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Charactéristics of Reinnervation in Control (C57BL/6J) and Dystrophic (dy²/j/dy²/j) Mouse Soleus Muscles

by

George Desypris

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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DEDICATION

This thesis is dedicated to the memory of my mother;
Effie Panagakos Desypris.
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I would like to thank the Physiology Department for allowing me to pursue my studies there, and for providing the atmosphere conducive to such an end.

A special thank you goes to Dr. David Parry, my supervisor and friend, for introducing me to a truly fascinating field, and also for exemplifying the essence of a Physiologist. His example has proven invaluable in terms of my own development into a scientist.

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STATEMENT OF THE PROBLEM

The purpose of this study is to examine some of the properties of reinnervated control (C57BL/6J) and dystrophic (C57BL/6J dy2j/dy2j) mouse soleus (SOL) muscle with respect to its nervous supply and muscle fiber type distribution.

Information gained from control animals could be useful in broadening our scope of the normal physiological events occurring during reinnervation. Then, by comparing reinnervation in dystrophic animals to controls, it may be possible to provide more insight into the etiology of murine dystrophy.

Three questions are being posed:

1) Do all motorneurons reinnervate the SOL following nerve transection?

To answer this the motorneurons will be counted in two different ways. One approach will be to count the number of stained motorneurons after labelling the SOL motornucleus retrogradely with horseradish peroxidase (HRP). Also, motor unit numbers will be estimated via the ventral root splitting technique.

2) Do all denervated muscle fibers become reinnervated?

If all muscle fibers are innervated, then the tetanic tension (Po) obtained by stimulating the muscle directly. Thus, the functional innervation ratio (FIR) i.e. the ratio of Po(indirect) to Po(direct) should provide an index of the degree of innervation of the muscle. This will be determined in
individual animals for both unoperated and reinnervated SOL.

3) Is there selective reinnervation?

As the SOL muscle is comprised of two fiber types and thus motorneuron types, it is conceivable that one may be more adept at reinnervating than the other.

To answer this the fiber type distribution will be compared between reinnervated and unoperated solei. Also the relationship between individual motor unit size and time to peak tension (TTP) of the twitch will be examined.

Refer to Project Overview, p. 38, for a more thorough description of experimental rationale.
ABSTRACT

Reinnervation of soleus (SOL) muscle was examined in control and dystrophic mice.

In control mice all motorneurons were successful in reinnervating the SOL. Thus the number of motorneurons, or motor units were the same in unoperated (u) and reinnervated (r) muscles (21 uSOL vs 21 rSOL). Most of the muscle fibers were in fact innervated as tetanic tension elicited by stimulation of the SOL nerve (indirect) did not differ significantly from that obtained by stimulating the muscle directly.

In dystrophic (dys) animals not all motorneurons were successful in re-establishing synaptic contact, although at least in the young group the motorneurons that did regrow and enter the muscle were able to capture most of the available territory. Hence the reinnervation ratios (RIR's) obtained on rSOLdys were comparable to that of rSOLcontrol. In an older dystrophic group reinnervation was variable. The variability however was lost if the analysis was done on the individual sexes comprising the group. In the male group reinnervation was complete as no difference was noted in the number of motor units between rSOLdys and uSOLdys (18 ± u(u) vs 16 ± 2(r)). Also most muscle fibers were innervated (RIR = .89 ± .13). In the females, fewer motor units were obtained in the reinnervated muscles (17 ± 2(u) vs 11 ± 2(r)). As well, the motor neurons that did reinnervate, were seemingly less "healthy" in terms of capturing the available
territory as illustrated by the very low values of RIR (.28 ± .27). Furthermore, in male animals that were castrated one week prior to denervation, reinnervation was impaired in a manner that was quantitatively similar to that seen in the female group.

Although all motoneurons did in fact regrow and reinnervate control SOL, it would appear that type I motoneurons were more successful in terms of expanding their innervating field. This is borne out in the following observations:

a) The isometric time to peak tension (TTP) of the twitch was significantly prolonged in rSOL (13.67 ± .52 msec uSOL vs 16.50 ± 1.05 msec rSOL).

b) In rSOL there was an increase in the proportion of muscle cross-sectional area that was occupied by type I fibers (.41 ± .07 uSOL vs .73 ± .05 rSOL).

c) Immunohistochemical evidence using monoclonal antibodies directed against type II and type I myosin is supportive of type II → type I fiber conversion in rSOL.

d) In motor unit studies the largest reinnervated units were all slow contracting while most (13 of 16) of the smallest units were fast contracting. No such correlation was found in units isolated from uSOL.

It was not possible to do the same analysis of motor unit properties on dystrophic animals. The main reason is as follows. Extensive amyelination in the lumbar spinal roots did not allow for the reliable isolation of single units. The resultant ephaptic activation of adjacent units led to counting of the same
unit more than once. Hence incremental stimulation of the sciatic nerve along with sciatic nerve split were employed. With these procedures it is not possible to obtain a TTP/unit size relationship which is crucial in delineating motor neuron susceptibility vis a vis the results on controls.

Also the co-existence of myosin types in individual fibers makes it difficult to reliably quantify the relative amounts of slow myosin.

However, the lack of increase in TTP of young rSOLdys compared to the unoperated muscle, along with an apparent lack of increase in slow myosin in rSOLdys, are suggestive of type I motor neuronal involvement.

To conclude, it appears that in control animals reinnervation is complete with most muscle fibers receiving innervation. Also, type I motor neurons are more adept at expanding their innervating fields once they have entered the SOL.

In dystrophics, success of reinnervation is variable. In older animals a sex difference was noted with the females reinnervating poorly, while in the males reinnervation was complete. The observation that reinnervation was impaired in castrated males suggests that perhaps a gonadal factor may play a protective role in the male group.

Nonetheless, in the young dystrophic group the lack of increase in TTP of rSOL along with no apparent increase in slow myosin suggests that type I motor neurons may be affected.
INTRODUCTION

Human Muscular Dystrophy: Evidence for neural involvement

Much of the difficulty encountered in the attempt to resolve the etiology of human muscular dystrophy stems from the inability to convincingly demonstrate a cause and effect relationship that can be irrefutably attributed to a primary disturbance in muscle or nerve.

The earliest description of dystrophy was by Meryon in 1852. He favored a myogenic etiology based on his observations of 'granular degeneration' of muscle with no evidence of pathological changes in anterior horn or motor roots. Soon thereafter Duchenne (1868) confirmed and extended these observations to include responses of muscle to electrical stimulation. The disease which now bears his name became the subject of intense investigation geared to characterising it in terms of distinguishing features.

In this regard it is noteworthy that Erb's (1891) histological description of dystrophy was so accurate that it is unchanged to date. He observed the presence of atrophic and hypertrophic muscle fibers in early stages, along with central nucleation and the accumulation of fat. No evidence for a neural defect was found.

In 1956 Kugelberg and Welander saw histological changes similar to dystrophy in the muscles of patients afflicted with spinal muscular atrophy, a disease of neural origin. This, along
with observations that chronically denervated muscle in many ways is similar to dystrophic muscle suggested that the idea of dystrophy as a pure myopathy may be incorrect.

Evidence for this was provided by McComas et al (1974). They examined motor units of human extensor digitorum brevis (EDB) muscle and found a reduction in their numbers and sizes in patients suffering from 4 types of dystrophy. Although this was support for a neural component to human dystrophy it did not prove a causality. These patients were already severely afflicted by the disease. It is thus conceivable that the neural defect is secondary to a primary myopathy in which for instance the muscle fibers that are affected lose their ability to sustain synaptic connectivity.

Of course, the possibility that the motor nerves are primarily affected remains.

This forms the basis of McComas' (1971) 'Sick Motor Neuron' Hypothesis where motor neurons are thought to exist in at least 3 states, healthy, dead, and in an intermediate stage, termed 'sick', where normal interactions with muscle cannot continue and a state of functional denervation ensues. Unfortunately though, this idea has not met with experimental support, at least in humans. The main reason for this is that the experiments that are needed to provide unequivocal proof on this matter are beyond ethical means.

This is why it has become necessary to search for animal
models whose phenotypic expression best approximates that which is seen in the human disorders.

**Animals Models**

There are many reports of hereditary 'dystrophy-like' myopathies in animals. I will review the most common ones.

The earliest published account of genetic dystrophy in non-humans was in 1955 when Michelson et al. reported a mutation (designated dy/dy) in a ReJ 129 inbred strain of mice.

The onset of clinical manifestations was rapid (2-3 wks. after birth) as was the progression of the disease, leading ultimately to death by 4-6 months of age.

All examined skeletal musculature was affected to some degree. Cross-sections of muscles stained by hematoxylin and eosin (H&E) showed a profile that was similar to Duchenne dystrophy with the exception of fat deposition. There were hypertrophied as well as atrophied fibers. Also, there was evidence of fiber splitting. Nuclei were centrally located and there was an apparent increase in their numbers. There was also an increase in the amount of connective tissue.

As no abnormalities were seen in peripheral nerve, it was concluded that this disorder was purely myopathic. This however has recently been challenged. Bradley and Jenkinson (1974) found large areas of amylolysis in the lumbosacral spinal roots of these animals. Also, reductions in both orthograde (Bradley and
Jaros 1973, Jablecki and Brimijoin 1974, Brimijoin and Schreiber 1982) and retrograde (Rutherford and Roegman 1982) axonal flow has been documented in peripheral nerves of these mice. Thus, the possibility that the dystrophy results from a trophic deficiency becomes tenable.

Indeed, the observations of functional denervation (McComas & Mrozek 1967, Law et al. 1976, Parry 1977) and impairment in reinnervation (Parry & Melenchuk 1981) of skeletal muscle in dystrophic mice may be consequential to a trophic deficiency.

For the moment though it will suffice to say that evidence exists for both a neural and muscular defect. Later on, in the 'Statement of the Problem' section, I will outline my thesis topic which addresses the etiology of murine dystrophy.

As far as the applicability of this model to human dystrophy is concerned, the fact that amyelination is not a feature of the disease in humans warrants caution when extrapolating data obtained with this model, to the human situation.

Also, because of the short life span and infertility of these animals this model is a somewhat cumbersome one to work with. In order to breed animals it is necessary to carry out ovarian transplants from affected animals to control counterparts thereby increasing the complexity of maintaining a viable colony of experimental animals.

This problem was alleviated in 1970, when Meier and Southard described a myopathy in a mutant strain of ReJ mice resembling
the dy/dy dystrophy, but unlike it in its rate of progression. Additionally these animals were longer lived (up to 1 1/2 years) and fertile, so that maintenance of a viable colony presented no major problems.

As the mutation in this myopathy was localised to the dy locus, it was designated dy²j. This allele was transferred to the control C57BL/6J background, thus producing the C57BL/6j dy²j/dy²j dystrophic mouse.

The myopathy is almost identical to that of the dy/dy strain. The main difference lies in its rate of progression. Clinical signs are apparent at 3-4 weeks of age and as in the dy strain include toe curling and extending when held suspended by the tail, and dragging of hindlimbs following pressure application to the hips. The histological profile is identical to that of the dy variety and the previously mentioned lack of myelination in segments of spinal root is also seen.

Later on in the Introduction I will be discussing more of this and the dy model and their contribution to our understanding of the etiology of dystrophy.

There are two other varieties of dystrophy that deserve note. These are that of the chicken and hamster.

Chicken dystrophy was first reported in 1956 by Asmundson and Julian. It was found to be autosomally recessive. Birds afflicted with the disease are easily distinguished by their smaller size, and weakness. Early on in their life they are
unable to elevate their wings above a horizontal plane. Later on the limb musculature is affected leading to an impairment in their gait.

Affected muscles show variable fiber size, along with progressive infiltration by fat. Centronucleation is also seen; however it is not likely an expression of the dystrophy per se for this happens to be a feature of normal chicken skeletal muscle.

As in the mouse myopathy this disease is progressive with some muscles being affected more than others. Unlike the mouse model, these animals usually live a normal lifespan.

Hamster dystrophy was first described by Homburger et al in 1962. They discovered an affected group of animals in their inbred line, BIO 1.50 Whitney, and went on to show that the disorder was genetic in origin.

First signs of symptoms were not apparent till 60-200 days following birth. Their life span was about 1/3 of normal or roughly 220 days.

The histopathological profile of affected muscle was similar to that of dystrophic mouse, but never progressed to the extent seen in the mouse. The reason for this is that along with skeletal muscle, cardiac muscle is also affected. The animals usually succumb to congestive heart failure, and they do so at a time when the disease has not had a chance to progress to the advanced stages as seen in the mouse. For this reason it is not
the best model of dystrophy to study. However, it has gained popularity as a model for congestive heart failure (Rajusz 1969).

Having introduced the more common examples of animal dystrophies, I would like to assess the relative roles of nerve and muscle involvement in their expression.

Before doing so, it would be instructive to review the literature pertinent to normal interactions between nerve & muscle. Let us start by examining the most fundamental component of muscle, the motor unit.

**The Motor Unit**

(i) Formation of a motor unit

We know that a motor unit is comprised of an alpha motorneuron plus all the muscle fibers that it innervates (Sherrington 1925). Usually each muscle fiber receives innervation from a single branch of the alpha motorneuron. The notable exception to this rule is in the case of vertebrate tonic muscles where multiple innervation is common (Hess 1970), and in neonatal mammalian muscles.

The first demonstration of multiple innervation in mammals was by Redfern (1970). He used a rat diaphragm preparation that was bathed in Ringer's solution with curare at a dosage that prevented contraction but allowed one to record subthreshold end plate potentials (EPP's). By stimulating the phrenic nerve at increasing voltages, and recording the EPP intracellularly he found that at birth each muscle fiber was innervated by at most 4
terminals. By 2½ weeks only one terminal was found per muscle fiber. Later on Bagust, Lewis and Westerman (1973) showed this in a more convincing fashion. They isolated ventral rootlets supplying axons to soleus (SOL) and flexor digitorum longus (FDL) muscles, and stimulated each separately. The sum of the tensions recorded exceeded that obtained by stimulation of the whole root. This phenomenon disappeared by about 3 weeks post-natally.

The disappearance of polynuronal innervation has been studied extensively and the general consensus is that increased neuronal activity is directly related to the loss. Basically, two experimental approaches have been used to investigate this.

In the first, neural activity was increased by artificial stimulation (O'Brien, Ostberg and Vrbova 1978), or removal of synergists (Zelena, Vyskocil and Jirmanova 1979). The result in both cases was an increase in the rate of elimination of polynuronal innervation. In contrast, tenotomy (Benoit and Changeaux 1975; Riley 1978), cordotomy alone (Miyata and Yoshioka 1980), or with deafferentation (Zelena et al 1979) along with nerve paralysis induced by chronic TTX application (Thompson et al. 1979) or blockage of neuromuscular transmission (Brown, Holland and Hopkins 1981) resulted in a persistence of polynuronal innervation.

Recently Dangain and Vrbova (1983) have provided further support for neural activity by examining the disappearance of this phenomenon in dystrophic mice. Because of the increased
neural activity in the hindlimbs of these mice one would expect the elimination of polyeuclidean innervation to proceed at a more rapid rate. This indeed was found. Unfortunately though, they did not measure EMG activity in these animals and thus were unable to provide any quantitative information on the actual amount of activity the muscle was receiving. It is thus conceivable that the dystrophy per se may precipitate a more rapid elimination of extra synapses. It should be pointed out however that because of the very small size of muscles in neonatal mice it would be technically difficult to quantitate EMG activity of single muscles. Perhaps a better internal control would be the forelimb muscles where this spontaneous activity is not present (Bateson 1982, Parry and Desypris 1983).

Once polyeuclidean innervation is lost there is a 1:1 correspondence between muscle fiber and endplate. It is from this point in time that a motor unit becomes a true unit in the sense that it undergoes trophic interactions and is electrically activated by a single alpha motor neuron.

(ii) Motor unit properties & fiber types

As the constituent muscle fibers of a unit are randomly distributed over a large area of muscle (Kugelberg et al 1970) one would expect the functional demands of each to be virtually identical. Given common innervation it is then logical to assume that each muscle fiber in a given motor unit should have
identical contractile and metabolic profiles. With the help of recent histochemical techniques, elucidation of this problem has been dealt with in a rigorous fashion, and the results are according to expectations. These experiments will be considered later on.

Perhaps the earliest observation of differences in muscle was by Ranvier (1874) who noticed the differences in color (red vs white) and sizes of fibers in various muscles. Later on, various histochemical techniques were developed to measure key enzyme activities. The basic principle of these procedures was that a colored product would be formed at a rate proportional to the enzyme activity. This product would be trapped on the section as it is formed, thus the enzyme activity would be roughly proportional to the staining intensity.

Nachmias and Padykula (1958) noted differences in staining intensity for succinic dehydrogenase, an enzyme marker of oxidative capacity, between individual muscle fibers. They also noticed that the smaller diameter fibers had a greater staining intensity than large ones which were quite pale in fact.

Later on, by combining this stain and others with one that would presumably indicate differences in the contractile apparatus, namely myofibrillar ATPase, a general classification arose based on differences in oxidative capacity, mitochondrial density, levels of glycogen, and contractile apparatus (Stein and Padykula 1962).
I do not wish to review all the classification systems of fiber types, as many exist and their review would only serve to confuse rather than instruct the reader.

A brief account will suffice to familiarise the reader with the events leading up to the formation of the classification system that is currently in wide use (refer to Table I).

Stein and Padykula (1962) divided muscle fibers into 3 groups: A, B, and C, also known as β, αβ, & α (Guth & Samaha 1969), white, intermediate, and red (Gauthier 1971). Each group was distinguished from the other on the basis of both ultrastructural and histochemical criteria.

Type A fibers are the largest in size with an average diameter of about 55 μm in the rat. They are poorly vascularised (Armstrong and Laughlin 1983), and low in oxidative capacity as assessed by succinic dehydrogenase (SDH) activity and mitochondrial content. In contrast, type C fibers are the smallest fibers with an average diameter of about 40 μm. Their vascular supply is the highest as is their oxidative capacity. The type B fibers are intermediate in all parameters. Their average diameter is about 45 μm.

The current popular classification stems from Dubowitz' (1960) study. He named fibers type I or II depending on their oxidative, glycolytic, and contractile profiles. Fibers rich in oxidative enzymes but low in glycolytic ones, and with a pale myofibrillar ATPase reaction at alkaline pre-incubation were
called type I. The reverse was true for type II fibers.

Brooke & Kaiser (1970) further divided the type II fibers into IIa and IIb depending on their relative staining intensity in the acid myofibrillar ATPase pre-incubation. Type IIa are pale at pH 4.5 while the IIb fibers are intermediate at this pH and pale at pH 4.3. In alkaline pre-incubation they are both dark. They also described a fourth fiber type, termed IIC in human biopsy samples. It was rare in appearance and was distinguished by its intermediate staining in acid pre-incubation.

Recently the use of antibodies directed against various myosin types has allowed for classification based on specific contractile apparatus (Gauthier and Lowey 1977). With this method, differences between fetal, neonatal, and adult fibers has been noted along with co-existence of myosin types in certain circumstances (Kelly and Rubinstein 1980, Whalen 1980, Billeter et al. 1981).

The greatest barrier to answering the question regarding the homogeneity of fibers within a single motor unit, has been the inability to isolate single fibers within any given unit.

With the advent of the glycogen depletion technique (Kugelberg and Edstrom 1968) the necessary tool for extracting this data, was at hand. The technique relies on the fact that if a muscle is repeatedly subjected to low frequency fatiguing schedules, its stores of glycogen will be exhausted. If a single
unit is mechanically isolated by ventral root splitting and treated in this manner, the depleted fibers will stand out as pale in a cross-section stained for glycogen. Serial sections can then be stained for myofibrillar ATPase, SDH, phosphorylase, and myosins, along with any other metabolic or contractile marker one wishes. The fibers of the unit can then be identified and analysed by fiber typing and quantitative microspectrophotometry.

I will review some of the key work that has examined the homogeneity of motor units.

Burke et al. (1971) isolated single units byimpaling motor neurons with a stimulating electrode. The histochemical properties could only be assessed on the last unit studied as only one unit per muscle could be identified by glycogen depletion. Three types of units were defined, fast fatiguing (FF), fast fatigue-resistant (FR), and slow (S). The speed refers to their isometric contraction times. Fatigue characteristics were obtained by comparing the tetanic tension (Po) prior to a fatiguing train of pulses, to that at the end of the fatiguing protocol. It was found that the FF units produced the greatest tetanic-tension (Po) and when analysed histochemically by the myofibrillar ATPase and SDH stain, all constituent fibers of the unit were identical. They corresponded to the typeIIb fibers of Guth and Samaha. FR units produced less tension than FF, but more than the S units. Histochemically they correspond to typeIIa fibers while the slow contracting S units
are the type I's.

Nemeth et al. (1981) measured malate dehydrogenase activity (an oxidative enzyme marker) in the fibers of motor units that were identified by glycogen depletion and found it to be identical in all the constituent fibers. In contrast, malate dehydrogenase activity showed wide variations when measured in fibers selected at random from other units, even though their ATPase sensitivity was the same.

As far as the contractile characteristics of single units is concerned, it is likely that contraction times or velocities of unloaded shortening are the same in constituent fibers. Unfortunately, the technical difficulties associated with measuring mechanical properties of single fibers in an identified motor unit are insurmountable at the moment. Thus, one can only make inferences from histochemical analysis of identified motor units, and contractile studies of histologically pure muscles.

On the basis of histochemical analysis the contractile apparatus has been shown to be the same in each fiber of a unit. Also, a direct correlation between myosin type or myofibrillar ATPase and isotonic speed of shortening has been found (Barany 1967, Close 1972). Lewis, Parry and Rowerson (1982) showed in the mouse soleus that fibers classified as type I by myofibrillar ATPase staining reacted with anti-slow antibodies, while the Ila fibers reacted with anti-fast antibodies. More recently the use of monoclonal antibodies, Schiaffino (unpublished) has allowed for
the raising of very specific antibodies with no cross-reactivity. Thus, the co-existence of myosin types in single fibers can be demonstrated with more certainty. Also with this technique, the existence of neonatal and fetal forms of myosin in certain pathological conditions of muscle has been documented.

**Nerve-Muscle Trophic Interactions**

One of the ways of studying the interactions between motor nerve and skeletal muscle is to dissect one from the other and observe the changes in properties of both muscle and nerve. A way to do this is by denervating the muscle and then measuring contractile, biochemical, and morphological properties. I will spend some time on this facet, but first I would like to discuss some of the evidence for early nerve-muscle interactions.

The earliest interaction between nerve and muscle ultimately leads to the formation of a functional synapse.

Using morphological data Couteaux (1963) suggested that the motor neuron exerts an influence on the muscle. This influence would probably be manifest as a manipulative one that led to the expression on the surface of the muscle, the postsynaptic receptive elements that keep the synapse in register. Alternatively, the muscle may influence nerve terminal differentiation and organisation (Peper et al. 1974). Unfortunately the causality is impossible to resolve solely on the basis of morphological data as both alternatives are equally plausible.

The use of tissue culture techniques has not solved the
problem, but has certainly gone a step further by providing us with more tangible information concerning nerve-muscle interactions.

For instance, in 1904 Harrison was the first to rule out the involvement of neuromuscular transmission (NMT) in synapse formation. He raised frog larvae in a medium that contained an anesthetic to paralyse the nervous system. When they were fully developed tadpoles he transferred them to a medium with no anesthetic. Following a short period of time, presumably reflecting washout of anesthetic, the tadpoles were able to swim.

More recently Crain and Peterson (1971) and Cohen (1973), using amphibian muscle showed normal synaptogenesis in a system where either postsynaptic acetylcholine (ACh) receptors were blocked, or release of transmitter inhibited.

If ACh receptors and transmitter release are not necessary for normal synapse formation, then at least three alternate possibilities exist.

1) A nerve trophic factor whose release is impulse independent.

2) A muscle trophic factor that somehow guides the nerve to the postsynaptic site.

3) A postsynaptic membrane component that attracts ingrowing neurons.

As far as the first possibility is concerned, up until now, there has not been any conclusive evidence of a non-impulse
related neural trophic factor whose function is to guide the neuron to its target. Even if such a mechanism existed it would be a rather awkward one since the neural factor would first have to notify the muscle that it was in its vicinity, and only then could the muscle release its 'homeing factor' to guide the neuron. It seems more sensible for the muscle to be sending out a homeing factor prior to being innervated. Once functional connections had formed, down-regulation of the expression of this factor would ensue.

This is not a farfetched idea as Brown et al (1981) have shown that partial denervation of mouse skeletal muscle leads to pre-terminal sprouting of remaining intact motor axons. Presumably the denervated fibers are sending out a signal. To this end Slack and Pockett (1982) have localised neurotrophic activity to the endplate region of denervated muscle fibers. The longevity of dissociated motor neurons obtained from the lumbo-sacral region of mice was greatly enhanced in the presence of this extract. If this factor is indeed the trophic factor that causes pre-terminal sprouting, then its absence in innervated fibers supports the concept of a muscle homeing factor that is down regulated by innervation.

If this is true, then pre-terminal sprouting may be the recapitulation of an early developmental process that has been released from tonic inhibition.

As far as the third possibility is concerned there is
evidence to suggest that the basal lamina of muscle fibers plays a role in the orientation of ingrowing neurons (Keynes et al. 1984). It is also of note that in a muscle that is deprived of its innervation and then allowed to reinnervate, new synaptic junctions will preferentially form on pre-existing post-synaptic sites (Guth and Brown 1965, Pecot-Dechavassine 1971, Jirmanova and Thesleff 1972, Gorio et al. 1983).

Thus, the bulk of evidence to date suggests that certain properties inherent in the muscle play a key role in attracting and sustaining synaptic connectivity.

Let us now turn our attention to the properties of denervated skeletal muscle. Early changes in membrane properties have been reported (Redfern and Thesleff 1971, Albuquerque et al. 1971, Thesleff 1974, Thesleff and Ward 1975), and include a decrease in resting membrane potential, the appearance of TTX resistant action potentials, along with the appearance of fibrillation potentials. This early membrane depolarization is of interest since it occurs before any change in miniature end plate potential frequency, is dependent on the length of sectioned nerve, and is not abolished by the application of drugs that interfere with axoplasmic flow (Albuquerque et al. 1972). Recently, Bray, Forrest and Hubbard (1982) have provided evidence that the normal membrane potential of muscle is maintained by non-quantal ACh release, through a Ca\(^{++}\) dependent mechanism. This finding may explain the early phenomenon of membrane
depolarisation following denervation, and would implicate ACh as the trophic agent.

In terms of structural changes, the most obvious is muscle atrophy. In the long run, up to 80% of initial weight can be lost. The decrease in muscle size is attributable to a decrease in the diameter of constituent fibers (Gutmann 1962). In denervated diaphragm a transient hypertrophy is seen due to stretch (Jirmanova and Zelena 1970). Eventually most of the muscle fiber bulk will be replaced by fat and connective tissue. In terms of fiber type susceptibility, type IIb fibers atrophy to the greatest degree (Engel 1962). Recently Davis and Kiernan (1981) showed that peripheral nerve extract significantly attenuated the atrophy of IIb fibers in denervated rat extensor digitorum longus (EDL) muscle. This finding supports the role of a neuronal trophic factor in maintaining the balance of anabolic and catabolic processes of skeletal muscle.

One of the early changes following denervation is an increase in the number of satellite cells (Ontell 1974). As they are involved in muscle regeneration, this may represent a release of their tonic inhibition.

In the later stages of denervation atrophy, nuclei become clumped and centrally located, and there is a loss of mitochondria and sarcoplasmic reticulum (SR). The loss of mitochondria and SR is also borne out in the histochemical and contractile characteristics of these muscles. Loss of enzymatic
profile is seen for SDH, phosphorylase, and lactate dehydrogenase (Nachmias and Padykula 1958, Romanul and Hogan 1965), while a slowing of twitch temporal characteristics and an increase in the twitch/tetanus ratio is also seen (Lewis 1972). The slowing is more pronounced in muscles that are typically fast and is more marked in the relaxation phase of the twitch.

Sreter (1970) showed that the initial rate of Ca\(^{2+}\) uptake was decreased in SR vesicles isolated from denervated fast and slow twitch muscle. If the in vitro situation is an accurate reflection of the properties of SR in vivo, then this observation would explain the prolongation of twitch temporal characteristics.

As denervation involves withdrawal of both activity and neurohumoral trophic influences, it is difficult if not impossible to assess the relative contribution of each to the changes seen. To answer this problem investigators have replaced one of the components at the expense of the other and observed the changes in muscle properties.

The crudest method of eliminating activity to a muscle is to immobilise it by casting in a shortened position. Not all activity is abolished however by this procedure and thus it becomes necessary to also perform a dorsal rhizotomy and spinal section.

Gallego et al. (1979) showed that metabolic changes in muscle resulting from inactivity could regulate a property of
motor neurons. It is known that motor neurons that innervate a slow muscle have a characteristic action potential whose duration of afterhyperpolarisation (AHP) is much longer than that of a motor neuron supplying a fast muscle. They immobilised the SOL of adult cats in shortened and lengthened positions and also performed a dorsal rhizotomy and thoracic section. Two weeks later they examined the muscles and the properties of motor neurons supplying the SOL. In the animals where the SOL was immobilised in a shortened position there was marked atrophy of the muscle and a decrease in the duration of AHP recorded in ventral horn cells. In contrast, when immobilised in the lengthened position, no change in weight of SOL was seen. Also the duration of AHP was not changed. Their results of muscle atrophy in a shortened position are in agreement with those of Goldspink (1977a,b), and suggest a role of passive stretch in the regulation of growth processes of muscles. This is borne out further on studies of stretched denervated muscles and rules out the nerve in the regulation of these processes. The change in duration of AHP is interesting in that it may represent a muscle-directed trophic influence whose purpose may be to set the maximum frequency of activation of the contractile apparatus.

It was mentioned that immobilisation in a shortened position results in muscle atrophy. In prolonged immobilisation a preferential atrophy of type I and IIa fibers has been noted (Karpati and Engel 1968). This observation supports the idea
that type IIb fibers may be more dependent on non-impulse related trophic factors than type I or IIa. The previously mentioned effect of nerve extract on type IIb fiber atrophy is consistent with this view.

Contrary to the slowing that is seen in denervated muscle, immobilised muscle has faster twitch temporal characteristics (Mann and Salafsky 1970). The speeding up is not due to fiber type transformation as there is no change in the histochemical profile and motor unit distribution of these muscles (Karpati and Engel 1968, Burke et al. 1975). It is unlikely that this property is under the control of trophic substances as the speeding up occurs despite the fact that innervation is intact. The idea that the activity pattern to the muscle may be important in the regulation of this property has received a lot of attention. Indeed, denervated fast muscle that receives artificial stimulation will slow in its contractile characteristics. The slowing is initially due to a change in SR properties, followed by changes in myosin type (Heilmann and Pette 1979, Salmons and Sreter 1976, Rubinstein et al. 1978). Also, cross transplantation of a nerve supplying a slow muscle with that of a fast one will lead to a speeding up of the isometric twitch response of the slow muscle and a slowing in the fast muscle (Buller, Eccles and Eccles 1960). Margreth et al. (1973) have shown that the SR in a SOL that has been cross-innervated by a fast nerve, has Ca++ sequestering characteristics
that resemble a fast muscle. As the change in SR properties occurs in both denervated and quiescent muscle, it appears that activity may be important in determining its expression. The myosin change in stimulated denervated muscles is probably a reflection of activity related metabolic changes in muscle that affect the expression of myosin type.

From the above it appears that neuronal influences in the form of activity and neurohumoral trophic interactions affect the expression of contractile and metabolic characteristics in skeletal muscle.

Muscle Reinnervation

If a specific neuron is able to program a muscle fiber, then it is not unreasonable to assume that if a muscle is denervated and then allowed to reinnervate, two possible outcomes may arise.

1) Motor neurons recognise previous endplates and thus reignervate at previous loci, resulting in no discernible change in histochemical profile.

2) Reinnervation is random and motor neurons re-specify muscle characteristics.

The first possibility can be ruled out immediately at least in mammals. The fiber type distribution of reinnervated muscle is unlike that of normally innervated muscle. It seems that ingrowing neurons capture and maintain synaptic association with fibers they encounter en passante because instead of seeing the
checkerboard pattern that is characteristic of unit fiber dispersion in normal muscles, there is type grouping of muscle fibers into clumps (Karpati and Engel 1968, Kugelberg et al. 1970). Also if the nerve supply to two muscles that differ greatly in their fiber type composition is switched and reinnervation allowed to proceed there is a conversion of their physiological and histochemical properties to resemble those of the muscle normally receiving that nerve supply. Thus the important variable in specifying muscle type seems to be the type of motor neuron innervating it.

Much evidence exists in favor of random reinnervation. This does not necessitate however that new synapses should be formed at newly formed end plates. For instance Gutmann and Young (1944) showed that in reinnervated rabbit peroneal muscles, synapses occurred at the sites of original endplates. When rat EDL was cross-innervated by the anterior tibialis muscle only 16% of the fibers had ectopic synapses (Bennett and Pettigrew 1976). In contrast, most muscle fibers of the slow SOL muscle were found to have ectopic synapses when cross-innervated by a fast foreign nerve (Frank et al. 1975). Taux (1983) cross-innervated the mouse EDL and SOL muscle with the sural nerve. In the reinnervated EDL less than 10% of the fibers had ectopic synapses while in the reinnervated SOL 90% of the fibers had ectopic synapses. Since the SOL is the only hindlimb muscle with a substantial proportion of type I fibers (Armstrong and Phelps...
1984) the above results support the notion that endplates of a fiber type prefer reinnervating axons of the same type. This does not mean that reinnervation will not occur if the match is not made. In this case, a new synapse will form at a site away from the original endplate zone.

Many studies have addressed the properties of motor units in reinnervated muscle, e.g. the distribution of motor unit sizes. Random reinnervation implies that motor nerves would capture muscle fibers on a 'first come, first served' basis. Those arriving earliest would have a head start on the others and would have more time to reinnervate. Thus, the latest arrivals would be left with less territory to reinnervate, and their sizes would consequently be diminished.

Bagust and Lewis (1974) examined the properties of reinnervated units in self-reinnervated cat SOL and FDL muscles. In brief, either the SOL or FDL nerve was sectioned and resutured (nerve-nerve resuture). Seven months later the mean motor unit tetanic tension was increased when compared to control. Also the range of sizes was broader than in control muscles. Since the average motor unit tetanic tension was larger, one can infer a decrease in the number of motor units. They did in fact mention that a number of axons did not functionally cross the point of nerve section. Gordon and Stein (1982) examined reinnervation of cat triceps surae group. Although muscle tetanic tension recovered to 80% of control there was a decrease in the number of
motor units from 204 in controls to 127 in reinnervates. The relative motor unit size was increased from 0.49% Po in controls to 0.79% Po in reinnervates thus illustrating the capacity of surviving motor neurons to expand their territories through collateral sprouting.

In another study Lowrie and Vrbova (1984) denervated rat SOL and EDL by crushing the sciatic nerve. Two months later the SOL had almost fully recovered, as maximum tetanic tension was 80% of control. The EDL however only recovered to 40% of control and also had a severely reduced number of muscle fibers. Their explanation for the discrepancy was that regenerating fast motor neurons may not be able to reinnervate all the available territory. Support for this is the observation that when SOL is crush denervated as above, there is a reduction in the number of motor neurons to SOL following reinnervation as demonstrated by HRP retrograde labelling of the cell bodies. Also, ATPase staining shows an almost homogeneous population of type I fibers in the reinnervated SOL whereas in the control the profile is mixed type I and IIa (Bearcroft et al. 1983).

In all the above studies the method of denervation, be it nerve crush or section followed by nerve-nerve resuture was performed at a site remote from the point of entry of the nerve into the muscle. For this reason the reduction in number of motor units of reinnervates warrants some caution in interpretation. For instance, it is possible that type II neurons grow back at a slower rate than type I neurons and
therefore would have little or no territory left to reinnervate once they reached the muscle. This would also explain the above results. It is of interest that Parry and Melenchuk (1981) found that by denervating mouse EDL and reapposing the nerve on the belly of the muscle, reinnervation was complete by 6 weeks. In fact in the younger group of animals, twitch tension was greater in the reinnervated muscle than control.

To summarise, at least 3 points can be stated with a fair amount of certainty regarding reinnervated muscle.

1) Ingrowing neurons do not show specificity for muscle fiber type, but do prefer previous endplates as sites for reinnervation.

2) Constituent fibers of a reinnervated motor unit tend to be clumped rather than scattered throughout the cross-section of muscle.

3) Re-specification of contractile and metabolic properties to that of the nerve type occurs following the formation of a new functional synapse.

**Murine Dystrophy: Myopathy or Neuropathy?**

Because of the similarity of murine dystrophy to that of the human Duchenne variety, it has gained enormous popularity as a model to study the dystrophic process and hopefully to permit extrapolation to the human situation.

As I mentioned in the beginning of the Introduction, most of
the earlier work pointed to a primary myopathy in human dystrophy. It was not until primary neuropathies such as lower motor neuron disease were described in which the involvement of muscle resembled that seen in dystrophy, that the inclusion of a neural component became tenable.

I will now review some of the work that has been done on the mouse model of dystrophy in an effort to pinpoint the abnormality in dystrophy.

There are basically three theories. The first was termed the vascular hypothesis. It was suggested that the degeneration that is seen in dystrophic muscle was consequent to an inadequate blood flow (Kure & Okinaka 1930). This theory has failed to be substantiated however as no impairment of blood flow in dystrophic patients has been observed (Bradley et al. 1975). Also, morphometrical analysis of small blood vessels is not quantitatively abnormal in dystrophic subjects (Jerusalem et al. 1974). In the second theory a primary disturbance in the muscle has been proposed. Thus, any coincident neural abnormality may either be a secondary manifestation of a primary myopathy or just simply the result of a second genetic defect, unrelated to the dystrophy. The third possibility is that a primary neural lesion leads to secondary pathological changes in skeletal muscle. There is of course a fourth possibility. It may be that the genetic locus that controls nerve and muscle is tightly coupled, thus a mutation may be reflected in altered function of both
muscle and nerve. In this case the initial defect would lie both in muscle and nerve, and progression of the disease would be consequential to altered nerve-muscle interactions.

Early attempts to resolve this dilemma focused on cross-transplantation experiments between phenotypically normal animals and dystrophic ones.

Salafsky's (1971) experiments on the ReJ 129 dy/dy mouse were supportive of a neural defect. He cross-transplanted minced normal and dystrophic anterior tibialis muscles and observed the extent of regeneration in the presence of the foreign environment. He found that there was no significant difference in regeneration between dystrophic and control regenerates in the control environment. Also, dystrophic minces regenerating in dystrophic hosts did not form functional neuromuscular connections. It was concluded from these results that the site of the defect was in the motor nerve.

Hironaka and Miyata (1975) provided further evidence to this end. They cross-transplanted whole EDL muscle between control and dystrophic mice. In dystrophic to control hetero/transplants and control homotransplants there was no significant difference in the parameters measured which included, isometric twitch and tetanic tension normalised to cross-sectional area. The increases in fiber diameter and weight were comparable in both control and dystrophic muscles. In contrast dystrophic, and control to dystrophic cross-transplants both fared poorly in the
above parameters. Although they concluded that the defect appears to lie in the nerve, their results of functional denervation only in the case of dystrophic to dystrophic reinnervation also points to muscle involvement. Since the animals used in this study were between 25 and 40 days old it is certainly conceivable that a secondary myopathic lesion can result thus rendering the muscles affected refractory to reinnervation.

To get around this problem Cosmos et al (1973) used sexually immature mice. They looked at regeneration of minced anterior tibialis muscle from control and dystrophic animals. From their results, the defect appeared to reside in the muscle, as cross-transplantation of dystrophic to control and control to dystrophic animals did not result in a conversion in muscle properties to resemble that of the host.

In an elegant experiment Law et al. (1976) joined control and dystrophic mice in parabiotic union and cross-innervated the SOL of one animal with the tibial nerve of the other. Thus the ability of nerve, be it normal or dystrophic, to affect the properties of muscle was examined. In a normal or dystrophic SOL cross-innervated by a normal tibial nerve there was a decrease in time to peak tension (TTP) and half-relaxation (½ RT) of the twitch and the presence of post-tetanic potentiation, a phenomenon that is exhibited by fast, but not slow muscle. Also, dystrophic nerves were capable of transforming normal muscle in
the same manner as the control tibial nerve. Control nerves were not able to reverse the dystrophic features of affected muscle, and likewise dystrophic nerves did not induce dystrophy in normal muscle. They concluded that normal neurotrophic interactions do not reverse any existing pathology, and thus the defect is inherent in the muscle.

More evidence to this end has been provided by the results of tissue culture experiments using co-cultures of embryonic muscle with spinal cord (Hamburgh et al. 1975, Peacock and Wilson 1973), but opposite results have also been obtained (Gallup and Dubowitz 1973).

Although not conclusive, perhaps the strongest evidence for an extramuscular etiology in murine dystrophy comes from the experiments of Peterson (1974). He used chimeras that were derived from the aggregation of normal and dystrophic pre-implantation embryos. The resultant mosaic was found to have healthy muscles of dystrophic genotype, and diseased muscle of normal genotype.

The conflicting results obtained on the same experimental preparation are illustrative of the difficulty in elucidating this problem, even in a controlled environment such as an animal model.

Regardless of the etiology, there is no doubt that both muscle and nerve are affected in murine dystrophy.

The most obviously affected of the two is muscle. Reports

In terms of neural involvement the previously mentioned amyelination in spinal roots has been extensively documented, (Bradley and Jenkinson 1974, Stirling 1975), but its role in the pathogenesis of murine dystrophy is not at all clear. As there is very little amyelination in cervical roots, the fact that some forelimb muscles of dy2j mice are just as severely affected as the hindlimb muscles casts doubt on the contribution of amyelination to the pathology. Also, amyelination has been recently demonstrated in the nerves of non-dystrophic C57RL-ob/+ mice (Beuche and Friede 1984). What seems more plausible is that the spontaneous activity resulting from ephaptic transmission in these amyelinated areas (Rasminsky 1978), can lead to changes in
hindlimb muscle properties such as increased TTP that are activity related rather than a consequence of the dystrophy per se (Parry and Desypris 1983).

In discussing Hironaka and Miyata's (1975) experiment I mentioned that dystrophic to dystrophic cross-transplants exhibited functional denervation. From their work, both a defect in nerve and muscle may be implicated, as previously mentioned. Alternatively a muscle involvement may be dismissed on the grounds that reinnervation may have been incomplete due to a slower regrowth of the nerve in conjunction with the larger barriers to regrowth offered in the form of connective tissue.

The existence, however, of functional denervation in dystrophic muscle that has not been manipulated in any way, raises the possibility of dual involvement of muscle and nerve. I will elaborate further on this later on. I would like to now address the evidence for functional denervation in dystrophic mouse muscle.

Evidence of impaired NMT in dystrophic muscle that was judged to be in good condition based on a resting membrane potential of between -60 and -80 mV (McComas and Mossawy 1965), prompted McComas and Mrozek (1967) to investigate the possibility that this situation may deteriorate to the extreme. In their study they stimulated single anterior tibialis and gastrocnemius muscle fibers of dy/dy mice directly with an intracellular microelectrode. They then stimulated the sciatic nerve and
looked for an indirectly evoked action potential. Their results showed that on the average, approximately 27% of the muscle fibers in the dystrophic animals did not respond to indirect stimulation, and were thus deemed functionally denervated.

Law and Atwood (1972) reported similar findings in an in vitro study of dy/dy mouse SOL muscle. In an in vivo study Law et al. (1976) confirmed the previous in vitro results. They recorded intramuscularly both at the endplate region of the fiber, and tendon of the muscle. The rational for this was that if an abortive non-propagating spike was recorded at the endplate there should be no response at the tendon region. Using this method they were able to find fibers that were completely functionally denervated (18%), along with normally innervated fibers (46%), and ones where the synaptic connectivity was presumably failing (36%).

Harris and Montgomery (1975) found no evidence of functional denervation in anterior tibialis of dy2j mice between 2 and 3 months of age. This is not surprising in light of the findings of Curran and Parry (1975), who showed that miniature end plate potential (mepp) frequency was not reduced in dy2j EDL until more than 100 days of age. Thus, functional denervation may not be expected to occur until at least that time. Indeed, Parry (1977) showed that functional denervation appears first in the dy2j EDL at approximately 10-26 weeks of age. He inferred functional denervation by measuring the functional innervation ratio (FIR)
which is the ratio of tension elicited by nerve stimulation to that obtained by stimulating the muscle directly. Thus, by 10-26 weeks the FIR was significantly reduced from age matched controls, and continued to fall until it reached a value of approximately 0.74. This corresponds to approximately 26% of the fibers being denervated and although in a different muscle and strain of mouse, agrees quite well with the values of McComas and Mrozek (1967).

The question one may ask now is, does the decrease in functional innervation occur as a result of motorneuron death and ineffective collateralisation by the remaining motor neurons, or do muscle fibers become refractory to innervation and lose their ability to sustain synaptic connections thus leading to functionally denervated fibers.

If the first possibility is correct, then, if one were to sample numbers of motor units a decrease should be found between controls and dystrophics. On the other hand, if the muscle becomes refractory to innervation, no change in number of motor units should be seen, although a reduction in their absolute size would be expected.

Law and Caccia (1975) found a reduction in both numbers and absolute sizes of motor units in dy/dy SOL muscle. They used incremental stimulation of the sciatic nerve to estimate the number of motor units. Unfortunately the validity of this technique has been criticised on the following grounds. It is
possible that adjacent motor neurons supplying the muscle in question have nearly identical thresholds for activation. If this is the case, then the increment in tension or EMG seen by an incremental rise in stimulus strength may represent the activation of more than one motor neuron and thus lead to an underestimate of the number of motor units.

Lewis and Parry (1979) estimated the number of motor units supplying control SOL with the ventral root splitting technique. Using this technique the problems of co-activation of more than one axon supplying the muscle under investigation are obviated. The reason for this is that the criteria for isolation of a single unit are stringent and include all or none EMG and twitch responses along with the all or none antidromic action potential.

Interestingly enough their estimate of numbers of motor units in control SOL was identical to that of Law and Caccia (1975) suggesting that perhaps in the mouse this technique is valid as an estimator of the number of motor units.

The reduction in both numbers and sizes of motor units observed by Law and Caccia (1975) support both a neural and muscle involvement. They suggested a neurotrophic deficiency, and the observed decrease in orthograde axonal flow (see Animal Models pg. 3) would provide a means whereby this can occur.

In the dy2j mutant Parry et al. (1982) used horseradish peroxidase (HRP) retrograde labelling of motor neuron cell bodies to estimate the number of motor units supplying the SOL. They
found a reduction from 25 in controls to 20 in the dystrophics. As they did not measure the number of motor units by direct means it is difficult to say how many of the motor units were in fact functional. It is conceivable that 'loosely connected' motor neurons that are incapable of NMT are able to transport HRP, thus overestimating the actual number of healthy motor neurons.

In another study Bateson and Parry (1983) found no change in the number and sizes of motor units of dy2j EDL, but a reduction in the absolute sizes of the units. Their results are supportive of a primary muscular involvement.

Thus, evidence exists for both neural and muscular involvement, but at the moment it is not possible to definitively state a causality.

Another approach to answer this question is to challenge the two components concerned, namely motor nerve and muscle. As this forms the basis of my thesis topic, I will now present the problem I am addressing.
PROJECT OVERVIEW

The aim of this project is to study reinnervation of skeletal muscle in control (C57BL/6J) and dystrophic (C57BL/6J \(dy^{2J}/dy^{2J}\)) mice.

The results obtained on control mice can then be accepted as reflecting the normal physiological response, and any deviations in the dystrophic animals may aid in further elucidating the etiology of murine dystrophy.

As previously mentioned in the Introduction, there is evidence for both muscle and nerve involvement in dystrophy. Of particular relevance to this project is the work of Parry and Melenchuk (1981). They examined the ability of dystrophic nerves to reinnervate \(dy^{2J}\) mouse EDL and SOL following surgical denervation. To quantify extent of reinnervation they measured the reinnervation ratio (RIR) see Materials and Methods pg. 45. They found that in control animals denervated at either young (4-6 wks.) or older (4-6 mos.) ages, reinnervation was complete by 6 weeks post-denervation. In the dystrophic animals reinnervation was clearly impaired in the EDL and SOL of the older groups, while only the EDL showed impaired reinnervation at a young age. They concluded that the observed impairment in reinnervation was consistent with, but not proof of a neurogenic defect in murine dystrophy.

The alternate possibility is that muscle fibers may become affected and thus refractory to reinnervation. As this would
occur randomly, and because the increase in connective tissue barriers could act to impede the regrowth of nerves, a decrease in RIR would also be anticipated.

To differentiate between the two possibilities it would be necessary to know if all the motor neurons did in fact regrow and reinnervate target tissue. Clearly, this would first have to be ascertained in control animals in order to establish the norm. This will be done in 2 ways.

The first method derives from the fact that neurons are capable of transporting horseradish peroxidase (HRP) retrogradely. Thus, by injecting the muscle under investigation with HRP and allowing sufficient time for the label to reach the cell bodies of the motor neurons supplying that muscle, one can obtain an estimate of the total number of motor neurons supplying the muscle. However it is conceivable that if a motor neuron is not functionally linked to muscle fibers (i.e. 'sick' see McComas et al. 1971, 1974) it may nonetheless be still capable of transporting HRP, thus leading to an erroneous estimate of the number of functional motor neurons. For this reason an alternate method that yields functional motor neurons is also required.

This leads to the second method i.e.: ventral root splitting. Using this technique, the number of functional α motorneurons, or motor units, can be obtained. A full description of this method along with the HRP protocol is found in the Materials & Methods section.
There is evidence of type I fiber involvement in murine
dystrophy (see Introduction pg. 32). It would thus be of
considerable value to know something about the normal
reinnervating capabilities of type I vs type II motorneurons,
since specific fiber involvement may simply reflect primary
motorneuronal impairment.

The fiber type distribution will be compared in unoperated
and reinnervated control muscles to see if any evidence for
preferential reinnervation exists. Fiber types will be
determined by conventional myofibrillar ATPase staining, along
with immunohistochemical staining with monoclonal antibodies
directed against the heavy chain of the various myosin types.
Also the size distribution of reinnervates vs unoperated motor
units in control animals can also be of use in assessing
preferential reinnervation.

To conduct this study I have chosen the soleus (SOL) muscle
as the muscle under investigation. In light of the fact that
dyj EDL was shown to be more affected in terms of functional
denervation (Parry 1977), and inability to reinnervate (Parry and
Melenchuk 1981), than SOL, my choice of this muscle deserves some
justification.

The two main reasons for choosing the SOL were as follows:
1) As I will be comparing anatomical motor neuron counts
(HRP) to physiological motor unit estimates (ventral root
splitting) the following problem emerges when using EDL.
One of the procedures in HRP retrograde labelling is a complete denervation of all hindlimb muscles except the EDL. This involves severing a branch of the tibial nerve that supplies the anterior tibialis muscle which also happens to be in close proximity to the EDL. The cut end of the nerve can then pick up HRP that has leaked out of the EDL and overestimates of motor neuron counts would result. This problem is not encountered with the SOL.

2) As previously mentioned, type I fibers have been implicated as being involved in murine dystrophy of the dy2j variety. This raises the possibility of selective dropout of type I fibers or neurons. As the SOL has a large proportion of type I fibers (40-60%) and thus units, it serves as a better candidate than EDL which at best contains about 3% type I fibers.
MATERIALS AND METHODS

Animals

The strains of mice used in this study were the control, (C57BL/6J) and dystrophic (C57BL/6J dy2j/dy2j) mutant. They were originally obtained from Jackson Laboratories, Bar Harbor, Maine, and since then have been bred at our own facilities.

Denervations

In all cases, the right soleus (SOL) of control (C57) or dystrophic (dy2j) mice was surgically denervated at either 3 weeks or 6 months of age, and allowed to reinnervate for periods of up to a year.

The following is a description of the denervation protocol. Mice were anesthetised with chloral hydrate (0.5 ml/gm) administered intraperitoneally.

The SOL muscle was surgically exposed with care taken not to damage nerve and blood supply.

The SOL nerve was then located, and sectioned as close as possible to its point of insertion in the muscle. The free end of the nerve was then apposed on the belly of the SOL. No attempt was made to position the nerve in its exact previous location, but an effort was made to locate the cut end of the nerve in the equatorial region of the muscle. In fact, visual inspection of the point of insertion of the reinnervating nerve
following experimentation, and prior to histological processing confirmed its location in successful reinnervates, to the equatorial region.

The leg was then sutured, and the animal allowed to recover.

Tension Measurements

All tension measurements were done in situ and under isometric conditions. The following procedure was employed. Mice were anesthetised as previously described. The right or left SOL was surgically isolated, and a stainless steel wire was tied to the distal tendon for subsequent attachment to the force transducer. Silk (7-0) thread was looped around the proximal tendon in order to facilitate clamping of the tendon. The proximal tendon had to be clamped in order to ensure that adequate isometric conditions would be established. In the case of dystrophic animals, the sciatic nerve was sectioned in the upper thigh. The reason for this is as follows. In these animals the lumbar spinal roots have segments that are amyelinated. Rasinsky (1978) has shown that these segments are foci for the activation of adjacent amyelinated axons. The constant twitching that is seen in the hindlimbs of these animals is secondary to this radial spread of excitation among alpha motorneurons, and also along the gamma-Ia-alpha loop. On this basis it was necessary to denervate the hindlimb, otherwise the tension measurements would be obscured by movement artifacts.
originating from adjacent muscle groups.

The animal was then transferred to the isometric tension measuring apparatus. The prepared leg was positioned in the bath and clamped with a pair of Dumont #5 stainless steel forceps. The proximal tendon was then clamped as above and the leg was sealed in the bath with cotton wadding soaked in a 5% aqueous Agar solution. The bath was then filled with Ringer solution and bubbled with 95% oxygen/5% CO₂. Temperature was maintained at 37°C by a thermostatically controlled heating coil that was located at the base of the bath.

The distal tendon was then severed, and the stainless steel wire was attached to the transducer. The transducer comprised a stainless steel cantilever beam, to which four semiconductor strain gauges (Kulite SUDP-350-160, Kulite Semiconductor Products, Ridgefield NJ) were bonded. The gauges were arranged as a full bridge circuit and the output, after suitable amplification was displayed on a 5111 storage oscilloscope (Tektronix, Beaverton, OR), and immediately analysed. The compliance of the transducer was 1.2 μm/gm and the unloaded natural frequency was 1.25 KHz.

Tension was elicited by stimulating the nerve (indirect) or muscle (direct). For nerve stimulation, platinum wire electrodes were used while platinum plate electrodes were utilised for direct stimulation. In order to establish the appropriate voltage for stimulation, the following protocol was employed.
Threshold voltage ie: the voltage necessary to elicit a contraction, was established, and was increased until maximum twitch tension was obtained. That voltage was noted and then multiplied by 1.5 to yield the operating voltage.

Once operating voltage was set, the next step was to position the muscle at its optimal length (Lo). The transducer was attached to the arm of a micrometer, thus facilitating manipulation of muscle length. Length was increased in steps 0.2 mm, followed by stimulation at the new length. Lo was reached when maximum twitch tension was obtained. The following parameters were read directly off the storage oscilloscope and entered into the data log book.

a) twitch tension (Pt) elicited both by direct and indirect stimulation

b) tetanic tension (Po) elicited both by direct and indirect stimulation

c) time to peak tension of the twitch (TTP)

d) time to half-relaxation of the twitch (½ RT)

From these parameters the functional innervation (FIR) and reinnervation ratio were derived as follows:

\[
\text{FIR} = \frac{\text{Pt(ind.} \text{)}}{\text{Pt(dir.} \text{)}} \quad \text{or} \quad \frac{\text{Po(ind.} \text{)}}{\text{Po(dir.} \text{)}}
\]

\[
\text{RIR} = \frac{\text{IR rein. side}}{\text{IR unoperated side}}
\]

If at any time during the experiment the value of Po fell to less than 90% of the initial Po, the experiment was discontinued.
At the end of the experiment the muscles were removed, trimmed free of fat and tendon, gently blotted, and weighed on a torsion balance to the nearest 0.1 mg. They were then processed for histological analysis.

**Histological Processing of Muscle**

Both solei of experimental animals were removed and mounted on a chuck, along with the anterior tibialis muscle (AT) which acted as a mechanical support. They were positioned alongside the AT as illustrated in the cross-sectional sketch below.

![Cross-sectional sketch](image)

Once mounted, the muscles were covered with embedding medium (OCT compound) immersed for 15 seconds in isopentane that was cooled in liquid nitrogen. They were then either stored at -70°C or processed immediately. Serial sections (10 μ thickness) were taken at the mid-belly region. The temperature of the cryostat
was set at -20°C. Sections were then processed for Hematoxylin and Eosin, myofibrillar ATPase at both acid (pH 4.5) and alkaline (pH 10.4) pre-incubations (Brooke and Kaiser 1970), and monoclonal antibody staining for myosin types (Appendix I). The primary antibodies used were monoclonal and directed against type I, IIa, and IIb myosins. They were generously provided by Dr. S. Schiaffino, Padua, Italy.

Quantification of Relative Cross-Sectional Area (CSA) Occupied by Type I Muscle Fibers

To determine the relative CSA occupied by type I fibers, black and white photographs of whole SOL were taken from sections that were stained for myofibrillar ATPase. Only muscle sections in which no freezing artifacts or tearing was present qualified for this analysis.

The total CSA of muscle was computed with the aid of a Zeiss MOP-3 (Morphological Optical Processor) image analyser. The CSA occupied by type I fibers was then similarly determined and expressed as a proportion of the total muscle CSA.

Horseradish Peroxidase (HRP) Retrograde Labelling of Soleus Motor Nucleus

Animals were anesthetised and the right and left solei were isolated as previously described. Bilateral denervation of all muscles adjacent to SOL was then done in order to minimise the
The chances of uptake of HRP by other motor neuronal pools.

The following nerves were sectioned as far as possible from the SOL: lateral popliteal, nerves to hamstrings, medial and lateral gastrocnemius, posterior tibial, and nerve to plantaris.

HRP (Sigma type VI) was dissolved in 0.9% saline with 1% Dimethyl Sulfoxide to make a 10% solution. A capillary tube was pulled on an electrode puller to make an electrode with a tip whose shank was about 1 cm. long and tip inside diameter between 1? and 14 μm. The electrode was sealed to the needle of a Hamilton microsyringe with wax. The electrode-syringe assembly was attached to the arm of a micromanipulator. HRP was drawn into the electrode and 5 - 10 μl were injected into each SOL. The injection was done along the long axis of the muscle, and over a period of 10 - 15 minutes. Following the injection, the electrode was withdrawn and the muscle gently blotted. The leg was sewn up with silk (5-0) sutures and the wound was protected from sepsis by dabbing it with cotton soaked in Cupersol. The animal was then left to recover.

20 - 24 hours later the animal was anesthetised, and the chest area was exposed. An incision was made in the right atrium and left ventricle. A 30 ml. glass syringe was filled with 0.9% saline and the animal was systemically perfused via the left ventricle with approximately 50 ml. This was followed by perfusion with 100 ml. of 5% glutaraldehyde in a 100mM Phosphate buffer at pH 7.4, and then 100 ml. of 10% sucrose in the same buffer at the same pH.
The skin from the back was then removed, and the spinal column was cleared of muscle. The spine was cut diagonally at the thoracic level. A diagonal cut was used in order to allow easy identification of the side concerned. The vertebrae were removed and the cord was taken out. The roots were peeled off, and the dura removed with the help of a pair of small dissecting scissors. A block of cord, with the lumbar enlargement included, was cut and put in 10% sucrose buffer to equilibrate for an hour.

The cord was then embedded in OCT compound at -15°C on the freezing stage of an IEC Minotome Cryostat. Longitudinal sections of 40 μm thickness were then taken. Individual sections were then floated in the compartments of an ice rack that was filled with the phosphate buffer. The sections were then transferred with a fine haired brush, to gelatine subbed slides that were wetted with buffer solution in order to aid in mechanical manipulation of the sections with minimal trauma. The mounted sections were left to dry at room temperature for approximately 45 minutes. They were then stained for HRP by the method of Mesulam (1978). Cell bodies were visualised under phase contrast microscopy and only ones with an identifiable nucleolus were counted.

Determination of Number of Motor Units with Ventral Root Splitting Technique

The right SOL of experimental, and control unoperated C57 or dy2j mice was isolated with care taken to minimise damage to both
nerve and blood supply. A longitudinal incision in the skin of the back was made from the neck region, down to the sacrum. The skin was pulled aside to expose the spine, rib cage, and sacrum. The sacrum was cleared of muscle in order to allow for its attachment to the spinal clamp device that was used to hold the animal in place. The spine was then cleared of muscle.

All the muscles of the hindlimb except SOL and some of the upper thigh muscles were then denervated. A loop of silk suture was placed around the proximal tendon of the SOL for the reasons previously mentioned. The distal tendon was isolated and a stainless steel wire with looped ends was tied to it. To check whether the denervation procedure was successful the sciatic nerve was stimulated in the upper thigh region and the muscles were observed under 20X magnification. Only the SOL should contract.

Once full denervation was achieved, the next step was to perform a laminectomy. Vertebrae were removed from L1 - L5 with gentle blotting of the spine where bleeding occurred. The animal was then transferred to the tension measuring apparatus where it was attached to a spinal column clamp as follows. The sacrum and mid-portion of the spine was clamped by opposing metal pins. The skin of the back was tied to the bent portion of the metal pins so as to form a pool that could be filled with the appropriate solution. The leg was clamped at the ankle and prepared in the same manner as for isometric tension measurements. The bath was
sealed with cotton soaked in agar solution. Ringer solution was poured into the back pool, and the dura was removed with the aid of fine forceps and dissecting scissors. The Ringers was then aspirated by vacuum suction, and replaced with paraffin oil. Paraffin was used in order to minimise current spread during stimulation of the ventral rootlets.

The dorsal roots on the right side were then exposed, cut, and reflected to the side. Ventral roots L3 and L4 were cut and mounted on a pair of platinum wire stimulating electrodes (central electrode). Another stimulating electrode was placed on the SOL nerve (distal electrode). Optimal muscle length was established by proximal nerve stimulation, and the IR was then determined.

Ventral roots L3 and L4 were then stimulated supramaximally, and the tension elicited was compared to that obtained by distal stimulation. Ideally, the two should be the same, but as the procedure is extremely delicate there is some chance of damage to the roots. This is particularly true in the case of dystrophic animals where large segments of root are amyelinated and consequently extremely fragile. Thus, occasionally the tension elicited proximally was less than that elicited distally. As this would involve random nerve damage and motor unit dropout, it should not bias the estimates of motor unit size.

The ventral roots were then placed on a stainless steel dissecting stage that was immersed in the paraffin pool, and then
split with the aid of very fine forceps. A split root was then mounted distally and stimulated supramaximally. The voltage was then lowered until threshold voltage was reached. Threshold voltage was taken as that voltage at which an all or none twitch is elicited 50% of the time. Ten pulses were given. If the tension elicited at threshold voltage was less than that at supramaximal voltage, the root was split further. This procedure was repeated until the two tensions were equal. The same protocol was done for the tetanus. If the all or none criteria were met, a single unit was considered to be isolated.

This was occasionally verified by another test, namely the all or none EMG elicited via proximal stimulation.

In the case of dystrophic animals, the conventional ventral root splitting technique proved to be inadequate for determining the number of motor units. It was found that whole muscle tension could be elicited by stimulation of a large number of individual rootlets. This was probably due to ephaptic activation in areas of amyelination that were distal to the site of the split. A likely consequence of this is that an individual unit could be isolated from several split rootlets. To alleviate any subsequent problems in interpretation of the data, instead of splitting the ventral roots, the sciatic was split in the upper thigh region. The protocol was essentially the same as ventral root splitting with the exception of the laminectomy and associated procedures.
Motor unit size was expressed as a percentage of the total tension elicited by stimulation of SOL nerve.

\[ \text{i.e. } \text{Size} = \frac{\text{Unit Po}}{\text{SOL Po}} \times 100 \]

The number of motor units was estimated by taking the average size and dividing it by the average SOL Po.

Thus \[ \# \text{ of Motor Units} = \frac{\text{Po SOL}}{\text{Po Unit}} \]

where Po is average tetanic-tension

Po SOL was checked after isolation of each unit, and if it dropped to less than 90% of initial Po SOL the experiment was terminated, and the most recently isolated unit was discarded.

In some instances incremental stimulation was used to estimate the number of motor units. The first and last 4 units obtained in the twitch were averaged, and divided into the twitch tension to yield the number of units.

\[ \text{i.e. } \# \text{ motor units} = \frac{\text{Pt}}{\text{P unit}} \]

As in the FIR determination, at the end of the experiment both solei were extirpated, cleaned of fat and tendon, and weighed on a torsion balance. The anterior tibialis was also removed and the muscles were prepared for histological examination in the fashion previously described.

Composition of Ringers Solution

NaCl: 7.071 g/L  NaHCO₃: 2.100 g/L
KCl: 0.354 g/L  KH₂PO₄: 0.068 g/L
CaCl₂: 0.1666 g/L MgCl₂·6H₂O: 0.047 g/L
Glucose: 2.000 g/L

In one experiment, 2 mg% d-tubocurare was added to the Ringer. The rational for this is as follows.

There was some concern that direct stimulation of the muscle may not depolarise all the muscle fibers directly. It is possible that the voltage used for direct stimulation is sufficiently large to depolarise the nerve terminals and not all the muscle fibers.

This was tested by first determining Po max by indirect and direct stimulation in normal Ringers solution. The normal Ringers was then replaced with Ringers that contained 2 mg% d-tubocurare to block neuromuscular transmission. Po max was elicited indirectly at 1/30 sec until it dropped to zero. The muscle was then stimulated directly. It was found that Po(ind)R = Po(dir)R = Po(dir)C, where R and C denote normal Ringers and curarised Ringers respectively.

On the basis of these results it was concluded that the voltage used for direct stimulation is adequate to activate all the muscle fibers directly.

Statistics

All values represent the means ± standard deviation (SD).

To ascertain the level of significance, the Students T-test was employed in the following fashion. All group to group comparisons were made using the unpaired T-test, while within group comparisons were analysed by using paired T-test.
RESULTS

General Morphology and Fiber Types

Table II compares the wet weights of reinnervated C57 and dy2j solei to that of the contralateral unoperated muscle. Although all reinnervated muscles recovered to statistically significantly less values than unoperated muscles, in the case of the older C57 group the difference was even more pronounced.

Also, there was no difference in the wet weights of young C57 compared to young dy2j SOL, and while the C57 SOL grew with age (7.58 mg vs 10.58 mg), the wet weight of dy2j SOL did not change significantly with age (6.72 mg vs 4.98 mg), and in fact a tendency to atrophy is noted. This was also borne out by visual inspection of cross-sections stained for H&E. Fig. 1 shows cross-sections of one month old C57, dy2j, and 6 month old dy2j SOL stained for H&E. No difference in morphology had been noted between young and old C57’s. Note the presence of centrally located nuclei, connective tissue infiltration, and atrophic fibers in dystrophic muscles. The older dy2j SOL is clearly more severely affected in this respect than the young one.

Reinnervated muscles are morphologically similar to their unoperated counterparts, however the fiber type distribution is altered in reinnervated C57 SOL (Fig. 2). Whereas the proportion of type I:type IIa fibers in C57 SOL is roughly 40:60, in reinnervated SOL this is increased to 75:25 (Table III).
fact, some of the "new" type I fibers also contain type IIa myosin (Fig. 3) and are thus presumably in the process of reprogramming. Co-existence of myosins was only rarely seen in C57 unoperated SOL.

The decrease in proportion of fibers staining dark for anti-type II antibodies as reinnervation proceeds (76% at one month vs 28% at 2 months), is evidence for the conversion of fibers from type IIa to type I (Fig. 3).

Also of interest is the fact that all fibers that stained intermediate for acid ATPase (Fig. 3b) were darkly staining for the anti-type II stain. In addition, not all dark staining fibers in the acid ATPase were pure type I as co-existence was shown in 9 of such fibers. Because of this inconsistency a note of caution should be heeded when making statements on fiber type composition based on ATPase staining alone. A more accurate description of the actual myosin type 's' contained in any given fiber can be obtained with the use of immunohistochemical techniques, and these should be given preference.

In contrast to the paucity of myosin co-existence in unoperated C57 SOL a large proportion of the fibers of dy2j SOL contain more than one myosin (Fig. 4). Furthermore, the existence of type IIb myosin in dy2j SOL was consistently noted. This is odd since C57 SOL rarely contains any type IIb myosin, and if so only a few fibers would contain it, perhaps the equivalent of one motor unit. What is also quite striking is the
vast amount of type IIa myosin, and the scanty amounts of type I myosin in the SOL of older dy2j animals. There were actually very few pure type I fibers in old dy2j SOL (Fig. 4a). Also, some fibers contained all 3 myosin types. In the case of reinnervates co-existence of myosins was extensive. All fibers contain some type II myosin and some fibers contained all 3 types (Fig. 4b).

**Contractile Characteristics**

Absolute twitch and tetanic tension of reinnervated SOL was significantly reduced in all groups with the exception of the old dy2j group where only tetanic tension was reduced. When normalised to the wet weight of the muscle, only the young C57 reinnervated SOL showed a significant reduction in twitch tension, while the old dy2j group was the only one to show a reduced normalised tetanic tension.

The values of twitch:tetanus ratio are shown in Table V. No difference is seen between reinnervated and unoperated SOL with the exception of the old dy2j group, where significantly higher values were noted when compared to both old unoperated and young dy2j reinnervates.

The twitch temporal characteristics of time to peak tension (TTP) and time to half-relaxation (IRT) are shown in Table VI. Note the increased TTP of reinnervated young and old C57 SOL versus unoperated muscles. This correlates well with the previously mentioned increase in proportion of type I fibers and
slow myosin. TTP was also increased in old reinnervated dy2j muscles as was the ½RT. Unfortunately ½RT's were not taken for the young C57 reinnervates, thus those data are not available. The old animals show an increase in this parameter.

Reinnervation Parameters

The functional innervation ratio (FIR) of all solei with the exception of the old dy2j reinnervated group was close to unity (Table VII). In the old dy2j group the FIR of the reinnervated SOL ranged from 0 to 1.00 with a mean of 0.58 ± 0.38. This is also borne out in the values of RIR where only the old dy2j group shows an impairment.

Upon closer inspection of the raw data it became apparent that the impairment in reinnervation was sex dependent. The male group reinnervated well (RIR= 0.89 ± 0.13(5)) while the females were clearly impaired in this parameter (RIR= 0.28 ± 0.27 (5)).

HRP Retrograde Labelling of Soleus Motor Nucleus

No difference in the number of motoneurons is seen between unoperated and reinnervated C57's thus indicating that all motoneurons do in fact regrow and capture territory (Table VIII). In the case of dystrophics a slight but significant reduction is noted in the case of reinnervates. The broad range indicates that this is somewhat variable. In fact, 2 of the 5 animals showed reduced numbers.

There was no difference in the numbers of motoneurons of unoperated dy2j's and control animals.
Motor Units

C57's

Out of seven old control C57 animals, 28 units were obtained. The motor unit sizes ranged from 2.2% to 8.6% of total tetanic tension (Po SOL) with a mean value of 4.71% thus yielding a total of 21 units. No relationship was seen between TTP and motor unit size (Fig. 5).

Seven old C57 reinnervates were examined. A total of 44 units were obtained. The range of sizes was 0.4% to 12.3% of Po SOL with a mean value of 4.98% thus yielding a total of 20 units. Although the mean unit sizes are not significantly different from reinnervate to control, the actual range of sizes is broader in the case of reinnervates (Fig. 6). There also is a relationship between TTP and size in reinnervated C57 SOL, that is only apparent in the region outside the normal range of sizes seen in unoperated muscle (Fig. 5). Out of the 44 reinnervated units, 16 had sizes ≤ 2.7% Po SOL, and out of these, 13 had TTP ≤ 12 msec. All the largest units i.e: those with Po > 8.6% were slow contracting (TTP ≥ 15 msec).

dy2j's

Out of 5 old dy2j mice, 11 units were obtained by sciatic split. Their average size was 4.83% Po SOL (Range: 2.0% - 11.5% Po SOL) yielding a total of 20 units. Unfortunately the sciatic split technique only yielded 3 units at best per animal thus making it impossible to estimate reliably the number of motor
units in each individual animal. Incremental stimulation of the sciatic was thus employed and the value obtained (18 ± 2) although slightly less, is still close enough to the value obtained by sciatic split to make it reliable.

Incidentally, in 3 animals the number of motor units was estimated by ventral root splitting. The problem associated with this technique was mentioned previously in the Methods Section pg. 54, however, what is of interest is that if all the units isolated (10 units) were used to determine the number of motor units, a value of 23 was obtained. If the units that were apparently activated more than once (3 units) are excluded, a value of 19 is obtained. This is close enough to 20 (sciatic split) and 18 (incremental stimulation) to lend some credence to these techniques as being fairly accurate indicators of the number of motor units.

In the one month post denervation group, the number of motor units was estimated by incremental stimulation of the sciatic nerve. For the whole group a value of 17 ± 1 (9) was obtained for the unoperated SOL, while the reinnervated SOL had 13 ± 3 (12) units. A sex difference was however noted. In the males, no difference was seen between unoperated (U) and reinnervated (R) muscles (18 ± 1 (4) U vs 16 ± 2 (6) R) while in the females fewer units were obtained in the reinnervated SOL (17 ± 2 (5) U vs 11 ± 2 (6) R) p < .001.

Some older reinnervates (3½ months post denervation) were
also examined. Out of 4 animals, 7 units were obtained by sciatic split. The sizes ranged from 3.6% - 21.3% of Po SOL. The average size was 7.38% Po SOL which translates into roughly 14 units.
Sex-Difference in Reinnervation of Old dy^2j^ SOL

As mentioned earlier on in the Results, a significant difference was seen in both FIR and RIR of the older dystrophic group when compared to younger dystrophics or older C57's. Even though the difference is significant (p < .05), the rather large values of standard deviation make it clear that a certain amount of variability does exist nonetheless. When the data were analysed with respect to the sex of the animal, an impairment in reinnervating ability was only apparent in the female mice (Table IX). Also, castration of male mice prior to denervation led to an impairment in their reinnervating ability that was quantitatively similar to that observed in the female group (Table IX; see also Fig. 7).

Other parameters that are normally affected in denervated muscle are TTP, JRT, and the twitch:tetanus ratio (Lewis 1972, Finol & Lewis 1975). It is thus not unreasonable to expect these parameters to be affected in muscles where a significant proportion of the fibers are functionally denervated. In non-pathological states, such data would lend to easier interpretation. In the dystrophic state, the aforementioned variables with the exception of twitch:tetanus ratio, are already altered by the disease process, this making interpretation of results hazardous at best. However, the fact that the increase in TTP, and JRT of the reinnervated female, and male castrated group was higher than in the male group leads me to suggest that the additional
increase is a reflection of the properties of muscle fibers that are functionally denervated.

This point is further emphasized when one looks at the values of twitch:tetanus ratio. In the male group, where reinnervation was complete, there is no significant difference between unoperated and reinnervated muscles. Reinnervated SOL of both females, and male castrates, had higher values of twitch: tetanus ratio than the unoperated muscles (Table X).

Lewis (1972) also noted that in some instances the twitch of denervated muscle was atypical in that after-contractions would occur during the relaxation phase. I also noted this, and only in animals where the FIR was severely reduced. A representative whole muscle twitch obtained by direct stimulation of dy2j reinnervated SOL is shown below. In this case the animal was female. Note the aftercontraction (arrow).

Since the impairment in reinnervation was only apparent in
dy2j females and castrated males, it would appear that a gonadal factor may play a role in reinnervation. If this is true, then it is somewhat curious that neither the C57 nor the young dy2j group showed any sex-difference in reinnervation.

A possible explanation for this is that through the progression of dystrophy in the dy2j mouse, motorneurons become affected in such a way so as to render them less able to resist a massive assault such as denervation. Ultimately, their capacity to regenerate would be diminished, but if a factor exists whose action is trophic in that it stimulates growth, then this deficiency would be masked, and reinnervation would appear unaffected.

As the data presented here points to a factor of gonadal origin as being involved, this subject is currently under investigation with particular emphasis on testosterone as the putative factor.
DISCUSSION

I will divide this section into two parts. In the first I will discuss the results pertaining to the slowing aspect of C57 reinnervated SOL. In the second part the question as to the etiology of murine dystrophy will be addressed with respect to the data obtained in this study.

(i) **Slowing of Reinnervated C57 Soleus**

The slowing referred to was manifest in the following ways: an increase in the TTP phase of the twitch, along with a parallel increase in the proportion of muscle fibers that contain type I myosin, or that stained type I positive in the myofibrillar ATPase stain (Table VI, Fig. 2, Table III, Fig. 3).

This slowing can be interpreted as resulting from either of two possibilities.

1) During reinnervation not all fast motorneurons were able to reinnervate target tissue. Their subsequent death is reflected by a decrease in the proportion of fast fibers in reinnervated muscles.

   OR

2) All motorneurons grow back and reinnervate target tissue, however the type I neurons are more adept at collateralising and consequently they leave less
available territory for the type II neurons.

Possibility #1 can be ruled out immediately as no difference was found between the number of motorneurons innervating reinnervated SOL versus the unoperated muscle (21.4 ± 1.1 control vs 21.6 ± 0.8 reinnervate). This was also borne out in the motor unit experiments where the numbers obtained correlate well with the HRP data (21 control vs 20 reinnervate).

Although other results show reductions in both motor units (Bagust and Lewis 1974, Gordon and Stein 1980) and motor neurons (Bearcroft et al. 1983), unlike in this study where denervation is performed at the point of entry of motor nerve into muscle, in those preparations, denervation was performed at a remote site from the point of entry of the nerve supply into the muscle. The possibility then arises that if type I and IIa motorneurons have differential rates of regrowth, with I > IIa then it would not be at all surprising to see a reduction in the number of reinnervating motor neurons, along with a slowing of contractile parameters. Bearcroft et al. (1983) also noted that the reinnervated rat soleus was almost entirely composed of type I fibers, whereas the unoperated muscle had roughly 20% of type IIa fibers.

On the basis of the lack of change in number of reinnervating motor neurons it is safe to say that, at least the first part of possibility #2, namely that all motor neurons grow back and reinnervate target tissue, is accurate.

The second part of possibility #2 is supported by the
following observations:

1) the slowing of TTP

2) the increase in proportion of type I fibers

3) immunohistochemical evidence for conversion of type IIa to type I fibers

4) the largest reinnervated motor units were all slow contracting

5) most (13 out of 16) of the smallest reinnervated units were fast contracting

On the aspect of slowing of TTP, Biscoe and Taylor (1967) derived a mathematical model that could predict the twitch temporal characteristics of a hypothetical muscle, given various proportions of fiber types. Using their model, an increase from 40% type I to 75% type I would result in a 26% increase in the TTP. Table VI shows that in the young C57 group TTP increased to 16.50 ± 1.05 msec in reinnervates compared to 13.67 ± 0.52 msec in unoperated muscles, an increase of approximately 22%. This value is in close agreement with the predicted value of Biscoe and Taylor, and the fact that it is not exactly 26% may be attributable to the co-existence of type II myosin in many of the fibers that contain slow myosin (Fig. 3).

On the co-existence of myosins an interesting point emerges when one compares 2 month to one month reinnervates.

At both one and two months following denervation all anti-type I cross reacting fibers stain uniformly. This is not the
case if the muscle is stained for type II myosins. A differential profile is seen. This is unlike the uniform pattern that is typical of unoperated SOL. Whereas at one month post denervation 19 of 25 fibers (76%) that show co-existence are dark, by 2 months only 12 of 42 (28%) are dark. The rest are either intermediate, or light in staining intensity (see Fig. 3). As the proportions of intermediate and light staining fibers to dark ones increases with duration of reinnervation it would appear that type IIa fibers are being converted to type I fibers. The persistence of type II dark fibers even at 2 months post denervation suggests that some fibers may be polyneuronally innervated. The reason for this is as follows.

The reinnervates are fully innervated by one month post denervation as I.R. = 1.0. This fact makes it difficult to explain the existence of fibers staining dark for both type I and II myosin by 2 months post denervation unless their myosin expression is influenced by at least 2 motorneurons of different type. Salmons & Sreter (1976) have shown that it takes approximately 4 weeks for re-specification of the contractile apparatus to occur following reinnervation by a foreign nerve. Based on this observation, by 2 months all fibers with co-existing myosins should stain paler for one of them. As this is not the case for some of the fibers it is likely that polyneuronal innervation persists for at least 2 months post denervation. Other investigators have shown persistence of
polyneuronal innervation following reinnervation for periods of up to 72 days (Frank et al. 1974, Brown and Irton 1978).

Thus far, the evidence presented is consistent with the notion that type I motorneurons are more adept at reinnervating. The motor unit studies add further credence to this idea. While the average reinnervated motor unit size was not different from unoperated muscles, the range exceeded both bounds. This has also previously been reported by others (Bagust and Lewis 1974, Gordon and Stein 1980). Furthermore, and more importantly with respect to preferential reinnervation is the fact that all the largest units (outside unoperated range) were slowly contracting, while most of the smallest units were fast contracting. It would have been ideal if I had been able to isolate the units by glycogen depletion. Unfortunately I was not able to perfect this technique in the mouse SOL. It would have been useful to correlate the unit size with myosin types. Nonetheless the data is certainly consistent with the notion that type I motorneurons can expand their reinnervating territory more successfully than type II motorneurons.

To conclude, the following points can be stated with a fair amount of certainty.

1) All reinnervating motorneurons form successful contacts, in some cases with more than one muscle fiber.

2) All muscle fibers become reinnervated.
3) Type I motorneurons appear to be more successful than type IIa motorneurons at collateralising and thus expanding their field of innervation.

(ii) Reinnervation of C57 and dy^2J SOL and the Etiology of Murine Dystrophy

It is not possible to state with certainty what the etiology of murine dystrophy is, from the results obtained. The bulk of the evidence however is supportive of both neural and muscular involvement.

There are basically 2 observations that are supportive of a "sick motorneuron" concept. The first concerns the reduced number of reinnervating motor units in old dy^2J mice. Before dealing with that aspect, I would like to first address the other piece of evidence in favor of altered motorneuron function, along with evidence for an intrinsic myogenic defect.

It was shown in Part (i) of the Discussion that the most likely explanation for the slowing of C57 reinnervated SOL was a more robust reinnervation by type I motorneurons. If this is indeed the case, then one would expect the properties of young reinnervated dy^2J SOL to shift in a parallel fashion, given of course that this response is an intrinsic property of type I motorneurons. This in fact did not occur. The twitch temporal characteristics of reinnervated dy^2J SOL did not differ from those of the unoperated muscle, and also no increase in type I myosin was noted. In addition, most of the fibers of
reinnervated $dy^{2J}$ SOL contained type II myosin thus indicating a more robust reinnervation by type IIa motor neurons. It is possible though that dystrophic SOL contains relatively fewer type I motor units than C57 SOL. If this were true then the results would not necessarily indicate motorneuron impairment. There are however 2 lines of evidence that would indicate that there likely is no difference in the proportions of fast to slow motorneurons in C57 SOL vs $dy^{2J}$ SOL. Firstly, the fiber type distribution of 3 week old (time of denervation of the young group) $dy^{2J}$ mouse SOL is virtually identical to that of age matched C57 mice (Wirtz et al., 1983 a,b, personal observations). Parry & Parslow (1981) found an increase in the amount of atypical (IIc) fibers in 3 wk $dy^{2J}$ SOL. They used ATPase histochemistry to identify the fibers. In a similar study Ovalle et al. (1983) found no difference in the number or proportion of atypical fibers in the soleus of 3 wk old $dy^{2J}$ mice. The discrepancy in these studies may be a reflection of the unreliability of ATPase staining as a sole criteria for fiber type identification. Wirtz (1983) combined oxidative, glycolytic and ATPase staining to arrive at 24 fiber classes. As previously mentioned no difference in fiber type proportions was seen between 3 wk C57 and $dy^{2J}$ mouse SOL in his study. So it is fairly safe to say that the proportion of fiber types is not significantly different between 3 wk C57 & $dy^{2J}$ SOL. Also in this study, no difference was found in the total number of
motorneurons to SOL between dy²J and C57 mice. It is thus reasonable to assume that the proportion of fast to slow motorneurons should be the same in C57 and dy²J mice. If then the robust reinnervation displayed by type I motorneurons is truly a normal physiological response, then its absence in dy²J SOL is consistent with a neural abnormality localised to type I motorneurons. Ovalle et al. 1983, showed that with age, the proportion of slow oxidative fibers in dy²J SOL decreased. As the absolute numbers of fast fibers did not change significantly up to 32 weeks of age, their observations are supportive of type I fiber involvement.

In context to the present discussion these observations can also be incorporated into a model where motorneurons are affected and lose their ability to maintain complete synaptic relations with their muscle fiber complement. This could be manifest in a number of ways, one of which may be the loss of a protein whose expression is dependent on neural influences. Buller, Eccles & Eccles (1960) showed that the development of slow muscle in the cat was largely dependent on neural influence. If motorneurons are already compromised in their ability to specify slow myosin synthesis, then it would not be surprising if these same motorneurons are less able to resist a stress such as denervation. The result would be a less robust reinnervation with the possibility of motorneuronal death, and very little change if any in fiber type composition.
Indeed, the number of motorneurons in reinnervated dy²J SOL was reduced relative to the unoperated side. Although, it is not possible to positively identify the affected motorneurons, a reduced number would be expected in the case of a neuropathy, and in context to the results presented thus far, it is not unreasonable to speculate that slow motorneurons may be involved. This indeed may be verifiable. Alpha motorneurons can be marked with HRP retrograde labelling. Then, if the demonstrated differences in oxidative enzymes (Sickles & Oblack 1984) or AChE isoforms (Gruber & Zenker 1978) between different alpha motorneuron types can be co-demonstrated by histochemical means, a method whereby the identity of affected motorneurons may be identified becomes tenable. Obviously, the viability of this approach is severely reduced if the measured parameters are themselves variably affected by the disease process.

It comes as somewhat of a surprise that the twitch temporal parameter of \( \delta RT \) is unaltered in the young dy²J group. \( \delta RT \) is a rough index of the rate of removal of Ca\(^{++}\) from the sarcoplasm. From denervation (Sreter 1970, Palekas et al. 1981), and foreign nerve reinnervation experiments (Margreth et al. 1973) on rat SOL, it has been demonstrated that the functioning of the sarcoplasmic reticulum (SR) is largely dependent on neural influences. It has also been shown that SR isolated from dystrophic muscles, has altered Ca\(^{++}\) pump activities (Sreter et al. 1967, Mrak & Baskin 1978). This could explain the increased
RT observed in dystrophic muscles by others (Harris & Montgomery 1975, Parslow & Parry 1981, Parry & Desyris 1983).

On the other hand, more recently Mrak & Fleischer (1982) used a gentler technique in isolating the SR from 2 month old 129B6F1/Jdy2J mice and found no difference in specific Ca++ pump activity. This may be the basis for the lack of increase in 1RT of dy2J mice that was obtained in this study. What is of note with respect to SR function is that the old dystrophic unoperated female group had significantly increased values of 1RT when compared to the old control unoperated SOL. This may be partly a manifestation of the intrinsic properties of denervated muscle fibers that exist in these muscles as their fIR's were lower. In addition it is possible that female dy2J motorneurons are less able to trophically sustain normal SR function.

Along with this evidence for neural involvement, there is also evidence for an intrinsic defect in tension generating capacity.

It was found that although the number of motorneurons, or units is not different from controls to dystrophics, the mean absolute motor unit size was decreased, as tetanic tension was significantly reduced. This may be interpreted as either an impairment in the ability of motorneurons to sustain larger numbers of fibers, or as an intrinsic weakness in the fibers themselves. It is difficult to reconcile this observation in terms of a neuronal impairment as if this were the case, then one
would expect to see functional denervation, at some later stage of the disease. This is not the case even in the older dystrophics. What seems more likely is that a defect may exist in the interaction of contractile proteins. There are reports of decreased amounts of troponin C and I in patients with Duchenne dystrophy (Ebashi & Sugita 1979) however it would be somewhat premature at this point to say that this is the case in the murine model of dystrophy. In this study it was observed that normalised twitch and tetanic tensions of the young dy²Ｊ group were significantly reduced compared to C57's. It is rather odd that this difference is not apparent in the older group. In this regard, Bressler et al. (1983), noted qualitatively similar findings in young and old dy²Ｊ mice vs C57's.

Thus, in the young group, the data would seem to suggest that a neural abnormality exists along with what appears to be an intrinsic myogenic defect. In the older group the neural defect is more pronounced, and as will be shown, the myogenic defect has also progressed.

The neural defect was clearly exposed in the older dy²Ｊ group. The impairment in reinnervation was manifest as a reduction in RIR and a tendency to lower numbers of motor units. As previously mentioned, a sex difference was noted, with male mice reinnervating well, both in terms of RIR and number of motor units. The female mice, on the other hand showed a clear impairment in reinnervation, as the RIR was significantly reduced.
along with the number of motor units.

The sex difference in reinnervation brings out some interesting points that support a neural involvement, but before considering that aspect it is also instructive to compare the old C57 group with the old dy²J group. What is interesting with respect to the twitch TTP and ½RT is that there is no difference in either of these parameters between C57's and the whole group or the males in the dy²J group. Females, on the other hand have significantly increased TTP and ½RT. As these muscles have identical FIR's it is possible that the change in these parameters is a result of the dystrophic process. The fact that there are not increased amounts of slow myosin in the female dystrophics suggests that the increased TTP may be the consequence of a longer duration of the active state which in itself could be brought about by a decrease in the efficacy of the S.R.

Why doesn't the male dystrophic express the same alterations? Perhaps a protective factor exists in these animals. Indeed, the degree of slowing is greater in reinnervated female and male castrated mice, than in the male group (Table X).

The most suggestive evidence that a protective factor of gonadal origin is involved in preventing full expression in males is seen in the reinnervated male castrated group. In these animals the TTP and ½RT of reinnervated SOL were not significantly different from the female reinnervated muscles.
Thus, any protective advantage that they normally would have had is lost upon removal of the gonads. Also the TTP and 4RT of the unoperated SOL of male castrated group was not significantly different from the dy2J unoperated male group thus indicating that 5 weeks of castration is not sufficient for the expression of the higher values of these parameters as seen in the female group. In terms of the identity of the putative factor, testosterone is one possibility. It's known anabolic actions may act to mask any existing impairment in motorneuronal function. Also, Breedlove & Arnold (1983) have recently shown that, motorneurons of the rat lumbar region show a sex difference in the accumulation of testosterone. Most male motorneurons readily accumulate the steroid, while very few female motorneurons were found to accumulate testosterone. It may be that in fact female motorneurons are not even capable of responding to the steroid. Thus "replacement therapy" in female mice would be expected to have little if any effect, while in male castrates, amelioration of the impairment should ensue if testosterone is the responsible factor. Such experiments are currently underway.

I mentioned previously that there was a sex difference in reinnervation. Indeed the number of motor units to reinnervated old dy2J females was significantly less than that of the male group. Also there was functional denervation, thus suggesting that the existing motorneuron population was not able to capture the denervated fibers. This evidence clearly supports a neural
involvement whose full manifestations are not apparent in all animals.

Is there any feature of the disease that is common to both male and female? There clearly is no doubt that male mice are affected by the disease. This is borne out in the histological profile of dystrophic male SOL which is indistinguishable from that of the female. Also, the reduction of normalised tetanic tension of the older $dy^{2J}$ group versus the older C57 group shows no sex dependence. It would seem then that the underlying factors in the decreased normalised tension are real manifestation of the disease that are either secondary to a primary neural defect (possibly trophic in origin) or primary disturbances devoid of any neural influence.

Nonetheless, the intrinsic strength of $dy^{2J}$ SOL appears to be reduced. If one looks at the average absolute motor unit sizes $\left( \frac{P_0}{\text{number of motor units or motor neurons}} \right)$, a reduction in young $dy^{2J}$ vs control muscles is seen (1.07 g/unit C57 vs 0.58 g/unit $dy^{2J}$). Ovalle et al. (1983) showed that in 7 wk $dy^{2J}$ mouse, the number of muscle fibers was approximately 750 while in the control C57 mouse there were roughly 890 fibers. Parry & Parslow (1981) also showed a 16% reduction in fiber number of 3 wk $dy^{2J}$ SOL. The expected percentage drop in tension should thus be approximately 16% from 1.07 g/unit to 0.90 g/unit, assuming there is no difference in fiber size and intrinsic
strength between C57's and dy2j's. In fact a 54% decrease is noted. As no difference in average fiber cross-sectional area (CSA) was found in young animals this would suggest that an intrinsic defect in force production exists. In the older group it is not possible to make a similar analysis. Although there is a reduction from 850 fibers in control SOL to 550 in the dystrophics (Ovalle et al. 1983), the variable fiber size makes it difficult to accurately estimate the actual drop in CSA due to muscle bulk.

A better approach would involve measuring the actual CSA occupied by muscle fiber and then expressing Po in a normalised fashion i.e. Force/mm².

It is rather odd however that no drop in normalized tetanic tension was seen between old C57 and old dy2j SOL (Table IV). This would seem to suggest that no difference in intrinsic strength exists between dystrophic and control animals. As previously mentioned, Bressler et al. (1983) noted qualitatively similar results in older dystrophic mice. Nonetheless it remains obscure why the intrinsic defect in force generation is not apparent in the older mice. Perhaps the observed slower rate of decay of the active state as observed by Saddadini and Baskin (1976) may be a contributing factor. Thus, the contractile apparatus would be active for a longer period of time, and this may allow for greater tension production. Of interest in this regard is that the older dy2j group shows a tendency for longer
1/2RT's. It would have been of considerable value to have measured half rise and decay times of the tetanus.

As this intrinsic myogenic defect is apparent at the young age where neuronal impairment was also shown, it is not possible to establish a causality. To this regard, it might be instructive to conduct motor unit experiments on very young (newborn?) animals to see if this tension deficit exists.

To conclude, the results of this thesis are supportive of both neural and muscular involvement in the dy^2J model of dystrophy however the temporal order is not clear.
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In all cases the values presented are means = Standard deviation with the value n in brackets.

The following designations are used to denote significance:

a: significantly different from unoperated muscle \( p < .001 \)
b: significantly different from unoperated muscle \( p < .01 \)
c: significantly different from unoperated muscle \( p < .05 \)

Young animals were denervated at 3 weeks of age \( \pm \) 2 days, and were examined one month later.

Old animals were denervated at 6 months of age \( \pm \) 1 week, and were examined one month later.
TABLE I
Popular classifications and Characteristics of Mammalian Fiber Types

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Vascular Supply</th>
<th>Isometric Contraction Times</th>
<th>Acid ATPase Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>β</td>
<td>White</td>
<td>IIB</td>
<td>low</td>
<td>40 μm</td>
<td>low</td>
<td>Fast</td>
<td>pH 4.3 pale</td>
</tr>
<tr>
<td>B</td>
<td>αβ</td>
<td>Intermediate</td>
<td>IIa</td>
<td>moderate to high</td>
<td>45 μm</td>
<td>intermediate.</td>
<td>Fast</td>
<td>pale pale</td>
</tr>
<tr>
<td>C</td>
<td>α</td>
<td>Red</td>
<td>I</td>
<td>high</td>
<td>55 μm</td>
<td>high</td>
<td>Slow</td>
<td>dark dark</td>
</tr>
<tr>
<td></td>
<td>IIC</td>
<td></td>
<td></td>
<td>moderate to high</td>
<td>--</td>
<td></td>
<td></td>
<td>intermediate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>dark</td>
</tr>
</tbody>
</table>

1. Stein & Padykula (1962)
2. Cuth & Sawa (1969)
3. Gauthier (1971)
4. Dubowicz (1960)
### TABLE II

Wet Weights of Unoperated & Reinnervated Solei from Young and Old C57 and dy2j Mice.

<table>
<thead>
<tr>
<th></th>
<th>Unoperated</th>
<th>Reinnervated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young C57</td>
<td>(7.58 \pm 1.32) (6)</td>
<td>(5.15 \pm 2.12) (6)c</td>
</tr>
<tr>
<td>Old C57</td>
<td>(10.58 \pm .76) (7)</td>
<td>(6.80 \pm 1.05) (7)a</td>
</tr>
<tr>
<td>Young dy2j</td>
<td>(6.72 \pm 1.54) (6)</td>
<td>(4.37 \pm 1.29) (6)c</td>
</tr>
<tr>
<td>Old dy2j</td>
<td>(4.98 \pm 1.03) (10)</td>
<td>(3.51 \pm 1.19) (10)b</td>
</tr>
</tbody>
</table>

### TABLE III

Ratio of the Cross-Sectional Areas (CSA) of type I fibers to Total Muscle CSA of Unoperated & Reinnervated C57 Solei

<table>
<thead>
<tr>
<th></th>
<th>C57 Unoperated</th>
<th>C57 Reinnervate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young Group</td>
<td>(.41 \pm .07) (5)</td>
<td>(.73 \pm .05) (5)a</td>
</tr>
<tr>
<td>Old Group</td>
<td>(.64 \pm .04) (5)</td>
<td>(.80 \pm .04) (4)a</td>
</tr>
</tbody>
</table>
### TABLE IV

Absolute and Normalised Twitch and Tetanic Tensions Obtained by Direct Stimulation of Unoperated and Reinnervated Solei from Control and Dystrophic Mice

<table>
<thead>
<tr>
<th></th>
<th>Unoperated</th>
<th>Reinnervated</th>
<th>g/mg wet wt.</th>
<th>Unoperated</th>
<th>Reinnervated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Twitch Tension</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young C57</td>
<td>2.74 ± 0.57(6)</td>
<td>1.25 ± 0.65(6)b</td>
<td>.36 ± .06(6)</td>
<td>.23 ± .07(6)b</td>
<td></td>
</tr>
<tr>
<td>Old C57</td>
<td>2.62 ± 0.64(7)</td>
<td>1.60 ± .90(7)c</td>
<td>.25 ± .05(7)</td>
<td>.23 ± .11(7)</td>
<td></td>
</tr>
<tr>
<td>Young dy2j</td>
<td>1.08 ± .33(6)*</td>
<td>0.50 ± .18(6)b*</td>
<td>.16 ± .04(6)+</td>
<td>.12 ± .05(6)</td>
<td></td>
</tr>
<tr>
<td>Old dy2j</td>
<td>1.47 ± .81(10)*</td>
<td>1.10 ± .57(10)</td>
<td>.28 ± .11(10)</td>
<td>.36 ± .22(10)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Unoperated</th>
<th>Reinnervated</th>
<th>g/mg wet wt.</th>
<th>Unoperated</th>
<th>Reinnervated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tetanic Tension</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young C57</td>
<td>22.47 ± 5.51(6)</td>
<td>13.19 ± 7.89(6)c</td>
<td>2.93 ± .42(6)</td>
<td>2.37 ± .58(6)</td>
<td></td>
</tr>
<tr>
<td>Old C57</td>
<td>23.16 ± 2.39(7)</td>
<td>12.50 ± 4.83(7)a</td>
<td>2.20 ± .17(7)</td>
<td>1.82 ± .58(7)</td>
<td></td>
</tr>
<tr>
<td>Young dy2j</td>
<td>12.26 ± 3.53(6)*</td>
<td>7.29 ± 2.09(6)c</td>
<td>1.81 ± .37(6)*</td>
<td>1.68 ± .26(6)</td>
<td></td>
</tr>
<tr>
<td>Old dy2j</td>
<td>9.80 ± 3.09(10)*</td>
<td>5.32 ± 4.71(10)c</td>
<td>1.94 ± .37(10)</td>
<td>1.44 ± .51(10)c</td>
<td></td>
</tr>
</tbody>
</table>

*: Significantly lower than either young or old C57

+: Significantly lower than young C57

### TABLE V

Twitch: Tetanus Ratio's for Unoperated and Reinnervated Solei of C57 and dy2j Mice

<table>
<thead>
<tr>
<th></th>
<th>Unoperated</th>
<th>Reinnervated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young C57</td>
<td>.12 ± .02 (6)</td>
<td>.10 ± .03 (6)</td>
</tr>
<tr>
<td>Old C57</td>
<td>.11 ± .02 (7)</td>
<td>.14 ± .05 (7)</td>
</tr>
<tr>
<td>Young dy2j</td>
<td>.09 ± .01 (6)</td>
<td>.07 ± .02 (6)</td>
</tr>
<tr>
<td>Old dy2j</td>
<td>.14 ± .04 (10)</td>
<td>.24 ± .08 (10)*b</td>
</tr>
</tbody>
</table>

*: Significantly higher than young dy2j reinnervate p < .001
TABLE VI

Twitch Times to Peak Tension (TTP) and Half-Relaxation (1/2 RT) from Unoperated and Reinnervated Solei of Young and Old C57 and dy2j Mice

<table>
<thead>
<tr>
<th></th>
<th>TTP (msec)</th>
<th>1/2 RT (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unoperated</td>
<td>Reinnervated</td>
</tr>
<tr>
<td>Young C57</td>
<td>13.67 ± 0.52(6)</td>
<td>16.50 ± 1.05(6)a</td>
</tr>
<tr>
<td>Old C57</td>
<td>13.86 ± 1.21(7)</td>
<td>19.29 ± 1.80(7)a</td>
</tr>
<tr>
<td>Young dy2j</td>
<td>14.50 ± 0.84(6)</td>
<td>14.17 ± 1.47(6)</td>
</tr>
<tr>
<td>Old dy2j</td>
<td>16.30 ± 3.68(10)</td>
<td>21.50 ± 4.58(10)b</td>
</tr>
</tbody>
</table>

*Significantly higher than young dy2j unoperated and reinnervated and unoperated old C57 p < .05.

TABLE VII

Functional Innervation Ratio (FIR) and Reinnervation Ratio (RIR) of Young and Old Control (C57) and Dystrophic (dy2j) MICE

<table>
<thead>
<tr>
<th></th>
<th>FIR</th>
<th>RIR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unoperated</td>
<td>Reinnervated</td>
</tr>
<tr>
<td>Young C57</td>
<td>0.98 ± .03(6)</td>
<td>0.85 ± .16(6)</td>
</tr>
<tr>
<td>Old C57</td>
<td>0.99 ± .02(7)</td>
<td>0.98 ± .03(7)</td>
</tr>
<tr>
<td>Young dy2j</td>
<td>0.98 ± .04(6)</td>
<td>0.93 ± .06(6)</td>
</tr>
<tr>
<td>Old dy2j</td>
<td>0.94 ± .06(10)</td>
<td>0.53 ± .37(10)b*</td>
</tr>
</tbody>
</table>

*Significantly different from both young dy2j and old C57 p < .05

TABLE VIII

HRP Retrograde Labelling of Motorneurons in Solei of Young C57 and dy2j Unoperated and Reinnervated Muscles

<table>
<thead>
<tr>
<th></th>
<th>Number of Motorneurons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>C57</td>
<td>21.4 ± 1.1(5)</td>
</tr>
<tr>
<td></td>
<td>18 - 24</td>
</tr>
<tr>
<td>dy2j</td>
<td>21.4 ± 1.0(5)</td>
</tr>
<tr>
<td></td>
<td>20 - 25</td>
</tr>
</tbody>
</table>

A: numbers below the mean ± SD represent the range
TABLE IX
FIR and RIR of Unoperated & Reinnervated Solei from 6 month Male, Female, and Male Castrated dy2j Mice

<table>
<thead>
<tr>
<th></th>
<th>Unoperated</th>
<th>FIR</th>
<th>Reinnervated</th>
<th>RIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Group</td>
<td>0.92 ± .13(17)</td>
<td>.49 ± .33(17)a</td>
<td>.53 ± .33(17)</td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>.97 ± .03(5)</td>
<td>.86 ± .14(5)</td>
<td>.89 ± .13(5)</td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>.91 ± .07(5)</td>
<td>.25 ± .24(5)a</td>
<td>.28 ± .27(5)*</td>
<td></td>
</tr>
<tr>
<td>Male Castrates</td>
<td>.88 ± .19(7)</td>
<td>.40 ± .24(7)a</td>
<td>.46 ± .24(7)*</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from males p < .01

TABLE (X)
TTP, \(\text{\textdagger RT}\), and Twitch: Tetanus Ratio of Unoperated and Reinnervated Solei from 6 month Male, Female, and Male Castrated dy2j Mice

<table>
<thead>
<tr>
<th></th>
<th>TTP</th>
<th>(\text{\textdagger RT})</th>
<th>Twitch/Tetanus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unoperated</td>
<td>Reinnervated</td>
<td>Reinnervated</td>
<td>Unoperated</td>
</tr>
<tr>
<td>Whole Group</td>
<td>14.94 ± 3.65(17)</td>
<td>22.24 ± 5.68(17)a</td>
<td>17.76 ± 5.07(17)</td>
</tr>
<tr>
<td>Males</td>
<td>14.20 ± 2.20(5)</td>
<td>18.60 ± 1.95(5)b</td>
<td>16.60 ± 4.51(5)</td>
</tr>
<tr>
<td>Females</td>
<td>18.40 ± 3.80(5)</td>
<td>24.40 ± 4.72(5)c*</td>
<td>22.80 ± 2.86(5)</td>
</tr>
<tr>
<td>Male Castrated</td>
<td>13.00 ± 2.77(7)</td>
<td>23.29 ± 7.25(7)b</td>
<td>15.00 ± 4.28(7)</td>
</tr>
</tbody>
</table>

*: Significant difference from males p < .05
Fig. 1

a  H & E of a control SOL of a one month old mouse
   - note absence of centronucleation

b  top: H & E of a dy2j SOL taken from a one month old mouse
   bottom: same as top except mouse was 6 months old
   - arrows point to centronucleation (CN) and connective tissue (CT)
   - H: hypertrophied fibers
   - A: atrophic fibers
Myofibrillar ATPase (10.4) of an unoperated and reinnervated C57 soleus.

- TTP is indicated at upper right hand
- refer also to Table III
- drawn line demarcates SOL from anterior tibialis muscle
Fig. 3

C57 old reinnervate 1 month post denervation
a anti - I myosin immunohistochemistry
b myofibrillar ATPase 4.5
c anti - II myosin immunohistochemistry

C57 old reinnervate, 2 months post denervation
d anti - I myosin immunohistochemistry
e anti - II myosin immunohistochemistry
- black dots indicate dark staining fibers where co-existence of myosins is found
- triangles indicate intermediate staining fibers where co-existence of myosins is found
- I: intermediate staining fibers showing co-existence of myosins
- L: light staining fibers showing co-existence of myosins
- arrow points to muscle spindle and is intended as a landmark
Fig. 4a

Immunohistochemical staining for anti type I (below), type II (opposite top), and type IIb (opposite bottom) myosins of old dy2j.

- numbers: fibers that are pure type I
- ▲ : fibers that contain type IIa and IIb myosin
- ● : fibers that contain type I and IIa myosin
- ■ : fibers that contain all 3 myosin types
Fig. 4b

Immunohistochemical staining for anti-type I (below), type II (opposite top), and type IIb (opposite bottom) myosins of old dy2j reinnervate.

- numbers indicate fibers that contain all 3 myosins.
Fig. 5

The relationship between time to peak tension and motor unit size of units isolated by ventral root splitting in unoperated and reinnervated C57 Soleus.
Fig. 6

Motor unit size / frequency histogram of same units as in Fig. 5. Frequency is expressed as a proportion of the total units.
Twitch and Tetanus Elicited Directly (DIR) and Indirectly (IND) on Unoperated and Reinnervated SOL of Castrated Male dy2j Mouse
APPENDIX I

Protocol for Immunohistochemistry with Monoclonal Antibodies

Phosphate Buffered Saline (PBS): Mix 25mM K₂HPO₄ and 25mM KH₂PO₄ to pH 7.4. Add NaCl to final concentration of 0.9% w/v

3′,3′Diaminobenzidine (DAP)/0.03% H₂O₂: Add 10 mg DAB to 10 ml of a 0.03% aqueous solution of H₂O₂.

1) PBS rinse: 3 X 1min., then 1 X 5 min.
2) Let cover slips dry.
3) Add drops of primary antibody (diluted 1:40 with 1% Bovine Serum Albumin in PBS) over sections and incubate in moist chamber for 16-18 hours at room temperature.
4) PBS rinse: 3 X 30 sec., then 1 X 10min.
5) Let cover slips dry.
6) Add drops of secondary antibody (rabbit anti-mouse IgG) diluted as above.
7) Incubate at 37°C for 1 hr. in moist chamber.
8) Repeat #4
9) Repeat #6 using Peroxidase-Anti-Peroxidase
10) Repeat #4
11) Incubate in DAB/H₂O₂ for 5 mins. (DAB/H₂O₂ must be prepared just prior to use)
12) Tap water wash: 10 mins.
13) 95% Ethanol: 2 X 1min.
14) 99% Ethanol: 2 X 1min.
15) Xylene: 2 X 2min.
16) Mount on slides with Permount (Fisher)

* Three primary antibodies were used:
   1) anti-type I myosin (BF-46)
   2) anti-type IIb myosin (BF-F3)
   3) anti-type IIa & IIb myosin (BF-34)