Université de Montréal

Influence of Fetal Tissue Transplant on
the Morphology of the Neuromuscular Junctions of
Tibialis Anterior and Medial Gastrocnemius
Following Spinal Transection in the Rat

par

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Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de

Maître en sciences de l'activité physique

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Ce mémoire intitulé :

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Following Spinal Transection in the Rat

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Summary

The present work examined the adaptations of the neuromuscular junction occuring in the tibialis anterior (TA) and the medial gastrocnemius (MG) following fetal tissue transplants in a rat model following spinal transection at T10-11. The cholinesterase-containing endplate was quantified using a modification of the method used by Pestronk and Drachman (1978) which also allows staining of the nerve terminals. The parameters measured included the endplate area, endplate longitudinal length, number of branch points and muscle fiber width, under conditions of a controlled state (CNTRL group, n=3), transected state (TRANS group, n=3) and transection with transplant state (TRNPL group, n=3). Both of these muscles were chosen on the basis that they elicit different actions but are of similar fiber-type composition. Another reason for the selection was to examine whether the function of the muscle plays a role in the results of transection or transplants. The present study reported no significant findings to embryonic tissue transplants post transection. Statistical analysis uncovered no significant difference between the TA and MG in terms of endplate area, endplate longitudinal length, number of branchpoints and muscle fiber width. Most mean results, excluding mean results for muscle fiber width, were found to be highest in the transection and transplant groups for both the TA and MG. Spinal transection did not cause muscle fibers to significantly atrophy in this experiment. Transection alone as well as the transplant condition did not result in any differences in the parameters measured.

The model in this experiment is significant in the sense that it allows researchers

to consider the effect of near-total inactivity on the motor endplate which is a problem in other models where inactivity is less complete. With a spinal transection there is no direct damage to the muscle and innervation of muscles is not physically disrupted; therefore, the interuption of transfer of electrical activity through the motoneurons likely stimulates changes that are observed (Salmons & Henriksson, 1981).

Spinal transection is of particular interest because it more closely mimics the human condition of spinal cord injury than spinal isolation and intact reflexes allow the study of the role of reflex-generated locomotor activity in attenuating the atrophic responses (Gardiner, 2001). Animals in the transplant group had a 4 week period of transplantation. The transplant aspect of the present model is of interest because fetal spinal cord tissue produces several growth and trophic factors during its development and it is possible that some of these may be released to have an influence upon the muscle directly or indirectly through action of the motorneurons (Houle et al.,1999). Dr. J. Houle has found that passive exercise seems to attenuate many muscle effects such as the decrease in gross muscle size and atrophy of individual muscle fibers following a spinal cord injury. His findings indicate that cycling exercises or fetal tissue transplantation can effectively limit the decrease in muscle size observed following a complete spinal cord lesion but neither approach affects changes in the expression of proteins important for muscle contractility. Though the present study uncovered no significant results, it does set the stage for future research eventually looking at the effects of passive exercise on any spinalectomy-induced changes in the NMJ.

Résumé

Cette expérience examine les adaptations des jonctions neuromusculaires du muscle antérieure (tibiale) et du (gastroc) médiale suivant la transplantation des cellules embryonnaires chez le rat transecté. La plaque motrice contenant de la cholinesterase était quantifiée par une modification de la méthode de Pestronk et Drachman (1978). Cette méthode permet de noircir les plaques motrices ainsi que les terminaisons nerveuses. Les paramètres mesurées étaient: l'aire de la plaque motrice, la longueur des plaques motrices, la largeur des fibres musculaires, et le nombre de points de bifurcations selon trois conditions. La première était pour un état contrôlé, la deuxième dans un état transecté et la troisième dans un état transecté avec une transplantation. Ces deux muscles a été choisies parce qu'ils provoquent des actions différentes et parce qu'ils sont d'une composition de fibres musculaires semblables. Ces muscles permettent à determiner si leur actions différents influencent les effets d'une transection ou d'une transplantation. La transplantation des tissus embryonnaires à la suite d'une transection a effectué aucun changement significatif pour cette expérience. Les resultats indiquent aucune différence statistiquement significative entre l'antérieure tibiale et le gastroc médiale pour les valeurs mesurées de l'aire de la plaque motrice, la longeure des plaques motrices, la largeure de la plaque motrice et le nombre de bifurcations. La plupart des valeurs, sauf celles pour la largeure des fibres musculaires, étaient plus élevées pour les états de transection et de transplantation pour l'antérieur tibiale et le gastroc médiale. La transection expérimentale n'a pas effectué l'atrophie des fibres musculaires pour cette étude. La modèle de l'éxperience est important à cause de l'inactivité presque complète de la

plaque motrice à la suite d'une transection, ce qui permet d'autre rechercheurs de l'examiner car d'autre modèles d'inactivité moins complète sont problématiques. Il n'y a pas d'endommagement directe au muscle ni d'interruption physique à l'innervation du muscle dans un cas de transection donc la transferation d'activité électrique à travers le motneurone n'est pas interompue. Ceci probablement stimule des changements (Salmons & Henriksson, 1981). La transection et sa vraisemblance à la condition d'une blessure de la moelle épinière chez l' humaine permet l'étude de locomotion généré par les reflexes pour diminuer l'atrophie des muscles (Gardiner, 2001). La transplantation est important parce que l'extrait de la moelle épinière foetale produit plusieurs facteurs de la croissance au cours du développement alors il est possible que le déchargement de ces facteurs influence les muscles directement ou indirectement par l'action des motoneurones (Houle et al., 1999). Selon les recherches de Dr. J. Houle, l'exercice passive semble attenuer les effets musculaires comme la réduction de la taille du muscle (en entier) et l'atrophie des fibres musculaires individuelles à la suite d'une blessure de la moelle. Les résultats de ses études indiquent que l'exercice du cyclisme ou la transplantation embryonnaire peut effectivement limiter la réduction en taille du muscle à la suite d'un transection complète. Ni l'un ni l'autre affectent les changements de l'expression des protéines nécessaire pour la contraction. Même si cette expérience a montré aucun résultat significatif elle est d'importance pour d'autres rechercheurs à la poursuite d'étudier les lésions spinales et les effets d'exercice passive sur les jonctions neuromusculaires à l'avenir.

Mots-clés : Jonction neuro-musculaire, blessure de la moelle épinière, transplantation de cellules fétales, types de fibres musculaires.

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List of Abbreviations

AChE	Acetylcholinesterase
AchR	Acetylcholine Receptors
ATPase	Adenosine triphosphatase
C	Celsius
ChE	Cholineserase
CNS	Central nervous system
CNTRL	Control
EDL	Extensor Digitorem Longus
EP	Endplate
FDL	Flexor Digitorem Longus
FG	Fast-twitch glycolytic
FOG	Fast-twitch oxidative glycolytic
FSC	Fetal Spinal Cord
HRP	Horseradish Peroxidase
IP	Intraperitoneal
MG	Medial Gastrocnemius
MHC	Myosin Heavy Chain
NMJ	Neuromuscular Junction
PDQ	Peroneus Digiti Quinti
PL	Plantaris
PN	Peripheral Nerve
RF	Rectus Femoris
SD	Standard Deviation of the mean
SO	Slow-twitch oxidative
Sol	Soleus
TA	Tibialis Anterior
TRANS	Transected
TRNPL	Transection + Transplant
μm	micrometer(s)
μm²	squared micrometer(s)
VL	Vastus Lateralis
VM	Vastus Medialis

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" The future influences the present just as much as the past. "
[Nietzche]

Review of literature

<u>General</u>

Adaptation to skeletal muscle fibers to increased and decreased use has been extensively investigated (Deschenes et al., 1994). However, little is known about the adaptations that occur to other components of the neuromuscular system (Deschenes et al 1994). The link of communication between motoneurons and muscle fibers is the neuromuscular junction (NMJ). The neuromuscular junction is specifically defined as the synaptic site between a motoneuron and muscle fibers belonging to a motor unit. It is the essential element of neuromuscular control of skeletal muscle (Sherrington, 1929). This dynamic structure undergoes both morphological and functional changes throughout the course of a lifetime (Sieck & Prakash, 1997).

<u>Historical Perspective – THE NEUROMUSCULAR JUNCTION</u>

Histologists in the early 1840's agreed with both Valentin and Emmert in 1836, as cited in Couteux, 1973, that nerves boasted bow-shaped endings in striated muscles with the nerve branches joining up and continuing in one another. This opinion, however, negated the existence of true nerve endings with direct connections between the motor nerve and each muscle fiber.

Wagner's observations in 1847, cited in Couteaux, 1973, projected doubt for the first time on the existence of terminal bows in striated muscles of vertebrates stated by Valentin and Emmert. Through his studies on the hyoidean muscles of the frog, Wagner was the first to reveal two fundamental features of the NMJ of vertebrates; he concluded that the motor nerve fiber, after branching, loses its myelin sheath and closely connects with the muscle fiber.

Subsequently discovered were two other important characteristics in various types of NMJs found in striated muscles. The first, described by Kuhne in 1862, showed that after piercing the sarcolemma on frog muscles, the motor nerve fiber branches again and provides a terminal arborization. The second, imparted by Rouget in 1862, reported the presence of a flattened heap of granular nucleated substance at the NMJ level, in the muscles of reptiles, birds, and mammals. He interpreted it as the spreading of the axis cylinder substance at the surface of the myofibrils and called it the endplate.

The following year Krause in 1863, described the terminal branching at the level of the endplate on the retractor muscle of the cat's eye, later termed it the motor endplate. The first researchers to really detail observations of the structure of the motor endplate were Ranvier and Kuhne. Further morphological research was based on the work of these two authors. Ranvier identified structural and functional differences between fast-twitch (type II) and slow-twitch (type I) mammalian muscles and Kuhne discovered the branched nerve terminal arborization, as cited in Couteaux, 1973.

Muscle fiber types and their recruitment during movement

Muscles of the rat hindlimb are composed of three distinct muscle fiber types in varying proportions. High proportions of slow-twitch oxidative fibers and fast-twitch glycolytic fibers are typically in the deepest regions of the hindlimb muscles. More superficially found are the fast-twitch glycolytic fibers (Armstrong & Phelps, 1984).

Most skeletal muscles are not homogeneous in their muscle fibre composition. The heterogeneity of muscle fibers within a muscle was first identified by the histochemical detection of different staining intensities of myosin ATPase and of oxidative and glycolytic enzymes in human and animal studies performed by Dubowitz and Pearce in 1960 and by Engel in 1962. Engel first classified muscle fibers into two types: type I and type II which correspond with slow twitch and fast twitch respectively. Muscle fibers have been classified according to myosin heavy-chain (MHC) protein that they possess. Adult mammalian limb muscles myosin heavy-chain species include types I, IIa, IIIx (also termed IId) and IIb (Gardiner, 2001).

Structural and functional differences between slow tonic and fast twitch mammalian muscles were identified more than a hundred years ago by Ranvier in 1874 and Grutzner in 1884. Since most mammalian muscles contain fast and slow twitch muscle fibers there is considerable heterogeneity of muscles and motor unit properties. This heterogeneity is functionally important for grading and controlling muscle force during normal movement (Vrbova, Gordon and Jones, 1995).

When movement is carried out whether it is running or walking, it is necessary that the appropriate muscles are activated for the specific movement and that the force

generated by those muscles is controlled. Henneman and Olson (1965) emphasized the functional matching of the slow and fast twitch muscle properties to their mode of recruitment during movement and made the astute observation that ' three heads are better than one ', in their description of the differences between the three heads of the triceps surae. The three muscles inserting into the Achille's tendon, act at the ankle joint, however the point of insertion at the ankle may be sufficiently different for the muscles possibly to control the rotation of the ankle joint differentially (Nichols et al., 1993). The more obvious difference is the fibers, the gastrocnemius muscles inserting on the femur and acting on knee joint to flex the limb during movement. The soleus muscle is a single joint muscle which stabilizes only the ankle joint. Finally the architecture of the synergistic ankle extensor muscles is quite different.

Many motor units varying widely in contractile force, speed and endurance and in the excitability, size and firing properties of their motoneurones, is the basis for the fine degree of control of force during movement. In a muscle which has a mixture of fiber types, motor units that have slow contractions and low susceptibility to fatigue are readily recruited and maintain force for long periods. In general, the large, fast motor units, particularly the fatiguable units, are recruited for brief, intermittant activities such as jumping, running or lifting (Burke 1981). The largest motor units, which are the most fatiguable, are recruited at high levels of force when the blood supply may be occluded. During repetitive activity , the discharge rate in motoneurones falls significantly concurrent with a slowing of the motor unit contractions and a decline of the fusion frequency.

Morphology of NMJ's of different fiber types

It has been found that NMJ's of different muscle fiber types manifest different structural and functional characteristics. Padykula & Gauthier (1970) were among the first investigators to detail muscle fiber type differences of NMJ's. Using the rat diaphragm they classified the muscle fibers according to cytochemical properties such as myoglobin and mitochondrial content, Z band width, and fiber diameter. These 3 criteria were the basis from which the 3 different fiber types were established: red, white and intermediate. These correspond to the slow-oxidative, fast-glycolytic, and fast-oxidative glycolytic classification generally used. They reported, through the use of electron microscopy that motor endplates on red (type I or IIa) fibers are smaller and less flat compared to endplates on white (type IIx or IIb) fibers. The junctional folds of the NMJ from red fiber were shorter and less abundant than those of white fiber. For the intermediate fiber the features were in-between those of the red and white fibers.

In 1957, Cole reported that some structural variation occurs in the endplate pattern of different muscles of the rat. He found the endplates of the diaphragm to be most irregular and difficult to classify. Other investigators observed that muscle fibers of larger diameter tend to have larger motor endplates. Gruber (1966) indicated that the length of a motor endplate, in the rat model, was directly related to the diameter of its motor nerve fiber (Padykula & Gauthier, 1970).

In the findings of Eccles et al. (1958), the axon diameter of motoneurons innervating fast-twitch muscle fibers was greater that that of motoneurons innervating slow-twitch muscle fibers. In addition, larger motoneuron fibers discharge 6 times faster

than smaller motoneurons. Considering this, Padykula & Gauthier (1970) then hypothesized that the difference in NMJ morphology of different muscle fiber types were related to structural and functional differences in the associated motoneurons. In studies of the diaphragm, spontaneous and evoked transmitter release per endplate increases with age according to Smith (1979), accompanied by an increase in the number of nerve terminal branches per endplate. However, there is an age-related decrease in branch number at the endplates of hindlimb muscles which was noted by other authors (Pestronk et al., 1980; Rosenheimer & Smith, 1985; Tuffery, 1971). The decrease is usually more pronounced in fast-twitch muscle than in slow-twitch muscle.

Padykula and Gauthier (1970) found in all three fiber types in the rat diaphragm branches of the motor nerve fibers terminated in an ultrastructural arrangement which is in general typical of neuromuscular junctions. The axonal ending lies in a depression of the muscle fiber surface ("primary synaptic cleft" or "synaptic gutter"). The sarcolemma of this region extends inward to form an elaborate system of infoldings ("secondary synaptic clefts" or "junctional folds"). The plasma membranes bounding the axon and muscle fiber are separated at all points along the primary synaptic cleft by a single basal lamina which presumeably represents a fusion of basal laminae from the two cells. An extension of this structure enters each secondary synaptic cleft as a single layer and continues along each wall of the cleft.

In Padykula and Gauthier's study it was demonstrated that the NMJ's of red, white and intermediate fibers could be distinguished by differences in shape and size of axonal endings and numbers of axoplasmic vesicles, by the distribution and spacing of junctional folds, and by the appearance of both axoplasmic and sarcoplasmic mitochondria.

Ogati & Yamaska (1985), using the more advanced technology of electron scanning microscopy, examined 3-dimensional characteristics of NMJ's of muscle fibers from intercostal muscle. Findings were in agreement to Padykula & Gauthier that different motor endplates characteristics were found on different fiber types.

In a more recent study by Sieck & Prakash (1997) a 3-color immunofluorescence technique was used to label motor axons, nerve terminals, motor endplates and MHC isoform expression. Clear images through microscopic inspection showed differences in the morphology of both nerve terminals and motor endplates on different fiber types of the rat diaphragm. It was found that the motor axons innervating type I fibers are the smallest and become progressively larger for the innervation of type IIa, IIx and IIb respectively. Such differences in the axonal diameter might be predicted based on the size principle of Henneman in 1957, whereby muscles composed of different motor unit types in varying proportions are capable of a number of functions involving selective recruitment of different motor unit types.

Effects of increased and decreased activity on the neuromuscular system

Skeletal muscles are well designed and matched for highly specific functions and, although they may be grouped as physiological flexors and extensors around any one joint, their architecture specialization is taken into account in the orchestration of any movement by the central nervous system.

Mammalian skeletal muscles with their high degree of specialization have a remarkable capacity for accommodating changes in demand. Muscles can acquire physiological and biochemical characteristics befitting the new functional requirements. A diminishment in functional demand can be a result of joint immobilization, bed rest, weightlessness, lesions of the spinal cord and dorsal roots, and neuromuscular or peripheral nerve block (Salmons & Henriksson,1981).

An increase in functional demand can be created either by the central nervous system (as in exercise or under hypergravity conditions) or by electrical stimulation of the peripheral nerve (Salmons & Henriksson, 1981). Using electrical stimulation as a means to subject selected muscles to an increased level of use has the advantage that the pattern of impulse can be closely defined by the experimenter. Chronic low-frequency stimulation of fast-twitch muscles has been achieved by implantable devices, externally mounted devices and external lead systems. Under these conditions a fast-twitch muscle undergoes an orderly sequences of change which ultimately bring about a complete transformation to a slow-twitch muscle by all criteria which have so far been applied. After the first week of stimulation there appeared to be increasing evidence of metabolic changes, consisting of an increase in the activity of enzymes of aerobic metabolism and a

decrease in the activity of enzymes of anaerobic metabolism.

In a number of respects, including contractile speed, specific activity of myosin ATPase, and calcium uptake by fragmented sarcoplasmic reticulum, the stimulated fast muscle is "slower" than the soleus, a typical slow muscle. A partial explanation for this overshoot lies in the total homogeneity of the transformed fast muscle: a normal rabbit soleus contains a small but finite number of fast fibers. Whether individual transformed fibers can acquire a normally slow combination of properties is a question which awaits the application of single-fiber techniques. Continuous low-frequency stimulation is accompanied by significant reductions in wet weight and cross-sectional area (Pette et al., 1976). The fibers seem to acquire diameters typical of those found in the slow soleus muscle. Since such a change would presumably facilitate the diffusion of oxygen from the capilllaries to the centers of the fibers, it can be viewed as an integral part of the adaptive process. The response to stimulation appears to be a reversible phenomenon.

Investigations have established that many of the normal properties of muscle fibers are maintained at least in part by muscle activity. A fall in resting membrane potential, an increase in input resistance, and spread of acetycholine receptors to extrajunctional sites can all be induced by eliminating muscle activity and prevented by direct stimulation of denervated muscle fibers (Snider & Harris, 1979).

Different models of disuse have been employed in investigating the muscle's response. Spinal isolation results in an alteration of neuromuscular activity with virtually no electrical or mechanical activity. Spinal transection reduces both electrical and mechanical activity. A reduction in mechanical and no change in electrical activity can be generated through limb immobilization and most likely, space flight. In spinal isolation,

all supraspinal, infraspinal, and peripheral input is eliminated to the motoneurons isolated in the lumbar region of the spinal cord. Spinal transection is an experimental method allowing the study of neuromuscular activity where there is no direct damage to the muscle and there is no physical disruption to the innervation of the muscle. The spinal cord is cut transversely whereby all sensations and voluntary movements are lost below the lesion. Motoneurons caudal to the lesion lose neural input which affects both their tonic and phasic firing patterns, and influences the electrophysiological and metabolic activities (Houle, 1988).

Spinal transection generally results in an atrophic response below the level of the lesion in a variety of muscles in humans (Grimby et al.,1976). The degree of atrophy is muscle-specific such that extensors atrophy more than flexors, and predominantly slow extensors are affected more than fast extensors (Roy & Acosta, 1986). Regardless of whether the transection occurs at an early age of development or as an adult atrophy will occur. According to a study by Roy and Acosta, the Sol and MG atrophy by 40% and 30%, respectively in cats transected at 2 weeks of age and by 45% and 15% respectively in cats transected as adults. In both of these groups, the TA, an ankle flexor, was minimally affected. Most muscles showed an increase in the percentage of fast-twitch fibers and a decrease in the number of slow-twitch fibers following spinal cord transection. A complete transection of the spinal cord in cats reduces the contraction time, relaxation time of the Sol but does not affect predominantly fast muscle at least up to several months after the lesion, this was observed by Buller et al. 1960, Edgerton et al.1980, and Gallego et al. 1978. Mayer et al. (1984) reported that decreases in wet weight were somewhat greater in Sol than in MG after spinal transection in the cat.

The limb immobilization model results in the elimination of weight-support activity without any surgical intervention which would compromise the nervous system. This model has been used extensively as a ground-based model in studying the effects of weightlessness on skeletal muscle properties. In a study carried out by Fahim (1989), the immobilization of the soleus was achieved by unilateral pinning of the ankle and the knee joints at right angles. His results suggest that immobilization can modulate the morphology of the NMJ, characterized by continual sprouting, retraction, and degeneration of presynaptic nerve terminals.

The hindlimb suspension model of altered activity uses a tail suspension technique whereby a tail cast (for rats) can be fastened to a harness and attached to the top of the cage by a swivel allowing 360 degrees of rotation. The suspension height can be adjusted allowing the rats to support their weight and move freely on their forelimbs, while the hindlimbs do not make contact with any surface. Hindlimb suspension progressively decreases the mass of some of the muscles in the hindlimb. The most rapid decrease occurs within the first 2 weeks of suspension (Desplanches et al., 1987). In other related experimental models in which atrophy occurs, the magnitude of the atrophic response is greater in predominantly slow extensors, which is greater than in predominantly fast extensors, which is greater than in predominantly fast flexors. The atrophy appears to be due only to a decrease in fiber size, not fiber number (Darr & Shultz, 1989). Hindlimb suspension is accompanied by a progressive decrease in the percentage of SO fibers in the Sol, whereas there appears to be minimal changes in fiber type composition of predominantly fast muscles such as the MG and TA (Graham et al.,

1989).

Weightlessness is a model of decreased use where the skeletal muscles are chronically unloaded due to the elimination of gravitational effects. During spaceflight, for the human, the tonic activity of the Sol is reduced, where as the tonic activity of the TA is enhanced during postural adjustments (Clement & Lestienne, 1988). Weightlessness results in a rapid atrophy of rat skeletal muscle, particularly those muscles that are comprised of predominantly slow fibers. All fiber types show some degree of atrophy following spaceflight (Baldwin et al., 1990).

The application of an exercise program would be a more physiological way of subjecting muscles to increased use. All forms of training involve both endurance and explosive aspects which are not easily separated. Exercise provides only an intermittent stimulus to the muscles. Another important consideration is in the way in which an increase in the normal level of activity is distributed along motor units within the muscle. Since motor units are recruited in an orderly manner the major effects of endurance exercise are likely to be concentrated in those motor units which are activated only infrequently under resting conditions but whose threshold is traversed repeatedly during exercise. A stimulus such as chronic exercise induces development of growth configurations without increasing the rate of fiber degeneration, could potentially be a positive intervention reducing the rate of (senile) muscular atrophy. The end result of exercise could increase the capacity of the muscle fibers to withstand age-related degeneration. A study investigating early events involved in regulating the muscles response to spinal transection and passive hindlimb exercise indicated that passive exercise can improve muscle atrophy after spinal cord transection and that cell activation

may play a role in muscle plasticity in response to transection and exercise (Houle & Reier, 1998).

Total disuse of the mammalian NMJ rapidly affects both pre- and post-synaptic characteristics, producing sprouting of terminals, enhanced synaptic transmission, and atrophy of post-synaptic folds of the perijunctional ' raised area ' within 3-7 days (Fahim & Robbins, 1986). A well-documented form of neuromuscular disuse which lasts 2-3 weeks, produces about 90% disuse and does not entail use of drugs or nerve injury, is obtained by limb immobilization. This procedure causes changes in synaptic transmission within 3-5 days. A rapid morphological response to subtotal disuse occurs within 5 days and consists of sprouting and longitudinal distortion of nerve terminals, flattening of primary grooves and partial loss of peri-junctional surface features. A brief period of partial disuse induces considerable and rapid synaptic plasticity in the adult nervous system.

The rate at which the process of atrophy develops can vary both within and among muscles. With this in mind, muscle-specific preferential fiber-type atrophy (Tuffery, 1971) and fiber-type conversion have been observed, suggesting that the fiber-type composition of muscle may influence the process and development of atrophy.

Sprouting of Motor Nerve Terminals

Motor nerve sprouting was first observed in response to partial denervation, and the possible consequences that follow partial denervation have all been postulated at one time or another as the sprouting stimulus. It has been possible to induce denervation-like changes in a muscle by simply blocking nerve-induced activity, when this is done sprouting is observed (Brown et al., 1980).

Motor nerve terminals have the ability to sprout under a variety of normal and pathological conditions. Continual sprouting occurs at the normal NMJ providing renewal of nerve terminals. Partial denervation of muscle tends to evoke growth of the nerve terminals. Accidental or experimental nerve injury result in sprouting of the intact motor nerves (Pestronk & Drachman, 1978).

Muscle activity exerts a trophic influence on motoneurones. It may be a factor in the regulation of sprouting (Snider & Harris, 1979). Brown and Ironton (1977) found fine, ultra terminal sprouts emanating from the endplates of muscles rendered inactive by chronic conduction block of the muscle nerve. Pestronk and Drachman (1978) observed increased motor nerve terminal branching and a consequent increase in endplate size in similar conditions.

The extent and nature of the motoneuron sprouting response have been shown to differ between slow and fast muscles (Brown et al., 1980). Duchen (1970) observed

dramatic differences in the sprouting response of soleus and gastrocnemius motor units and even amongst motor units from different regions of the same muscle (gastrocnemius and plantaris) when mouse motoneurons sprouted in muscles that had been paralyzed by botulinum toxin. His data showed extensive sprouting in the soleus, somewhat less extensive in the deep regions of the gastrocnemius and plantaris, and absent in the superficial regions, which were composed of almost exclusively of large type II b muscle fibers. These differences persisted for up to 4 weeks at which point the deep portion of the fast mixed muscles (plantaris and gastrocnemius) resembled soleus in regard to the number of sprouts, terminal morphology and cholinesterase distribution.

Axonal withdrawal has been implicated as a causation factor for fiber atrophy associated with aging muscles. The peripheral nervous system is also active in the process that reduces fiber atrophy. This process known as terminal sprouting, occuring throughout the life-span, is believed to be a mechanism involved in end-plate growth and reconstruction. Outgrowths (sprouts) on the motor axons migrate toward and eventually innervate the parent end-plate.

The process of terminal sprouting and withdrawal may act to sustain and reorganize motor end-plate morphology in response to changes in functional demand during growth, aging or disease (Cardasis & Padykula, 1981). Increases in neuromuscular contact through terminal sprouting helps to maintain integrity of the NMJ.

In a study by Cuppini et al., (1993), muscle reinnervation, after motor nerve lesion, is a reparative process model that can be reproduced in the peripheral nervous system. Proximal stumps of axotomized axons can regenerate and growing axons reach the muscles to reinnervate them. On average, branchpoints per endplate was higher in

reinnervated muscles than in normally innervated muscles. Terminal sprouting is stimulated by muscle inactivity or, alternatively, by some nerve degeneration products while nodal sprouting necessarily requires nerve degeneration products (Brown et al. 1981). When muscle, which has been inactive for several days, is reached by regenerating axons, it most likely stimulates terminal sprouting. As reinnervation proceeds, the stimulus for terminal sprouting decreases.

Spinal cord injury

The spinal cord and the brain make up the CNS. The spinal cord coordinates the body's movement and sensation. Unlike nerve cells, or neurons, of the PNS, which carry signals to the limbs, torso and other parts of the body, neurons of the CNS do not regenerate after injury (Fawcett, 1992).

An injury to the spinal cord used to be considered a fatal condition. If one did not die as a direct result of the injury he/she would die within a few weeks or months from complications, such as a kidney infection, respiratory problems or badly infected skin sores (National Spinal Cord Injury Association, 2000).

A spinal cord injury disconnects the major conduits through which sensory and motor signals pass from the body to the brain and vice versa (Li et al. 1994). It was believed this

condition was irreversible because it was thought that the environment of the central nervous system was inhibitory to neuron growth. The potential for regeneration and extension of the CNS axons was recognized by the turn of the century (Ramon y Cajal, 1928). If a permissive environment is provided, axons are able to grow for long distances following a CNS lesion.

Repair of the injured mammalian spinal cord by neurotransplantation procedures has been the subject of considerable studies. Neural tissue transplant experiments have

been devised to investigate the capacity for promoting regrowth and integration after injury to the CNS (Houle, 1992). Results from neural tissue transplantation studies indicate that both central and peripheral neurons have the capacity for regrowing their axonal process after injury and that PN grafts can promote and guide axonal regrowth for long distances toward specific target regions. Mature nerve cells cannot divide to heal a wound as skin cells can. Replacement of nerve cells requires transplantation of new nerve cells into the site of injury with the hope that they will mature and integrate into the host nervous system.

Researchers have been in agreement that transplantation of adult nerve tissues does not work, while embryonic or fetal transplantation can be quite successful. Fetal spinal cord tissue implants can rescue neurons from injury-induced cell death as well as provide a source of tissue capable of supporting neogenic axonal growth (Houle, 1991). Attempting to repair injured mammalian spinal cord through neurotransplantation procedures has been under considerable study recently.

Houle (1992) conducted a study whereby fetal spinal cord tissue was transplanted into a hemisection lesion site of the adult rat spinal cord that had been produced 3, 6 or 11 weeks prior to grafting. Glial scar tissue formation following surgical manipulation was excised prior to transplantation of FSC tissue. Results support the hypothesis that FSC tissue will affect the persistence of glial scar tissue in a chronic lesion site as well as limit the extent to which a new scar is formed, thereby facilitating axonal contact between the transplant and host spinal cord (Houle and Reier, 1988). This establishes that FSC tissue transplantation grafts can survive and mature within this potentially histopathological environment.

Regeneration of dorsal root axons could be enhanced if their proximal cut end was immediately apposed to an intraspinal transplant of fetal spinal cord tissue. Transplanted fetal CNS tissue can provide an environment conducive to promoting structural reorganization of the injured spinal cord. Extensive axonal growth could be initiated even after a considerable delay between the time of injury and the placement of fetal tissue into the lesion site. A potential exists for regrowth of certain axonal types from within the chronically injured spinal cord (Houle & Reier, 1989).

Houle (1991) designed an experiment to determine if neurons associated with a chronic spinal cord injury retain the capacity to regenerate their axonal process for an extended time period after injury. True blue was injected into the adult rat lumbar spinal cord to label neurons with axons coursing through this region. Spinal cord tissue surrounding the injection sites was removed 7 days later, creating a hemisection cavity. Four weeks later scar tissue lining the cavity was removed prior to grafting segments of autologous tibial nerve to the rostral and caudal surfaces of the cavity wall, leaving the distal end of each peripheral nerve graft ligated and unapposed to spinal cord tissue. Four weeks later the distal end was exposed to nuclear yellow to retrogradely label neurons that had grown an axon into the graft. Neurons containing both TB and NY were deemed capable of axonal regeneration while in a chronically injured state. Most neurons were located within 10mm of the lesion with the majority caudal to the lesion. Such results indicate that certain neurons associated with a chronic spinal cord injury have the potential to regenerate their axonal process for at least 4 weeks after sustaining a direct injury (Houle, 1991).

Studies on regeneration in spinal cord injury

Researchers are applying new knowledge to approach regeneration in animal

models of spinal cord injury. Some strategies include grafting peripheral nerve segments

and fetal tissue into the damaged area of the spinal cord, administration of growth factors,

genetic manipulation of cell death, and bypassing or neutralizing natural growth

inhibiting substances (National Institute of Neurological Disorders and Stroke).

Considering that demyelination plays an important role in the pathology of spinal

cord injury, a new strategy of restoring function in a spinal cord injury has been suggested

by transplanting myelin forming glial cells to demyelinated parts of the injured spinal

cord. Remyelination can enhance conduction in demyelinated axons (Honmou et

al.,1996; Imaizumi et al.,1998).

Animal studies have suggested that some trophic factors may also play a role in

stimulating axons to regenerate (Schnell et al., 1994). Neurotrophin-3, when combined

with an antibody _IN-1 that blocks myelin inhibitory proteins, facilitates increased axonal

growth or sprouting in axotomized corticospinal fibers.

The problem of CNS response to injury is incredibly complex. No one theory or

approach will overcome all of the effects of SCI, and many scientists now believe that the

"cure" will not be found in a single approach, but rather in a combination of techniques

(National Institute of of Neurological Disorders and Stroke, 2000). Consequently, it is important for all possible research areas to be addressed so overall knowledge about how the system works may eventually lead to a cure for SCI.

Rationale for the current study

When the spinal cord is damaged and loss of motor function occurs, it becomes almost inevitable for muscles to undergo atrophy. Slow-twitch muscles, which under normal conditions are recruited more frequently, seem to atrophy more than fast-twitch muscles (Roy & Acosta 1986). It has been shown in studies that slow-twitch muscle fibers tend to take on more of a fast-twitch character in SCI (Graham et al., 1989).

This study measured the morphological parameters of nerve terminals from control, transected and transection-transplanted groups. Both the MG and TA are fast muscles. They were selected for the following reasons:

i) The MG and TA perform different actions (flexion and extension of the lower limb);

ii) The MG and TA have a similar fiber-type make up but the TA has a slightly higher percentage of fast-twitch fibers;

A question of particular interest since MG and TA are of similar fiber-types was, does

the function play a role in the results of transection or transplants?

METHODOLOGY

SUBJECTS

Experiments were performed conforming to the guidelines of the Canadian Council on Animal Care and were sanctioned by the animal ethics committee of Université de Montréal. Nine adult (16 to 24 weeks old) male Sprague-Dawley rats (from University of Arkansas) were given a standard rat diet (ProLab RMh 4018) and water ad libitum. They were housed in controlled surroundings (12 hours light-12 hours dark cycle, 22°C). This experiment comprised of 3 groups of subjects: a control group (group CNTRL, n=3), a spinal transection group (group TRANS, n=3) and a spinal transection with fetal transplant group (TRNPL, n=3).

SURGICAL PROCEDURES

Rats from the University of Arkansas were subjected to the following surgical procedures.

Transection of Spinal Cord

An intraperitoneal injection of ketamine/xylazine was given to anesthetize the rats. A dorsal laminectomy of vertebral bone was then performed exposing thoracic level T9-T10. Upon opening the dura mater the dorsal spinal vein was cauterized at two sites, separated by about 6 mm, preventing extensive bleeding. An incision through the pia mater was made. Spinal cord tissue was aspirated using a glass micropipette until a cavity of about 2mm in length was produced. The cavity was enlarged extending to the lateral and ventral borders of the meninges. Gelfoam was placed in the cavity to control bleeding and left there in preparation for transplantation (group TRNPL). Suturing of the dura mater was done using 10-0 silk thread. Subjects received an IP injection of glucose-saline post-op and antibiotics for one week post-op. The urinary bladder was manually depressed 2-3 times per day for 2 weeks. Daily monitoring was done to detect urinary tract infections or self-mutilation of the limbs.

Embryonic tissue transplant

A 4% solution of chloral hydrate (1 ml/100 gram body weight) was used to anesthetize a pregnant dam. Once removed from the uterus, embryos were immersed in Hank's balanced salt solution. The embryo was pinned to a wax plate so as to expose the dorsal side. The spinal cord was excised from the embryo with microforceps and sharp tungsten wires. Spinal cord tissue was sectioned into small segments and passed through a graded series of smaller hypodermic needles, up to about 28 gauge. This created a mixture of tissue easily transferred by micropipette to the cavity created in the adult rat spinal cord. It is crucial that no air bubbles be present in the tissue after the transplantation procedure. Suturing of the dura mater was done using 10-0 silk thread. As with TRANS rats, the TRNPL rats were given an IP injection of glucose-saline post-op and received antibiotics for 1 week post-op.

The urinary bladder was manually depressed 2-3 times per day for about 2 weeks, in order to eliminate accumulation of urine. Daily monitoring was done to detect urinary tract infection or self- mutilation of limbs. Animals in the TRNPL group had a transplant for 4 weeks, +/- three days.

TISSUE PREPARATION

Electrophysiological recordings from motoneurones were performed under ketamine/xylazine anesthetic for 12 hours following the 4- week treatment period. Rats were then sacrificed using an overdose of anesthetic. After the excision of the MG and TA, these muscles were placed on cork sheeting and submerged in isopentane for freezing. Muscles were then wrapped in aluminum foil and kept in a freezer at $-80°$ C.
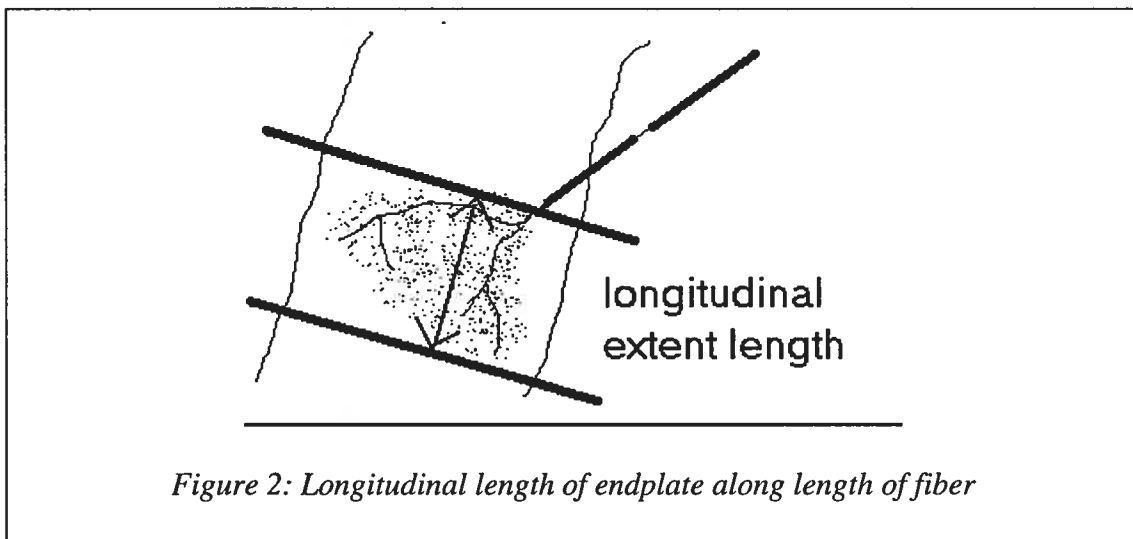
HISTOCHEMICAL PROCEDURES

Muscle samples were allowed to warm up for 30 minutes in a cryostat (-15° C). Sections were cut 50 μm in thickness longitudinally, placed on glass slides and dipped in a 3% solution of EDTA cooling in an ice bath. Slides dried at room temperature.

Staining of the endplates was established using a procedure modified from Pestronk and Drachman's technique (1978) (refer to appendix A). Nerve terminals were stained by a technique using a silver nitrate solution (refer to appendix B). The combination of these two methods allowed the demonstration of both the endplate and its contrasting nerve terminals.

The following measurements were taken:

I. Area of the end-plate, outlined by the blue cholinesterase stain (Figure 1);

II. The longitudinal length of each endplate, outlined by the cholinesterase stain, parallel to the length of the muscle fiber (Figure 2);

III. The number of nerve terminal branch points within each end plate (Figure 3);

IV. Muscle fiber width (Figure 4), demonstrating atrophy of the muscle in question.

Figure 1. Endplate area outlined by cholinesterase staining

longitudinal
extent length

Figure 2: Longitudinal length of endplate along length of fiber
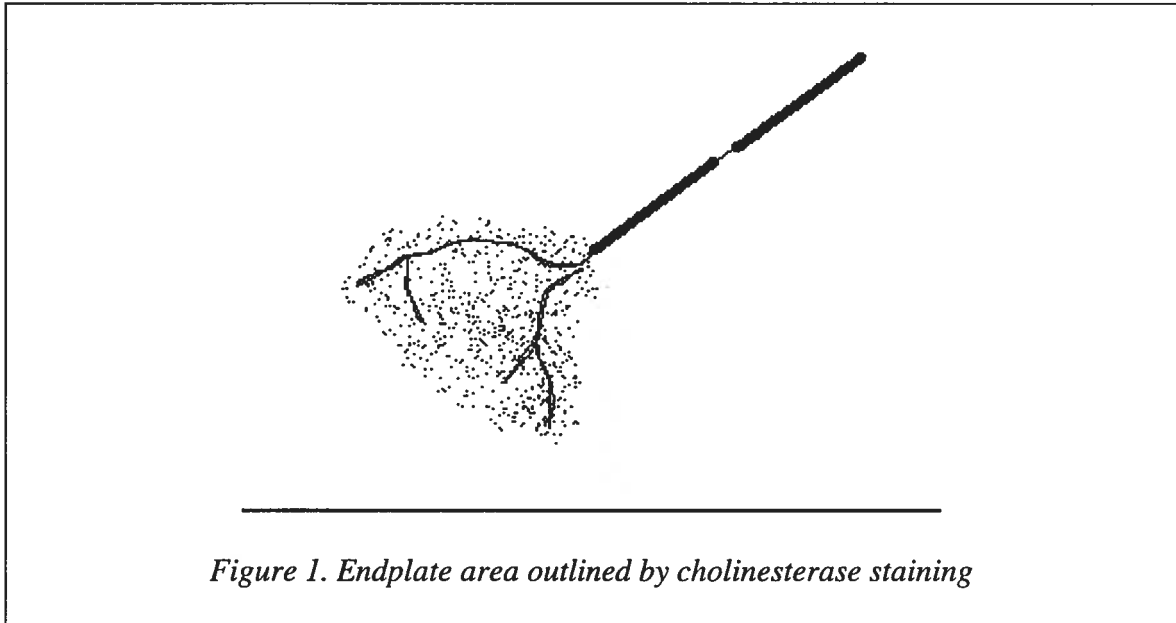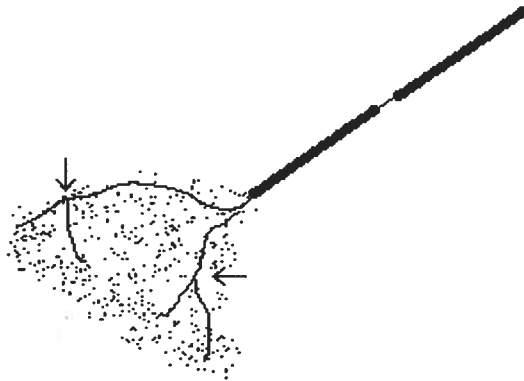
Figure 3: Endplate branchpoints as indicated by ↑
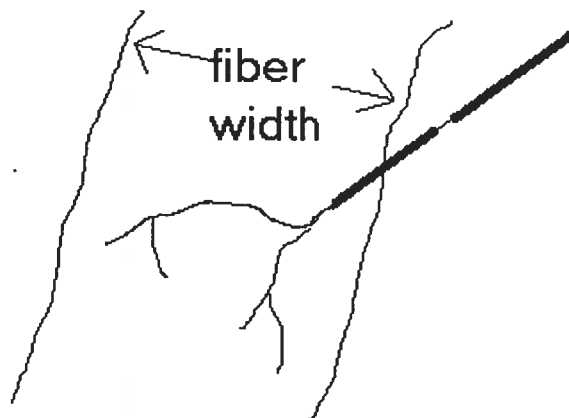
fiber
width

Figure 4: Muscle fiber width

Data were collected and recorded for each parameter (ie: EP area, EP length, branch points and fiber width) until 145 entries for each muscle were obtained. A maximum of 4 endplates were measured from each image.

An endplate was selected if a single parameter was clearly measurable.

A Nikon Optiphot-2 light microscope, equipped with a JVC TK-5210 video camera (TK-A240 power unit supply) was connected to a video monitor (Javelin). The screen was linked to a PC-based 83386 microcomputer (Jaripel) equipped with a digital image processing softward (Image Pro II version 2.0). The PhotoShop software permitted the measurement of end-plate area, longitudinal length and nerve terminal branch points. Images were captured on hard drive and disk for later analysis at varying magnifications.

STATISTICAL ANALYSIS

Data was analyzed using one-way ANOVA, to determine the effects of transection alone and transection with transplant. When a significant main effect was found, a post hoc test was used to determine significance of the difference between specific means. A probability level below .05 was considered significant.

RESULTS

FIBER WIDTH

MUSCLE DIFFERENCE

Statistical analysis uncovered no significant main effect (p>0.05) between the fiber widths of the TA and MG. Mean muscle fiber widths of TA were only slightly higher than those of MG in all groups except for the TRANS group (Table I).

TREATMENTS

Statistical analysis uncovered no significant main effect (p>0.05) between the different treatments. Spinal transection did not cause the muscle fibers to atrophy significantly in this experiment. Transected TA muscle fiber widths were shown to be 21% lower than control TA muscles. Muscles undergoing transection plus fetal tissue transplant were shown to have muscle fiber lengths 18% lower than controls. It was expected that fetal tissue transplants would delay the onset of muscle atrophy, however, a difference of 3% in terms of muscle fiber width for TA between the TRANS group and the TRNPL group is insignificant. Fiber widths of transected MG muscles were found to be 20% lower than those of the control group. Those of the TRNPL group were shown to be 20% lower than the control group as well.

TABLE I. Muscle fiber widths (μm) [+] SD

GROUPS			
MUSCLES	CNTRL	TRANS	TRNPL
Tibialis Anterior	31.7 ± 5.0 (43)	25.0 ± 5.7 (67)	25.8 ± 5.4 (48)
Medial Gastrocnemius	31.4 ± 6.8 (43)	25.4 ± 7.0 (63)	25.1 ± 5.7 (32)

Values are means $\pm$ SD, n=number of fiber widths measured in ().

EP LONGITUDINAL LENGTH:

MUSCLE DIFFERENCE

Statistical analysis uncovered no significant main effect (p>0.05) between the end-plate lengths of the TA and MG. Very little difference between endplate lengths of TA muscle and the MG muscle was detected within each condition group. (Table II).

TREATMENTS

Statistical analysis uncovered no significant main effect (p>0.05) between the different treatments. For the TA muscle, endplate lengths of the transected group and the transplant group were 5.6% longer than the control group. Endplates of the MG for both the transected and transplant group were also 5.6% longer than the control group.

TABLE II: EP lengths (µm) + SD

GROUPS			
MUSCLES	CNTRL	TRANS	TRNPL
Tibialis Anterior	28.9 ± 3.4	30.4 ± 3.8	30.8 ± 3.4
Medial Gastrocnemius	28.4 ± 6.1	30.1 ± 4.6	30.1 ± 7.3

EP AREA:

MUSCLE DIFFERENCE

Statistical analysis uncovered no significant main effect (p>0.05) between the endplate areas of the TA and MG (Table III).

TREATMENTS

Statistical analysis uncovered no significant main effect (p>0.05) between the different treatments. According to the data obtained, endplate area is greater in the transected and the transplant group for both the TA and the MG. Since there was no significant main effect discovered it is questionable whether transection causes the endplate to enlarge. Although endplates in the transplant group seem to be smaller than the transected group, it is still indefinite whether a transplant can counter the effects of transection.

TABLE III. Endplate area (μm^2) $^+$ SD

MUSCLES	CNTRL	TRANS	TRNPL
Tibialis Anterior	583.5 ± 272.7 (46)	649.8 ± 218.3 (65)	635.9 ± 277.2 (53)
Medial gastrocnemius	595.7 ± 252.3 (50)	661.9 ± 231.6 (57)	641.4 ± 287.2 (46)

Values are means $\pm$ SD, n=number of endplates in ( ).

NUMBER OF BRANCH POINTS

MUSCLE DIFFERENCE

Statistical analysis uncovered no significant main effect (p>0.05) between the number of branch points of the TA and MG (Table IV).

TREATMENTS

Statistical analysis uncovered no significant main effect (p>0.05) between the different treatments. The number of branchpoints did not differ between the TA and MG. There was a 16% increase in the number of branchpoints in the transected group in comparison to the control group. The transplant group showed an increase of 11% in number of branchpoints compared to the control group for both the TA and MG muscles.

TABLE IV. Branch points + SD

MUSCLES	CNTRL	TRANS	TRNPL
Tibialis Anterior	1.6 ± 0.7 (52)	1.9 ± 0.7 (52)	1.8 ± 0.7 (46)
Medial Gastrocnemius	1.6 ± 0.9 (51)	1.9 ± 0.7 (52)	1.8 ± 0.7 (50)

N=number of branchpoints obtained in ().

DISCUSSION

The purpose of this study was to determine whether the different functionality of similar fiber type muscles plays a role in the results of transection or transplantation on the morphology of the nerve terminal. The anterior tibialis and the medial gastrocnemius muscles were chosen in particular for this study because they are responsible for different functions of the lower limb. The TA muscle contracts in order to flex the ankle whereas the MG muscle contracts to extend the ankle and flex the knee (Gordon & Mao, 1994). Both muscles are considered to be predominantly fast muscles with at least 60% of the muscle fibers being type II. They are considered to be phasic muscles, which are mainly recruited during strenuous activities (Armstrong & Phelps,1984). Fast twitch muscles are usually active less often than slow muscles but respond with greater magnitude to exercise training. Because of this, they seem to be least affected by conditions of inactivity (Eldridge et al., 1981). In a case where the leg has a loss of function due to spinal transection the extent of muscle atrophy of predominantly fast twitch muscles is less than the muscle atrophy in slow-twitch muscles (Roy et al., 1986). This may be due partly to the fact that slow twitch muscle is recruited more often since it is a tonic muscle. When transection disrupts the impulse transmission, tonic muscles are no longer recruited leading to muscle atrophy. Phasic muscles, on the other hand, can undergo a longer period of disuse or inactivity before showing any muscle atrophy.

It has been noted in previous studies (Buller et al. 1980 Mayer et al. 1984, Roy et al. 1986) that fast extensors atrophy more than fast flexors. A tendency toward this result occurred in this study although means were not significantly different. In terms of

muscle fiber width, transected muscles had lower fiber widths (24.9 $\pm$ 5.7 μm) than the

control muscles (31.7 $\pm$ 5.0 μm) for TA. The transected MG also had lower fiber widths

(25.4 $\pm$ 7.0 μm) compared to the control group (31.4 $\pm$ 6.8 μm). Fiber width results

showed no significant difference between the TA and MG. The average mean of muscle

fiber widths showed no significant differences among the three groups. (Refer to TABLE

I).

In a study involving spinal transection of cat muscles, Mayer et al. (1984) noted

decreases in muscle fiber size of the MG in comparison to control values. His results

were based upon 21-23 weeks post transection. Less overall atrophy in type I fibers than

type II fibers was also noted.

Motor endplate area, which is demonstrated by cholinesterase staining, is related

to muscle fiber size. There is a linear relationship between muscle fiber diameter and

endplate size (Harris, 1954). Larger fibers tend to have larger endplates (Crockett et al.

1976). Endplates in the TA of the rabbit model seemed to have very little alteration after

3 weeks of tenotomy compared to controls. The gastrocnemius, as well, showed no

visible atrophy according to a study conducted by Dias (1979). Fibers of fast twitch

muscles are generally larger. TA muscle fiber width was larger than MG in control

groups. The TA has more fast twitch fibers than the MG (Armstrong & Phelps, 1984).

The size of an individual endplate within a muscle group is correlated with the diameter

of the fiber on which it is located. Different muscle groups have their own ratios of

endplate size to fiber diameter, which might be regulated by the motor neurons

innervating the muscle and/or the physical activity of the muscle (Oda, 1985). In a study

by Finkelstein et al. the fiber cross-sectional area of MG decreased by 25% for denervations up to 7 days. When compared to longer periods of denervation there was a 50-60% decrease in fiber area. In the present study there were no significant findings when comparing the endplate area of TA ($649.8 \pm 218.3 \ \mu m^2$)and MG (661.9 ± 231.7 μm^2) in the transected group. Although average mean values for endplate area were slightly higher for MG for all three groups (Refer to TABLE III).

Muscles undergoing transplantation of embryonic cells had lower muscle fiber widths than the control. No significant findings were obtained when comparing results of transected muscles and transplant muscles (Refer to TABLE I). According to Howland et al. (1995), intraspinal transplants of fetal spinal cord enhance the development of locomotor performance after the spinal cord of newborn animals has been either completely transected or hemi-sected. In adult recipients limited recovery is observed when transplants were placed into the site of a complete spinal cord transection (Miya et al.1994). The mechanisms by which transplants enhance locomotor performance are unknown. It was expected that transplantation of embryonic cells would have an impact on diminishing the extent of muscle atrophy in the condition of transection, unfortunately this did not occur in the present experiment. Intraspinal transplant of fetal spinal cord tissue affects myofiber size following complete transection. In a study conducted by Houle et al. 1999 it was noted that fast twitch fibers were significantly larger in rats receiving transplants in comparison to transection alone. It remains to be determined whether fetal tissue transplants can reverse the loss in muscle fiber size following SCI or whether it can prevent the reduction in size.

Spinal cord transplants in adult rats may serve as a relay to convey supraspinal input to spinal cord levels caudal to the transplant. It has been shown in previous studies that after spinal cord lesions in either newborn or adult rats, the presence of a transplant of fetal spinal cord tissue increases the extent of recovery of locomotor function beyond that seen in lesion-only control animals.

Nerve terminals respond to the presence of denervated or paralyzed muscle fibers by sprouting. Following the loss of even a portion of the motor supply, sprouting maintains muscle strength through the innervation of denervated fibers (Son et al., 1996). In the rat adult model, stress presented in the form of exercise training resulted in endplates becoming more branched in hindlimb muscles (Rosenheimer & Smith, 1985; Gardiner et al., 1984). Rosenheimer and Smith found that age-related changes in endplate architecture of sedentary control animals seem to be quite different in functionally diverse muscles. At 25 months of age both fast-twitch phasic EDL and slow-twitch tonic soleus muscles, which were recruited less often with age, exhibited a significant decrease in terminal branch number compared with younger (10-month) animals. Branch number, in contrast, increased with age in endplates of the fast-twitch diaphragm, which remains constantly active (Rosenheimer, 1985).

The branch number (number of branch points) provides an index of nerve terminal complexity. One would assume that more branching would occur as a result of

transection or transplantation. In a study conducted by Rosenheimer (1985) branch number within endplates was either elevated or reduced depending on the functional characteristics of different muscles. However, there is an age-related increase in terminal branch number at the endplates within the hindlimb muscles. The decrease generally being more pronounced in fast twitch phasically activated muscles (Pestronk et al.,1980). Changes in terminal branch number are linked with corresponding alterations in releasing acetylcholine. It may be assumed that a 4-week period of transplantation may not be long enough to manifest a counter effect to transection. Average branch points were highest in the transection group for both muscles. For the MG the average branch point for transected muscle was 1.9 ± 0.7 whereas the control group the average branch point was 1.6 ± 0.9. For the TA the average branch point for the transected group was 1.9 ± 0.7 whereas for the control it was 1.6 ± 0.7. (Refer to TABLE IV). In comparison, control branch point values from Rosenheimer (1985) in which he looked at the EDL and Sol his findings were 1.25 ± 0.004 and 1.63 ± 0.008 respectively. According to Tomas et al. (1992) the percentage of branch points in controlled muscles were higher in the fast-twitch EDL compared to the Sol.

Following spinal cord lesions and transplants of fetal spinal cord tissue at birth, there is extensive growth of host descending axons into the transplants (Bregman et al.,1997). Host axons extend throughout the territory provided by the transplant, and both regenerating axons and sprouting axons contribute to this growth. Although descending axons project into the a transplant of fetal spinal cord tissue after injury in the adult, despite the fetal CNS environment within the transplant, host axons terminate within a

few hundred microns of the host/transplant border. After spinal cord lesions in adult rats, for each of the pathways examined, exogenous application of BDNF, NT-3, or NT-4 increased both the distance of axonal growth and the density of axonal growth within transplants of fetal spinal cord tissue placed at the lesion site. It is suggested that after injury in the mature CNS, neurotrophic factors can exert a neurotrophic influence on mature CNS neurons in vivo, increasing the extent of axonal growth. As the age of an animal at time of injury increases, the capacity for axonal growth decreases and the extent of axonal growth becomes restricted.

Why did neither transection nor transplant have any effect on the parameters measured?

In assuming that both muscles are composed of similar fiber types, the TA being composed of a slightly greater percentage of fast twitch, glycolytic fibers than the MG (Armstrong & Phelps, 1984), it seems very likely that the muscles would show no distinct differences in terms of muscle atrophy. Muscle fiber width may be a less accurate measure of muscle fiber atrophy because contractile properties of predominantly fast muscles are relatively unaffected by a decrease in activity (Herbert et al., 1988). In a study by Finkelstein et al. (1993) mean fiber area of MG decreased by 25% following denervations of up to 7 days. Although some studies found that slow twitch muscle shows greater atrophy in a case of muscle disuse or inactivity (Gordon & Mao, 1994), there was no significant difference found in this experiment between the TA and the slower MG. Both muscles underwent the same conditions of transection and transplantation without showing any significant differences in muscle fiber width or endplate area. It is likely that a 4-week period of tissue transplant is not enough time to produce any morphological changes. Another reason as to why differences were not observed with transplantation may be explained by the findings of Das (1981). He reported that spinal cord tissue from 16-18-day-old fetuses did not persist in the injured spinal cords of adult rats. Reier et al. (1983) determined that the best growth and long-time survival of fetal spinal cord transplants were obtained with donor tissue taken from fetuses ranging in age from 12-15 days of gestation. It would be expected that since the TA and MG are responsible for

different functions that they would elicit different responses to transection and to transplantation however this was not observed in this experiment. Gordon & Mao (1994) found that in animal models with spinal cord injuries there is more severe atrophy of extensor muscles, especially slow–twitch muscles that cross a single joint. In a study by Roy & Acosta (1986) there was a differential atrophic effect on the muscles below the level of lesion, 6- 12 months after transection, ie., extentensors atrophied more than flexors. It was noted that the TA was minimally affected by transection whereas the MG atrophied by 15% in comparison to the control group. Jiang et al. (1991) also noted that the MG of the cat atrophied by 12% 6 months following spinal transection. When neuromuscular activation is reduced the relative atrophy of muscles is as follows: atrophy in the slow extensor is greater than the fast extensor which is greater than the fast flexor (Roy et al. 1991). Considering that these muscles are fast- twitch they are usually recruited less often during rest as opposed to slow twitch muscles. During activity these fast-twitch muscles are recruited more. Regardless of their difference in functionality these two muscles in a condition of inactivity responded similarly. Seeing as though both the MG and TA are predominantly fast muscles, the change in activity pattern through transection equally affected the muscles. A general observation is that atrophy is related more to the function of the muscle than to its fiber composition (Roy & Acosta, 1986). If this holds truth then the expected outcome for this experiment would be for the transected MG (slower extensor) fibers to atrophy more than those of the TA (fast flexor), however, the length of disuse or inactivity needs to be taken into account.

According to Buller et al., (1960) complete transection does not affect predominantly fast muscles up until several months after the lesion in the cat model. In a

study by Duchen et al. (1970) muscle fibers of the gastrocnemius became progressively atrophied six weeks or more following botulinum toxin injection in the mouse model. Repairing damaged human nerves can be more complex than inflictions used in experimental procedures. Damage may not always be clearly defined and infection or extensive crushing of the nerve can further complicate recovery.

CONCLUSION

Research has been advancing towards very promising directions in one day regenerating the spinal cord. Transplantation of fetal tissue presents an important method in which to study problems related to spinal cord damage and regeneration. It has been established in other studies that such transplants survive, differentiate and become integrated with the CNS of neonatal and adult rat recipients (Reier et al, 1983). Although the present study reported no significant findings to embryonic tissue transplants post transection, several limitations may have been present. These limitations include: a small sample size, assumptions in the duration of transection and transplants because of off-site procedures, unknown age of donor fetus, human inaccuracies in the quantification of endplates from computer screen resolution and microscope magnification. Most mean results, excluding mean results for muscle fiber width, were found to be highest in the transection and transplant groups for both the TA and MG. The study however provides valuable information in recreating conditions promoting regeneration experiments. With rapidly expanding information, researchers may have better understanding of growth factors, inhibitory factors and roles that transplants can play in improving the environment of an injured spinal cord.

References

Andonian, M.H., Fahim, M.A., *Effects of Endurance Exercise on the Morphology of Mouse Neuromuscular Junctions During Aging.* J. Neurocytol. 16(5):589-599, 1987.

Armstrong, R.B., Phelps, R.O., *Muscle Fiber Type Composition of the Rat Hindlimb.* Am. J. Anat. 171:259-272, 1984.

Baldwin, K.M., Herrick, R.E., Ilyina-Kakeuva, E., Oganov, V.S., *Effects of Zero Gravity on Myofibril Content and Isomyosin Distribution in Rodent Skeletal Muscle.* FASEB J. 4:79-83, 1990.

Balice-Gordon, R.J., Lichtman, J.W., *In Vivo Visualizations of the Growth of Pre- and Postsynaptic Elements of Neuromuscular Junctions in the Mouse.* J. Neurosci. 10(3):894-908, 1990.

Bell, G.J., Ayer, K., Gordon, T., Devashayam, S., Martin, T.P., *Recovery of Rat Tibialis Anterior Motor Unit Properties Following Partial Denervation.* Can. J. Physiol. Pharmacol. 70:1324-1329, 1992.

Betz, W.J., Caldwell, J.H., Ribchester, R.R., *Sprouting of Active Nerve Terminals in Partially Inactive Muscles of the Rat.* J. Physiol. 303:281-297, 1980.

Bregman, B.S., McAtee, M., Dai, H.N., Kuhn, P.L., *Neurotrophic Factors Increase Axonal Growth after Spinal Cord Injury and Transplantation in the Adult Rat.,* Experimental Neurology 148:475-494, 1997.

Brenner, H.R., Rudin, W., *Effect of Muscle Activity on the End-Plate Membrane in Denervated Mouse Muscle.* J. Physiol. 410:501-512, 1989.

Brown, M.C., Holland, R.L., Ironton, R., *Nodal and Terminal Sprouting From Motor Nerves in Fast and Slow Muscles of the Mouse.* J. Physiol.(Lond). 306:493-510,1980.

Brown, M.C. and Ironton, R., *Motor neuron sprouting induced by prolonged tetrodotoxin block of nerve action potentials.* Nature, 182: 801-802, 1977.

Buller, A.J. , Eccles, J.C., Eccles, R.M., *Differentiation of Fast and Slow Muscles in the Cat Hindlimb.* J. Physiol. (Lond)., 150:399-416, 1960.

Burke, R.E., *Motor Units: Anatomy, Physiology and Functional Organization.*, Handbook of Physiology. The Nervous System, Am. Physiol. Soc. Sec., 1981.

Cardasis, C.A., Padykula, H.A., *Ultrastructural Evidence Indicating Reorganization at the Neuromuscular Junction in the Normal Rat Soleus Muscle.* The Anatomical Record 200:41-59, 1981.

Clement, G., Lestienne, F., *Adaptive Modifications of Postural Attitude in Conditions of Weightlessness.* Exp. Brain Res. 72:381-389, 1988.

Couteaux, R., (revision by Bourne, G.H ,) *Motor End Plate Structure.* The structure and Function of Muscle-Second Edition,volume II,Structure Part 2, (482-527),1973.

Crockett, L., Edgerton, V.R., Max, S.R., Barnard, R.J., *The Neuromauscular Junction in Response to Endurance Training.* Experimental Neurology 51: 207-215, 1976.

Cuppini, R., Cecchini, T., Ciaroni, S., Ambrogini, P., Del Grande, P., *Nodal and Terminal Sprouting by Regenerating Nerve in Vitamin E-Deficient Rats.*, Journal of Neurological Sciences.,117:61-67,1993.

Darr, K.C., Schultz, E., *Hindlimb Suspension Suppresses Muscle Growth and Satellite Cell Proliferation.* J. Appl. Physiol. 67:1827-1834, 1989.

Das, G.D., *Neural Transplants in the Spinal Cord of the Adult Rat.* Anat. Rec. 199: 65A (abstr.), 1981.

Deschenes, M., Covault, J., Kraemer, W.J., Maresh, C.M, *The Neuromuscular Junction: Muscle Fiber Type Differences, Plasticity and Adaptability to Increased and Decreased Activity.* Sports Med. 17:358-372, 1994.

Desplanches, D., Mayet, M.H., Sempore, B., Flandrois, R., *Structural and Functional Responses to Prolonged Hindlmb Suspension in Rat Muscle.* J. Appl. Physiol. 63: 558-563, 1987.

Dias, P.L.R., *Effects of tenotomy on the Motor End Plates of Fast and Slow Twitch Muscles of the Rabbit.* J. Anat. 129(2): 399-404, 1979.

Duchen, L.W., *Changes in Motor Innervation and Cholinesterase Localization Induced by Botulinum Toxin in Skeletal Muscle of the Mouse: Differences Between Fast and Slow Muscles.* J. Neurol. Neurosurg. Psychiat. 33:40-54, 1970.

Dupont-Versteegden , E.E., Houle, J.D., Gurley, C.M., Peterson, C.A., *Early Changes in Muscle Fiber Size, Myosin Heavy Chains and Regulatory Factors with Spinal Cord*

Transection and Exercise. APStracts 5:0189C ,1998.

Eldridge, L., Liebhold, M., Steinbach, J.H., *Alterations in Cat Skeletal Neuromuscular Junctions Following Prolonged Inactivity*. J. Physiol. 313:529-545, 1981.

Fahim, M.A., *Rapid Neuromuscular Remodelling Following Limb Immobilization*. Anat. Rec. 227(1): 102-109,1989.

Fahim, M.A., Robbins, N., *Remodelling of the Neuromuscular Junction after Subtotal Disuse*. Brain Research 383:353-356, 1986.

Fawcett, J.W., *Spinal Cord Repair: Future Directions*. Paraplegia 30(2):83-85, 1992.

Finkelstein, D.I., Dooley, P.C., Luff, A.R., *Recovery of Muscle After Different Periods of Denervation and Treatments*. Muscle & Nerve 16:769-777, 1993.

Gardiner, P.F., Neuromuscular Aspects of Physical Activity. U.S.A., Human Kinetics, 2001.

Gardiner, P.F., Michel, R., Iadeluca, G., *Previous Exercise Training Influences Functional Sprouting of Rat Hindlimb Motoneurons in Response to Partial Denervation*. Neurosci. Lett. 45:123-127, 1984.

Gordon, T., Mao, J., *Muscle Atrophy and Procedures for Training After Spinal Cord Injury*. Phys. Ther. 74:50-60, 1994.

Graham, S.C., Roy, R.R., Hauschka, E.O., Edgerton, V.R., *Effects of Weight Support on Medial Gastrocnemius fibers of Suspended Rats*. J. Appl. Physiol. 67:945-953, 1989.

Grimby, G., Broberg, C., Krotkiewska, I., Krotkiewska, M., *Muscle Fiber Composition in Patients with Traumatic Cord Lesion*. Scand. J. Rehab. Med. 8:37-42, 1976.

Harris,C., *The Morphology of the Myoneural Junction as Influenced by Neurotoxic Drugs*. Am. J. Pathol. 30:501-519, 1954.

Hasan, S.M., Jennekens, F.G.I., Wieneke,G., Veldman, H., *Elimination of Superfluous Neuromuscular Junctions in Rat Calf Muscles Recovering From Botulinum Toxin-Induced Paralysis*. Muscle & Nerve17:623-631, 1994.

Hashizume, K., Kanda, K. and Burke, R.E., *Medial Gastrocnemius Motor Nucleus in the Rat: Age-Related Changes in the Number and Size of Motoneurons*. J. Comp. Neurol. 269:425-430, 1988.

Herbert, M.E., Roy, R.R., Edgerton, V.R., *Influence of one week of Hindlimb Suspension and Intermittent High Load Exercise on Rat Muscles*. Exp.Neurol.102:190-198,1988.

Honmou,O., Felts,P.A., Waxman, S.G., Kocsis, J.D. *Restoration of Normal Conduction in Demyelinated Spinal Cord Axons in the Adult Rat by Transplantation of Exogenous Schwann Cells.* J.Neurosci.16:3199-3209, 1996.

Houle, J.D., *Demonstration of the Potential for Chronically Injured Neurons to Regenerate Axons into Intraspinal Peripheral Nerve Grafts.* Exp. Neurol. 113:1-9, 1991.

Houle, J.D., *The Structural Integrity of Glial Scar Tissue Associated With a Chronic Spinal Cord Lesion Can Be Altered by Transplanted Fetal Spinal Cord Tissue.* J.Neurosci. Res. 31:120-130, 1992.

Houle, J.D., Reier , P.J., *Transplatation of Fetal Spinal Cord Tissue Into the Chronically Injured Adult Rat Spinal Cord.* J. Comp. Neurol. 269: 535-546, 1988.

Houle, J.D., Reier, P. J., *Regrowth of Calcitonin Gene-related Peptide (CGRP) Immunoreactive Axons from the Chronically Injured Rat Spinal Cord into Fetal Spinal Cord Tissue Transplants.* Neuroscience Letters. 103: 253-258, 1989.

Houle, J.D., Morris ,K., Skinner, R.D., Garcia-Hill, E., Peterson, C.A., *Effects of Fetal Spinal Cord Tissue Transplants and Cycling Exercise on the Soleus Muscle in Spinalized Rats.* Muscle & Nerve 22:846-856, 1999.

Imaizumi,T., Lankford, K.L., Waxman, S.G., Greer, C.A., Kocsis, J.D., *Transplanted Olfactory Ensheathing Cells Remyelinate and Enhance Axonal Conduction in the Demyelinated Dorsal Columns of the Rat Spinal Cord.* J.Neurosci.18:6176-6185, 1998.

Jiang, B., Roy, R.R., Navarro, C., Nguyen, Q., Pierotti, D., Edgerton, V.R., *Enzymatic Responses of the Cat Medial Gastrocnemius Fibers to Chronic Inactivity.* J.Appl. Physiol. 70:231-239, 1991.

Li,Y., *Axonal Sprouting in the Hemi-sected Adult Rat Spinal Cord.* Neuroscience 61(1):133-139, 1994.

Mayer, R.F., Burke, R.E., Toop, J., Walmsley, B., Hodgson, J.A., *The Effect of Spinal Cord Transection on Motor Units in Cat Medial Gastrocnemius Muscles.* Muscle & Nerve 7:23-31, 1984.

Miya, D.Y., Clark, B., Tessler, E., Murray, M., *Transplanted Mediated Behaviour Recovery of Weight Support and Interlimb Coordination in Normal, Spinal and Spinal + Transplant Rats.* Soc. Neurosci. Abstr. 20:1706, 1994,

Munson, J.B., Foehring, R.C., Lofton, S.A., Zengel, J.E., Sypert, G.W., *Plasticity of Medial Gastrocnemius Motor Units Following Cordotomy in the Cat.* J. Neurophys.

55(4):619-634, 1986.

National Institute of Neurological Disorders and Stroke., *Spinal Cord Injury :Emerging Concepts.* http.//www.ninds.nih.gov/health_and_medical/pubs/sci_report.htm., 2000.

National Spinal Cord Injury Association., *Spinal Cord Injury Treatment and Cure Research.* http://www.spinalcord.org/resource/Factsheets/factsheet5.html., 2000.

Oda, K., *The Relationship Between Motor Endplate Size and Muscle Fiber Diameter in Different Muscle Groups of the Rat.* Japanese Journal of Physiology 35:1091-95, 1985.

Padykula, H.A., Gauthier, G.F., *The Ultrastructure of the Neuromuscular Junctions of Mammalian Red, White and Intermediate Skeletal Muscle Fibers.* J. Cell Biol. 46:27-41, 1970.

Pestronk, A., Drachman, D.B., *A New Stain for Measurement of Sprouting at Neuromuscular Junctions.* Muscle & Nerve 1:70-74, 1978.

Pestronk, A., Drachman, D.B., Griffin, J.W., *Effects of Aging on Nerve Sprouting and Regeneration.* Exp. Neurol. 70: 65-82, 1980.

Pette,D., Muller, W., Leisner, E., Vrobova, G., *Time dependent Effects on Contractile Properties, Fibre Population, Myosin Light Chains and Enzymes of Energy Metabolism in Intermittently and Continuously Stimulated Fast Twitch Muscles of the Rabbit.* Pflugers Arch. Eur. J. Physiol. 364:103-112, 1976.

Prakash, Y.S., Sieck, G.C., *Age-Related Remodelling of Neuromuscular Junctions on Type-Identified Diaphragm Fibers.* Muscle & Nerve 21:887-895, 1998.

Reier, P.J., Perlow, M.J., Guth, L., *Development of Embryonic Spinal Cord Transplants in the Rat.* Developmental Brain Research 10: 201-219, 1983.

Rosenheimer, J.L., Smith, D.O., *Differential Changes in the End-plate Architecture of Functionally Diverse Muscles During Aging.* J. Neurophys. 53(6): 1567-1581, 1985.

Rosenheimer, J.L., *Effects of Chronic Stress and Exercise on Age-Related Changes in End-Plate Architecture.* J. Neurophys., 53:1582-1589, 1985.

Roy, R.R., Acosta, L., *Fiber Type and Fiber Size Changes in Selected Thigh Muscles Six Months After Low Thoracic Spinal Cord Transection in Adult Cats: Exercise Effects.* Exp. Neurol. 92:675-685, 1986.

Roy, R.R., Baldwin, K.M., Edgerton., V.R., *The Plasticity of Skeletal Muscle: Effects of Neuromuscular Activity.* Exercise and Sport Sciences Reviews. 269-311, 1991.

Salmons, S., Henriksson, J., *The Adaptive Response of Skeletal Muscle to Increased Use.* Muscle & Nerve 4:94-105, 1981.

Schnell, L., Schneider, R., Kolbeck, R., Barde, Y.A., Schwab, M.E., *Neurotrophin-3 Enhances Sprouting of Corticospinal Tract During Development and After Adult Spinal Cord Lesion.* Nature 367:170-173, 1994.

Sieck, Gary C., Prakash, Y.S, *Morphological Adaptations of Neuromuscular Junctions Depend on Fiber Type.* Can. J. Appl. Physiol. 22(3):197-230, 1997.

Snider, W.D., Harris,G.L., *A Physiological Correlate of Disuse-Induced Sprouting at the Neuromuscular Junction.* Nature 281:69-71, 1979.

Son, Y.J., Trachtenberg, J.T., Thompson, W.J., *Schwann Cells Induce and Guide Sprouting and Reinnervation of Neuromuscular Junctions.* Trends Neurosci. Jul:19(7): 280-285, 1996.

Stebbins, C.L., Schultz, E., Smith, R.T., Smith, E.L., *Effects of Chronic Exercise During Aging on Muscle and End-plate Morphology in Rats.* J. Appl. Physiol. 58(1):45-51, 1985.

Tissenbaum, H.A, Parry, D.J., *The Effect of Partial denervation of Tibialis Anterior (TA) on the Number and Sizes of Motoneurons in TA motornucleus of Normal and Dystrophic (C57BL dy 2j/dy 2j) Mice 1.* Can. J. Physiol. Pharmacol. 69:1769-1773, 1991.

Tomanek, R.J., Tipton, C.M., *Influence of Exercise and Tenectomy on the Morphology of a Muscle Nerve.* Anat.Rec. 159:105-114, 1976.

Tuffery, A.R., *Growth and Degeneration of Motor End-plates in Normal Cat Hind Limb Muscles.* J. Anat. 110(2):221-247, 1971.

APPENDIX A

STAINING FOR QUANTITATIVE MEASUREMENT OF NMJ

TISSUE PROCESSING

1. Cut longitudinal frozen sections (50um) thick with cryostat (temp \15°C).
2. Place on AES charged slides.
3. Use 3% disodium EDTA on slides.

CHOLINESTERASE-STAINING PROCEDURE

1. Turn on bath 37°C.
2. I mmersse slides in 20% solution of sodium sulfatefor 3 minutes.
3. After this and each succeeding step, wash slides in deoinized water.
4. To stain sections for acetylcholinesterase, incubate at 37°C for 14 min. 30 sec. - 15 minutes in the following solutions:

- 5 bromoindoxyl acetate	0.004g
- Ethanol	0.3ml
- Potassium ferrocyanide	0.063g
- Potassium ferricyanide	0.050g
- Tris-base (HCL=7.2)	0.033g
- Deionized water	30.0ml

(Tris solution mixed to pH 7.2 with calcium chloride and water can be pre-mixed and kept as stock solution)

APPENDIX B

NERVE-STAINING PROCEDURE

1. Dehydrate in 70% and 100% ethanol for 1-2 min.

2. Fix for 30 min. at room temperature in the following buffered formal-saline solution at pH 7.0:

- 37-47% formaldehyde	20.0ml
- Sodium chloride	4.25g
- Acid sodium phosphate monohydrate	0.80g
- Anhydrous disodium phosphate	1.30g
- Deionized water	180.0ml

3. Soak for 30 min. at 37° C in 10% chloral hyddrate with 1% pyridine.

4. Incubate for 40 min. in 20% silver nitrate containing 0.1% cupric sulfate with 0.085g of calcium carbonate at the bottom of staining jar. (Solution must be made fresh)

5. Develop in solution of 1% hydroquinone and % % sodium sulfite. Use two baths, the first for 10 sec. And the second for 4 min. (Solution should be made fresh)

6. Fix for 2 min. in 5% sodium thiosulfate.

7. Tone for 4 minutes in 0.2% sodium tetrachloroaurate containing one drop of glacial acetic acid per 100ml. This solution may be used multiple times it the edges and the backs of slides are cleaned before immersion. (Must be discarded if precipitate forms)

8. (OPTIONAL) Darken axons by immersing in 1% oxalic acid 2 min.

9. Immediately fix again for 5 min. in 5% sodium thiolsulfate

10. Dehydrate in 70% and 100% ethanol, then Histoclear for 1 min.

11. Mount slides using drops of permount.