Université de Montréal

Morphology of soleus and EDL Neuromuscular Junctions in the Transected Rat Model Following Fetal Tissue Transplant

par

Rémy J. Pilon

Département de Kinésiologie

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

Maîtrise en sciences (M.Sc.)
en sciences de l'activité physique

Août 2003

Université de Montréal
Faculté des études supérieures

Morphology of soleus and EDL Neuromuscular Junctions in the Transected Rat Model Following Fetal Tissue Transplant

présenté par :

Rémy Pilon

a été évalué par un jury composé des personnes suivantes :

Dr. Phillip Gardiner - Directeur de recherche

Dr. Louise Béliveau – Membre de jury

Dr. Martin Brochu – Membre de jury

Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de
Maîtrise en sciences (M.Sc.)
en sciences de l'activité physique

Août 2003

ABSTRACT

Recently many studies have tried to add to the ever-growing body of knowledge with regards to spinal cord transection by introducing embryonic stem cells at the site of injury with the goal of providing an environment that could promote the restoration of physical function in the transected model.

The present study examined the effect of transection of the spinal cord on neuromuscular junction (NMJ) parameters in rat hind limb muscles (SOL and EDL) 4 weeks post-lesion at the lower thoracic level and the influence of transplanted embryonic stem cells into the transection site. A histological technique modified from that previously described by Pestronk and Drachman (1978) was used for endplate staining. This method allowed for the quantification of the cholinesterase-containing end plate as a demarcated transparent blue area, against which the silver-stained nerve-terminals stood out. This study was composed of three groups of rats: an unoperated control group (group CNTRL, n=3), a spinal transection group (group TRANS, n=3) and a spinal transection plus fetal transplant group (TRNPL, n=3). The NMJ parameters quantified included end-plate area, end-plate longitudinal length, number of nerve terminal branch points and muscle fiber width.

Post transection, the soleus had significant increases in end-plate length, number of branch points and a significant decrease in muscle fiber width. The end-plate length was the only SOL parameter that was attenuated with the transplant post-transection.

Similar to the soleus, the EDL underwent a significant decrease in muscle fiber width after transection. With regards to end-plate (EP) length, EP area and branch points, the parameters were not significantly different from CNTRL values ($p > 0.05$). The transplant procedure did not influence any of these properties. This could be attributed to the more fast-twitch nature of the EDL when compared to the SOL, thus taking longer to undergo end-plate morphological changes.

Though the reproducibility and relevance of these findings have yet to be determined, embryonic stem cell transplants and their influence on neuromuscular junctions hold much interest and promise for future research in the restoration of physical function to those who suffer from spinal cord injuries.

RÉSUMÉ

Récemment, plusieurs études ont utilisé des cellules embryonnaires pour fournir un environnement permettant le rétablissement de fonction physique au modèle qui ont subit une transection expérimentale.

Cette étude a examiné l'effet d'une transplantation des cellules embryonnaires sur des paramètres de jonctions neuromusculaires des muscles SOL et EDL qui ont subi une transection expérimentale au niveau du thorax inférieur.

Une méthode histologique a été modifiée de celle décrite par Pestronk et Drachman (1978) pour teindre les plaques motrices. Cette méthode permet la quantification de la cholinestérase de la plaque motrice comme une région bleue en mettant en contraste les terminales des nerfs rendus argentés par la teinture.

Cette étude est composée de trois groupes de rats: un groupe contrôle (non opéré) (CNTRL, n=3), un groupe transecté (TRANS, n=3) et un groupe transecté avec transplantation fœtale (TRNPL, n=3). Les paramètres de la jonction neuromusculaire (NMJ) comprennent l'aire de la plaque motrice, la longueur longitudinale de la plaque motrice, le nombre de branches neuronales (« branch points ») et la largeur des fibres musculaires.

Après la transection, une augmentation de la longueur de la plaque motrice a été observée pour le soléaire ainsi qu'une augmentation du nombre de branches neuronales et une diminution de largeur des fibres musculaires. Avec la transplantation, la longueur de la plaque motrice a été le seul paramètre atténué.

Comme le soléaire, l'EDL a subi une diminution en largeur de la fibre musculaire. Eu égard à la longueur de la plaque motrice, à l'aire et aux points de branche, les paramètres n'étaient pas significativement différents des valeurs contrôles ($p > 0,05$).

La procédure de transplantation n'a eu aucune influence sur ces propriétés. Ceci peut être attribué au caractère rapide du EDL en comparaison avec le soléaire, d'où une durée plus longue pour le changement morphologique des plaques motrices.

Même si le pertinance ou l'abilité de reproduire ces résultats sont ndeterminé jusqu'à maintenant, l'effet d'une transplantation des cellules embryonnaires surdes jonctions neuromusculaires est un domaine qui devraient être étudier plus à fonds.

TABLE OF CONTENTS

LIST OF DIAGRAMS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATONS

CNS	Central Nervous System
CTRL	Control group
EP	End-Plate
EDL	Extensor Digitorum Longus
ESC	Embryonic Stem Cells
IP	Intraperitoneal
MyHC	Myosin Heavy Chain
NMJ	Neuromuscular junction
SC	Stem cells
SCI	Spinal Cord Injury
SOL	Soleus
TRANS	Transection
TRNP	Transplant

ACKNOWLEDGEMENTS

First I would like to thank Dr, Phillip Gardiner for his inexhaustible enthusiasm and encouragement throughout my studies at the Université de Montréal. His dedication has made my time spent with him a most enriching and rewarding experience, and for this I am truly grateful.

I would also like to dedicate this prolonged work to my parents, Lillian and René Pilon, for all their immeasurable support throughout the years. From a young age, they instilled within me the desire and the "justification for higher education." Thanks to their guidance and tutelage, they have motivated me in every facet of my life, including my recent move to Ottawa, to pursue a career in the pharmaceutical industry. Words simply cannot express how thankful I am to be their son.

I gratefully acknowledge the rest of my family and friends for providing support in their own special ways, and of course my fiancée Catherine Chan, who has inspired me in her own right. So a special thank you to Peter Tzavaris for convincing me to apply and for Dr. Phil Gardiner for pairing Catherine and I together. Sometimes life's greatest surprises can be right before your very eyes.

Lastly, I would also like to thank Dr. Houle, from the University of Arkansas, for generously providing the transected and transplant subjects used in this study.

REVIEW OF LITERATURE

Every sensation, action and thought revolves around the complicated processes of the central nervous system (CNS), which consists of the spinal cord and the brain. Despite over a century of exhaustive research into spinal cord injury (SCI), we still can offer no cure. The Christopher Reeves Paralysis Foundation estimates 250,000 spinal cord injured individuals in the United States, with an additional 11,000 new injuries occurring every year. The majority of these cases occur between the ages of 16 to 30, with the average age of injury being 31. Adaptation to skeletal muscle fibers to increased use and decreased use has been extensively investigated.

Little is known about the adaptations that occur to other components of the neuromuscular system. This review of literature will summarize the current literature regarding spinal cord injury, the transected experimental model and the morphology of the neuromuscular junction. The neuromuscular junction is specifically defined as the synaptic site between a motoneuron and muscle fibers belonging to a motor unit. The NMJ is a dynamic structure that undergoes both morphological and functional changes throughout the course of a lifetime (Sieck & Prakash, 1997).

Though extremely small and measured in micrometers, the NMJ plays a critical role in all neuromuscular functions and its morphology post-transection with fetal tissue transplant has yet to be studied.

SPINAL CORD INJURY

The Christopher Reeves Paralysis Foundation estimates 250,000 spinal cord injured (SCI) individuals in the United States, with an additional 11,000 new injuries occurring every year. The majority of these cases occur between the ages of 16 to 30, with the average age of injury being 31.

The spine supplies the main defense for the spinal cord, providing a protective barrier against injury. A fluid filled area, known as the syrinx, offers additional protection by absorbing shocks (Christopher Reeve Paralysis Foundation, 1999).

Though generally extremely beneficial, both these defenses cause complications upon injury. Swelling induces additional damage to the spinal cord as pressure builds in the confined space between the cord and vertebrae. Though the cord is not usually completely severed during injury, the swelling impedes vital blood supply to neurons, resulting in cell death. The syrinx contributes to the scar tissue that builds up around the area of injury, blocking the neurons from reconnecting once the cord has been severed (Brown and Ironton, 1978).

The complex interactions between the brain and neurons, in combination with the enormous number of individual neurons and synapses, make reconnection of the nerve cells post-transection extremely difficult (Christopher Reeve Paralysis Foundation, 1999).

The types of disabilities associated with spinal cord injury vary greatly depending on the severity of the injury, the segment of the spinal cord at which the injury occurs, and the particular nerve fibers that are damaged. The destruction of nerve fibers that carry motor signals from the brain to the torso and limbs leads to muscle paralysis, while damage to afferent fibers leads to loss of sensation such as touch, pressure, and temperature. There are

numerous other serious consequences which include exaggerated reflexes; loss of bladder and bowel control; sexual dysfunction; lost or decreased breathing capacity; impaired cough reflexes; and muscular spasticity (Christopher Reeve Paralysis Foundation, 1999).

A severe spinal cord injury disconnects the major conduits through which sensory and motor signals pass from the body to the brain and vice versa. It was believed this condition was irreversible because it was thought that the environment of the central nervous system was inhibitory to neurite growth. The potential for regeneration and extension of the CNS axons was recognized by the turn of the century by Ramon y Cajal (1928). Cajal and Camillo Golgi received the Nobel Prize in 1906 for introducing the silver-chromate stain. With his reduced silver nitrate technique he was able to demonstrate neurons and their connections quite easily. His introduction of his gold chloride-mercury bichloride technique to demonstrate astrocytes was a monumental contribution as was his work on degeneration and regeneration of the nervous system. Cajal and Golgi (1928) hypothesized that in a permissive environment, axons would be able to grow for long distances following a CNS lesion.

This is a hypothesis, though nearly a century old, still raises questions and encourages researchers to further investigate the morphology of the neuromuscular junction.

RESTORATION OF FUNCTION FOLLOWING TRANSECTION

The inability of the axons of the central nervous system to regenerate across lesions in the spinal cord is an extremely puzzling phenomenon. The sprouting nerves will simply not traverse the lesion and consequently cannot reach their targets to form connections and restore function. Contrastingly, a lesion of a peripheral nerve need not be followed by permanent disability. Peripheral axons have been shown to reinnervate skeletal muscles (Bregman et al., 1993; Kunkel-Bagden & Bregman, 1989), allowing for controlled movements post lesion. This discrepancy between central and peripheral nerves that exists in the mammalian species, does not exist in lower vertebrates or invertebrates. The leech for example, after complete transection of the CNS, can grow and reconnect nerves and targets with astonishing precision (Nicholls, 1987).

McDonald and colleagues (1999) from Washington University School of Medicine successfully implanted embryonic stem cells in laboratory rats. They induced mid-thoracic spical cord injury in the rat using a 2.5 mm diameter metal rod in diameter resulting in paralysis. Nine days after the injury, McDonald et al. transplanted roughly 1 million ES cells in the cavity. Two weeks after the transplantation ES cells filled the area normally occupied by glial scarring. After five weeks the stem cells had migrated further away from the implantation site. Although a number of them had died, there was still enough raw material to have a growing supply of neurons and glial cells. Most of the surviving cells were oligodendrocytes and astrocytes, but some neurons were found in the middle of the cord. The rats had actually regained limited use of their legs. Paralysis had apparently been cured at least partially.

Though McDonald's work represented new successes in stem cell technology, there is much work ahead of us before we might test any of this technology in humans. Scientists are achieving results, though they do not fully understand the mechanisms or factors behind controlled nerve regeneration. McDonald et al. (1999) hypothesized that the regaining of function was attributable to the few differentiated neurons. Perhaps function was regained due to embryonic stem cells producing growth factors. Complications could also arise from such technology. One example pertains to the rejection of foreign bodies by the host's immune system. Cyclosporin is most commonly used in rats, but the human body promises to be much more complicated. The brain and spinal cord are complex and mysterious, and until science can predict the exact effects of our evolving technology, no testing on humans can occur.

But before these questions can be answered, a successful restoration of physical function post-transection must be achieved on animal models prior to any ethical research on humans may be attempted.

SPINAL CORD TRANSECTION (EXPERIMENTAL MODEL)

Transection is a preferred experimental method by which to study neuromuscular inactivity since there is no direct damage to the muscle and the innervation is not physically disrupted. Spinal cord transection is a transverse cutting of the spinal cord resulting in loss of all sensations and voluntary movements inferior to the lesion (Dupont-Versteegden et al., 1998). Except for reflex movement, transection produces unmistakable lesions that result in qualitative and quantifiable motor deficits (Houle, 1988).

Typically, following a laminectomy in an anesthetized animal, the pia mater is cut and a small glass micropipette is used to aspirate spinal cord tissue until a cavity about 2 mm long is created. Motoneurons caudal to the transection lose neural input, affecting both their tonic and phasic firing patterns, and influencing their electrophysiological and metabolic activities (Houle and Reier, 1988).

Characteristic changes in muscles after spinal cord injury include decrease in gross muscle size, atrophy of individual myofibers, and altered expression of myosin heavy chain proteins, such that the type II isoforms become prevalent (Lieber et al., 1986). The rapid transformation from a slow to a fast phenotype, after a spinal cord transection, supports the observation that appropriate neural activity is an important component in maintaining the expression of slow characteristics of the muscle (Lieber et al., 1986). Neural input or muscular activity (active or passive) are not the sole regulatory mechanism of muscular phenotypic expression. Humoral signals, particularly thyroid hormones, can positively influence fast fiber transformation, independent of the state of innnervation (Iannuzo et al., 1977).

REGENERATION IN PERIPHERAL VS CENTRAL NERVOUS SYSTEM

The CNS environment does not favour axonal growth. However in the PNS, severed axons sprout to restore normal function, partly due to the assistance of Schwann cells. These cells have been associated with assisting extending nerve processes in response to denervation and were found to be associated with most nerve sprouts in partially denervated muscle. Schwann cells have been shown to both induce and guide the process of axonal sprouting (Son and Thompson, 1995).

Schwann cells that make up the myelin do not die post-transection, but in fact stay where they are allowing for a new sprouting axon to use the previously existing pathway (Son and Thompson, 1995). When the axons start to grow, they can travel through the same "tunnel" that an axon previously occupied, and eventually reach the same location of the skin or muscle.

Contrastingly, in the CNS, the most axons can grow is a few mm. Part of the problem is that the Schwann cell homologue in the CNS is the oligodendrite, which does not remain to assist in the regrowth of severed axons. To make matters worse, oligodendrites possess molecules on their surface that are inhibitory to axonal growth cones (Schwab, 1988). This inhibitory effect can be neutralized by monoclonal antibodies. Schwab used these antibodies in the transected cat and axons were seen to regenerate past the lesion for distances over 1 centimeter, which was significantly greater than in the control groups. Though the number of regenerated axons was deemed too few to have any significant functional effect, Schwab (1990) concluded that the antibodies were clearly making the CNS environment less inhibitory to axonal growth.

In an attempt to promote favorable conditions for axonal growth around the site of an injury in the CNS, numerous experimental approaches have been utilized (Guth et al., 1993). There are two main strategies currently being used to overcome this inability of the neurons in the CNS to regenerate new axons thus reinnervating the appropriate post-synaptic neurons.

The ***first*** consists of manipulating the molecular environment to facilitate regeneration. It has been found that there are a number of molecules that will either promote or inhibit axonal regeneration (Guth et al., 1993). These generally include a number of nerve growth factors that work primarily by increasing the expression of certain genes necessary for axon elongation.

The ***second***, of great interest to this report, centers around the bridging of the lesion with the utilization of transplant tissue that is conducive to growth into the lesioned cord. In this strategy, the transplant acts as a "bridge" for the central neurons to grow on. Transplants have resulted in axonal growth, a reduction of muscle atrophy and even reinnervation (Reier et al., 1983; Howland et al., 1995(a); Miya et. al., 1997; Houle et al., 1999).

It has been reported that spinal lesions sparing even small amounts of tissue permit considerable function in the cat (Blight and DeCrescito, 1986), therefore one would assume that increased function might be achieved when the spinal cord is repaired by a transplant, even without complete restitution of all the original pathways (Miya et al., 1997). Despite having a significantly diminished number of intact ascending and descending axons, patients with subtotal spinal cord transection generally have limited function. Fawcett (1992) hypothesizes that perhaps what might seem an insignificant number of axons, should they be of the right kind and reconnecting the right areas, just might be of "considerable functional benefit." He goes on to add that this might be arranged by the addition of Schwann cells or peripheral nerve grafts. "The limitations of such grafts pertain to the fact that the axons that

grow through the graft cannot penetrate far enough into spinal cord tissue distal to the graft to make useful connections (Kuhlengel et al., 1990).

Younger subjects generally possess the ability to accept transplanted fetal tissue and regrow axons better than senescent individuals. Therefore, it has been hypothesized that fetal donor tissue would hold more promise than adult tissue transplant since it possesses a greater ability for growth and sprouting (Kunkel-Bagden & Bregman, 1989).

A fetal transplant into the site of spinal transection can influence both regeneration of axons and recovery of motor functions in the rat. Howland et al. (1995b) used embryonic spinal transplants that were placed into the site of a complete midthoracic spinal transection in kittens, and observed that it permitted the development of locomotion that exceeded that of littermates with transections alone.

Fawcett (1992) postulated that this problem may be somewhat improved by employing a combination of treatments, including but not limited to: nerve growth factors to neuronal cell bodies and axons to enhance outgrowth, blockers of oligodendrites inhibition and astrocyte active factors for regeneration of axons leaving the graft to penetrate the spinal cord thus making the appropriate neural connections.

TISSUE TRANSPLANT:

A MEDIUM TO ENCOURAGE AXONAL REGENERATION

Since the early studies of Ramon y Cajal (1928), there has been much speculation that immature mammalian CNS might show regeneration after spinal injury. A trend in spinal cord research centers around transplanting pieces of fetal spinal cord tissue into the site of the lesion of transected recipients (Houle al., 1999; Kunkel-Bagden & Bregman 1989; Miya et al. 1997). The strategy behind transplanting grafts involves establishing a "bridge" which might assist axon elongation through and beyond the injury. It has been proposed that the transplants provide an environment that supports growth of axotomized and late developing axons and a rescue of neurons destined to undergo retrograde cell death (Miya et al., 1997). Transplants are thought to rescue severed neurons by serving as a surrogate source of target-derived neurotrophic factors (Bregman and Reier, 1986).

Researchers have been in agreement that transplantation of adult nerve tissues does not work, while embryonic or fetal transplantation can be quite successful (Houle, 1991). In other words, transplants may restore some elements of spinal circuitry or otherwise affect a reorganization of host tissues.

There is evidence that axonal integration between the transplant and host spinal cord occurs, either directly by growth of host axons through the transplant or indirectly by a relay of mechanisms involving synaptic contacts between host and transplant neurons (Bregman et al., 1997; Houle and Reier, 1988).

In many cases, the growth of descending pathways into the fetal transplants was substantially greater after lesions at birth than at maturity, in both rats and cats (Bregman et al., 1993; Bregman and Goldberger, 1982). The capacity for the CNS neurons for axonal regrowth after injury decreases as the age of the animal at the time of injury increases. In the adult, Bregman et al. (1993) demonstrated that the growth is restricted to within 200 um of the host/transplant border. Studies utilizing neonatal spinal rats given transplants seemed to produce optimism for the transplant theory in rats (Iwashita et al., 1994). When a transplant was inserted into a lesioned cord of a newborn, the host axons descend into the transplant and exhibit extensive regeneration growth into and beyond the transplant.

Houle et al. (1999) compared the effects of a fetal transplant graft versus passive hindlimb cycling in the SOL. They found that the graft had no effect on myosin heavy chain (MyHC) expression, though it did limit muscle atrophy after complete transection of the spinal cord. Muscle fiber cross sectional areas (CSA) of the transection (TRANS) group was 50% that of the control (CNTRL), while the transplant (TRNPL) and exercise groups were 77% and 74% when compared to CNTRL. It was suggested that these results support the notion that a different regulatory mechanism for the control of muscle size and for the expression of proteins that are considered important for muscle contractility.

Studies have shown that spinal lesion sparing small amounts of tissue permits considerable function (Blight and DeCrescito, 1986). One might postulate that function may be possible with a transplant, even without full restoration of normal pathways. Intraspinal transplants of fetal spinal cord have been shown to enhance the development of locomotor performance after complete transection in the newborn rat (Diener and Bregman, 1994; Miya et al., 1997).

MORPHOLOGY OF THE NEUROMUSCULAR JUNCTION

It has long been demonstrated that activity directly affects the morphology of the mammalian motoneuron (Mann, 1894). The neuromuscular junction is specifically defined as the synaptic site between a motoneuron and muscle fibers belonging to a motor unit. This dynamic structure undergoes both morphological and functional changes throughout the course of a lifetime (Sieck & Prakash, 1997).

Various studies have demonstrated that, in some ways, the structure of the NMJ is specific to muscle fiber type (Brown et al., 1980). The literature has shown that both the morphology and the physiology of the NMJ have been shown to adapt to both increased and decreased levels of activity (see review: Panenic and Gardiner, 1998). Increased activity for instance aerobic or anaerobic types of exercise stimulates adaptations that enhance neuromuscular transmission, thus improving muscular performance. On the contrary, decreased activity of this myoneural synapse, due for instance to limb immobilization or spinal cord transection initiates morphological changes that can impair transmission and function.

Both complete and incomplete muscle disuse appears to bring about similar signs of NMJ degeneration and regeneration (Fahim, 1989; Fahim and Robbins, 1986). Adaptations to disuse include the number and size of structural components (Deschens et al., 1993), as well as the functional characteristics (Robbins and Fischbach, 1971; Brenner and Rudin, 1989) of the NMJ.

Some of the structural adaptations from muscle disuse include motor end-plate expansion (Eldridge et al.1981), enlarged presynaptic nerve terminal area, amplified branch

number, increases in sprouting and complexity of nerve terminals (Tomas et al., 1989). Motor units seem to exist in a dynamic state and display a high degree of plasticity (Mendell, 1994). This plasticity is inherent in the motor unit and has been attributed to the developmental process and to changes in the adult, which are under continual influence by external stimuli (Mendell, 1994; Navarrette and Vrbova, 1993).

SPROUTING OF MOTONEURONES

Total disuse rapidly affects both pre- and post-synaptic characteristics of the mammalian NMJ. The most prevalent changes include increased sprouting at nerve terminals, enhanced synaptic transmission, and atrophy of post-synaptic folds. Sprouting is the production of new processes (outgrowths) by nerve cells. During the embryonic stages sprouting is referred to as undergoing primary differention, and redifferentiating when it occurs in the adult neurons in response to nervous tissue damage or in dissociated neurons.

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. Brown et al. (1980) also observed sprouting upon inducing denervation-like changes in muscle by simply blocking nerve-induced activity.

Motor nerve terminals have the ability to sprout under a variety of normal and pathological conditions. Continual sprouting occurs at he 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.

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.

It has been suggested that that this atrophy is initiated by the disruption of the NMJ that leads to progressive end-plate degeneration and gradual withdrawal of the terminal axon (Stebbins et al., 1985). 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.

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

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 innervating 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.

Sprouting is evident at the microscopic level by formation of new nerve growth either along the axon or endplate. Motoneuron sprouting was documented in the late eighteen hundreds and was thought to be a possible explanation for the lack of degenerating fibers observed in partially denervated muscle (Exner, 1884).

Following partial denervation, fast muscles motoneurons showed more sprouting than their slow muscle counterparts, though the production of sprouts was similar subsequent to complete paralysis with botulinum toxin (Brown et al., 1980). Brown & Ironton (1977) hypothesized that the signal for terminal sprouting originated from the denervated muscle but also proposed that the origins for nodal sprouting may be linked to nerve degeneration.

The only common denominator for the sprouting stimulus has been reported as either an inactive muscle or a muscle state showing characteristic of denervated muscle (Brown et al., 1980). Brown & Lunn (1988) later revised their conclusions to include central changes such as chromatolysis in the cell body, loss of afferent input and in addition to peripheral changes, altered neuronal interactions.

Van Mier and Lichtman (1994) have published convincing evidence for the presence of a growth signal emanating from inactive muscles that attracts nearby axons to form new connections. Their results showed that damaged muscle fibers provided a directional sprouting signal that was detected by nearby axons. It was also noted that in cases where the original endplate areas were left intact, new sprouts avoided the areas but nonetheless made contact with the regenerating fibers. The sprouts were subsequently displaced by

reinnervation of the original axon at the original site. However, if both the fiber and its original terminal site were destroyed, there was more extensive reinnervation of the fiber and some evidence for a protracted competition between newly arrived sprouts, until one contact became the permanent and sole contact.

During the mid-nineteen hundreds, new nerve growth was observed to be "sprouting" from the Nodes of Ranvier and from the terminals of intact nerves of partially denervated muscles (Hoffman, 1950). In addition, Hoffman observed these new growths made contact with the denervated muscle fibers and underwent remyelination, suggesting that these were new *functional motor units*. This reorganization by the residual systems is seen as an attempt to restore functions to damaged areas and has been shown to restore functions in the partially denervated model.

Sprouting occurs relatively rapidly and its extent is quantifiable by the measuring of branch points. The literature re-iterates that it is important to note that these are probably overestimates of the time required for the sprouting response to occur because the laboratory techniques utilized may not be sensitive enough to pick up early morphological changes.

FIBER TYPE DIFFERENCES WITH REGARDS TO THE NMJ

Differences between type I and type II muscle fibers have long been documented. Granit et al. (1957) discovered that the nerves innervating white muscle (fast/type II) were larger than those innervating red muscle (slow) and suggested a functional implication for the difference in diameter.

Postulations for these differences between the types of fibers pertained to the intrinsic speed differences of the muscle that are at least partially regulated by neural mechanisms (Buller et al., 1960). Wigston (1989) suggested that the degree of remodeling post-transection of the NMJ may vary among fast and slow twitch muscles, perhaps attributable to the pattern of use.

Duchen (1970) reported that this distinction in sprouting, between fast and slow muscles, persisted up to 4 weeks after transection. The deep portion of the fast muscles (plantaris and gastrocnemius) resembled the soleus in terms of the number of sprouts, terminal morphology and cholinesterase distribution 28 days post-transection. The superficial regions of the fast mixed muscles began to show some sprouting but still contained a predominance of non-sprouted nerve terminals.

Dramatic differences were observed in the sprouting responses of motor units from paralysed (botulinum toxin) soleus and gastrocnemius muscles and even amongst motor units from different regions of the same muscle (gastrocnemius and plantaris). The data showed nearly immediate extensive sprouting in slow soleus, somewhat less extensive in the deep regions of the gastrocnemius and plantaris, and absence in the highly concentrated type IIb superficial regions (Duchen, 1970).

In addition, fast and slow muscles responded differently to periods of paralysis induced by injection of tetanus toxin, a presynaptic blocking agent. The injection of tetanus toxin elicited terminal sprouting in the soleus, though these new growths were not observed in the extensor digitorum longus muscle (Duchen and Tonge, 1973).

It has been suggested by Brown et al. (1980) that the relatively fast muscles have less sprouting upon denervation than when compared to their slow muscle counterparts. This reduction of nerve morphology can be attributed to their higher resistance from a reduced neuronal input.

Traditionally, the sprouting of the soleus is seen as a direct result of changes in muscular activity and/or neural input as in transection or limb-immobilization. Whereas the traditional sprouting for the EDL is generally associated with the natural process of aging. This has been attributed to the fact that slow-twitch fiber is mainly responsible for postural and everyday functions, whereas the utilization of the fast-twitch muscles generally decline with senescence. For a more detailed analysis of morphological adaptation differences between fiber types, including sprouting, see Sieck and Prakash (1997).

SUMMARY OF THE LITERATURE

Damage to central nervous system axons is devastating. There has been much effort to increase regenerative capacity experimentally. The utilization of transplant tissue to enhance reparation has been most promising in recently. Specifically, transplant tissue inserted into the transected spinal cord has spared muscle tissue innervated by axons caudal to the transection. Though this is an extremely promising step for spinal cord repair, the mechanisms remain unknown.

The effect of transplant tissue on transected nerve terminal morphology remains completely unknown.

PURPOSE OF THE STUDY

Numerous studies have looked at the potential of fetal transplants for CNS restoration in the spinal cord transection model. However, in comparison, only modest research has studied the morphological effects of these transplants on the NMJ.

The purpose of this study was to analyze morphological parameters of the neuromuscular junctions in hindlimb muscles (soleus and EDL) of rats following spinal cord transection, with and without a fetal transplant procedure performed at the time of transection. Due to the differing nature of fast and slow muscle fibers (Sieck and Prakash, 1997), one would expect the embryonic transplant to elicit different responses in the SOL and EDL end-plates. Due to its slow-twich properties, it is hypothesized that the transplant would attenuate the EP morphological changes generally seen post-transection in the SOL more so than in the EDL.

The parameters quantified were endplate area, end-plate longitudinal length, branch points within the endplate and muscle fiber width.

WHY WERE THE EDL AND SOLEUS SELECTED?

The present investigation assessed different morphological parameters of nerve terminals from control (CNTRL), transected (TRANS) and transection-transplanted groups (TRNP). The slow-twitch soleus and the phasic fast-twitch EDL muscles were selected for several specific reasons:

a) The soleus and EDL perform functionally different actions (flexion and extension, respectively);

b) There are differences in their firing patterns during rest (Fischbach and Robbins, 1969; Navarrette and Vrbova, 1980);

c) The EDL and soleus are composed primarily of types II and I fibers, respectively (Fahim et al., 1984; Panenic and Gardiner, 1991).

MATERIALS AND METHODS

1. SUBJECTS

All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the animal ethics committee of the University of Montreal. A total of 9 adult (16 to 24 weeks old) female Sprague-Dawley rats (6 from University of Arkansas, 3 from Charles River, St-Constant, Quebec) were studied. The animals received a standard rat diet (ProLab RMH 4018) and water ad libitum. They were housed in an environmentally controlled facility (12 hours light- 12 hours dark cycle, 22°C).

Female rats were chosen because they tend to gain weight less rapidly than males when eating ad libitum, and therefore there is less of a difference in body weight between controls and treated rats when the treatment influences food intake (Harpur 1980).

The study was composed of three groups: an unoperated control group (group CNTRL, n=3), a spinal transection group (group TRANS, n=3) and a spinal transection plus fetal transplant group (TRNPL, n=3).

2. SURGICAL PROCEDURES

The rats from the University of Arkansas underwent the following surgical procedures.

2.1 Spinal Cord Transection

The rats were anesthetized using an intraperitoneal injection of ketamine/xylazine and the vertebral column at the thoracic level T9-T10 was exposed by a dorsal laminectomy of vertebral bone. After opening the dura mater the dorsal spinal vein was cauterized at two places, separated by about 6 mm, limiting extensive bleeding. A slit through the pia mater was made and a small glass micropipette was used to aspirate spinal cord tissue until a cavity about 2 mm long was created. The cavity extended completely to the lateral and ventral borders of the meninges. Bleeding in the cavity was controlled with gelfoam, which was left in place while the tissue for transplantation was being prepared (in the case of the TRNPL rats). The dura mater was sutured closed with 10-0 silk thread. Animals received antibiotics for 1 week post-op and an IP injection of glucose-saline immediately post-op. The urinary bladder required manual expression 2-3 times per day for about 2 weeks. Rats were monitored daily for signs of urinary tract infection or self-mutilation of paralyzed hind limbs (Houle, 1988).

2.2 Transplanted embryonic tissue

A pregnant dam was anesthetized with chloral hydrate (4% solution, 1 ml/100 gram body weight). 1-2 embryos were removed from the uterus and immersed in a Hank's balanced salt solution. The embryo was pinned to a wax plate, dorsal side up, and the spinal cord was carefully dissected from the embryo with microforceps and tungsten wires that had been sharpened. Spinal cord tissue was cut into small segments and passed through a graded series of smaller hypodermic needles, up to about 28 gauge. This created a coarse slurry of tissue that can be transferred by micropipette to the lesion cavity in the adult rat spinal cord. The cavity was kept fairly dry at the time of transplantation and it was critical that no air bubbles be present in the tissue after the transplantation procedure. The dura mater was sutured closed with 10-0 silk thread. As with TRANS rats, the TRNPL rats received antibiotics for 1 week post-op and an IP injection of glucose-saline immediately post-op. The urinary bladder required manual expression 2-3 times per day for about 2 weeks. Rats were monitored daily for signs of urinary tract infection or self-mutilation of paralyzed hind limbs. All operated animals were either transected, or had a transplant for 4 weeks, +/- three days (Houle, 1988).

3. HARVESTING OF TISSUES

Following the treatment period (4 weeks, +/- 3 days), rats underwent a 12-hour terminal experiment under ketamine/xylazine anesthesia which involved electrophysiological recording from spinal motoneurones. These experiments were not part of the present work but were performed off site by Dr. Houle (Arkansas). At the end of the experiments, the rat was killed with an overdose of anesthetic. Hindlimb muscles were quickly removed, laid on ¼-inch cork material such that all visible slack was removed, covered with paraffin embedding compound, and frozen in melting isopentane. Muscles frozen in this manner included soleus (SOL) and the extensor digitorum longus (EDL). Tissue samples were subsequently transferred to a storage freezer kept at a temperature of –80 °C, where they were kept until later cutting and histochemical processing.

4. HISTOCHEMICAL ANALYSIS

Excised muscles were removed from the freezer (-80 °C) and were placed into the cryostat (-14°C) 30 minutes prior to cutting. Longitudinal sections, 50um thick, were cut in the cryostat, and placed on plastic slides. They were submerged in cooled 3 % EDTA solution for at least 1 minute to prevent contraction, and were subsequently allowed to air-dry at room temperature.

Histochemical processing of the end plates used a technique modified from that previously described by Pestronk and Drachman (1978). Cholinesterase staining was performed by a bromoindoxylacetate technique detailed in Appendix A. Nerve terminals were demonstrated by the technique detailed in Appendix B. This method allowed for the quantification of the cholinesterase-containing end plate as a demarcated transparent blue area, against which the silver-stained nerve-terminals stood out.

The following measurements were taken:

- Area of the end-plate, as outlined by the blue cholinesterase stain (Figure I);
- The longitudinal length of each endplate, as outlined by the cholinesterase stain, parallel to the length of the muscle fiber (Figure II);
- The number of nerve terminal branch points within each end plate (Figure III);
- Muscle width, as a measure of atrophy of the muscle in question (Figure IV).

Reference: Pestronk and Drachman (1978)

Diagram 1: Cholinesterase Staining of End-Plate Areas

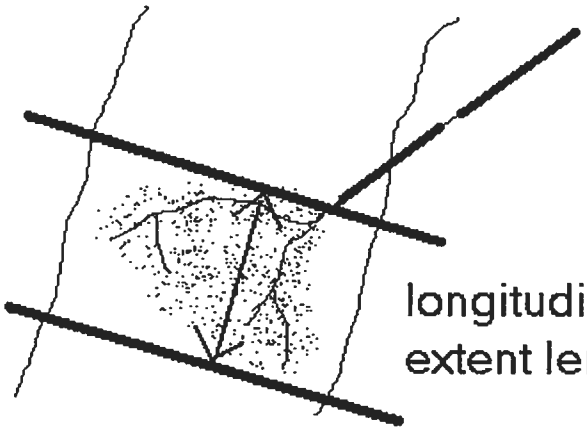


Diagram 2: Measurement of End-Plate Longitudinal Length

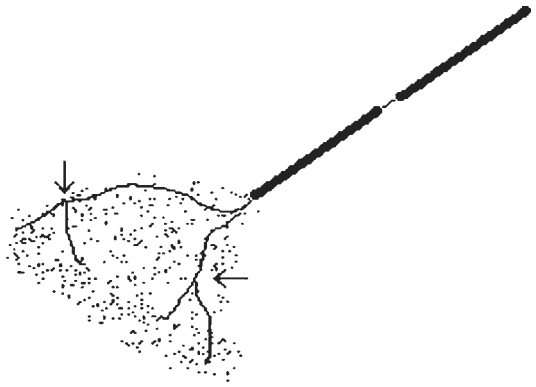


Diagram 3: Calculation of End-Plate Branch Points
*(within the end-plate: **indicated by** ↑)*

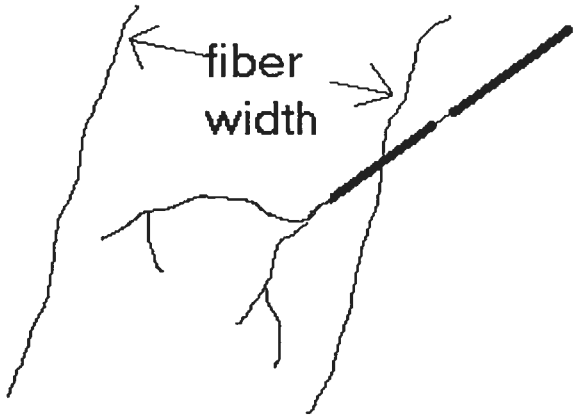


Diagram 4: Measurement of Fiber Width

Data were collected and recorded until the pool of data of each parameter (ie: EP area, EP length, branch points and fiber width) attained a minimum of 145 entries. No more than 4 EPs were measured from any one slide. An EP was selected if a least 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 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.

5. 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 (Scheffe's test) was used to determine significance of the difference between specific means. A probability level below .05 was considered significant.

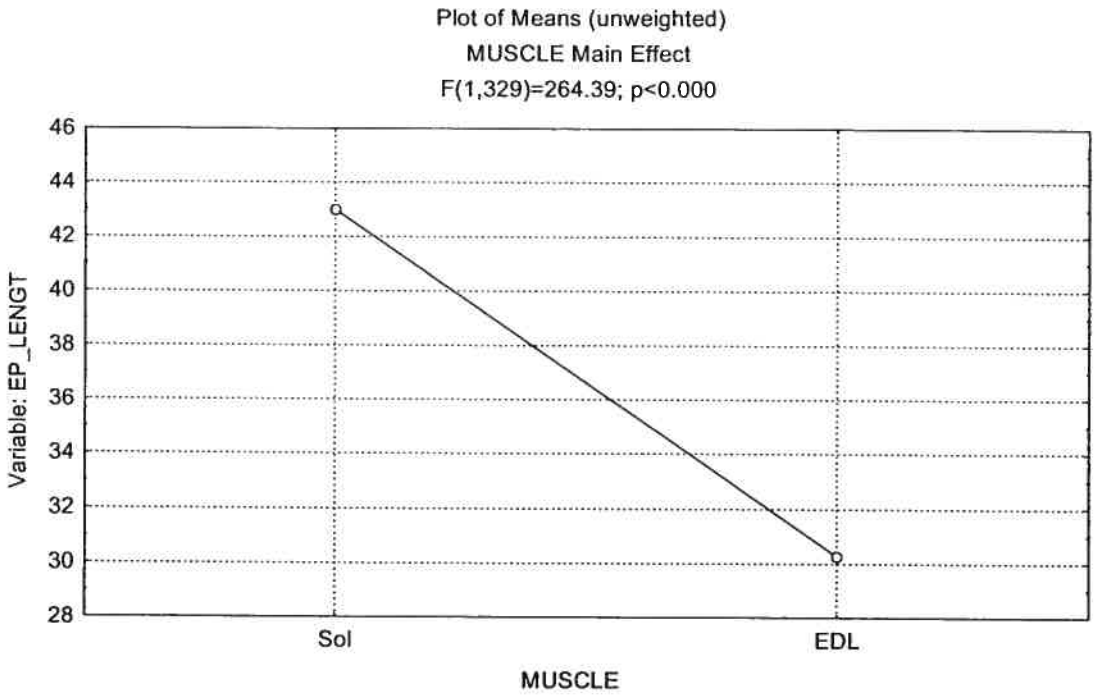
RESULTS

EP_LONGITUDINAL_LENGTH:

MUSCLE DIFFERENCE

Statistical analysis uncovered a significant main effect ($p<0.05$) between the end-plate lengths of the EDL and SOL (Figure 1). The longitudinal lengths of the soleus endplates were on average 43.3% larger than those of EDL.

Figure 1: Muscle Main Effect on End-Plate Length

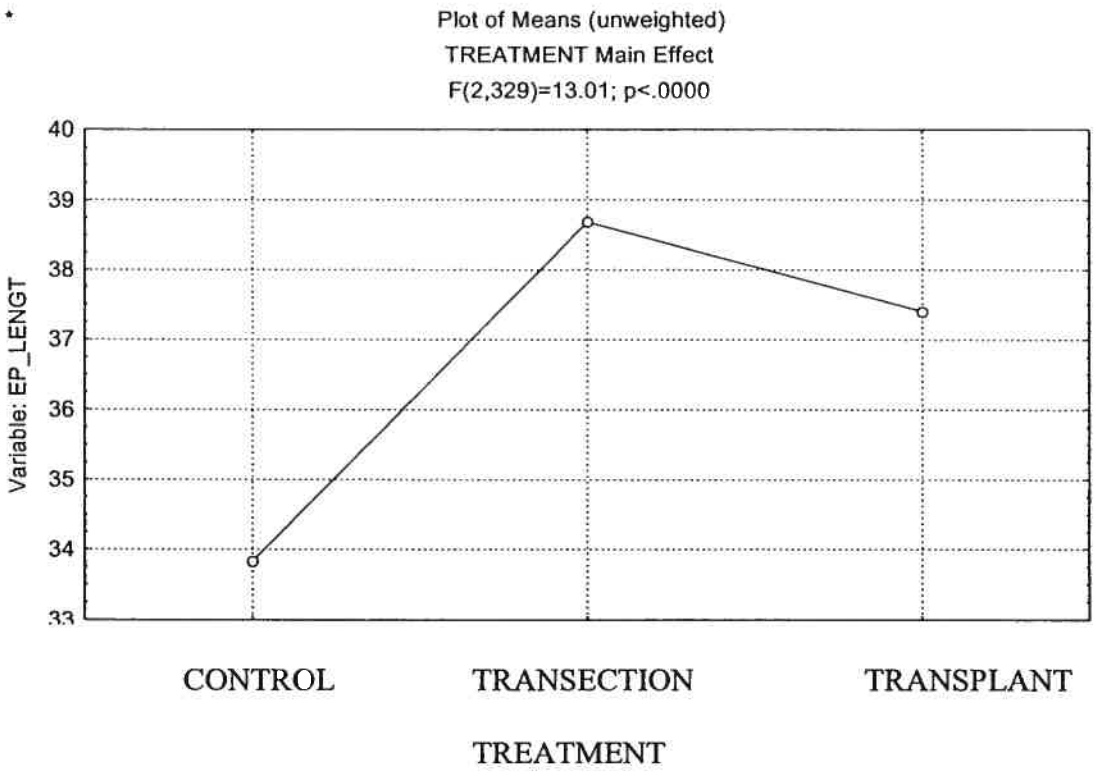


Sol: Soleus
EDL: Extensor Digitorum Longus

TREATMENTS

Statistical analysis uncovered a significant main effect ($p<0.05$) between the different treatments (Figure 2). TRANS and TRNPL groups were significantly different (larger) than control. The TRANS and TRNPL groups were not significantly different from each other.

Figure 2: Treatment Main Effect on End-Plate length



Soleus:

Table 1 lists the EP lengths (±SD) for the data presented in the Figures 3 and 4. The Scheffe test found significant difference between the SOL CNTRL and SOL TRANS groups ($p<0.05$). There was no significant difference between the TRNPL and either the CNTRL or TRANS groups ($p>0.05$; $p=0.069$, $p=0.43$, respectively), indicating that the transplant procedure tended to attenuate the increase in sol endplate length that occurred with transection alone.

EDL:

There was no significant difference found between any of the EDL groups.

TABLE 1. EP lengths for SOL and EDL (μm) ± S.D. of data from Figures 3 & 4.

	GROUPS		
MUSCLES	**CNTRL**	**TRANS**	**TRNPL**
SOL	39.0 ± 8.1	46.4 ± 8.6 *	43.7 ± 8.6
EDL	28.7 ± 4.3	31.1 ± 4.8	31.2 ± 5.5

*** Significantly different from CNTRL Value ($p < 0.05$)**

Figures 3 & 4 illustrate the distribution of data for SOL and EDL EP lengths for the three treatments according to their respective percentile.

Figure 3: SOL EP length distribution according to percentile for CNTRL, TRANS and TRNPL.

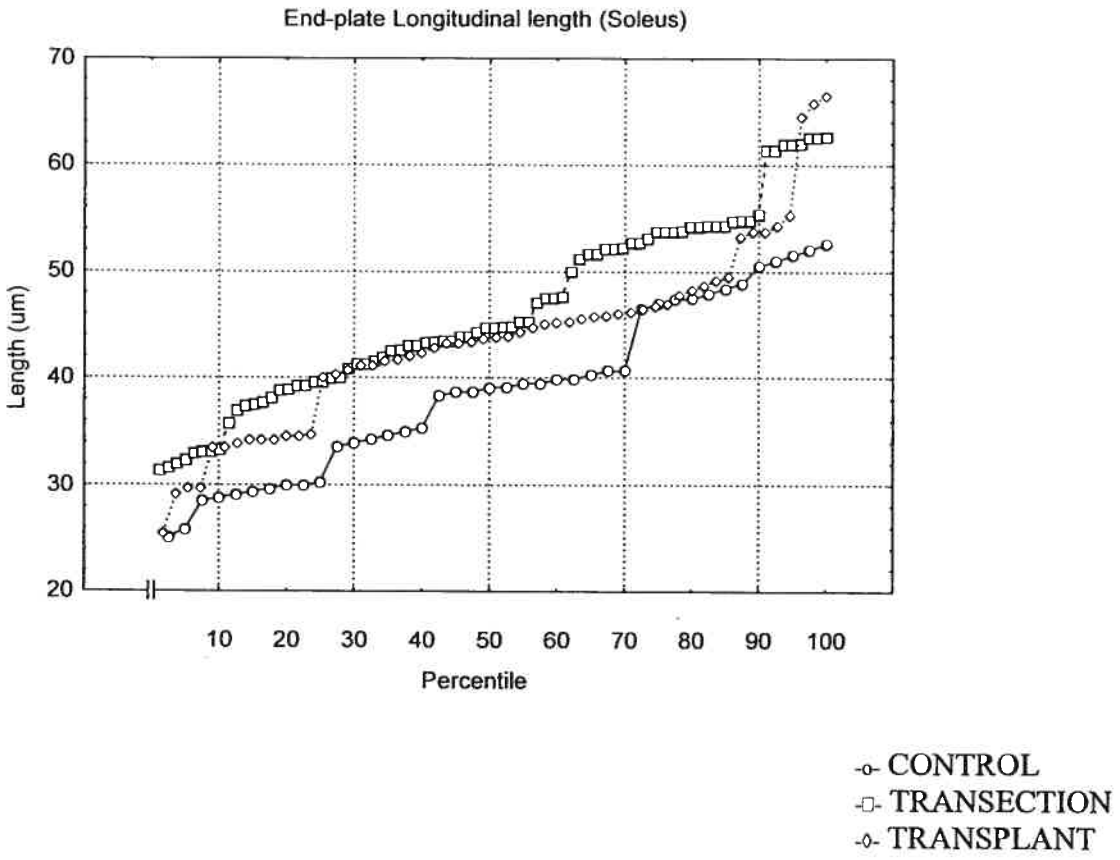
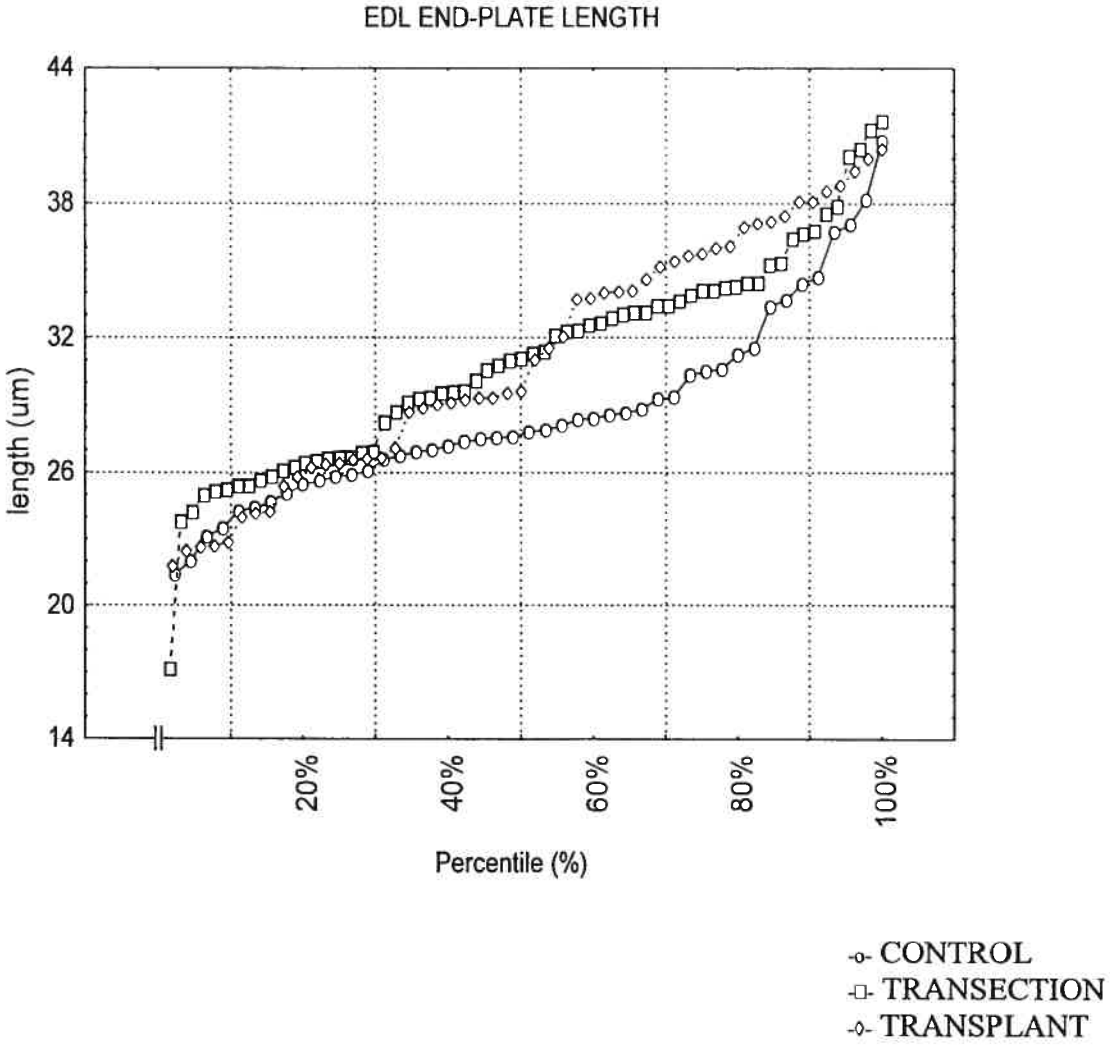


Figure 4: EDL EP length distribution according to percentile for CNTRL, TRANS and TRNPL.

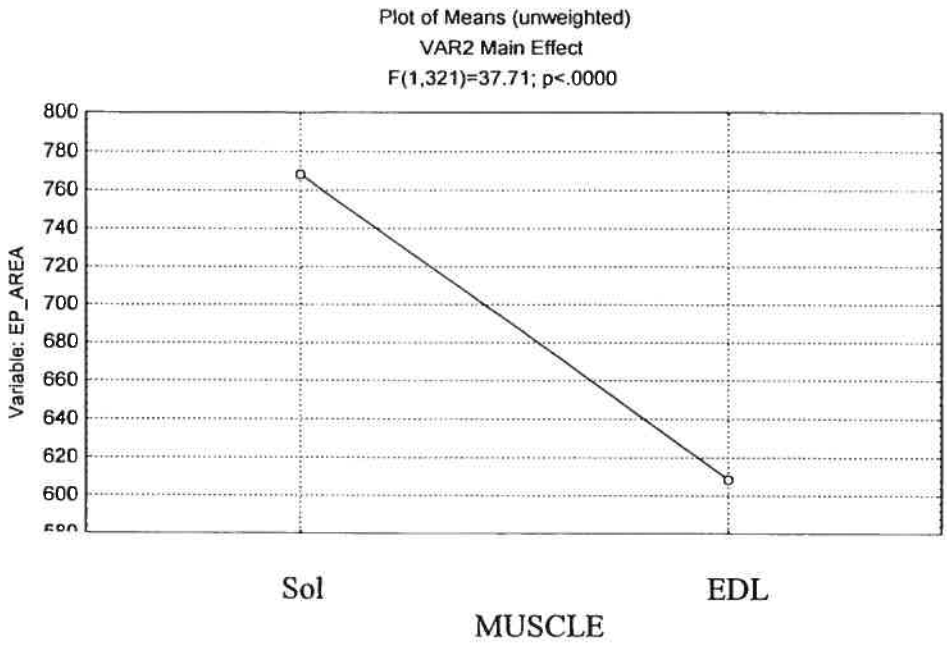


EP AREA:

MUSCLE DIFFERENCE

Statistical analysis uncovered a significant main effect ($p<0.05$) between the end-plate areas of the EDL and SOL (Figure 5). The area of the SOL endplates was 26.2% greater than that for EDL endplates.

Figure 5: Muscle Main Effect Of End-Plate Area

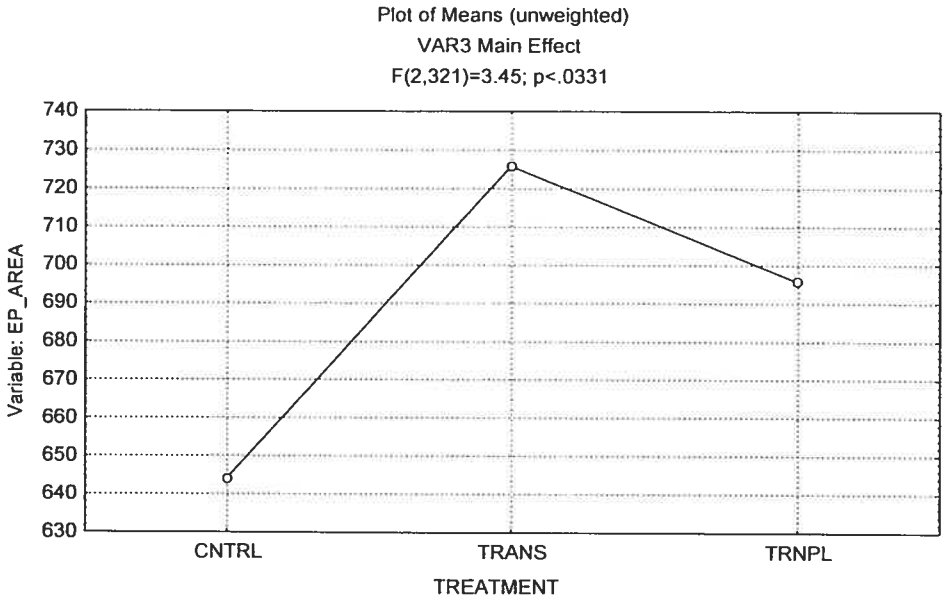


Sol: Soleus
EDL: Extensor Digitorum Longus

TREATMENT

Statistical analysis uncovered a significant main effect ($p<0.05$) between the end-plate areas with regards to the treatments (Figure 6).

Figure 6: Treatment Main Effect on end-plate area



CNTRL: CONTROL
TRANS: TRANSECTION
TRNPL: TRANSPLANT

Soleus:

Table 2 lists the EP areas (±SD) for the data collected from the SOL & EDL with regards to end-plate area. The Scheffe test found no significant difference between any of the SOL groups ($p>0.05$).

EDL:

There was no significant difference found between any of the EDL groups ($p>0.05$).

TABLE 2. EP areas (μm^2) ± S.D.

	GROUPS		
MUSCLES	**CNTRL**	**TRANS**	**TRNPL**
SOL	716.9 ± 278.3	824.7 ± 268.1	763.4 ± 215.7
EDL	571.3 ± 239.3	626.9 ± 176.7	628.0 ± 208.1

Figure 7: SOL EP area distribution according to percentile for CNTRL, TRANS and TRNPL.

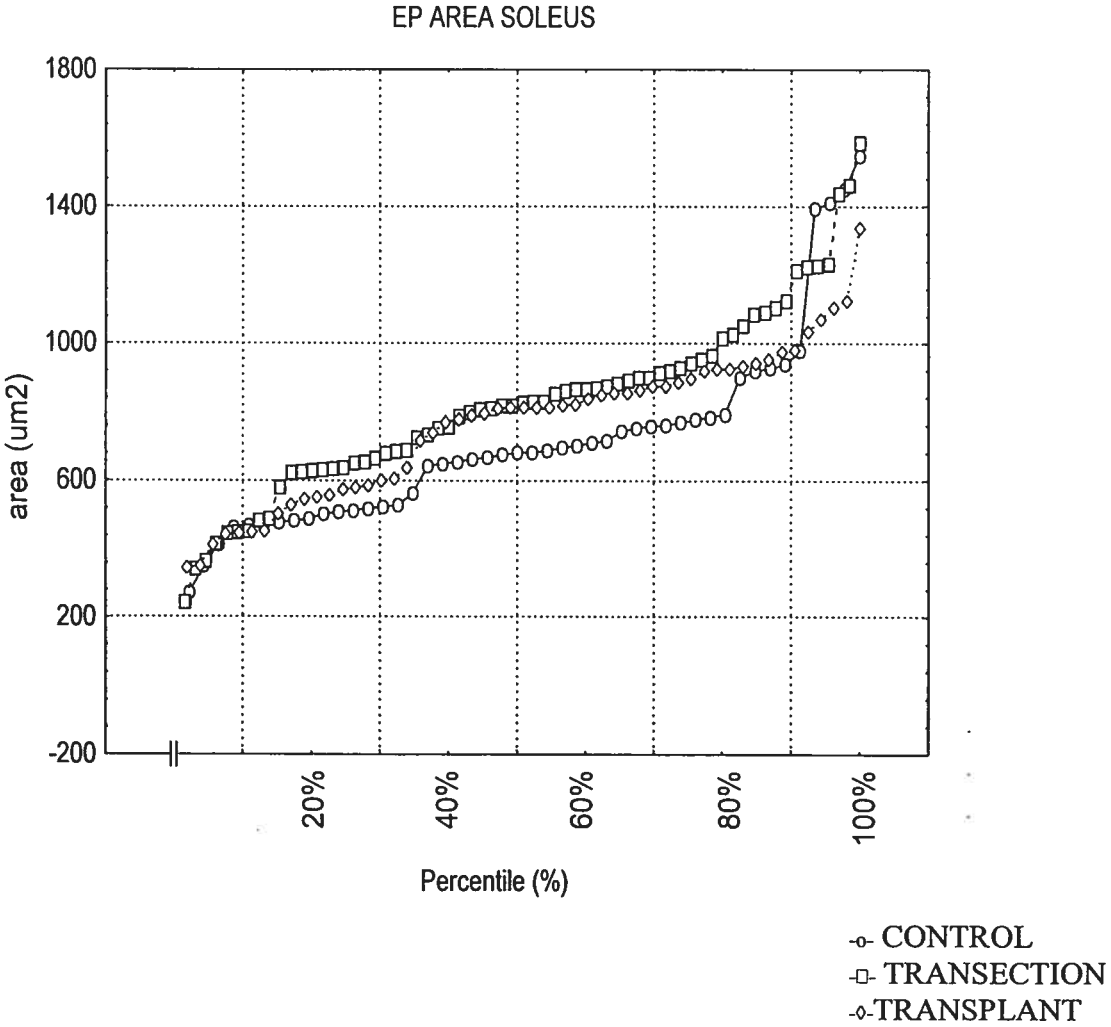
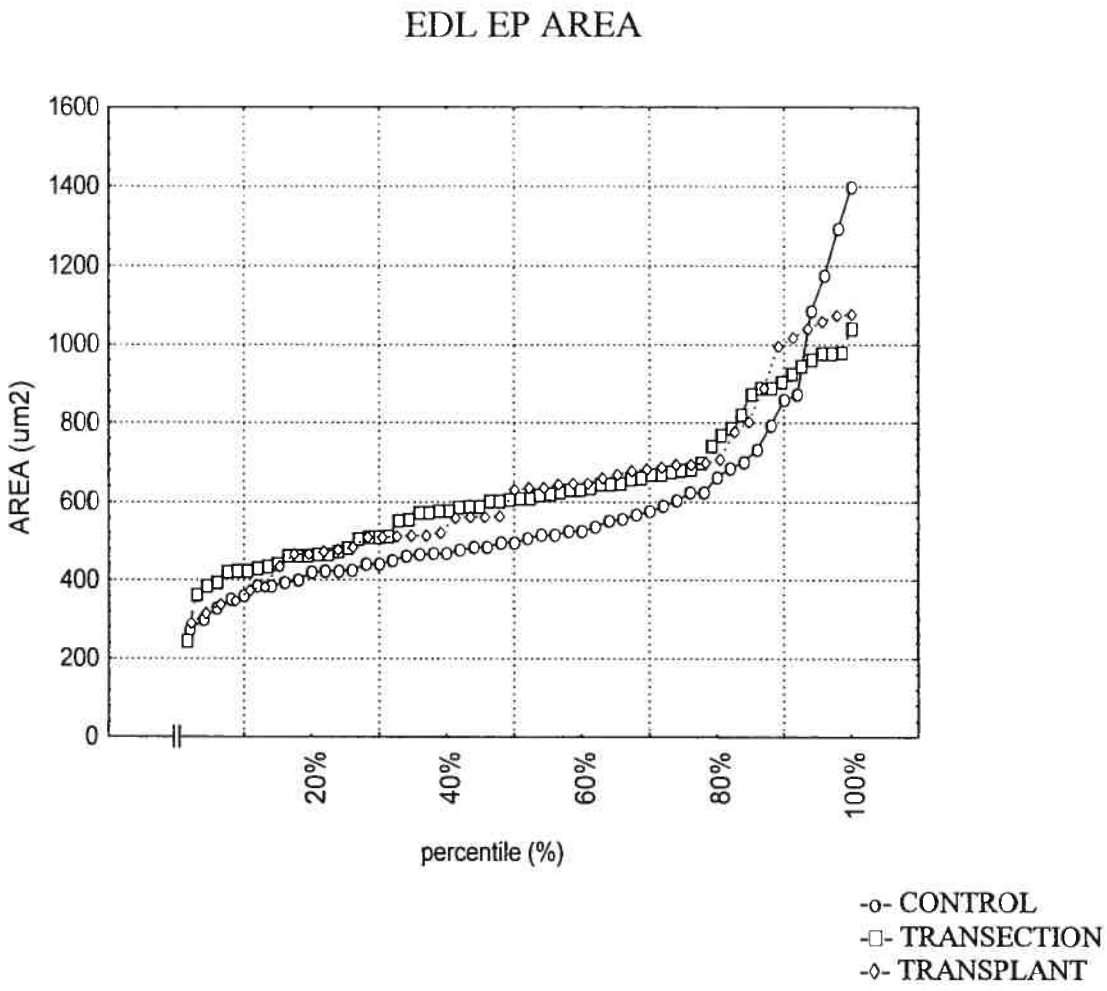


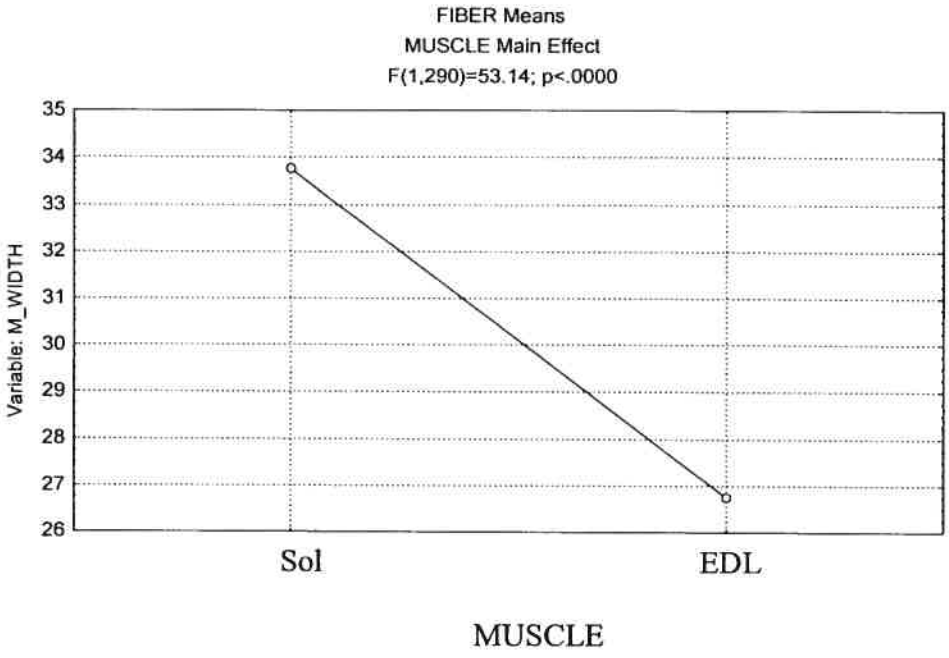
Figure 8: EDL EP area distribution according to percentile for CNTRL, TRANS and TRNPL.



MUSCLE FIBER WIDTH:

Statistical analysis uncovered a significant main effect ($p<0.05$) between the fiber widths of the EDL and SOL (Figure 9). Soleus fiber widths were 25.9% larger than those of EDL.

Figure 9: Muscle Main Effect On Muscle Fiber Width

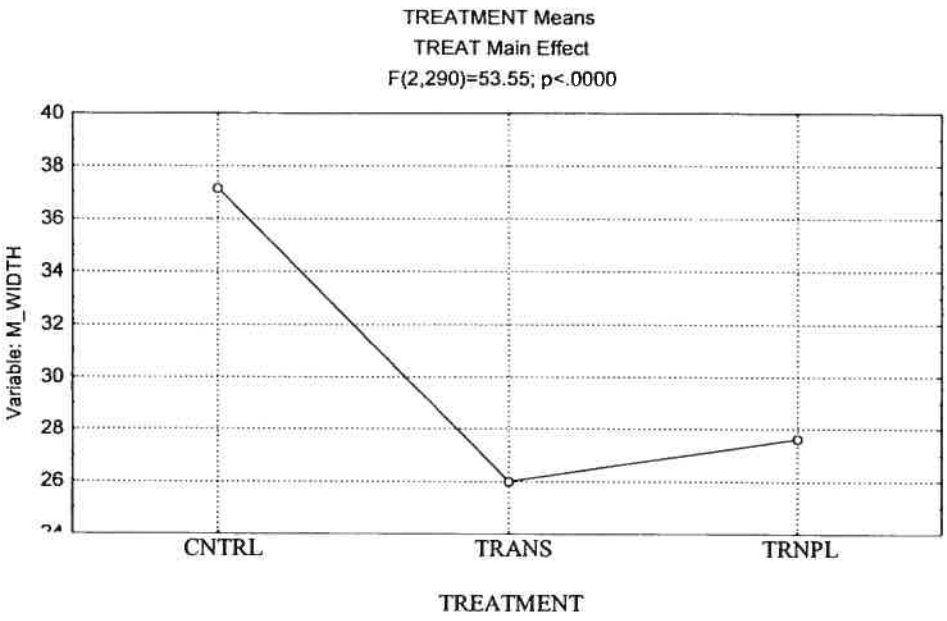


Sol: Soleus
EDL: Extensor Digitorum Longus

TREATMENT

Statistical analysis uncovered a significant main effect ($p<0.05$) between the fiber widths with regards to the treatment (Figure 10). The TRANS and TRNPL groups were not significantly different from each other.

Figure 10: Treatment Main Effect on Fiber Width



CNTRL: CONTROL
TRANS: TRANSECTION
TRNPL: TRANSPLANT

Soleus:

Table VI lists the fiber widths (±SD) for the data presented in Figures 6 & 7. The Scheffe test found significant differences between the SOL CNTRL group and both the SOL TRANS and TRNPL groups ($p<0.05$; $p=0.00$, $p=0.00$). No significant difference was found between the TRANS and TRNPL groups ($p>005$; $p=0.153$).

EDL:

Significant differences were found between the EDL CNTRL group and both the EDL TRANS and TRNPL groups ($p=0.02$, $p=0.02$ respectively). No significant difference was found between the TRANS and TRNPL groups ($p=0.99$).

TABLE 3. Fiber width (μm) ± S.D.

	GROUPS		
MUSCLES	**CNTRL**	**TRANS**	**TRNPL**
SOL	43.4 ± 8.4	26.8 ± 11.4*	31.1 ± 6.7*
EDL	31.0 ± 6.1	25.2 ± 6.6*	24.1 ± 5.0*

*** Significantly different from CNTRL Value ($p < 0.05$)**

Figure 11: SOL FIBER WIDTH distribution according to percentile for CNTRL, TRANS and TRNPL.

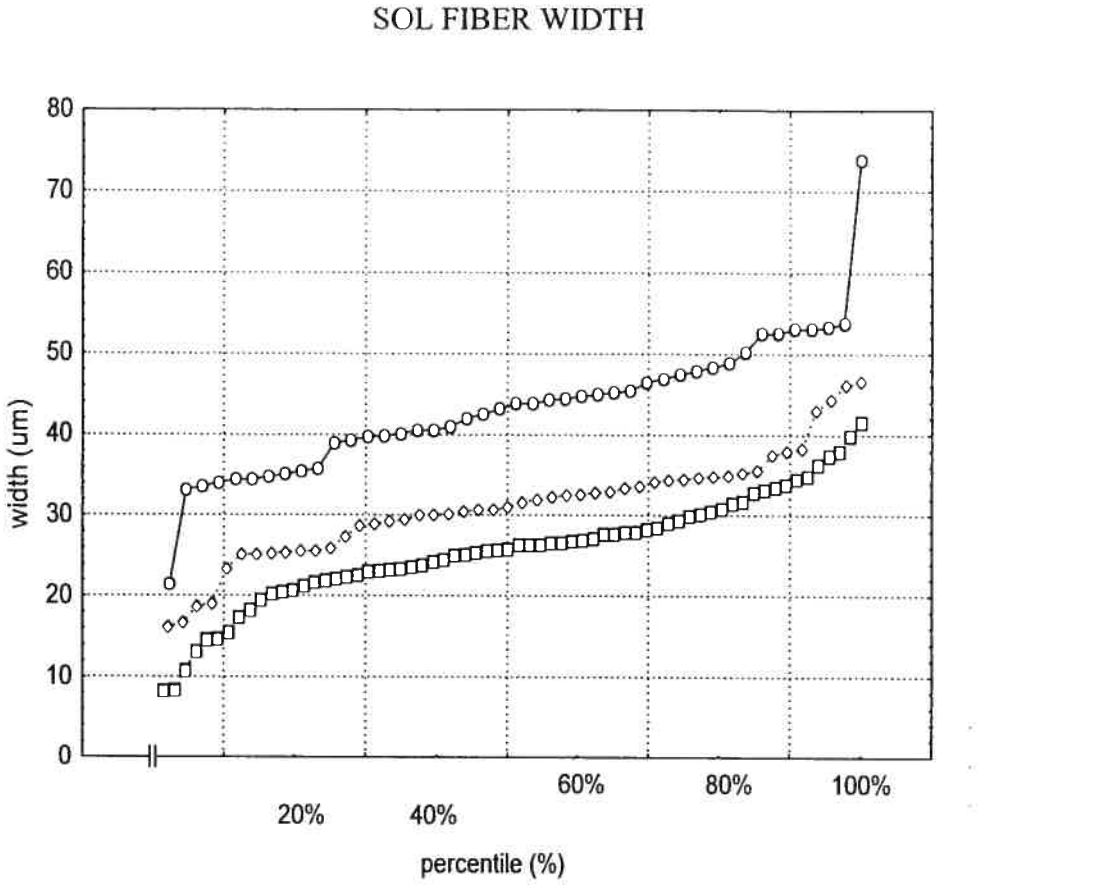
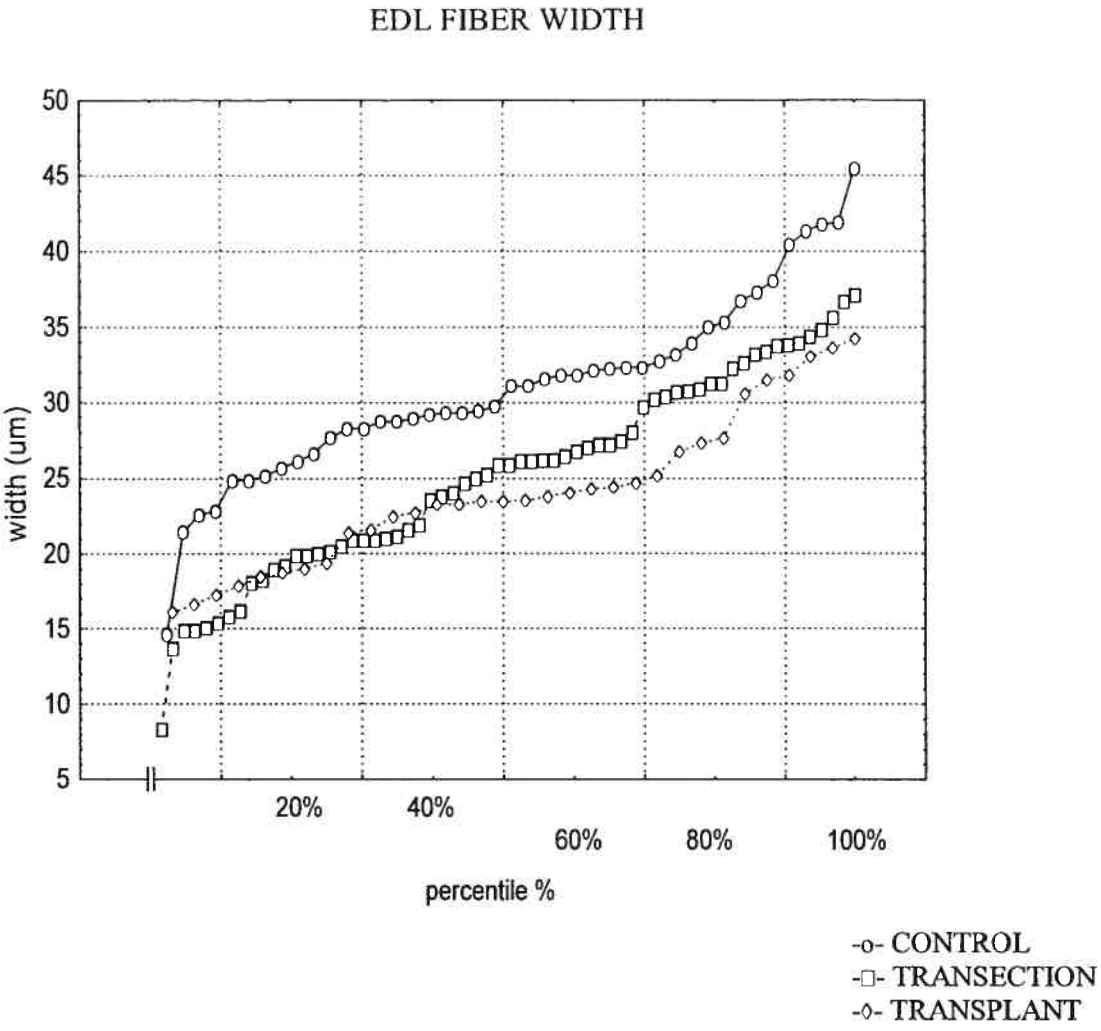


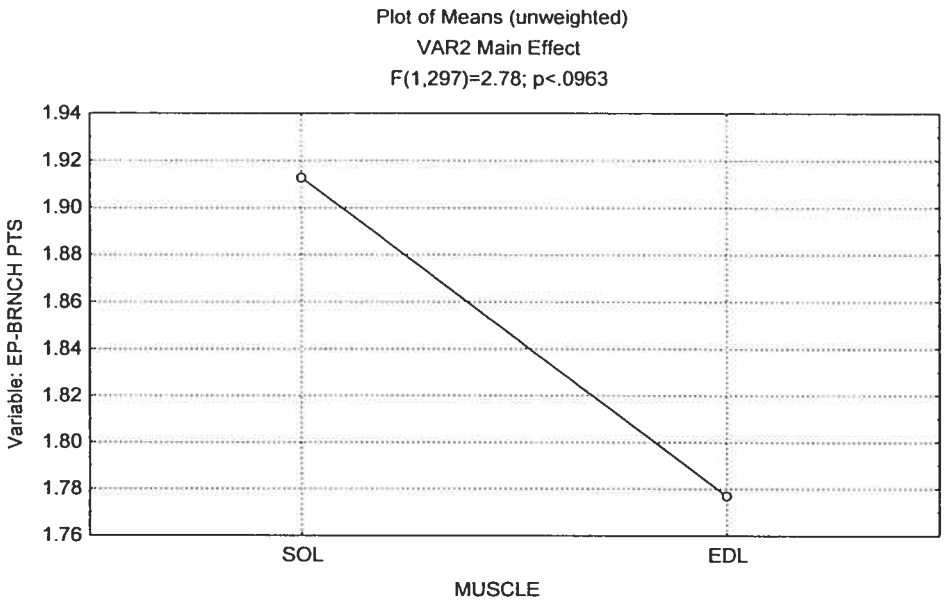
Figure 12: EDL FIBER WIDTH width distribution according to percentile for CNTRL, TRANS and TRNPL.



BRANCH POINTS

Statistical analysis did not uncover a significant main effect between the branch points of the EDL and SOL (Figure 13).

Figure 13: Muscle Main Effect of Branch Points

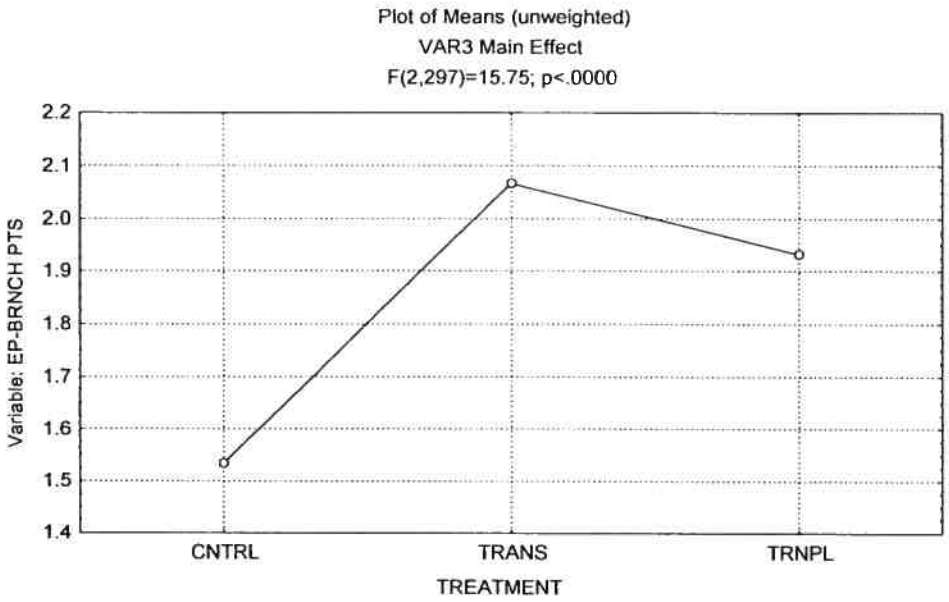


Sol: Soleus
EDL: Extensor Digitorum Longus

TREATMENT

Statistical analysis uncovered a significant main effect ($p<0.05$) among the treatment groups with regards to branch points (Figure 14).

Figure 14: Treatment Main Effect on end-plate branch points.



CNTRL: CONTROL
TRANS: TRANSECTION
TRNPL: TRANSPLANT

Soleus:

Table 4 lists the branch points (±SD) for branch point data collected. The Scheffe test found significant differences between the SOL CNTRL group and both the SOL TRANS and TRNPL groups ($p=0.0001$, $p=0.006$). No significant difference was found between the TRANS and TRNPL groups ($p=0.978$).

EDL:

The Scheffe test showed no significant effect between any of the EDL groups.

TABLE 4. Branch points ± S.D.

	GROUPS		
MUSCLES	**CNTRL**	**TRANS**	**TRNPL**
SOL	1.48±0.64	2.19±0.77*	2.07±0.74*
EDL	1.59±0.75	1.94±0.67	1.80±0.67

**** Significantly different from CNTRL Value (p<0.05)***

SUMMARY PRINCIPLE RESULTS

1) TRANS SOL EP length was larger than in the TRNPL or CNTRL groups, suggesting that the transplant procedure tended to attenuate the increase in SOL EP length that occurs with transection.
2) SOL and EDL EP AREAS had no statistically significant differences between the CNTRL, TRANS or the TRNPL groups.
3) Both SOL and EDL TRANS and TRNPL groups had significantly larger widths than their respective CNTRL groups, suggesting that the transplant had no effect on attenuating the fiber width atrophy attributable to transection.
4) SOL TRANS and TRNPL groups had significantly more branch points than the CNTRL group, suggesting that the transplant had no effect on attenuating the SOL EP sprouting related to transection.

DISCUSSION

The goal of this study was to determine what effect, if any, a fetal graft transplant would have on the morphology of fast and slow neuromuscular end-plates of hindlimb muscles (SOL and EDL) following spinal cord transection. The findings indicate that the transection induced some of the expected structural adaptation of motor end-plate expansion (Eldridge et al.,1981), such as an increase in sprouting and complexity of nerve terminals (Tomas et al., 1989). As expected, the transection of the spinal cord induced muscle fiber with atrophy, end-plate expansion and neuronal sprouting in both the SOL and EDL. The transplant attenuated the increase in SOL end-plate length post-transection when compared to transection alone.

The muscle fiber width, EP area, and number of branch point parameters, for both the SOL and EDL, were not attenuated by the transplant post-transection. Therefore it was concluded that the embryonic cell transplant had no significant change when compared to control subjects, such may be due to the duration of the transplant before muscles were excised. Perhaps a duration of 28 days is not long enough for the transplant to elucidate any changes within the neuromuscular junction.

CNTRL EP length values

The CNTRL values for the SOL EP lengths were consistent with the literature. SOL EP length of 39.0 μm ± 8.1 is in the same range as Pestronk and Drachman's (1978) EP length of 38 μm ± 2 and Fahim's (1989) EP length of 48.4 μm ± 2.0. The EDL EP length of 28.7μm ± 4.3 is comparable with the range of 25-55 μm repeated by Eldridge, Liebhold and Steinbach (1981).

CNTRL EP Area values

The CNTRL values for the rat SOL and EDL EP areas were consistent with the literature. Our SOL EP area of 716.87 ± 278.33μm^2 is consistent with EP areas reported by Rosenheimer and Smith (1985) (941.20 ± 6.35 (SE)) and by Duchen (1970) (838 ± 297μm^2) (range: 200-1600μm^2).

The EDL EP areas in this work of 571.31 ± 239.26 μm^2 is also comparable with Rosenheimer and Smith's (1985) EP area of 601.24 ± 2.54 (SE), and Duchen (1970) 813 ± 267μm^2 (range: 100-2200μm^2).

CNTRL Fiber width values

The CNTRL values for the SOL and EDL fiber widths were consistent with the literature. Our SOL fiber width of 43.36 ± 8.38μm is comparable with Fahim and Adonian's (1990) 50 ± 2 (SE) and Fahim and Robbins's (1986) 44 ± 0.7 SE and Oda's (1985) fiber diameter range of 30-70 um. Our EDL fiber width of 30.963 ± 6.072μm is consistent with Fahim and Adonian's (1990) 39 ± 2 (SE).

Houle et al. (1999) observed that the graft attenuated the extent of muscle atrophy in the soleus post transection. Their soleus transected cross-sectional area (um^2) was found to

be 50% that of the CNTRL (888:1681 um^2), while the TRNPL was only 77% that of the CNTRL (1155:1681 um^2). The present study found the average SOL TRANS width to be 62% of the CNTRL while the TRNPL width was determined 72% of control. The Scheffe test did not reveal a statistical difference between the TRANS and TRNPL groups.

One reason why we did not observe a significant attenuating effect from the TRNPL group with regards to fiber width could be that Houle (1999) compared another indicator of atrophy, myofiber cross-sectional area, and not the width of the fiber. Another difference in the protocol was that Houle used a duration of treatment of 90 days, whereas our subjects were sacrificed 4 weeks post-tranplant.

CNTRL EP branch point values

Our SOL EP branch points 1.48 ± 0.64 is comparable with Rosenheimer and Smith's (1985) branch points of 1.63 ± 0.01 (SE), and Pestronk and Drachman's (1978) 2.6 ± 0.01

Our EDL EP branch points 1.59 ± 0.75 is consistent with Rosenheimer and Smith's (1985) branch points of 1.25 ± 0.004 (SE) and Tomas et al. (1989) branch points of 2.99 ± 1.29.

WHY WERE THESE 2 MUSCLES INFLUENCED DIFFERENTLY?

The most marked atrophic responses following are typically seen in postural muscles that possess a relatively high percentage of type I fibers, and which experience the greatest change from their normal activity pattern.

The EDL and soleus are composed primarily of types II and I fibers respectively (Fahim et al., 1984), perform different actions (flexion and extension, respectively) and exhibit differences in firing patterns (Fischbach and Robbins, 1969; Navarrette and Vrbova, 1980).

Brown et al. (1980) suggest that the relatively fast muscles generally have less sprouting when denervated when compared to their slower fiber counterparts attributable to their higher resistance to the effects of nerve degeneration. Slow muscles that are used for postural maintenance, tend to have a more continuous firing pattern than fast muscles and would suffer the greater change in firing pattern post-transection, and undergo greater morphological changes.

The current literature shows that after transection, the soleus atrophies markedly, while the EDL seems substantially more resistant to the decrease in activity (Dupont-Versteegden et al., 1998). This was also evident in the results of the present study, where the impact of transection was seen to be greater on SOL parameters when compared with respective control values.

It is hypothesized that transplants may assist the host by providing an environment that supports growth of axotomized and the rescue of neurons destined to undergo retrograde cell death. Transplants are thought to rescue severed neurons by serving as a surrogate source of target-derived neurotrophic factors (Bregman and Reier, 1986). The greater vigor

in regenerating axons from embryonic rather than adult neurons is one of the reasons why much of the current research employs embryonic donors in an attempt to regain function. In this study, the transplant seems to have contributed in attenuating the increase in SOL longitudinal length resulting from transection. In other words, the transplant may have restored some elements of spinal circuitry, thus assisting in the reorganization of the host tissue.

Studies have shown that spinal lesion sparing small amounts of tissue permits considerable function (Blight and DeCrescito, 1986). Therefore, in this study, it is hypothesized that small amounts of spinal tissue may have been spared, resulting in an attenuated SOL end-plate growth post transection.

Though the transplant had little effect on end-plate morphology post-transection, several mechanisms have been suggested by which transplants placed in transected rats could permit repair of injured spinal cord. These mechanisms include axonal regeneration, the rescue of severed neurons destined to die, and trophic effects on remaining circuitry, thus improving the substrate through which compensatory mechanisms operate and thus permit development improvement motor control.

Presently, trophic molecules are being tested and applied together with antibodies against growth-inhibitory molecules, grafts of Schwann cells are being introduced into lesion sites, and embryonic cells are being implanted. It has been shown that some trophic agents such as NGF, FGF and BDNF are able to boost axon regeneration in vitro (Lindsay, 1988; Bahr et al., 1989).

Future research should include introducing various components such as neurotrophins, thus providing an environment that could promote neuronal growth. Other

aspects that might alter NMJ morphology would include passive exercise, electrostimulation and changing the underlying gene expression, which alters axonal growth with increased age.

These questions are raised not to be pessimistic or to dampen enthusiasm about prospects for spinal cord repair, but to raise relevant points for future research.

STUDY LIMITATIONS

Some of the study limitations would include:

1. Small sample sizes might have reduced the statistical power of some tests. Marginal changes could have become statistically significant if the number of rates per groups had been larger.
2. Animals used were female Sprague-Dawley rats.
3. Assumptions were made as to the duration of transection and transplant periods since part of the protocol was done off-site;
4. Quantification of end-plate parameters relied on human precision, computer screen pixel resolution and microscope magnification standardizations.
5. Conclusions are restricted to SOL and EDL muscles.

CONCLUSIONS

Utilization of spinal tissue for transplantation is an extremely promising area for future research. The transplant tissue is thought to rescue severed neurons by serving as a surrogate source of target-derived neurotrophic factors and by providing "spare parts" thus assisting in the restoration of neuronal pathways.

In this study, the parameters measured included EP area, EP length, muscle fiber width and number of branch points, all were seen to undergo the morphological changes expected post-transection. These included an increase in EP area, EP length and branch point number within the EP, while a decrease in muscle fiber width signified muscle fiber atrophy.

The transplant had no effect on attenuating the transection response of the EP area, muscle width and branch points of the EDL or SOL. The SOL longitudinal length response was the only parameter attenuated post-transection by the transplant. Though the relevance and reproducibility of these findings has yet to be determined, this finding should propogate further studies with regards to the NMJ post-tranplant.

REFERENCES

Aguayo, A.J., Dickson R., Trecarten, J., Attiwell, M. Ensheathment and myelination of regenerating PNS fibers by transplanted optic nerve glia. *Neurosci Lett* 9: 97-104, 1978.

Bahr M., Vanselow J., Thanos S. Ability of adult rat ganglion cells to regrow axons in vitro can be influenced by fibroblast growth factor and gangliosides. *Neurosci Lett* 96: 197-201, 1989.

Blight, A.R. and DeCrescito V. Morphometric analysis of experimental spinal cord injury in the cat: the relations of injury intensity to survival of myelinated axons. *Neuroscience* 19: 321-341, 1986.

Bregman, B.S. NT factors increase axonal growth after and tranplantation. *Experimental Neurology* 148: 475-494, 1997.

Bregman, B.S. and Goldberger, M.E. Anatomical plasticity and sparing of function after spinal cord damage in neonatal cats. *Science* 217: 553-555, 1982.

Bregman, B.S., Kunkel-Bagden, E., Reier, P., Dai, McAtee, M. and Goa, D. Experimental Neurology 123: 3-16, 1993.

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. *Exp Neurol*; 148: 475-494, 1997.

Bregman, B.S., Reier, P.J. Neural tissue transplants rescue axotomized rubrospinal cells from retrograde death. *J. Comp. Neurol*; 244: 86-95, 1986.

Brenner, H.R. and Rudin, W. On the effect of the muscle activity on the end-plate membrane in denervated mouse muscle. *J. Physiol.*, 410: 501-512, 1989.

Brown, M.C., Holland, R.I. and 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.

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

Buller, A.J., Eccles, J.C. and Eccles, R.M. Differentiation of fast and slow muscles in the cat hind limb. *J. Physiol. (Lond.),* 150:399-416, 1960.

Burke, R.E. Motor units anatomy, physiology and functional organization. Handbook of Physiology. The Nervous System, MD, *Am. Physiol. Soc.* Sec. 1, Vol. 4, Chapt. 10: 345-422, 1981.

Cajal, S. R. Degeneration and Regeneration of the nervous system. *Trans. R.M. London:* Oxford Univ Press. 1928.

Carlstedt, T., Cullheim, S., Risling, M., & Ulfhake, B. Nerve fibre regeneration across the PNS-CNS interface at the root-spinal cord junction. *Brain Res Bull* 22: 93-102, 1992.

Christopher Reeve Paralysis Foundation: http://paralysis.apacure.org/progress/facts.html , 1999.

David, S., Aguayo, A.J. Axonal elongation into peripheral nervous system bridges after central nervous system injury in adult rats. *Science* 241: 931-933, 1981.

Diener, P.S., Bregman, B.S. Neurotrophic factors prevent the death of CNS neurons after spinal cord lesions in newborn rats. *Neuroreport*; 5:1913-1917, 1994.

Deschens, D.M., Mayet, H., Senpore, B. and Flandoris, R. Structural and functional responses to prolonged hindlimb suspension in rat muscle. *J. Appl. Physiol.,* 63(2): 558-563, 1987.

Deschens, D.M., Marsh, C.M., Crivello, J.F., Armstrong, L.E., Kramer, W.J., and Covault, J. The effects of exercise training of different intensities on neuromuscular junction morphology. *J. Neurocytol.,* 22: 603-615, 1993.

Duchen, L.W. Changes in motor innervation and cholinesterase localization induced by toxin in skeletal muscle of the mouse: differences between fast and slow. *J. Neurol. Neursurg. Psychiat.,* 33: 40-54, 1970.

Duchen, L.W. and D.A. Tonge. The effects of tetanus toxin on neuromuscular transmission and on the motor end-plates in slow and fast skeletal muscle of the mouse. *J. Physiol. (Lond.),* 228:157-172, 1973.

Dupont-Versteegden, E.E., Houlé, J.D., Gurley, C.M. and Peterson, C.A. Early changes in muscle fiber size and gene expression in response to spinal cord transection and exercise *Am. J. Physiol Cell Physiol., 275*:C1124-C1133, 1998.

Eccles, J.C. and Sherrington, C.S. Numbers and contraction-values of individual motor units examined in some muscles of the limb. *Proc R. Soc. B.*, 106

Eldridge L., Liebold M., Steinbach J.H. Alterations in the cat skeletal neuromuscular junctions following prolonged inactivity. *Journal of Physiology* 313: 529-545, 1981: 326-357, 1930.

Exner, S. Die innervation des hohlkopfes. *S.B. Akad Wiss. Wien*, 89(3): 63-118, 1884.

Fahim MA. Rapid neuromuscular remodeling following hindlimb immobilization. *Anatomical Record* 224: 102-109, 1989.

Fahim, M.A. and Andonian N. Effect of chronic corticosterone treatment on the morphology of fast and slow twitch muscle. *Anat Rec* May: 227(1):132-137, 1990.

Fahim MA and Robbins N. Remodeling of the neuromuscular junction after subtotal disuse. *Brain Research* 383: 353-356, 1986.

Fawcett, J.W, Housden, E., Smith-Thomas, L., Meyer, R.L. The growth of axons in three dimensional astrocyte cultures. *Dev Biol* 135: 449-458, 1989.

Fawcett, J.W. Spinal cord repair: Future directions. *Paraplegia* 30(2): 83-85, 1992.

Fischbach, G.D. and Robbins, N. Changes in contractile properties of disused soleus muscles. *Journal of Physiology* 201, 305-320, 1969.

Granit, R., Pascoe, J.E. and Steg, G. The behavior of tonic alpha and gamma motoneurones during stimulation of recurrent collaterals. *J. Physiol. (Lond),* 138: 381-400, 1957.

Guth, L., P.J. Reier, J.P., Barrett, C.P. and Donati, E.J. Repair of the mammalian spinal cord of the rat. *J. Neurosci.* 51: 73-86; 1983.

Harpur, R. The rat as a model for physical fitness studies *Comp. Biochem. Physiol.* [A] 66: 553-574, 1980.

Hoffman, H. Local reinnervation in partially denervated muscle: A histo-physiological study. *Aust. J. Exp. Biol. Med. Sci.,* 28: 383-397, 1950.

Houle, J.D., Morris, M.D. Skinner, R., Garcia-Rill, E., Peterson, C. Effects of fetal spinal cord tissue transplants and cycling exercising on the soleus muscle in spinalized rats. *Muscle & Nerve*, July 846-856, 1999.

Houle, J.D., Reier, PJ. Transplantation of fetal spinal cord tissue into chronically injured adult rat spinal cord. *J. Comp Neurol;* 269: 535-547, 1988.

Howland D.R., Bregman B.S., Tessler, A., Goldberger, M.E. Development of locomotor behavior in the spinal kitten. *Neurol* 135: 108-122, 1995(a).

Howland D.R., Bregman B.S., Tessler, A., Goldberger, M.E. Transplants enhance locomotion in neonatal kittens whose spinal cords are transected. *Exp. Neurol* 135: 123-145, 1995(b).

Iannuzo, D., Patel, P., Chen, V., O'Brien, P., Williams, C. Thyroidal trophic influence on skeletal muscle myosin. *Nature* 270: 74-76, 1977.

Iwashita, Y., Kawaguchi, S., Murata, M. Restoration of function by replacement of spinal cord segments in the rat. *Nature* 367: 167-170, 1994.

Kuhlengel KR, Bunge MB, Bunge RP, Burton, H. Implantation of cultured sensory neurons and Schwann cells into lesioned neonatal rat spinal cord. II. Implantation characteristics and examination of corticospinal tract growth. *J Comp Neurol* 293: 74-91, 1990.

Liddell, E.G.T and C.S. Sherrington. Recruitment and some other factors of reflex inhibition. *Proc R. Soc. London Ser. B.*, 97:488-518, 1925.

Lieber, R.L., Friden, J.O, Hargens, A.R., Feringa, E.R. Long-term effects of spinal cord transection on fast and slow rat skeletal muscle. *Exp. Neurol*, 91-435-448, 1986.

Lindsay, R.M. Nerve growth factors (NGF, BDNF) enhance axonal regeneration but are not required for survival of adult sensory neurons. *J Neurosci* 8: 2384-2405, 1988.

Lomo, T. and Westgaard, R.H. Control of Ach sensitivity in rat muscle fibers. *Cold Springs Harbor Symp. Quant. Biol.*, 40:263-274, 1976.

Mann, G., Histological changes induced in sympathetic, motor and sensory nerve cell by functional activity. *J. Anat. and Physiol.*, 29:100-108, 1894.

McDonald, J.W., Xiao-Zhong, L., Qu, Y. Su, L., Mickey, S.K. Transplanted embryonic stem cells survive, differentiate and promote recovery in the injured rat spinal cord. *Nature Medicine* 5, 12: 1410-1412, 1999.

Mendell, L.M., Collins, W.F. and Munson, J.B. Retrograde determination of motoneuron properties and their synaptic input. *J. Neurobiol.,* 25(6): 707-721, 1994.

Miya, D., Giscter, S., Mori, F., Adipudi, V., Tessier, A., and Murray, M. Fetal Transplants Alter the Development of Function after Spinal Cord Transection in Newborn Rats. *The Journal of Neuroscience*, 17(12):4856-4872, 1997.

Munson, J., B., Foehring, R.,C., Mendell, L.M., Gordon, T. Fast-to-slow conversion following chronic low-frequency activation of medial gastrocnemius muscle in cats. Motoneuron properties. *J. Neurophysiol.*, 77(5): 2605-15, 1997.

Navarrette, R., and Vrbova, G. Electromyographical activity of rat slow and fast muscle during post-natal development. *Journal of Physiology* 305, 33-34P, 1980.

Navarrette, R., and Vrbova, G. Activity-dependent interactions between motoneurons and muscles: Their role in the development of the motor unit. *Prog. Neurobiol.,* 41: 93-124, 1993.

Nicholls J.G. The search for Connections: Studies of Regeneration in the Nervous System of the Leech. *Sinauer, Sunderland*, MA, 1987.

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.

Panenic, R. and Gardiner, P. The case for adaptability of the neuromuscular junction to endurance exercise training. *Canadian Journal of Applied Physiology* 23(4): 339-360, 1991.

Pestronk, A. and Drachman. Motor Nerve Sprouting and Acetylcholine Receptors. *Science,* Vol. 199, pp 1223-1225, 1978.

Reier and Perlow. Development of embryonic spinal cord transplants. *Dev Brain Research Vol.* 10, pp 201-219, 1983.

Robbins, N. and Fischbach, G.D. Effect of chronic disuse of rat soleus neuromuscular junctions on presynaptic functions. *J. Physiol.,* 34: 570-578, 1971.

Rosenheimer, Julie and Smith, D. Differential Changes in the end-plate architecture of functionally diverse muscles during aging. *Journal of Neurophysiology*, Vol. 53, No.6, pp1567-1581, June 1985.

Schwab, M.E. and Caroni, P. Oligodendrocytes and CNS myelin are nonpermissive for neuronal cell adhesion and fiber outgrowth. *Jounal of Neuro Science* 9:1126-1133, 1988.

Sickles, DW and Oblak, TG. Metabolic variation among alpha-motoneurons innervating different muscle fiber types. Oxidative enzyme activity. *J. Neurophysiol.*, 51(3): 529-537, 1984.

Sieck and Prakash. Morphological adaptations of MNJ depends on fiber type. *Canadian Journal of Applied Physiology*, 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 and Thompson,W.J. Nerve sprouting in muscle is induced and guided by processes extended by Schwann cells. *Neuron*, 14: 133-141, 1995.

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.

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

Tomas, J., Fenoll, R., Santafé, M., Batlle, J., Mayayo, E. Motor nerve terminal morphologic plasticity induced by small changes in locomotor activity of the adult rat. *Neurosci. Lett.* 106:137-140, 1989.

Tomas J., Santafe, M., Fenoll, R., Mayayo, E, Batlle, J., et al. Pattern of arborization of the motor nerve terminals in the fast and slow mammalian muscles. *Biology of the Cell* 74: 299-305, 1992.

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

Van Mier, P. and Lichtman, J.W. Regenerating muscle fibers induce directional sprouting from nearby nerve terminals: Studies in living mice. *J. Neurosci.*, 14(9): 5672-5686, 1994.

Wigston, D. Remodeling of Neuromuscular Junction in adult mouse soleus. *The Journal of Neuroscience.* Feb 9(2): 639-647, 1989.

APPENDIX A

STAINING FOR QUANTITATIVE MEASUREMENT OF NEUROMUSCULAR JUNCTION

TISSUE PROCESSING

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

CHOLINESTERASE-STAINING PROCEDURE

1. *TURN ON BATH @ 37 °C.*
2. Immerse slides in 20% solution of sodium sulfate ***[129: A1]*** for 3 minutes.
3. *AFTER THIS AND EACH SUCCEEDING STEP, WASH SLIDES IN DEIONIZED WATER.*
4. To stain sections for acetylcholinesterase, incubate at 37 °C for 18 minutes in the following solutions:

- 5-bromoindoxyl acetate 4.0 mg ---------***[D3]***
- Ethanol 0.3 ml

- Potassium ferrocyanide-------63.0 mg--------***[96: A1]***
- Potassium ferricyanide-------50.0 mg---------***[95: A1]***
- Tris-Base (HCl=7.2) 46.0 mg--------***[A1]***
- Calcium chloride---------- 33.0 mg--------***[23B: A1]***
- Deionized water 30.0 ml

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

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 formol-saline solution at pH 7.0:

    - 37-47% formaldehyde ; 20.0 ml ; ***[across]***
    - Sodium chloride ; 4.25 g ; ***[115A; A1]***
    - Acid sodium phosphate monohydrate ; 0.80 g ***; [122; A1]***
    - Anhydrous disodium phosphate ; 1.30g ; ***[124 ; A1]***
    - Deionized water ; 180 ml
      (sol'n must be less than one week old)

3. Soak for 30 min. at 37°C in 10% chloral hydrate ***[27:A1]*** with 1% pyridine ***[A3]***

4. Incubate for 40 min. in 20% silver nitrate ***[1:A1]*** containing 0.1% cupric sulfate ***[32; A1]***, with 100 mg calcium carbonate ***[24;A1]*** at the bottom of staining jar. ***(Sol'n should be made fresh each day)***

5. Develop in solution of 1% hydroquinone ***[55:A1]*** and 5% sodium sulfite ***[130:A1].*** Use two baths, the first for ***10 sec*** and the second for ***4 min***. **Discard solutions when cloudy.**

6. Fix for ***2 min*** in 5% sodium thiosulfate ***[132:A1].***

7. Tone for ***3 minutes*** in 0.2% sodium tetrachloroaurate ***[134:A1]*** containing one drop of glacial acetid acid per 100 ml.
   This solution may be used multiple times if the edges and backs of slides are cleaned before immersion.

   (Must be discarded if a precipitate forms.)

8. (OPTIONAL) Darken axons by immersing in 1% oxalic acid ***(30 secs)*** ***[76B;A1]***

9. Immediately fix again for ***5 min*** in 5% sodium thiosulfate ***[132:A1].***