; Desypris 1986). Harris et al. (1972) found reduced numbers of axons of all sizes innervating the TA of the dy/dy mouse. Similarly, Law and Atwood (1974) have shown the dy/dy mouse had a 20% reduction in the number of myelinated nerves innervating the soleus muscle when compared to the normal ReJ 129 mouse. Montgomery and Swenarchuk (1977) examined the number of myelinated axons in the normal and dy2j dystrophic mouse. In the myelinated axon counts, the soleus muscle was used as well as the fast-twitch plantaris muscle. Axon counts were progressively reduced in the nerves to both muscles. However, in the soleus muscle, a significant reduction was observed between one and two weeks of age, while in the plantaris, significant reductions were only seen after the mice reached six weeks of age. In the soleus muscle a 20% reduction was observed while in the plantaris only a 10% decrease was shown. These studies provide additional support of a neuropathy associated with the dystrophic process.

It should be mentioned that there is evidence of a myopathy associated with murine dystrophy as well. Montgomery and Swenarchuk (1977) also examined muscle fiber counts to examine any myopathic effects of the dystrophy on the mouse. Muscle fiber counts were made at 1, 2, 4, 15, 24, and 72 weeks in both the slow-twitch soleus muscle and fast-twitch extensor digitorum longus muscle (EDL) muscle. Early reductions were seen in the dystrophic muscle fiber counts in both the soleus and EDL. In the soleus muscle, the reductions did not become significant until fifteen weeks of age where a 37.4% reduction was noted. At 72 weeks a significant reduction was still observed. In the EDL muscle, a somewhat larger

reduction in muscle fibers occurred. The reduction seemed to start at a later stage of development in the fast-twitch muscle. The EDL showed no significant reductions until 15 weeks of age when the reduction was 46% and persisted up to 72 weeks. There seems to be a more drastic reduction in the muscle fiber counts when compared to the changes in the nerve axon counts with dystrophy. Whether this is a direct effect of the dystrophy on the muscle or the fact that there is a lack of neuromuscular connection with the muscle (indirect effect), is not conclusive.

Gallup and Dubowitz (1973) examined the dystrophic process by using a tissue culture technique. Four series of nerve/muscle cultures were used consisting of an explant of a cross-section of spinal cord and a bundle of muscle fibers from either a normal or dystrophic mouse. The four series used were as follows: normal nerve/normal muscle; normal nerve/dystrophic muscle; dystrophic nerve/normal muscle; and dystrophic nerve/dystrophic muscle. The results show two particular items of interest. First, with normal nerve and either normal or dystrophic muscle, muscle in the tissue cultures appeared normal morphologically. Secondly, when a dystrophic spinal cord was placed with either normal or dystrophic muscle, regeneration was severely affected with very few myotubes present in any of the cultures. Since all humoral, vascular and higher central nervous system inputs were absent, these experiments suggest that neurons in the spinal cord are capable of producing the myopathic state of the muscle in dystrophy. The authors stated that in the dystrophic spinal cord cultures, the neurons appeared normal with normal neuritic outgrowths but failed to form neuromuscular junctions. They also suggest that the study

seems to indicate a neuropathy associated with muscular dystrophy.

Tissue culture experiments showing the converse have also been extensively found. These include the studies of Hamburg et al. (1975) where co-cultures of embryonic muscle and spinal cord showed that when normal muscle was cultured with either dystrophic foetal or normal spinal cord, regeneration occurred. When control nerves were coupled with both dystrophic and normal muscle, the control nerves could not reverse the dystrophic signs present in the affected muscle. These studies show the continuing controversy as to the etiology of muscular dystrophy. Whether the cause is a primary neuropathy or a primary myopathy, experimental results indicate that a neural abnormality is associated with muscular dystrophy.

b) Motoneuron Sizes

i) Lack of bimodality

Figures 2-5 show that bimodal distributions were not evident when TA motoneuron areas were plotted as the raw data and as the % of the mean. There are several factors that should be explored to determine why evidence for a bimodal distribution among the TA motoneuron areas was lacking. Three types of motoneurons have been shown to exist. These are the *alpha*, *gamma*, and *beta* motoneurons. The *alpha* motoneurons also called the skeletomotor motoneurons are the largest of the three types of motoneurons and innervate the extrafusal muscle fibers. The *gamma* or fusimotor motoneurons are the smallest and responsible for innervating the intrafusal muscle fibers (the muscle spindles). *Beta* motoneurons are intermediate in size, with their range in between the size of the *alpha*

and *gamma* motoneurons. *Beta* motoneurons have been shown to innervate both the intrafusal and the extrafusal muscle fibers. The *alpha* and the *gamma* motoneurons have been extensively documented in mammalian muscles. While the *beta* or skeletofusimotor motoneurons have been found extensively in the lower vertebrates (amphibian-Murthy and Taylor 1978; reptiles-Proske 1973; Cliff and Ridge 1973; Proske and Ridge 1974; Proske and Walker 1975), the evidence for *beta* innervation in mammals is limited to fewer species and fewer muscle groups. In mammalian studies, *beta* motoneurons have previously been shown to exist in lumbrical muscles of cat (Bessou et al. 1965) and rabbit (Emonet-Denand et al. 1970), in the cat hindlimb muscles (Emonet-Denand et al. 1975, Emonet-Denand and Laporte 1975; Brown and Butler 1976; Burke and Tsairis 1977; Grill and Rymer 1987; Scott 1987) and in the caudal tail muscles of the rat (Kidd 1964; Andrew and Part 1974). A unimodal distribution might be seen if the TA has *beta* motoneurons contributing to its innervation. Since these motoneurons are intermediate in size, when the cell areas are plotted as a frequency distribution, an apparently unimodal distribution may result from the overlapping of these three populations.

Absence of a bimodal distribution in the TA is not uncommon. When Peyronnard and Charron (1983) used the retrograde labeling technique to examine the TA motoneuron pool in the rat, they reported that only three out of five animals showed a bimodal distribution with a clear separation of the small and large motoneurons. Similarly both Krishnan et al. (1985) and Lowrie et al. (1987) reported unimodal distributions with the rat TA motoneuron pool.

HRP studies on other hindlimb muscles, confuse the situation even more. Bimodal distributions have been shown in the motoneuron pools of feline medial gastrocnemius and soleus muscles (Burke et al. 1982), murine soleus muscle (Parry et al. 1982) and medial and lateral gastrocnemius muscles in the rat (Swett et al. 1986). On the other hand, unimodal distributions have been shown in the forelimb motornucleus of mice (Parry and Falconer 1986), the tibial and peroneal motoneuron pools of the rat sciatic nerve (Swett et al. 1986) and the rat plantar motoneuron pool (Crockett et al. 1987).

Masking of a bimodal distribution might have occurred if there was mechanical error in the processing of the data, in the data collection itself, or the technical procedure itself. As described briefly in the results section, the soleus motoneuron pool was examined with intramuscular injection of HRP in three animals. Since this muscle has successfully shown a bimodal distribution in its motoneuron supply with this technique in our lab (Parry et al. 1982, Parry and Falconer 1986), if there were errors made with the technical procedure, then unimodal distributions should be seen in the soleus motoneuron pool. The results showed a mean of 29 motoneurons in the pool which is a similar estimate to the previous work done on this muscle (Parry et al. 1982, Lewis et al. 1982). When the cell sizes were measured and plotted as a frequency distribution, the graph suggests that a bimodal distribution exists even with such a small sample size.

Without a bimodal distribution, it is very difficult to determine the proportion of *alpha* and *gamma* motoneurons innervating the TA muscle.

Without the clear estimate of the number of alpha motoneurons, a morphological estimate of motor unit number and size becomes very difficult. Since the number of alpha motoneurons cannot be determined, a morphological estimate of motor unit size becomes impossible.

ii) C57 vs. dy2j motoneurons

Although the results from these experiments show a 20% reduction in the number of motoneurons in the dystrophic mice when compared to the age-matched control mice, there does not appear to be any apparent difference in the size of the neurons between the two groups. Previous studies from this lab on the soleus (Parry et al. 1982) and the flexor carpi ulnaris (Parry and Falconer 1986) found that the 20% reduction in motoneuron number was accompanied by an overall increase in the mean soma size of the dystrophic motoneuron population. One may ask whether the increase in motoneuron size is a compensatory response to the decreased number of motoneurons responsible for innervating the dystrophic muscle. However, no increase in soma size was seen in the dystrophic TA. Perhaps part of the reason the discrepancy lies in ~~the~~ fact that the tibialis anterior is a much larger muscle than the soleus and the FCU. This could contribute to some variability in the motoneuron pool distribution and as such, mask any significant increase in the soma areas. Another possibility may be that the TA is clearly compartmentalized. The superficial portion of the muscle seems to be completely unaffected by the dystrophy while the deep portion shows clear signs of the disease. The TA motoneuron pool could therefore have a percentage of the motoneurons of completely normal

size and when combined with the diseased neurons, the increased soma size of these motoneurons could be masked.

2) Effect of Partial Denervation

In the statement of the problem, it was outlined that the original aim of this thesis was to examine the effect of partial denervation on dystrophic mice to assess the ability of their nerves to sprout collaterals causing an increase in motor unit size. Two methods for measuring motor unit numbers and sizes were to be performed, one physiological and the other morphological.

a) Physiological Recordings

The first attempt at measuring the number and size of motor units following partial denervation was, using the method of ventral root splitting described in the Methods section. Several problems occurred with this procedure in the TA since it was impossible to isolate a filament which contained a single motor unit. Based on the motoneuron numbers determined in this thesis with HRP, the TA muscle might be expected to contain approximately 100 motor units (approximately 70% of the total number of labeled motoneurons). It seems likely that when the ventral root is split randomly, it will require to be split in half as many as six times before a single motor unit is isolated. For example: the first split will give 50 units, the second 25, then 12, 6, 3 and finally 1. Unfortunately, the ventral roots in the mouse are very small and splitting the root six times successively is extremely difficult. With muscles

having fewer motor units, this difficulty should not arise as indicated by the successful reports of ventral root splitting on the mouse EDL (Bateson and Parry 1983) and on the mouse soleus (Lewis et al. 1982, Desypris 1986).

Since the lateral popliteal nerve innervates the EDL muscle along with the tibialis anterior, the surgical procedure will also partially denervate the EDL muscle. Following the unsuccessful attempts at ventral root splitting with the TA, this procedure was tried on the EDL muscle since as already indicated, this has been previously performed successfully (Bateson and Parry 1983). However, soon after the length-tension relationship for EDL was established with the muscle set at optimal length, the tension following indirect stimulation dropped. This occurred over a period of ten to twenty minutes. The tension decreased. Consequently, these experiments were discontinued and determination of the number and sizes of motor units by a morphological technique was attempted.

B) Morphological Methods

The morphological determination of the average motor unit size for each individual animal was as follows:

$$\frac{\# \text{ of innervated muscle fibers in the tibialis anterior muscle}}{\# \text{ of } \alpha \text{ motoneurons}}$$

The introduction discussed the changes that occur in motor unit size in normal animals following partial denervation. Since the calculations derived from the formula above only give an average motor unit size, there

cannot be any detailed description of the actual size of the individual motor units. The morphological method would simply show if any changes occurred in the mean motor unit size with partial denervation.

i) Horseradish Peroxidase Retrograde Labelling

As previously mentioned, when the cell areas were plotted as a frequency distribution, a unimodal distribution was seen. This limits the possibility of obtaining a clear morphological estimate of motor unit size since this measurement requires the number of *alpha* motoneurons. The exact number of each of the two populations could be found if a tracer was available that would exclusively label either the *alpha* or the *gamma* motoneurons. However, no tracer of this kind exists at present. The only method that could be used is to estimate the size of the *gamma* motoneurons from previously known bimodal populations. There is a possibility that the number of *alpha* motoneurons could be obtained simply by dividing the frequency distribution at this appropriate size. The soleus muscle could be used since a clear bimodal distribution has already been established from this lab, using the same technique that is used in these experiment on the TA. However, the soma sizes of the soleus motoneurons (Parry et al. 1982) were quite different from those in this thesis.

In Figures 2-5, the motoneuron soma areas are plotted as a frequency distribution in the unoperated TA and with partial denervation. In both the C57 and the dy2j mice, partial denervation does not seem to selectively eliminate any particular size of motoneuron, but simply shifts

the distribution to the right. However, without the exact proportion of the *alpha* and *gamma* motoneurons within the TA muscle, it is very difficult to examine any selectivity that may occur with reinnervation, whereby one motoneuron type increases its proportion of total innervation with recovery from nerve manipulation. Following a three month recovery from sciatic nerve crush, Brushart and Mesulam (1980) examined the number and sizes of the motoneurons in the rat peroneal motoneuron pool by intramuscular injection of HRP. Upon recovery, only 69% of the motoneurons had regained normal connections with the periphery. This decreased motoneuron supply was due to a specific reduction in the number of small diameter motoneurons. However, there was no change in the size of the remaining motoneurons in the operated pool. The authors suggest that with reinnervation there may be some problems with the extent of *gamma* motor control in the muscle and the specificity of muscle reinnervation.

Previous work on changes in motoneuron profiles following nerve injury include studies examining the presence of skeletofusimotor or *beta* innervation in cat muscles. Briefly, mammalian muscle spindles (intrafusal muscle fibers) will receive innervation from specific fusimotor (*gamma*) axons and from *beta* axons. *Beta* axons will distribute terminal branches to both extrafusal and intrafusal fibers (Jami et al. 1982). Brown and Butler (1976) compared the reinnervation pattern in both the cat tenuissimus and peroneus longus muscles after sciatic nerve crush and following sciatic nerve section. Upon recovery, they found an increase in functional *beta* innervation in the nerve sectioned-animals when compared with both normal animals and those with sciatic nerve crush.

However, this work was criticised by Scott (1987) who showed that if the data from the two different muscles were separated, the results were contradictory. Following nerve section, the peroneus longus muscle showed an increase in *beta* innervation while the tenuissimus muscle showed a decrease in *beta* nerve supply when compared with the normal and sciatic crush animals. For this reason, Scott (1987) examined the effect of sciatic nerve crush and sciatic nerve section on the peroneus tertius (PT) muscle. The peroneus tertius was chosen since the *beta* nerve supply had been previously mapped by Jami et al. (1982). Scott (1987) found that there was no change in the proportion of *beta* innervation of the cat PT muscle spindles following recovery from either sciatic nerve section or sciatic nerve crush when compared to normal animals. Although these results do provide some controversy, one should note that the possibility of increased *beta* innervation in the muscle spindles following nerve section does exist. Since this thesis uses a technique of partial transection of the lateral popliteal nerve, the possibility of an increased *beta* innervation is important. However, since *beta* innervation has not yet been shown to exist in the mouse hindlimb, no conclusions can be made regarding any change in the *beta* innervation pattern.

The lack of bimodality within the TA motoneuron population limits the possibility of examining a preferential loss of either small (presumed *gamma*) or large (presumed *alpha*) motoneurons.

ii) Muscle Fiber Counts

The morphological estimate of the average motor unit size not

only requires the number of *alpha* motoneurons innervating the TA, but the total number of innervated muscle fibers in the muscle as well. From histological sections of the TA muscles stained with Hematoxylin and Eosin (H and E), photographic composites were made to obtain an accurate count of the number of innervated muscle fibers within the TA. The counts are listed in Table 5. Some caution must be taken when examining these numbers. Histological sections of dystrophic muscle contain connective tissue and muscle fibers of variable sizes (see Figure 10). With the added factor of partial denervation, many of the muscle fibers in the sections from the dystrophic mice, were so small that it was very difficult to assess whether the fibers were degenerating, regenerating, denervated or reinnervated. The counts listed in Table 5 for the dy2j mice include all of these very small fibers and therefore the number of muscle fibers in the TA of the dy2j mouse may be overestimated, particularly in the partially denervated TA.

Some criteria were necessary to identify denervated muscle fibers. To determine the morphological characteristics of denervated muscle fibers, a series of experiments was performed in which the TA was completely denervated and then examined histologically two months later. However, when those sections were examined, many of the muscle fibers appeared 'normal' in both the C57 (Figure 9d) and the dy2j mice. Therefore, even the counts of the TA from the C57 mice requires some added attention. It does not appear that reinnervation was responsible for the appearance of these 'normal' muscle fibers. Prior to removal of the muscle, the sciatic nerve was stimulated in the region where the

denervation occurred. No contractions were seen in the TA muscle, following indirect stimulation in all four mice. Moschella and Ontell (1987) found similar results when they examined the effects of chronic denervation on the ReJ 129 normal and dy/dy dystrophic mice. After 100 days of chronic denervation, there was a loss of large muscle fibers. However, this loss was accompanied by the appearance of many apparently 'normal' fibers. They stated that almost 50% of the muscle fibers were of 'normal' fiber size. The results from the completely denervated TA experiments clearly make it difficult to accurately count the number of innervated muscle fibers in both the C57 and the dy2j TA muscles simply on the basis of size or other morphological characteristics in H and E stained sections. It seemed clear at this juncture, that a staining procedure that would preferentially mark either the denervated or innervated fibers would be needed in order to obtain a true number of innervated muscle fibers in the TA muscle.

iii) Neural Cell Adhesion Molecule Experiments

NCAM, or neural cell adhesion molecule, has been shown to be present on the surface of denervated and paralysed muscle fibers. NCAM seems to disappear when the muscle fibers become innervated (Covault and Sanes 1985). If the muscle sections are stained with antibodies directed against NCAM, then the number of denervated muscle fibers in the partially denervated and completely denervated muscle sections can be assessed.

Covault and Sanes (1985) were amongst the first to examine the presence of NCAM in mammalian muscle. They described NCAM as an integral

membrane glycoprotein present under specific conditions on the surface of most peripheral and central nervous system nerves. Its appearance seems to be regulated by the state of innervation of the muscle. Tissues that contain a lot of NCAM include embryonic (Rutishauser et al. 1983), denervated and paralysed (Covault and Sanes 1985) muscle fibers, while samples from normal and reinnervated muscle will not stain positive for NCAM (Covault and Sanes 1985; Moore and Walsh 1986). In denervated muscles, NCAM is expressed on the sarcolemma within the muscle fiber itself and between the muscle fibers (Rutishauser et al. 1983; Covault and Sanes 1985; Moore and Walsh 1986). NCAM is thought to mediate adhesion of neurons to cultured myotubes (Covault and Sanes 1985). More recently, Booth and Brown (1987) have suggested that NCAM may provide a stimulus for terminal sprouting that occurs following partial denervation and after paralysis.

The figures reveal some unexpected results with the NCAM immunohistochemistry. There seem to be some fibers in the diaphragm that are NCAM positive (presumed denervated). These muscle fibers of the diaphragm do not show any reaction simply with the secondary antibody as well indicating an immunohistochemical response is occurring with the first antibody. This clearly warrants some caution in interpreting the results. With partial denervation, there is clear non-specific staining as well. This made it very difficult to assess the total number of innervated muscle fibers even with this stain. In the muscle from the dy2j mice, once again the situation was confusing with non-specific staining. The results seem to indicate that the very small, bright fibers may be the

denervated muscle fibers. There seem to be very few of these fibers in either the C57 or the dy2j partially denervated muscle fibers.

Tables 1 and 3 shows the effect of partial denervation on the number of motoneurons in the TA muscle. The results show that after a two month recovery, the majority of the animals have 40-60% of their original motoneuron pool in contact with the muscle. Since the actual sizes of the remaining motor units could not be measured, some attempts were made to assess the sprouting ability of the TA motor nerves in the control C57 animal. This was performed by staining partially denervated TA sections with anti-NCAM to determine whether any denervated muscle fibers remained. One C57 mouse had only 20% of its original motoneuron supply remaining following partial denervation and recovery. When stained with anti-NCAM, only a few small muscle fibers were positively stained. This seems to indicate that the TA motor axons in the C57 mouse can increase their peripheral field size by five times. Motor axons supplying the TA muscle may be capable of enlarging their peripheral field by more than five times, but since none of the mice used exhibited more than an 80% loss of motoneurons, this could not be determined in these experiments. As reviewed in the introduction, past studies examining the expansion of the peripheral field following partial denervation have suggested that motor units can increase their size by at least a factor of five (Thompson and Jansen 1977; Brown and Ironton 1978; Guth et al. 1980; Gorio et al. 1983). Therefore, the results presented in this thesis seem to confirm these findings in the tibialis anterior of the mouse.

Luff et al. (1988) as previously cited, studied the motor units

in cat fast-twitch (flexor digitorum longus, FDL) and slow-twitch muscle (soleus) 100 days after partial denervation. Using the technique of ventral root splitting, they examined both the size and type of the motor units following the recovery period. When the individual motor unit types was examined in the FDL, the force development of the type S units were not affected by the partial denervation surgery. In the fast-twitch motor units, the FF type units increased their motor unit force up to eleven times when compared to the normal FDL units. However, the majority of these fast units increased between two to four times. The fast-twitch non-fatiguable, FR, motor units elicited force increases up to 19 times, with most of the units increasing between two to seven times. In the soleus, the S units increased up to sixteen times the normal force with most units, increasing in force from five to 12 times the normal. Luff et al. (1988) concluded that the largest motor units that were observed in this study were the maximum values that an individual motor unit could sprout. They based their conclusions on the fact that when less than 15% of the motor axons remained following partial denervation, small diameter (presumed denervated) fibers were present. In the soleus muscle; this was accompanied by a decrease in the force produced by the muscle. They concluded that there is a definite limit to the extent that motor axons could sprout. These results are important since this was the first physiological study to show definite limits to motor unit expansion following partial denervation. However, it should be noted that these authors used the presence of small, atrophic fibers as the only index of denervation. The results presented in this thesis using the anti-NCAM

suggest that caution should be used in making such an assumption.

iv) Immunohistochemistry

Following reinnervation after nerve section and crush, Karpati and Engel (1968) were the first to observe that the motor units are clumped into groups rather than being randomly dispersed in the muscle. As reviewed in the introduction, 'Type-grouping' has only been shown following partial denervation when extensive numbers of motor axons are removed (Kugelberg et al. 1970; 1981; Narusawa 1985). To examine the presence of 'type-grouping' with reinnervation, following partial denervation in my experiments, the TA muscle sections were stained with three antibodies, each recognizing a different myosin heavy chains (MHC). These monoclonal antibodies were specific for type I (XIII₆), type IIA (VII₁₁) and type IIB (F₃) myosin heavy chains. In this thesis, from 40-60% of the motor axons remained in most animals following partial denervation. When muscle sections were stained with the monoclonal antibodies, small compact groups of muscle fibers of the same type were not clearly shown (Figure 17). The figures show that there seem to be a few very small clumps of fibers of the same type in the superficial region of TA. This is where the groups could be expected since the NCAM sections (4 day post-partial denervation) have shown that the muscle fibers that become denervated from the surgery are in this region (Figure 12). As stated by Kugelberg (1981), perhaps only when the majority of the axons are removed, will partial denervation surgery induce 'type grouping' in the reinnervated muscle.

c) Effect of Gender on recovery from partial denervation

In the experiments of Desypris (1986), following acute denervation of the soleus, in the female dy2j mice reinnervation was impaired when compared with the male dy2j mice. When the male dystrophic mice were castrated, sex differences no longer occurred. Yu and Yu (1983) examined the effect of testosterone propionate on the hypoglossal nerve of young rats following nerve crush. Steroids were given to both the male and female animals during the recovery period. Following recovery, steroid treatment significantly promoted axonal outgrowth as measured by an increase in the proportion of labelled hypoglossal neurons. In this thesis, the males and females were examined separately in both the C57 and dy2j populations. However, no difference occurred in any of the measured parameters. This enabled the male and female sample groups to be placed together, increasing the sample size from 6 to 12.

3) Motoneuron Size and the Size of the Peripheral Field

a) Plasticity in adults

In the Introduction of the thesis, several physiological parameters were related to the size of the cell body in the spinal cord. Upon reviewing the studies of factors related to motoneuron size, there have been several suggestions of a relationship between the size of the cell body in the spinal cord and the size of the peripheral field. The peripheral field refers to the number of muscle fibers that are innervated by that particular motor unit. As previously stated, Henneman et al.

(1965) were the first to suggest this relationship when they correlated the diameter of a motor axon with the number of muscle fibers that it innervates. This was done indirectly using conduction velocity as an indicator of axonal diameter and tension production for motor unit size. This suggests that motor axon size is directly related to motor unit size. Since the diameter of an axon has already been shown to be a good indicator of soma size (Cullheim 1978), one can relate the motor unit size to motoneuron size. This seems quite plausible since a large motor unit must have sufficient axonal substance to give off a large number of terminals (Henneman et al. 1965).

Tables 2-3 show that there was a significant increase in the mean soma area of the surviving motoneurons after partial denervation. Although every animal, both normal and dystrophic, showed an increase in the mean soma area individually, it was not always significant. Partial denervation effectively lowers the motoneuron supply to the tibialis anterior muscle and has been shown to cause the remaining motor units to increase in size (Thompson and Jansen 1977; Brown and Ironton 1978). From this one may ask, does this increase in motoneuron area reflect a compensatory response to an increase motor unit size as a result of partial denervation?

Murphey et al. (1986) examined the morphology of axotomized motoneurons which had reinnervated their target tissue. Axotomy or nerve section, was performed on the cat trochlear nerve which innervates the superior oblique muscle. Following a 2-3 month recovery, intramuscular injection of horseradish peroxidase was used to determine the number of

motoneurons which had reestablished their connections to the periphery. In normal cats, the trochlear motoneuron pool contained approximately 1200 neurons. Following the recovery period, the axotomised motoneuron pool was significantly reduced to fewer than 200 neurons. This reduction was accompanied by a significant increase in the soma size of the operated side motoneurons when compared to the normal cell areas. Murphey et al. concluded that the loss of motoneurons due to the surgery reduced competition for the target tissue (peripheral field) which resulted in hypertrophy of the surviving cells.

Klueber (1987) examined neuronal size within the mouse extensor digitorum longus (EDL) motor nucleus after orthotopic transplantation of the muscle. The extensor digitorum longus muscle was removed from mice and then soaked in bupivacaine, a known myotoxic agent and then reattached to its tendons. After a 100 day recovery period, intramuscular injection of horseradish peroxidase was performed to assess the number and sizes of motoneurons in the operated muscle pool. The results indicated that there was a significant reduction in the numbers of motoneurons in the transplanted muscle pool accompanied by a significant increase in the soma size of these remaining neurons. Many of the *alpha* motoneurons supplying the grafts were twice the average size of the control population. Klueber suggested that the increase in the number of large motoneurons resulted from hypertrophy of neurons which had successfully reinnervated the muscle.

In the experiments of Klueber (1987) and Murphey (1986), a technique of axotomy was used. According to both Lieberman (1971) and

Kandel and Schwartz (1981), within 2-3 days following nerve section of motor axons or dorsal root ganglion cells, the parent motoneurons swell. Various changes occur within the motoneuron including movement of the nucleus and breakdown of the rough endoplasmic reticulum. Biochemically, there is a massive increase in protein synthesis necessary for regeneration of the axons. Chromatolysis lasts from one to three weeks and ends either in proper connections of the severed axons being restored or in atrophy and death of the motoneuron. Since both these experiments employ nerve section techniques, whereby damaged nerves regain their connections, chromatolysis may play a role in the results presented with the hypertrophy of the operated motoneurons.

Although chromatolysis has not been documented following partial denervation, it is possible that it may account for the hypertrophy of the motoneurons seen in the experiments in this thesis. Since the technique of partial denervation in this thesis involves direct contact of the scalpel with some of the remaining intact motor axons, the possibility exists that some of the remaining axons were damaged. If so, the increased soma area may simply be a transient chromatolytic response although since there it is not clear as to how long the motoneurons will remain swollen following axotomy. Therefore, if the animals were left to recover for a longer period of time, the cell soma sizes should return to control values. A group of animals were left to recover for a period of 18 months following partial denervation. The results are listed in Table 6. In both the dy2j (N=4) and the C57 (N=4) mice, there was a similar reduction of motoneurons was shown as in the two month recovery animals. This decreased number of

motoneurons in the operated side accompanied by a significant increase in the mean soma area of these motoneurons. Since the increased soma size remained even 18 months after the surgery, the results suggest that this was a permanent state for the motoneuron, unlike the transient state of chromatolysis. It seems possible that the increased soma size that occurs following partial denervation is a compensatory response to an increased motor unit size as a result of the partial denervation. Such an increased motor unit size may be inferred from the decreased number of TA motoneurons unaccompanied by a reduction in the number of innervated muscle fibers (Table 5, Figure 14).

It should be emphasized that the technique of Murphey (1986) and Klueber (1987) differs from that employed in this thesis, since with partial denervation, the increased soma size occurs in motoneurons that are left intact. Both Klueber's and Murphey's results are similar to the results of partial denervation presented in this thesis insofar as all three reveal an increased motoneuronal area associated with a reduction in the number of neurons innervating the muscle. Do the results suggest that the hypertrophy of the motoneurons is in fact caused by an increased motor unit size? If so, this would lead one to speculate that motoneuronal area may be linked in some way to the number of muscle fibers innervated (i.e. motor unit size).

To test this hypothesis, a second set of experiments were performed on both C57 and dy2j mice. In this study, the mice were subjected to a partial denervation (PD) of TA, as described in the methods section but this was accompanied by a partial muscle extirpation (PE) of

the TA. If there is a relationship between motoneuron size and the size of the peripheral field as suggested by the results, then since both the number of axons and muscle fibers will be reduced, motoneuron area should not increase.

Before examining the results of these experiments, previous studies on the relationship between motoneuron size and the size of the peripheral field in development will be discussed.

b) Developmental studies

When nerve crush is performed on animals during development, the effects are more drastic than in adult animals. Following nerve crush immediately after birth, Romanes (1946) was the first to show that fewer ventral horn cells were counted on the operated side when compared to the control side in the rat. Similarly, following nerve crush at birth, Zelená and Hník (1963), found fewer motor units in the soleus muscle when compared to the control side. This could be partly attributed to the observations that there was a decreased number of nerve fibers supplying the soleus and the muscle itself showed a great loss of weight. The sizes of the remaining axons was reduced as well.

Bearcroft et al. (1983) performed sciatic nerve crush on the rat soleus muscle at birth. Four weeks later, the motoneuron pool revealed a drastic reduction in the number of motoneurons in the operated soleus muscle when compared with the contralateral control side. When the soma areas were measured, the reinnervated side revealed a slight reduction in

size when compared to the control side. However, when a morphological estimation of motor unit size was done, the motor units in the normal soleus had a mean size of 70-90 fibers/neuron, while the motor units on the sciatic crush side had a greatly reduced motor unit size, only 20-50 fibers/neuron. They also observed an increase in the number of type I fibers in the muscle on the operated side. They concluded that the motoneurons supplying type I fibers might have a better chance at surviving early deprivation of their target tissue. However, they are not capable of occupying their original peripheral field.

Krishnan et al. (1985) examined the effect of reducing the peripheral field on motoneuron development in the rat. This was accomplished by examining the effects of sciatic nerve crush and of partial removal of a muscle on rats at 5-6 days of age. In the first set of experiments, the sciatic nerve was crushed in the mid thigh region. In the other group, a longitudinal section of the TA and extensor digitorum longus (EDL) muscles were removed. The animals were left to recover for 4-5 weeks. The method of retrograde axonal transport of horseradish peroxidase was used to assess the number and sizes of motoneurons supplying innervation to the TA-EDL muscle. The nerve crush experiments revealed no change in the number of motoneurons supplying the TA-EDL. The partial extirpation experiments, revealed a reduction of motoneurons in some animals although the group means were not significantly different. However, in all the animals, the overall mean soma area had decreased and there was clear evidence of the largest motoneurons shifting towards an intermediate size. The frequency distributions of the motoneuron areas

lacked any presence of bimodality.

Lowrie et al. (1987) examined the effects of sciatic crush in rats during the first two weeks of life. Reinnervation was assessed in both fast-twitch and slow-twitch muscles physiologically and by morphological analysis using HRP to label the motoneurons. It was found, that the earlier the nerve injury was performed, the more impaired was the neuronal development. Sciatic nerve crush performed at birth resulted in the greatest change in physiological and morphological characteristics. After a one-two month recovery from nerve crush at birth, the results showed that the TA-EDL motoneuron pool had decreased by 41% while the soleus motoneuron pool decreased 31%. This was accompanied by a slight reduction in the size of the motoneurons as well. It was also shown that the slow-twitch soleus muscle always recovered better than the fast-twitch TA-EDL muscle.

The studies on young animals discussed above, show that when the sciatic nerve is injured during the first two weeks of life, a decreased number of motoneurons with either normal or reduced soma areas will develop. However, when this is performed in the adult, as shown with the studies of Klueber (1987) and Murphey (1986), the decreased number of motoneurons have larger soma areas. During embryogenesis, there is a critical stage when motoneuron survival depends on contact with the peripheral field (Oppenheim et al. 1978;1984;1988). Krishnan et al. (1985) suggest that there is an intermediate period of development in the early days of life, when the motoneuron would survive but it would be permanently damaged if temporarily disconnected from its target. The

damage would decrease the number of muscle fibers the motoneuron could reinnervate. Since, Krishnan and associates (1985) found the same results with partial extirpation and with nerve crush on young animals, they suggested that the decreased number and sizes of the motoneurons was more a reflection of a reduced peripheral field occupied by each motoneuron. This could have been induced by some permanent damage to the motoneuron following early experimental manipulation. Similarly, Zelena and Hnik (1963) suggest that the reason that they observed a reduced motor unit number with decreased sizes after nerve crush at birth was due to the inability of the motoneurons to reoccupy their original territory due to their permanent impairment.

Krishnan et al. (1985) offer another suggestion for the selective loss of the large motoneurons that was found in their experiments. They suggest that a large number of muscle fibers supplied by the neurons were unable to survive the loss of contact with the motor nerves. Lowrie et al. (1982) found after nerve crush on rats at 5-6 days, there were no large, glycolytic, non-oxidative fibers, i.e. those normally supplied by large motoneurons. Lowrie and Vrbova (1984) showed that a substantial number of muscle fibers were lost from the fast muscles shortly after reinnervation following nerve crush on rat TA-EDL muscles at birth. Krishnan et al. (1985) suggest that these results show the territory occupied by the large motoneurons could be affected to a much greater extent than any other sized motoneurons. Parry (1988) showed that in the rat following sciatic nerve crush during early life, the IIB fibers are most affected. They showed with immunocytochemistry, that if innervation is withdrawn before

expression of Iib myosin, then development of the Iib fibers will be affected.

It seems that when nerve section or crush is performed at an early age, motor units numbers are usually reduced. This is accompanied by either a decrease or no change in the motoneuron soma size. Zelená and Hnik also revealed that the muscle size is reduced. This suggests that in early development the motoneurons cannot increase their motoneuron size since part of the muscle is lost as a result of the nerve manipulation as well. As such, the relationship between motoneuron size and the size of the peripheral field exists in early development as well. The experiments show that there is a limited time in the early days of life where critical development of the nervous system is still occurring. If manipulation is performed during this time, permanent damage will occur. However, once fully developed, the animals will respond to a number of stresses imposed on the nervous system.

c) Partial Denervation/ Partial Extirpation Experiments

i) Motoneuron Numbers

The number of motoneurons supplying the TA on the unoperated side of C57 and dy2j mice were not significantly different in the two sets of experiments (PD and PD/PE). As such, the two groups were placed together and the numbers discussed previously include a total of 24 animals as discussed earlier.

With PD/PE, the motoneuron pool was reduced approximately 45% as seen in Table 3. This could be attributed in part to both surgical

procedures. Partial denervation removes a number of the motor axons and reduces the motoneuron supply to the muscle as seen in the first set of experiments in this thesis. However, removal of part of the TA could conceivably eliminate some of the motoneurons if all their muscle fibers were removed. Some of the motoneurons may have had part of their supply eliminated by the surgery and as such, the amount of HRP transported to the spinal cord would be too small to detect.

ii) Motoneuron Sizes

Motoneuron size was determined as previously described with the partial denervation experiments. In tables 3 and 4 the results of these experiments are presented. With PD, a decreased number of motoneurons is associated with an increased cell soma area. With PD/PE, a decreased number of motoneurons, supplying a comparably reduced number of muscle fibers, resulted in no change in motoneuron size. This seems to indicate that there is a relationship between motoneuron size and the size of the peripheral field in adult mice.

If there is a relationship between motoneuron size and the size of the peripheral field, what are the possible mechanisms? How does this effect the etiology of murine dystrophy, since this relationship seems to exist in dystrophic mice? The next section will review some related evidence that could help to explain this relationship.

4) The Trophic Theory

A direct relationship between the size of the cell soma in the

spinal cord and the size of the peripheral field is indicated by the results in this thesis. This implies that the motoneuron is dependent on the target tissue for its maintenance and integrity. There have been numerous studies describing the target dependency that exists in the motoneuron during embryogenesis and examining target-derived trophic factors that regulate neural development. These studies can be discussed in relation to those results in an attempt to discover how this relationship can be maintained. Briefly, based on evidence from experiments examining neuronal cell death during development and on discovery of neurotrophic factors, it seems that the relationship between motoneuron size and the size of the peripheral field may stem from a muscle-derived trophic factor that travels by retrograde axonal transport to the motoneuron.

Purves (1977) defined the 'trophic theory of neural connections' as follows: formation of synaptic contacts will be maintained by interactions in which nerve terminals compete for target-derived molecules available in limited supply. The 'trophic theory', defining target-dependent activities can be exemplified in the situation of neuronal cell death during embryonic development.

Hamburger (1953) was the first to record that massive neuronal cell death exists during the development of the vertebrate nervous system. This occurs during embryogenesis where a surplus number of neurons in the embryonic nervous system is reduced to the adult number by means of competitive interactions between the neurons. This competition appears to be at the level of the neuronal target such that the number of motoneurons

in the population adjusts itself according to the size of the target tissue. Therefore, the number of motoneurons in the adult motoneuron pool ultimately depends on the size of the peripheral field available for innervation during embryogenesis.

Clarke (1985) has recently reviewed the relationship between neuronal cell death and the size of the target tissue. Normally, in the ciliary ganglion (CG), the lumbar motoneurons (LMN) and the isthmo-optic nucleus (ION), approximately 50% of the motoneurons will die during the neuronal cell death period in embryogenesis. When experiments were performed that increased competition for the target tissue such as reduction in the peripheral field size or when input to the target was blocked or eliminated, more than 50% of the motoneurons died. Conversely, if competition was decreased for the target tissue, such as by increasing the size of the target tissue (Hollyday and Hamburger 1976), more than 50% of the motoneurons remained following neuronal cell death in all three areas. If the target tissue was completely destroyed in all three areas, the CG, LMN, and ION, 100% of the motoneurons were destroyed. These results suggest that connections of the neuron cell body to the target tissue are essential for survival.

It is possible that this relationship is regulated via the retrograde transport of a target-derived factor(s) necessary for normal development of the cell body. This is in agreement with Berg (1984), who suggested that production of growth factor by the target tissue necessary for the morphological and functional differentiation of the responsive neurons, could account for the dependency of the motoneuron on the post-

synaptic target.

According to Hofer and Barde (1988), to classify a protein as a neurotrophic factor, it must regulate neuronal survival during development *in vivo* by increasing neuronal numbers and/or decreasing neuronal death. Such a situation arose with the discovery of Nerve Growth Factor (NGF). The isolation of NGF (Hamburger and Levi-Montalcini 1951, for review Levi-Montalcini 1982) brought attention to the trophic theory whereby target-derived factors mediate cell growth. Berg (1984) stated that the discovery of NGF emphasized the likelihood that neuronal development, particularly target-dependent neuronal cell death, might be mediated by diffusible molecules. Further studies have shown that when NGF is administered *in vivo*, it can reverse the naturally occurring and experimentally induced cell death in the sympathetic and sensory neurons (see review Levi-Montalcini 1982). More recent experiments with NGF have examined its effects on spinal motoneurons (Yan et al. 1988). NGF receptors in spinal motoneurons can bind NGF, internalize it and transport it retrogradely but these receptors do not mediate motoneuronal cell death (Yan et al. 1988).

NGF is the only known trophic factor to have been completely isolated and characterized. Since the discovery of NGF, there have been extensive studies searching for other neurotrophic factors. Brain-derived neurotrophic factor (BDNF) exists in extremely small quantities in the central nervous system (Hofer and Barde 1988). In a recent study, following injection of BDNF into quail embryos, Hofer and Barde (1988) found that neuronal survival had increased in the nodose and dorsal root ganglia populations of the neural crest. They concluded that BDNF has

trophic actions on the sensory neurons and appears to be a potential candidate for a neurotrophic factor.

Oh et al. (1988) isolated a muscle-derived trophic factor examined its effects on the target dependent events of the development and survival of motoneurons. Through biochemical purification, the factor appeared to be a protein which with immunochemical experiments, was unlike any other neurite-promoting factor. When incubated with neurons from the peripheral and central nervous system, the factor caused a dramatic increase in the neurite outgrowth activity. This led Oh et al. (1988) to speculate that since the increased neurite outgrowth activity is known to occur when muscle is denervated, and was observed following administration of the muscle-derived factor, then it is possible that this factor plays an important role in motor nerve growth *in vivo* and in motor nerve sprouting.

A very recent study of great importance was that of Oppenheim et al. (1988) where naturally occurring cell death was reduced *in vivo* by a target-derived neurotrophic factor. In these experiments Oppenheim et al. (1988) treated chick embryos *in vivo* with crude and partially purified extracts from embryonic hindlimbs (day 8-9) during normal cell death period (day 5-10). The results showed a significant number of motoneurons were rescued from degeneration with the survival rate being dose-dependent. While the extract significantly affected the motoneuron survival, no effect on the survival of sensory, sympathetic, parasympathetic and cholinergic sympathetic preganglionic neurons was shown. These experiments are the first to show that the target dependency of massive motoneuronal death is indeed linked to the competition of the

motoneurons for a neurotrophic factor produced from the muscle. These results indicate that *in vivo* a target-derived neurotrophic factor is involved in the regulation of motoneuron survival and can explain the trophic theory as it pertains to motoneuron cell death in embryogenesis.

PD and PD/PE Experiments

The results presented in this thesis suggest that a relationship exists between the size of the motoneuron in the spinal cord and the size of the peripheral field. This is similar to the relationship that appears in motoneuronal cell death in embryogenesis. With the study of Oppenheim et al. (1988), neurotrophic factors derived from the target tissue seem to be the source of the competitive reduction of motoneurons in development. If these factors exist during embryogenesis when they travel by retrograde axonal transport from the target tissue to the motoneuron, is it not possible that these exist throughout life?

In order for motoneuron size to be influenced by the size of the peripheral field, there must be communication between the two. It is plausible that this could exist in the form of a trophic factor released from the muscle and which, through retrograde axonal transport, reaches the motoneuron. With partial denervation, the competition for the target tissue is reduced. An increased amount of trophic factor would then be available for the remaining motoneurons. This could travel to the reduced number of motoneurons and signal that an increased peripheral territory is available. The motoneurons would in turn increase their peripheral field. The resulting increase in motoneuron size is a reflection of the increased

territory that the motoneuron now must maintain. With PD/PE, there is a reduced supply of motoneurons with a reduced peripheral field so that no change should be observed in the size of the motoneurons since the motor unit size is the same.

These experiments suggest that in the adult, motoneurons may still be controlled by factors derived from the peripheral field. Further studies should isolate a muscle-derived trophic factor that influences the motoneuron in the adult as in the experiments of Oppenheim et al. (1988).

5) Do Dystrophic Motor Nerves Sprout?

In this thesis, partial denervation of the dy2j mouse resulted in an increase in the mean soma area of the reduced motoneuron pool. This was the same response that was shown ~~with~~ partial denervation in the C57 mouse. If the direct relationship between motor unit size and motoneuron size is suggested, then the results might be interpreted as indicating that motor unit size does indeed increase in the dy2j mouse following partial denervation. However, in the dy2j mice, a relationship between motor unit size and the size of the peripheral field cannot be inferred as in the C57 mice since the muscle from the dy2j mice did not stain well with the anti-NCAM. The H and E sections of the partially denervated muscle of the dy2j mouse also revealed large areas of very small muscle fibers. Therefore, any suggestion as the sprouting response or the relationship between motoneuron size and the size of the peripheral field in the dy2j mouse cannot be justified.

6) Concluding Remarks

The results of the PD and PD/PE experiments suggest that in the C57 mouse, the size of the motoneuron is related to the size of the peripheral field (number of muscle fibers innervated). However, in the dy2j mouse, the relationship is not conclusive, no assessment can be made as to the number of muscle fibers innervated in the dy2j mice. Further studies on the number and sizes of motor units following partial denervation must be done with the dystrophic animal.

REFERENCES

- Andrews, C.J. and N.J. Part. (1974). The division of control of muscle spindles between fusimotor and mixed skeletofusimotor fibres in a rat caudal muscle. *Q. Jl. exp. Physiol.* 59:331-349.
- Appelberg, B. and F. Emonet-Dénand. (1967). Motor units of the first superficial lumbrical muscle of the cat. *J. Neurophysiol.* 30:154-160.
- Bagust, J. (1974). Relationships between motor nerve conduction velocities and motor unit contraction characteristics in a slow-twitch muscle of the cat. *J. Physiol. (Lond.)* 238:269-278.
- Bagust, J. (1979). The effects of tenotomy upon the contraction characteristics of motor units in rabbit soleus muscle. *J. Physiol. (Lond.)* 290:1-10.
- Bagust, J., Knott, S. Lewis, D.M., Luck, J.C. and R.A. Westerman. (1973a). Isometric contractions of motor units in a fast-twitch muscle of the cat. *J. Physiol. (Lond.)* 231:87-104.
- Bagust, J., Lewis, D.M., and R.A. Westerman. (1973b). Polyneuronal innervation of kitten skeletal muscle. *J. Physiol. (Lond.)* 229:241-255.
- Bagust, J., Lewis, D.M. and R.A. Westerman. (1974). The properties of motor units in a fast and a slow twitch muscle during post-natal development in the kitten. *J. Physiol. (Lond.)* 237:75-90.
- Bagust, J., Lewis, D.M. and R.A. Westerman. (1981). Motor units in cross-innervated fast and slow twitch muscle of the cat. *J. Physiol. (Lond.)* 313:223-235.
- Ballantyne, J.P., and S. Hansen. (1974). New method for the estimation of the number of motor units in a muscle. Part 2 (Duchenne, limb-girdle and facioscapulohumeral, and myotonic muscular dystrophies. *J. Neurol. Neurosurg. Psychiat.* 37:1195-1201.
- Barker, D. and M.C. Ip. (1966). Sprouting and degeneration of mammalian axons in normal and de-afferented skeletal muscle. *Proc. R. Soc. Ser. B.* 163:538-544.
- Barrett, J.N. and W.E. Crill. (1971). Specific membrane resistivity of dye injected cat motoneurons. *Br. Res.* 28:556-561.
- Bateson, D. (1982). M.Sc. Thesis. University of Ottawa.

Bateson, D.S. and D.J. Parry. (1983). Motor units in a fast-twitch muscle of normal and dystrophic mice. *J. Physiol. (Lond.)* 345:515-523.

Bearcroft, C.P., Lowrie, M.B., and D.C. Peterson. (1983). Selective effect of postnatal nerve crush on 'fast' motor units in the rat. *J. Physiol. (Lond.)* 338:12P.

Berg, D.K. (1984). New Neuronal Growth Factors. *Ann. Rev. Neurosci.* 7:149-170.

Bessou, P. and Y. Laporte. (1966). Observations on static fusimotor fibres. In: *Muscular Afferents and Motor Control*. Nobel Symposium I, 81-89. R. Grant (Editor). Almqvist Wiksell, Stockholm.

Bessou, P. and B. Pages. (1975). Cinematographic analysis of contractile events produced in intrafusal muscle fibres by stimulation of static and dynamic fusimotor axons. *J. Physiol. (Lond.)* 252:397-427.

Bessou, P., Emonet-Dénand, F. and Y. Laporte. (1963). Relation entre la vitesse de conduction des fibres nerveuses motrices et le temps de contraction de leur unités motrices. *C.J. Hebd. Séanc. Acad. Sci., Paris.* 256:5625-5627.

Bessou, P., Emonet-Dénand, F. and Y. Laporte. (1965). Motor fibers innervating extrafusal and intrafusal fibers in the cat. *J. Physiol. (Lond.)* 180:649-672.

Betz, W.J., Caldwell, J.H., and R.R. Ribchester. (1980). The effects of partial denervation at birth on the development of muscle fibers and motor units in rat lumbrical muscles. *J. Physiol. (Lond.)* 303:265-279.

Binder, M.D., Cameron, W.E. and D.G. Stuart. (1978). Speed-force relations in the motor units of the cat tibialis posterior muscle. *Am. J. Phys. Med.* 57#2:57-65.

Booth, C.M., and M.C. Brown. (1987). Innervated muscle fibers in partly denervated mouse muscles transiently express N-CAM. *J. Physiol. (Lond.)* 386:160P.

Booth, C.M., Brown, M.C., and S.K. Kemplay. (1987). Reduction of paralysis-induced terminal sprouting in mouse muscles with antibodies to N-CAM. *J. Physiol. (Lond.)* 390:177P.

Bradley, W.C. and M. Jenkison. (1973). Abnormalities of peripheral nerves in murine muscular dystrophy. *J. Neurol. Sci.* 18:227-247.

Brown, M.C. (1984). Sprouting of motor nerves in adult muscles: a recapitulation of ontogeny. *Trends in Neurosci.* 6:1-10.

Brown, M.C. (1987). A comparison of motor axon regrowth and collateral sprouting. *J. Anat.* 152:247-248.

Brown, M.C., and R.G. Butler. (1976). Regeneration of afferent and efferent fibres to muscle spindles after nerve injury in adult cats. *J. Physiol. (Lond.)* 260:253-266.

Brown, M.C., and R.L. Holland. (1979). A central role for denervated tissues in causing nerve sprouting. *Nature* 282:724-726.

Brown, M.C., Holland, R.L. and W.G. Hopkins. (1981A). Motor nerve sprouting. *Ann. Rev. Neurosci.* 4:17-42.

Brown, M.C., Holland, R.L., Hopkins, W.G. and R.J. Keynes. (1981B). An assessment of the spread of the signal for terminal sprouting within and between muscles. *Br. Res.* 210:145-151.

Brown, M.C., Holland, R.L. and R. Ironton. (1980). Nodal and terminal sprouting from motor nerves in fast and slow muscles of the mouse. *J. Physiol. (Lond.)* 306:493-510.

Brown, M.C., Hopkins, W.G., Keynes, R.J. and I. White. (1982). A comparison of early morphological changes at denervated and paralyzed endplates in fast and slow muscles of the mouse. *Br. Res.* 248:382-386.

Brown, M.C. and R. Ironton. (1978). Sprouting and regression of neuromuscular synapses in partially denervated mammalian muscles. *J. Physiol. (Lond.)* 278:325-348.

Bruschart, T.M. and M.M. Mesulam. (1980). Alterations in connections between muscle and anterior horn motoneurons after peripheral nerve repair. *Science* 208:603-605.

Buller, A.J., Eccles, J.C., and R.M. Eccles. (1960). Interaction between motoneurons and muscles in respect of the characteristic speeds of their responses. *J. Physiol. (Lond.)* 150:417-439.

Burke, R.E. and P. Tsairis. (1977). Histochemical and physiological profile of a skeletofusimotor (beta) unit in cat soleus muscle. *Br. Res.* 129:341-345.

Burke, R.E., Levine, D.N., Tsairis, P. and F.E. Zajac III. (1973). Physiological types and histochemical profiles in motor units of the cat gastrocnemius. *J. Physiol. (Lond.)* 234:723-748.

Burke, R.E., Levine, D.N., Salzman, M. and P. Tsairis. (1974). Motor units in cat soleus muscle: physiological, histochemical and morphological characteristics. *J. Physiol. (Lond.)* 238:503-514.

Burke, R.E., Dum, R.P., Fleshman, J.W., Glenn, L.L., Lev-Tov, A., O'Donovan, M.J., and M.J. Pinter. (1982). An HRP study of the relation between cell size and motor unit type in cat ankle extensor motoneurons. *J. Comp. Neurol.* 209:17-28.

Cajal, Ramón, Y. S. (1909). *Histologie du système nerveux de l'homme et des vertébrés.* Paris, Maloine. Vol. 1.

Cangiano, A. and L. Lutzemburger. (1977). Partial denervation affects both denervated and innervated fibers in the mammalian skeletal muscle. *Science* 196:542-545.

Clarke, P.G.H. (1985). Neuronal death in the development of the vertebrate nervous system. *Trends in Neurosci.* 7:345-349.

Cliff, G.S. and R.M.A.P. Ridge. (1973). Innervation of extrafusal and intrafusal fibres in snake muscle. *J. Physiol. (Lond.)* 233:1-18.

Close, R.I. (1972) Dynamic properties of mammalian skeletal muscles. *Physiol. Rev.* 52:129-197.

Covault, J., and J.R. Sanes. (1985). Neural cell adhesion molecule (NCAM) accumulates in denervated and paralyzed skeletal muscles. *Proc. Natl. Acad. Sci. USA.* 82:4544-4548.

Crockett, D.P., Harris, S.L., and M.D. Egger. (1987). Plantar motoneuron columns in the rat. *J. Comp. Neurol.* 265:109-118.

Cullheim, S. (1978). Relations between cell body size, axon diameter, and axon conduction velocity of cat sciatic α -motoneurons stained with horseradish peroxidase. *Neurosci. Lett.* 8:17-20.

Cullheim, S. and J.-O. Kellerth. (1978A). A morphological study of the axons and recurrent axon collaterals of cat sciatic motoneurons after intracellular staining with horseradish peroxidase. *J. Comp. Neurol.* 178:537-558.

Cullheim, S. and J.-O. Kellerth. (1978B). A morphological study of the axons and recurrent axon collaterals of cat α -motoneurons supplying different hind-limb muscles. *J. Physiol. (Lond.)* 281:285-299.

Cullheim, S. and J.-O. Kellerth. (1978C). A morphological study of the axons and recurrent axon collateral of cat α -motoneurons supplying different functional types of muscle units. *J. Physiol. (Lond.)* 281:301-313.

Desypris, G. (1986). M.Sc. Thesis. University of Ottawa.

Douglass, T.C., Davenport, H.A., Heinecker, P., and G.H. Bishop. (1934). Vertebrate nerves: some correlations between fiber size and action potentials. *Am. J. Physiol.* 110:165.

Dum, R.P. and T.T. Kennedy. (1980). Physiological and histochemical characteristics of motor units in cat anterior tibialis and extensor digitorum longus muscles. *J. Neurophysiol.* 43#6:1615-1630.

Dum, R.P., Burke, R.E., O'Donovan, M.J., and J. Toop. (1979). The properties of whole FDL muscle and of individual FDL muscle units after cross-innervation by soleus motoneurons in cat. *Neurosci. Abs.* 5:766.

Duchenne, G.B. (1861). *De L'Electrisation l'ocalisée et son application à la pathologie et la therapeutique.* 2nd. ed. Paris.

Edds, M.V. (1953). Collateral nerve regeneration. *Q. Rev. Biol.* 28:260-276.

Edström, L. and E. Kugelberg. (1968). Histochemical composition, distribution of fibres and fatiguability of single motor units. Anterior tibialis muscle of the rat. *J. Neurol. Neurosurg. Psychiat.* 31:424-433.

Emonet-Dénand, F. and Y. Laporte. (1975). Proportion of muscle spindles supplied by skeleto-fusimotor axons (beta axons) in the peroneus brevis muscle of the cat. *J. Neurophysiol.* 38:1390-1494.

Emonet-Dénand, F., Jankowska, E., and Y. Laporte. (1970). Skeleto-fusimotor fibres in the rabbit. *J. Physiol. (Lond.)* 210:669-680.

Emonet-Dénand, F., Laporte, Y. and U. Proske. (1971). Contraction of muscle fibers in two adjacent muscles innervated by branches of the same motor axon. *J. Neurophysiol.* 34:132-138.

Emonet-Dénand, Jami, L., and F. Laporte. (1975). Skeleto-fusimotor axons in hindlimb muscle of the cat. *J. Physiol. (Lond.)* 249:153-166.

Erb, W.H. (1883). Ueber die "juvenile form" der progressiven Muskelatrophie ihre beziehungen zur sogenannten pseudohypertrophie der muskeln. *Dtsch. Arch. Klin. Med.* 34:467-480.

Erlanger, J. and H.S. Gasser. (1927): The role played by the sizes of the constituent fibers of a nerve trunk in determining the form of its action potential wave. *Am J. Physiol.* 80:522-547.

Erlanger, J. and H.S. Gasser. (reprinted 1968). Electrical signs of nervous activity. Philadelphia University Press, Philadelphia. 2^{ed} edition.

Gallup, B. and V. Dubowitz. (1973). Failure of "dystrophic" neurones to support functional regeneration of normal and dystrophic muscle in culture. *Nature* 243:287-289.

Gardiner, P.F. and R.E. Faltus. (1986). Contractile responses of rat plantaris muscles following partial denervation, and the influence of daily exercise. *Pflügers Arch.* 406:51-56.

Gardiner, P.F., Michel, R., and G. Iadeluca. (1984). Previous exercise training influences functional sprouting of rat hindlimb motoneurons in response to partial denervation. *Neurosci. Lett.* 45:123-127.

Gerlach, R.I., Stauffer, E.K., Goslow, G.E., and D.G. Stuart. (1976). Relation between nerve axon size and muscle unit size and speed in motor units of cat hind limb muscles. *Electromyography and Clinical Neurophysiology.*

Goldberger, M.E. (1972). Restitution of function in the central nervous system. *Exp. Br. Res.* 15:79-96.

Gordon, T. (1987). Muscle Plasticity during sprouting and reinnervation. *Amer. Zool.* 27:1055-1966.

Gorio, A., Cariguoto, G., Finesso, M., Polato, P. and M.G. Nunzi. (1983). Muscle Reinnervation.-II., Sprouting, synapse formation and repression. *Neuroscience* 8:403-416.

Göthlin, G.F. (1907). Experimentella underötninger ag ledningens natur i den haita nervsubstansen. Akad. Afhandling. Uppsala, Almquist and Wiksell. 57P.

Grill, S.E., and W.Z. Rymer. (1987). β -Contributions to fusimotor action in triceps surae muscles of decerebrated cats. J. Neurophysiol. 57#2:574-595.

Grinnell, A.D. and A.A. Herrera. (1981). Specificity and plasticity of neuromuscular connections: Long-term regulation of motoneuron function. Prog. Neurobiol. 17:203-282.

Gurney, M.E.; Apatoff, B.R., and S.P. Heinrich. (1986): Suppression of terminal axonal sprouting at the neuromuscular junction by monoclonal antibodies against a muscle-derived antigen of 56,000 daltons. J. Cell Biol. 102:2264-2272.

Guth, L., Smith, S., Donati, E., and E. Albuquerque. (1980). Induction of intramuscular collateral nerve sprouting by neurally applied colchicine. Expl. Neurol. 67:513-523.

Habgood, M.D., Hopkins, W.G., and J.R. Slack. (1984). Muscle size and motor unit survival in mice. J. Physiol. (Lond.) 356:303-314.

Hamburger, V. and R. Levi-Montalcini. (1949). J. Exp. Zool. 111:457-502.

Hamburgh, M., Peterson, E., Bornstein, M.B., and C. Kirk. (1975). Capacity of foetal spinal cord obtained from dystrophic mice (dy2j) to promote muscle regeneration. Nature 256:219-220.

Harris, J.B. and R.R. Ribchester. (1978). Neuromuscular transmission is adequate in identified abnormal muscle fibres. Nature (Lond.) 271:362-364.

Harris, J.B. and R.R. Ribchester. (1979). Muscular dystrophy in the mouse: Neuromuscular transmission and the concept of functional denervation. Ann. N. Y. Acad. Sci. 313:152-170.

Harris, J.B., Wallace, C., and J. Wing. (1972). Myelinated nerve fiber counts in the nerves of normal and dystrophic mouse muscle. J. Neurol. Sci. 15:245-249.

Harris, J.B. and P. Wilson. (1971). Mechanical properties of dystrophic mouse muscle. J. Neurol. Neurosurg. Psych. 34:512-520.

Hatcher, D.D., Luff, A.R., Westerman, R.A., and D.I. Finkelstein. (1985). Contractile properties of cat motor units enlarged by motoneurone sprouting. *Exp. Br. Res.* 60:590-593.

Henneman, E. (1981). Recruitment of motoneurons: The Size principle in Motor Unit Types, Recruitment and Plasticity in health and disease. J.E. Desmedt (editor). *Progress in Clinical Neurophysiology.* 9:26-60. S. Karger Publications.

Henneman, E., Somjen, G. and D.O. Carpenter. (1965). Functional significance of cell size in spinal motoneurons. *J. Neurophysiol.* 28:560-580.

Herbison, G.J., Jaweed, M.M., and J.F. Ditunno Jr. (1986). Electrical stimulation of sciatic nerve of rats after partial denervation of soleus muscle. *Arch. Phys. Med. Rehabil.* 67:79-83.

Hofer, M.M. and Y.A. Barde. (1988). Brain-derived neurotrophic factor prevents neuronal death in vivo. *Nature* 331:261-262.

Holland, R.L. and M.C. Brown. (1981). Nerve growth in botulinum toxin poisoned muscles. *Neuroscience* 6#6:1167-1179.

Hollyday, M. and V. Hamburger. (1976). Reduction of the naturally occurring motor neuron loss by enlargement of the periphery. *J. Comp. Neurol.* 170:311-320.

Hopkins, W.G. and J.R. Slack. (1981). The sequential development of nodal sprouts in mouse muscles in response to nerve degeneration. *J. Neurocytol.* 10:537-556.

Hoffman, H. (1950). Local re-innervation in partially denervated muscle: a histophysiological study. *Aust. J. Exp. Biol. Med. Sci.* 28:383-397.

Hoffman, H. (1952). Acceleration and retardation of the process of axon-sprouting in partially denervated muscle. *Aust. J. Exp. Biol. Med. Sci.* 30:541-566.

Huizar, P., Kuno, M., Kudo, N., and Y. Miyata. (1977). Reaction of intact spinal motoneurons to partial denervation of the muscle. *J. Physiol. (Lond.)* 265:175-191.

Hursh, J.B. (1939). Velocity and diameter of nerve fibers. *Am. J. Physiol.* 127:131-139.

Jami, L. and J. Petit. (1975). Correlation between axonal conduction velocity and tetanic tension of motor units in four muscles of the cat hindlimb. *Br. Res.* 96:114-118.

Jami, L., Murthy, K.S.K., and J. Petit. (1982). A quantitative study of skeletofusimotor innervation in the cat peroneus tertius muscles. *J. Physiol. (Lond.)* 325:125-144.

Kandel, E. R. and J.H. Schwartz. (1981). Principles of neural science. Volume 1. Elsevier Science Publishing Co. Inc. New York.

Karpati, G. and W.K. Engel. (1968). "Type-grouping" in skeletal muscles after experimental reinnervation. *Neurology* 18:447-455.

Kemplay, S. and C. Stolkin. (1981). Terminal sprouting in rat sternocostalis muscle following partial denervation. *Cell Tissue Res.* 217:189-198.

Kernell, D. and B. Zwaagstra. (1981). Input conductance, axonal conduction velocity and cell size among motoneurons of the cat. *Br. Res.* 204:311-326.

Kidd, G.L. (1964). Excitation of primary muscle spindle endings by beta axon stimulation. *Nature* 203:1248-1251.

Klueber, K.M. (1987). Morphometric analysis of neuronal soma size within the motor nucleus of a transplanted muscle. *Anat. Rec.* 217:402-406.

Klueber, K. (1987b). Role of muscle neurotization in the reinnervation of murine muscle grafts. *Anat. Rec.* 219:429-433.

Krishna Murthy, K.S. (1978). Vertebrate fusimotor neurones and their influences of motor behavior. *Prog. Neurobiol.* 11:249-307.

Krishnan, S., Lowrie, M.B. and G. Vrbová. (1985). The Effect of reducing the peripheral field on motoneurone development in the rat. *Dev. Br. Res.* 19:11-20.

Kristensson, K. and Y. Olsson. (1971). Retrograde axonal transport of protein tracers. *Acta. Neuropathologica* 17:127-138.

Kugelberg, E. and L. Edström. (1968). Differential histochemical effects of muscle contractions on phosphorylase and glycogen in various types of fibres: Relation to fatigue. *J. Neurol. Neurosurg. Psychiat.* 31:415-423.

Kugelberg, E., Edström, L., and M. Abruzzese. (1970). Mapping of motor units in experimentally reinnervated rat muscle. *J. Neurol. Neurosurg. Psych.* 33:319-329.

Kugelberg, E. (1981). The Motor unit: morphology and function in Motor Unit Types, Recruitment and Plasticity in health and disease. J.E. Desmedt (editor). *Progress in Clinical Neurophysiology*. 9:1-16. S. Karger Publications.

Lapicque, L. and R. Legendre. (1913). Relation entre le diamètre des fibres nerveuses et leur rapidité fonctionnelle. *Compt. rend. Acad. Sci.* 157:1163.

Law, P.K. and H.L. Atwood. (1972). Nonequivalence of surgical and natural denervation in dystrophic mouse muscles. *Expl. Neurol.* 34:200-209.

Law, P.K. and H.L. Atwood. (1974). Does axonal sprouting occur in dystrophic mouse muscles? *Experientia* 30#2:155-156.

Law, P.K., and M.R. Caccia. (1975). Physiological estimates of the sizes and the numbers of motor units in soleus muscle of dystrophic mice. *J. Neurol. Sci.* 24:251-256.

Law, P.K., Atwood, H.L., and A.J. McComas. (1976). Functional denervation in the soleus muscle of dystrophic mice. *Expl. Neurol.* 51:434-443.

Leiberman, A.J. (1971). The axon reaction: a review of the principle features of perikaryal responses to axon injury. *Int. Rev. Neurobiol.* 14:49-124.

Levi-Montalcini, R. (1982). Developmental neurobiology and the natural history of nerve growth factor. *Ann. Rev. Neurosci.* 5:341-362.

Lewis, D.M. and D.J. Parry. (1979). Properties of motor units in mouse soleus. *J. Physiol. (Lond.)* 292:90P.

Lewis, D.M., Parry, D.J., and A. Rowlerson. (1982). Isometric contractions of motor units and immunohistochemistry of mouse soleus muscle. *J. Physiol. (Lond.)* 325:393-402.

Lowrie, M.B., Krishnan, S. and G. Vrbová. (1987). Permanent changes in muscle and motoneurons induced by nerve injury during a critical period of development of the rat. *Dev. Br. Res.* 31:91-101.

Lynch, G., Gall, C., and C. Cotman. (1977). Temporal parameters of axon "sprouting" in the brain of the adult rat. *Expl. Neurol.* 54:179-183.

Luff, A.R., Hatcher, D.D., and K. Torkko. (1988). Enlarged motor units resulting from partial denervation of cat hindlimb muscles. *J. Neurophysiol.* 59#5:1377-1394.

Maehlin, J. and A. Nja. (1986). Sprouting after partial denervation in the superior cervical ganglion; preganglionic nerve stimulation. *Acta Physiol. Scand.* 105:18A-19A.

Mastaglia, F.L. and B.A. Kakulas. (1969). Regeneration in Duchenne muscular dystrophy: a histological and histochemical study. *Brain* 92:808-818.

McComas, A.J. (1975). The neural hypothesis. In: *Recent advances in myology.* 152-158. W.G. Bradley, D. Garner-Brown, and J.N. Walton (Editors). *Excerpta Medica: Amsterdam.*

McComas, A.J. (1977). *Neuromuscular function and disorders,* Butterworths: London.

McComas, A.J., and K. Mrozek. (1967). Denervated muscle fibres in hereditary mouse dystrophy. *J. Neurol. Neurosurg. Psychiat.* 30:526-530.

McComas, A.J., Campbell, M.J., and R.E.P. Sica. (1971a). Electrophysiological study of dystrophia myotonica. *J. Neurol. Neurosurg. Psychiat.* 34:132-139.

McComas, A.J., Fawcett, P.R.W., Campbell, M.J., and R.E.P. Sica. (1971b). Electrophysiological estimation of the number of motor units within a human muscle. *J. Neurol. Neurosurg. Psychiat.* 34:121-131.

McComas, A.J., Preswick, G., and S. Garner. (1988). The sick motoneurone hypothesis of muscular dystrophy. *Prog. Neurobiol.* 30:309-331.

McComas, A.J., Sica, R.E.P., and M.J. Campbell. (1971c). 'Sick' motoneurons. A unifying concept of muscle disease. *Lancet* 1:321-325.

McComas, A.J., R.E.P. Sica, and S. Currie. (1971d). An electrophysiological study of Duchenne dystrophy. *J. Neurol. Neurosurg. Psychiat.* 34:461-468.

McComas, A.J., Sica, R.E.P., and K. Tononaga. (1978). Incidence, severity and time course of motoneurone dysfunction in myotonic dystrophy: their significance of an understanding of anticipation. *J. Neurol. Neurosurg. Psychiat.* 41:882-893.

McDonagh, J.C., Binder, M.D., Reinking, R.M., and D.G. Stuart. (1980A). Tetrapartite classification of motor units of cat tibialis posterior. *J. Neurophysiol.* 44:696-712.

McDonagh, J.C., Binder, M.D., Reinking, R.M., and D.G. Stuart. (1980B). A Commentary on muscle unit properties in cat hindlimb muscles. *J. Morphology* 166:217-230.

McPhedran, A.M., Wuerker, R.B., and E. Henneman. (1965). Properties of motor units in a homogeneous red muscle (soleus) of the cat. *J. Neurophysiol.* 28:71-84.

Meier, H. and J.L. Southard. (1970). Muscular dystrophy in the mouse caused by an allele of the dy-locus. *Life Sci.* II 9:137-xx.

Meryon, E. (1852). On granular and fatty degeneration of the voluntary muscles. *Medico-Chirurgical Trans. (Lond.)* 35:73-84.

Mesulam, M.M. (1978). Tetramethylbenzidine for horseradish peroxidase neurohistochemistry.: A non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. *J. Histochem. Cytochem.* 26:106-117.

Mesulam, M.M. (Editor). (1982). Tracing Neural connections with horseradish peroxidase. *IBRO Handbook Series: Methods in the neurosciences.*

Michelson, A.M., Russel, E.S., and P.J. Harman. (1955). Dystrophic muscularis. A hereditary primary myopathy in the house mouse. *Proc. Nat. Acad. Sci.* 41:1079-1084.

Montgomery, A., and L. Swenarchuk. (1977). Dystrophic mice show age related muscle fibre and myelinated axon losses. *Nature* 267:167-169.

Montgomery, A. and L. Swenarchuk. (1978). Further observations of myelinated axon numbers in normal and dystrophic mice. *J. Neurol. Sci.* 38:77-82.

Moore, S.E. and F.S. Walsh. (1986). Nerve dependent regulation of neural cell adhesion molecule expression in skeletal muscle. *Neuroscience* 18#2:499-505.

Morse, J.K., Scheff, S.W., and S.T. DeKosky. (1986). Gonadal steroids influence axon sprouting in the hippocampal dentate gyrus: a sexually dimorphic response. *Expl. Neurol.* 94:649-658.

Moschella, M.C. and M. Ontell. (1987). Transient and chronic neonatal denervation of murine muscle: A procedure to modify the phenotypic expression of muscular dystrophy. *J. Neurosci.* 7#7:2145-2152.

Murphey, E.H., Klions, D.E., DiRienzo, R. and R. Baker. (1986). Morphology of axotomized motor neurons which reinnervate their target. *Neurosci. Abs.* 12:1576.

Murray, M. and M.E. Goldberger. (1974). Restitution of function and collateral sprouting in the spinal cord: the partially hemisectioned animal. *J. Comp. Neurol.* 158:19-36.

Murray, J.G. and J.W. Thompson. (1957). The occurrence and function of collateral sprouting in the sympathetic nervous system. *J. Physiol. (Lond.)* 135:133-162.

Murthy, K.S.K. and A. Taylor. (1978). Muscle spindle response to active muscle shortening in the amphibian. *Expl. Neurol.* 60:175-188.

Nature. (1973). Muscular Dystrophy and the neurogenic hypothesis. *News and Views-Nature.* 243:258-259.

Oh, T. H., Markelonis, G.J., Dion, T.L., and S.L. Hobbs. (1988). A muscle-derived substrate-bound factor that promotes neurite outgrowth from neurons of the central and peripheral nervous systems. *Dev. Biol.* 127:88-98.

Oppenheim, R.W. (1981). Cell death of motoneurons in the chick embryo spinal cord. V. Evidence on the role of cell death and neuromuscular function in the formation of specific peripheral connection. *J. Neurosci.* 1:141-151.

Oppenheim, R.W. (1984). Cell death of motoneurons in the chick embryo spinal cord. VII. Motoneurons prevented from dying in the embryo persist after hatching. *Dev. Biol.* 101:35-39.

Oppenheim, R. W., Chu-Wang, I.W., and J.L. Maderdrut. (1978). Cell death of motoneurons in the chick embryo spinal cord. III. The differentiation of motoneuron prior to their induced degeneration following limb-bud removal. *J. Comp. Neurol.* 177:87-111.

Oppenheim, R.W. and R. Nunez. (1982). Electrical stimulation of hindlimb increases neuronal cell death in chick embryo. *Nature* 295:57-59.

Oppenheim, R.W., Haverkamp, L.J., Prevet, D., McManaman, J.L., and S.H. Appel. (1988). Reduction of naturally occurring motoneuron death *in vivo* by a target-derived neurotrophic factor. *Science* 240:919-922.

Panayiotopoulos, C.P., Scarpalezos, S., and T. Papapetropoulos. (1974). Electrophysiological estimation of motor units in Duchenne muscular dystrophy. *J. Neurol. Sci.* 23:89-98.

Panayiotopoulos, C.P., and S. Scarpalezos. (1975). Electrophysiological estimation of motor units in limb-girdle dystrophy and chronic spinal muscular atrophy. *J. Neurol. Sci.* 24:95-107.

Papapertopoulos, T.A. and W.G. Bradley. (1972). Spinal motor neurones in murine muscular dystrophy and spinal muscular atrophy. *J. Neurol. Neurosurg. Psychiat.* 35:60-65.

Parry, D.J. (1977). A study of functional denervation in fast and slow muscles of dystrophic mice of various ages. *Expl. Neurol.* 55:556-566.

Parry, D.J. (1988). Effects of sciatic crush on young rats and mice on fibre type distribution. *UCLA Symposium Developmental Biology-Abstract.*

Parry, D.J., and A. Falconer. (1986). Number and size of motoneurons in a forelimb motor nucleus of normal and dystrophic (C57BL/6J dy2j/dy2j) mice. *Expl. Neurol.* 91:183-192.

Parry, D.J., and S. Melenchuk. (1981). Rate and extent of functional reinnervation in fast-twitch and slow-twitch muscles of the dystrophic mouse (C57BL/6Jdy2j). *Expl. Neurol.* 72:446-461.

Parry, D.J., McHanwell, S., and N. Haas. (1982). The number and size of motoneurons in the soleus motor nucleus of the normal and dystrophic (C57BL/6Jdy2j) mouse. *Expl. Neurol.* 75:743-754.

Parry, D.J. and H.G. Parslow. (1981). Fibre type susceptibility in the dystrophic mouse. *Expl. Neurol.* 73:674-685.

Petrofsky, J.S. and C.A. Phillips. (1979). Determination of the contractile characteristics of the motor units in skeletal muscle through twitch characteristics. *Med. & Biol. Eng. & Comput.* 17:525-533.

Peyronnard, J.M. and L. Charron. (1983). Motoneuronal and motor axonal innervation in the rat hindlimb: a comparative study using horseradish peroxidase. *Exp. Br. Res.* 50:125-132.

Pockett, S., and J.R. Slack. (1982). Source of the stimulus for nerve terminal sprouting in partially denervated muscle. *Neuroscience* 7:3173-3176.

Prewitt, M.A. and B. Salafsky. (1970). Enzymic and histochemical changes in fast and slow muscles after cross-innervation. *Am. J. Physiol.* 218:69-74.

Proske, U. (1973). An Electrophysiological analysis of responses from lizard muscle spindles. *J. Physiol. (Lond.)* 205:289-304.

Proske, U. and R.M.A.P. Ridge. (1974). Extrafusal muscle and muscle spindles in reptiles. *Prog. Neurobiol.* 3:3-29.

Proske, U. and P.M.E. Waite. (1976). The relation between tension and axonal conduction velocity for motor units in the medial gastrocnemius muscle of the cat. *Exp. Br. Res.* 26:325-328.

Proske, U. and B. Walker. (1975). Responses of muscle spindles in a tortoise. *Br. Res.* 91:79-88.

Purves, D. (1977). The formation and maintenance of synaptic connections. In: *Function and Formation of Neural Systems.* 21-49. G.S. Stent (Editor). Dahlem Konferenzen, Berlin.

Purves, D. (1986). The trophic theory of neural connections. *Trends in Neurosci.* 8:486-489.

Raisman, G. (1969). Neural plasticity in the septal nuclei of the adult rat. *Br. Res.* 14:25-48.

Raisman, G. and P.M. Field. (1973). A quantitative investigation of the development of collateral reinnervation after partial denervation of the septal nuclei. *Br. Res.* 50:241-264.

Rasminsky, M. (1978). Ectopic generation of impulses and cross-talk in spinal nerve roots of dystrophic mice. *Ann. Neurol.* 3:351-357.

Robbins, N., Karpati, G., and W.K. Engel. (1969). Histochemical and contractile properties in the cross-innervated guinea pig soleus muscle. *Archs. Neurol.* 20:318-325.

Romanes, G.J. (1946). Motor localization and the effects of nerve injury on the ventral horn cells of the spinal cord. *J. Anat.* 80:117-131.

Romanul, F.C.A. and J.P. Van Der Meulen. (1967). Slow and fast muscles after cross-innervation: Enzymatic changes and physiological changes. *Arch. Neurol.* 17:387-402.

Rosene, D.L. and M.M. Mesulam. (1978). Fixation variables in horseradish peroxidase neurohistochemistry. I. The effects of fixation time and perfusion procedures upon enzymatic activity. *J. Histochem. Cytochem.* 26:28-39.

Rowe, R. and G. Goldspink (1969). Muscle fibre growth in five different muscles in both sexes of mice II. Dystrophic mice. *J. Anat.* 104:531-538.

Rutihauser, U., Grumet, M., and G.M. Edelman. (1983). Neural cell adhesion molecule mediates initial interactions between spinal cord neurons and muscle cells in culture. *J. Cell Biol.* 97:145-152.

Sears, T.A. (1987). Structural changes in intercostal motoneurons following axotomy. *J. Exp. Biol.* 132:93-109.

Scott, J.J.A. (1986). The Repair and regeneration of damaged peripheral nerves. *Biologist* 33#4:227-234.

Scott, J.J.A. (1987). The reinnervation of cat muscle spindles by skeletofusimotor axons. *Br. Res.* 401:152-154.

Sherrington, C.S. (1906). *The integrative action of the nervous system.* New Haven, Yale University Press. reprinted 1946.

Sherrington, C.S. (1925). Remarks on some aspects of reflex inhibition. *Proc. R. Soc. London (Biol.)* 97:519.

Sriratana, D. (1979). Anatomy and physiology of the cat's flexor digitorum longus and flexor hallucis longus. Ph. D. Thesis. Monash University, Clayton, Australia.

Strick, P.L., Burke, R.E., Kanda, K., Kim, C.C., and B. Walmsey. (1976). Differences between *alpha* and *gamma* motoneurons labeled with horseradish peroxidase by retrograde transport. *Br. Res.* 113:582-588.

Swett, J.E., Wikholm, R.P., Blanks, R.H.I., A.L. Swett and L.C. Conley. (1986). Motoneurons of the rat sciatic nerve. *Expl. Neurol.* 93:227-252.

Thompson, W. (1978). Reinnervation of partially denervated rat soleus muscle. *Acta Physiol. Scand.* 103:81-91.

Thompson, W. and J.K.S. Jansen. (1977). The Extent of sprouting of remaining motor units in partly denervated immature and adult rat soleus muscle. *Neuroscience* 2:523-535.

Torigoe, K. (1985). Distribution of motor nerve sproutings in the mouse gastrocnemius muscle after partial denervation. *Br. Res.* 330:273-282.

Van Harreveld, A. (1945). Re-Innervation of denervated muscle fibers by adjacent functioning motor units. *Am. J. Physiol.* 144#4:477-493.

Walsh, F.S., and S.E. Moore. (1985). Expression of cell adhesion molecule, N-CAM, in diseases of adult human skeletal muscle. *Neurosci. Lett.* 59:73-78.

Wasserschaff, M. and D. Kleinebel. (1988). The activity pattern of reinnervated muscle in relation to the effective motoneuron pool. *Eur. J. Physiol.* 411:267 (Supplement 1).

Weddell, G., Guttman, L., and E. Guttman. (1941). The localization of nerve fibers into denervated areas of skin. *J. Neurol. Psychiatry* 4:206-225.

Westerman, R.A. (1978). In: *Studies in Neurophysiology*. R. Porter (Editor). Cambridge University Press, Cambridge.

Westerman, R.A., Dennett, X., Chan, H.S., Jedwab, J.D., and D. Sriratana. (1979). Muscle hypertrophy after partial denervation: a feline model. In: *Proceedings of a symposium of the foundation of life sciences*, edited by A.D. Kildman, and J.K. Tomkins, Amsterdam:Excerpta Med.

Wines, M.M. and E.C.B. Hall-Craggs. (1986). Neuromuscular relationships in a muscle having segregated motor endplate zones. II. The response to partial denervation. *J. Comp. Neurol.* 249:152-156.

Wueker, R.B., McPhedran, A.M., and E. Henneman. (1965). Properties of motor units in a heterogeneous pale muscle (M. gastrocnemius) of the cat. *J. Neurophysiol.* 28:85-99.

Yan, Q., Snider, W.D., Pinzone, J.J., and E.M. Johnson, Jr. (1988). Retrograde transport of nerve growth factor (NGF) in motoneurons of developing rats: assessment of potential neurotrophic effects. *Neuron* 1:335-343.

Yu, W.A. and M.C. Yu. (1983). Acceleration of the regeneration of the crushed hypoglossal nerve by testosterone. *Expl. Neurol.* 80:349-360.

Zelená, J. and P. Hník. (1963). Motor and receptor units in the soleus muscle after nerve regeneration in very young rats. *Physiol. Bohemoslov.* 12:227-289.