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CENTRAL NERVOUS SYSTEM ADAPTATIONS TO EXERCISE TRAINING

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LOIS ANNE KAMINSKI

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By

LOIS ANNE KAMINSKI

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ABSTRACT

CENTRAL NERVOUS SYSTEM ADAPTATIONS TO EXERCISE TRAINING

By

Lois A. Kaminski

Exercise training causes physiological changes in skeletal muscle that results in enhanced performance in humans and animals. Despite numerous studies on exercise effects on skeletal muscle, relatively little is known about adaptive changes in the central nervous system. This study investigated whether spinal pathways that mediate locomotor activity undergo functional adaptation after 28 days of exercise training. Ventral horn spinal cord expression of calcitonin gene-related peptide (CGRP), a trophic factor at the neuromuscular junction, choline acetyltransferase (Chat), the synthetic enzyme for acetylcholine, vesicular acetylcholine transporter (Vacht), a transporter of ACh into synaptic vesicles and calcineurin (CaN), a protein phosphatase that phosphorylates ion channels and exocytosis machinery were measured to determine if changes in expression occurred in response to physical activity. Expression of these proteins was determined by western blot and immunohistochemistry (IHC). Comparisons between sedentary controls and animals that underwent either endurance training or resistance training were made. Control rats received no exercise other than normal cage activity. Endurance-trained rats were exercised 6 days/wk at 31m/min on a treadmill (8% incline) for 100 minutes. Resistance-trained rats supported their weight plus an additional load (70-80% body weight) on a 60^0

incline (3x3 min, 5 days/wk). CGRP expression was measured by radioimmunoassay (RIA). CGRP expression in the spinal dorsal and ventral horn of exercise-trained animals was not significantly different than controls. Chat expression measured by Western blot and IHC was not significantly different between runners and controls but expression in resistance-trained animals assayed by IHC was significantly less than controls and runners. Vacht and CaN immunoreactivity in motor neurons of endurance-trained rats was significantly elevated relative to control and resistance-trained animals. Ventral horn CaN expression by immunoblot was found to be significantly elevated (1.7 fold) in endurance-trained animals relative to sedentary controls. C-terminal Vacht immunoreactivity was greater in runners than controls and hangers. The number of Vacht immunoreactive "C" terminals was greater in exercise-trained animals than controls. It is hypothesized that an increase in CaN could lead to a decrease in release of ACh from the motor terminal by preventing phosphorylation of exocytosis machinery. Thus, preventing desensitization of ACh receptors and depletion of ACh stores. The elevation in motor neuron Vacht immunoreactivity may indicate a functional increase in ACh recycling. The increase in Vacht terminal staining in the spinal ventral horn in exercise trained animals suggests that sprouting of excitatory cholinergic terminals was induced by activity affecting motor neuron excitability. Thus, the findings are evidence that a relatively short period of exercise training can produce adaptive changes in motoneurons and associated ventral horn neural circuits.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5-HT	serotonin
a.h.p.	after hyperpolarization potential
ACh	acetylcholine
AChE	acetylcholine esterase
AChR	acetylcholine receptor
ATP	adenosine triphosphate
CaN	calcineurin
CGRP	calcitonin gene-related peptide
Chat	choline acetyltransferase
CNS	central nervous system
CP	creatine phosphate
CS	citrate synthase
CsA	cyclosporin A
EDL	extensor digitorum longus
EMG	electromyography
FDL	flexor digitorum longus
FF	fast fatiguable
FFR	fast fatigue resistant
FG	fast glycolytic
FOG	fast oxidative glycolytic
FTR	fast twitch red
FTW	fast twitch white
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
IHC	immunohistochemistry
LC	myosin light chain
LDH	lactate dehydrogenase
LTP	long-term potentiation
m2	Muscarinic receptor subtype 2
MEPP	miniature end-plate potential
MHC	myosin heavy chain
NE	norepinephrine
NeuN	neuronal nuclear specific protein
NMDA	N-methyl-D-aspartate
NMJ	neuromuscular junction
PBS	phosphate buffered saline
PFK	phosphofructokinase
SDH	succinate dehydrogenase
SO	slow oxidative
TBS	trizma buffered saline
TBST	trizma buffered saline with tween
TTX	tetrodotoxin
Vacht	vesicular acetylcholine transporter

Chapter One

Introduction

The overall aim of this study is to investigate changes that may occur in motor neurons in response to physical activity. The effects of physical activity on the human body have been extensively studied. Areas that have received the most attention are the structure and function of skeletal muscle, changes in cardiovascular and respiratory functions, and metabolic changes in the respective systems. Studies of changes in the central nervous system associated with physical activity are limited, especially regarding effects of exercise on the brain and motor neurons. The brain receives sensory information and generates motor signals, while the motor neurons serve as the vehicle that excites the muscle to bring about movement.

Anecdotal data, often cited in textbooks, suggest that changes occur in motor output from the spinal cord in response to physical activity, but this has not been systematically evaluated. Textbooks commonly refer to these changes as an increase in "neural drive" (McArdle et al., 1996; McComas, A., 1996; Berne and Levy, 1998). It seems logical that there would be adaptive changes in spinal motor neurons in response to the demands placed on muscles during physical activity, and it is reasonable to hypothesize that as the demands for information processing increase with an increase in physical activity, changes occur in the brain and spinal motor neurons to meet these demands.

Sherrington (1906) stated that motor neurons are the final point of integration, the "final common pathway" between descending pathways from the brain and the skeletal

muscle that is innervated by motor neurons. A single motor neuron and all of the muscle fibers that are innervated by it are defined as a motor unit, the functional unit of the motor system. This is important because it dictates how muscles are recruited, which in turn is determined by the mechanics of the desired movement. The motor unit has a central nervous system component, the motor neuron, and a peripheral component, the axon of the motor neuron and the skeletal muscle fibers it innervates. Thus, motor neurons are a bridge of communication between the central nervous system and the periphery. As a result, it is a logical point at which to focus an investigation of neural adaptation to exercise training.

The physiological demands placed on the body during physical activity are dependent on the type of activity performed. Both animals and humans show rapid changes in the performance of a physical task within the first few exposures to the task. Changes also occur with long-term exposure to specific physical activity protocols.

The initial changes that occur with performance cannot be accounted for by changes in the skeletal muscle. One aspect of the adaptive process may involve changes in "neural drive" which are often described in terms of: 1) an increase in communication between the central motor command centers in the brain and motor neuron pools, 2) a change in the order of recruitment of motor units, 3) an increase in the firing frequency of motor neurons, or 4) a decrease in co-activation of antagonistic muscles during muscular contraction.

The motor neuron is the last site at which integration can occur; it may also be one of the first sites at which adaptation occurs. There are a number of specific adaptations that may occur in motoneurons in response to physical activity. These may

reflect changes in motor neuron excitability, or a change in protein expression within the motor neuron that would allow the neuromuscular junction to operate more efficiently. Possible adaptive responses include: 1) change in ion channel distribution that would modify the electrical potential of the motor neuron membrane, 2) alterations in neurotransmitter pools, and/or 3) an increase in neurotrophic factors that govern signal propagation from the motor neuron to the neuromuscular junction, and neurotransmitter production.

This study examined adaptive changes in motor neurons in response to systematic physical training in rats using immunohistochemical and biochemical assays of protein markers. The following proteins were investigated: calcitonin gene-related peptide (CGRP), choline acetyltransferase (Chat), vesicular acetylcholine transporter (Vacht) and calcineurin (CaN). Chat and Vacht support the production and packaging of acetylcholine (ACh), which is the principal neurotransmitter between motor neurons and muscle. CGRP and CaN are molecules that modulate the release and actions of ACh. The specific aim of the study was to determine whether the expression of CGRP, Chat, Vacht and CaN by motor neurons changes in response to endurance and resistance training. It was hypothesized that different training protocols would produce differences in the expression of these protein markers in motor neurons.

The study provides information about how motor neurons adapt to the increased demands of systematic physical activity. Such information is generally limited due to the lack of techniques that allow access to and analysis of the spinal cord. The study may also contribute to understanding the concept of "neural drive" in response to physical training. The expression of functional proteins can modify neural drive.

Chapter 2

Background and Significance

Introduction

Sherrington (1906) stated that motor neurons are "the final common pathway" in motor control. What Sherrington meant by this statement was that motor neurons are the last site at which both voluntary and reflexive control of motor activity can occur. Once a motor neuron is excited, an action potential is generated. All integration of input from higher brain centers, sensory input, negative feedback and feedback from surrounding neurons in the ventral horn of the spinal cord must occur before the action potential is generated, once generated the action potential cannot be stopped. Figure 1 illustrates the final common pathway. Motor centers in the brain formulate complex motor commands from sensory information and transmit this information via the corticospinal tract and other descending pathways to motor neurons located in the ventral horn of the spinal cord.

Spinal motor neurons are relatively large cells (up to 100μm in diameter) with extensive dendritic trees spanning the gray matter of the ventral horn (Cajal, 1995). Their *dendrites* provide a large surface area for synaptic inputs from muscle afferents, descending nerve tracts, and local interneurons (Motorina, 1992). The *cell body* houses the nucleus and organelles vital to the functioning of the neuron and provides additional surface area for synaptic inputs. The *axon hillock* is a region of the cell that has a high concentration of sodium channels and is the site at which action potentials are initiated. The *axon* is an insulated conduit that propagates the action potential to the neuromuscular

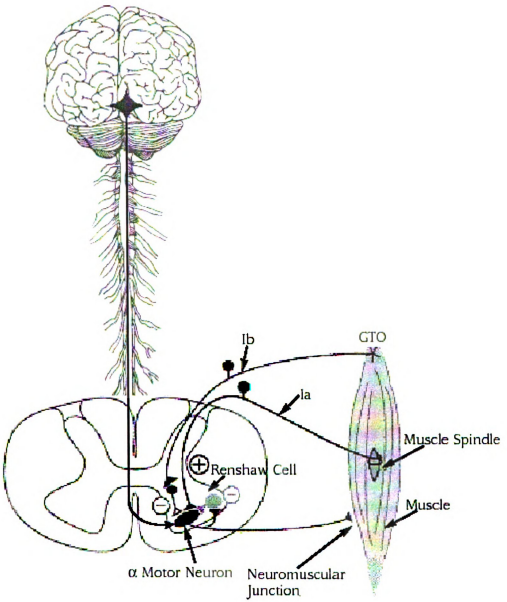


Figure 1. Depiction of Sherrington's "Final common pathway" demonstrating some major inputs that motor neurons integrate prior to stimulating the muscle to contract. ♦ Symbol in the brain represents all the higher brain centers that are involved in generating voluntary movement and modulating reflex activity. Modified form Berne, R. and Levy, M (1998) Physiology 4th Ed. Mosby. St. Louis, MO.

junction (NMJ) and houses the transport system that brings enzymes, nutrients and trophic factors from the cell body to the NMJ. A motor neuron and all of the muscle fibers that are innervated by it are defined as a *motor unit*, the functional unit that produces movement. Motor units differ physiologically, biochemically and anatomically (see below).

When the membrane potential of a motor neuron is depolarized to the point of threshold, an action potential is initiated and propagated down the axon toward the neuromuscular junction where the neurotransmitter acetylcholine (ACh) is released from the motor terminals. ACh binds to receptors on the postsynaptic membrane of the muscle and causes opening of cation channels permeable to Na^+ and K^+ that depolarize the muscle membrane and initiate muscle contraction. The mechanisms underlying the activation of muscle are reviewed by Gardiner (2001) and McComas (1996).

Motor neurons are under direct control by two types of *interneurons*, Renshaw cells and Ia interneurons (see Fig.1). Renshaw (1941,1946) postulated the existences of inhibitory interneurons that inhibit the firing of motor neurons. Eccles et al. (1954) provided physiological evidence of these neurons, which were subsequently named Renshaw cells, and showed that their inhibition is mediated by the hyperpolarizing action of γ-aminobutyric acid (GABA) on the motor neuron membrane. Renshaw cells provoke feedback inhibition, thus controlling the firing frequency of motor neurons. Ia interneurons are also inhibitory and are involved in reflex pathway control.

The motor system constantly adapts to feedback that it receives from the sensory system. Adaptive changes in the motor system are arbitrarily and generally divided into peripheral and central alterations. Peripheral changes refer to those that occur in skeletal

muscle, motor nerve axon and neuromuscular junction both structurally and chemically. Central changes encompass those that occur in the brain and in the spinal cord.

All adaptations that occur in skeletal muscle with training are not considered in this review. Rather, the focus is on changes in muscle fiber types, histochemistry, composition, myoglobin concentration, metabolic characteristics, and internal structure in response to physical training.

Fiber typing and enzymatic characteristics of skeletal muscle

Research over the last fifty years has highlighted differences in the physiology, biochemistry and structure of muscles (Table 1). Ranvier (1873) initially described histological differences between Type I and Type II skeletal muscle fibers. Type I fibers appear red due to the high concentration of myoglobin (an oxygen-carrying molecule in muscle similar to hemoglobin) while Type II appear white due to a relative lack of myoglobin. With the development of histochemical techniques, chemical differences between fiber types were more easily analyzed. [A third type of muscle fiber was discovered through these techniques; it was designated Type IIA, with Type II subsequently renamed IIB and has properties intermediate to Type I and II fibers (Staron, 1997)].

Skeletal muscle fibers differ metabolically, and staining for oxidative and glycolytic enzymes distinguishes between fibers types. Oxidative enzymes are found in mitochondria and produce adenosine triphosphate (ATP) by way of the Krebs cycle. ATP is the energy form that muscles need to perform work. Type I fibers have high concentrations of mitochondria and oxidative enzymes (Table 1). Examples of aerobic

Table 1. Muscle fiber characteristics

Characteristic	Type I	Type IIA	Type IIB
SDH activity & ATPase activity	SO Slow-oxidative	FOG, FTR Fast-oxidative glycolytic	FG, FTW Fast glycolytic
Motor unit type **Force development**	S Slow	FFR Intermediate	FF Fast
Histochemistry classification Low ATPase High ATPase	 Yes NO	 Some Yes	 No Yes
Myosin Heavy Chain Homodimer Isoform	MHC I some MHC IIA	Mostly MHC IIA some MHC IIB	Mostly MHC IIB some MHC IIA
Myosin Light Chain combinations Heterodimers (2) essential Homodimers (2) regulatory	 (2) LC1s (2) LC2s	 (2) LC1f or (2) LC3f or LC1f, LC3f (2) LC2f	 (2) LC1f or (2) LC3f or LC1f, LC3f (2) LC2f
Myoglobin content	Intermediate-High	High	Low
Mitochondria	Many	Many	Few
Glycogen content	Low	High	Very High
Gross muscle color	Red	Red	White
SDH enzyme activity	High	High (but variable)	Low
Peak twitch force	Low (≤ 2g)	Medium (~10g)	High (~50g)
Time to peak isometric twitch force	Slow (100ms)	Fast (<25ms)	Fast
Tetanic fusion frequency	Low (15hz)	High (> 50hz)	High
Properties of motor neurons Axon Diameter Conduction Velocity Excitability Fatigability	 Small Fast High (low rheobase) Low	 Large Very Fast Low (interm. rheobase) Moderate	 Large Very Fast Low (high rheobase) High

SO slow-oxidative, FOG fast oxidative, FTR fast-twitch red fiber, FG fast glycolytic, FTW fast-twitch white, S slow twitch, FFR fast fatigue resistant, FF fast fatigable, LC1s light chain 1, LC2s light chain 2, LC1f light chain 1 fast, LC3f light chain 3 fast, LC2f light chain 2 fast, SDH succinate dehydrogenase.

enzymes include succinate dehydrogenase, cytochrome C, malate dehydrogenase, and 3-hydoxyacyl-CoA-dehydrogenase (Edgerton, et al. 1969; Terjung et al., 1973; Hickson et al., 1975; Baldwin et al., 1977; Miller et al., 1992). Type I fibers have high oxidative enzyme levels, Type IIA have intermediate levels, and Type IIB have very low levels. Type IIA fibers have greater capillary density than Type IIB fibers, but less than Type I fibers (Table 1). A high capillary density allows more blood-born oxygen into the muscle and increases the oxidation of glucose and fatty acids.

Glycolysis is an anaerobic process, which utilizes glycogen as its fuel source and does not require oxygen to generate ATP. Glycogen is stored in muscle fibers and the liver. Both Type II fibers contain high levels of glycogen, but Type IIB utilizes glycolysis as its main energy source whereas Type IIA uses oxidation for this purpose. Glycolytic enzymes include phosphofructokinase (PFK) and lactate dehydrogenase (LDH) (see Table 1).

The development of electromyography (EMG) and electrophysiological techniques has allowed more detailed study of the physiological properties of skeletal muscle fibers. Type I fibers produce small amounts of force very slowly and can sustain the force developed during tetanic stimulation for a long period of time (Table 1). Type IIA fibers are classified as fast twitch and fatigue resistant. They produce high twitch force and possess a moderate fatigue level. Type IIB fibers are called fast fatigable because they produce a high amount of twitch force but fatigue very rapidly.

Muscle contractile unit

Another way to describe the physiological differences between muscle fiber types is by examining the proteins that make up the muscle contractile unit, the sacromere (Figure 2). There are two filamentous contractile proteins in skeletal muscle, which make up the contractile machinery, *myosin* and *actin*. Myosin is a very large protein (480 kD), whereas actin is much smaller (42 kD). The sarcomere is an orderly arrangement of thick and thin filaments with distinct banding patterns, which effect overlap of the two contractile filaments. At rest, there is very little overlap between the two contractile elements. The myosin heavy chain component is a dimer molecule made from a combination of two myosin heavy chain variants wrapped around each other. The dimer structure forms the myosin tail, which attaches to the M-line of the sarcomere. The head of the myosin contractile protein contains four myosin light chain molecules: two to control shortening velocity of the myosin crossbridge cycle, and two to regulate the phosphorylation of the myosin molecule. The globular head contains enzymes that generate the power stroke when actin and myosin bind, causing the filaments to slide across one another (Figure 2).

When an electrical impulse is triggered in the muscle, calcium enters the muscle fiber and binds to *troponin*, which causes a conformational change in the actin filament arrangement and exposes the binding site of actin to myosin. Release of phosphorylated by-products of ATP results in sliding of the myosin filament over the actin filament. The two proteins stay bound to each other until another ATP molecule releases them. Once released the cycle can start again. For peak muscle force generation, there must be

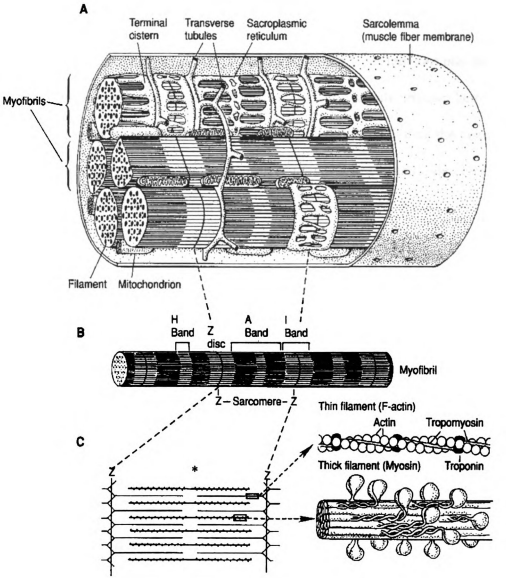


Figure 2. Structure of a sarcomere. From Kandel, Schwartz and Jessell (1991) Principles of Neural Science, 3rd Ed. p 549. * If a vertical line were drawn through the sarcomere structure at the asterick, it would show where the myosin filaments attach to the sarcomere, called the M-line.

optimal overlap between the contractile proteins otherwise peak muscle force capabilities are diminished.

Both myosin and actin have a range of contractile protein variants that have biochemical properties ranging from slow to fast. For example, myosin heavy chain IIA, IIX and IIB are found in adult skeletal muscle with Type IIB having the fastest myosin ATPase cycling capabilities. This variability in myosin heavy and light chain types allows for a wide range in myosin contractile properties (Table 1).

Effects of exercise training on muscle

Systematic exercise training improves performance by: 1) improving knowledge about task requirements, 2) increasing muscle strength, 3) increasing endurance, and/or 4) increasing motor efficiency. This section focuses on improvements in performance associated with changes in the response of skeletal muscle to exercise training. Two types of training protocols are considered, *endurance* and *resistance training*. Endurance training involves repetitive, long-term activation of the skeletal musculature to increase the duration that a task can be performed (e.g., distance swimming and running), whereas resistance training involves repetitively lifting a load that is heavier than one is accustomed to lifting in order to increase strength.

Studies in both humans and animals have shown that skeletal muscle undergoes adaptive changes in response to endurance training. An increase in capillary beds around muscle fibers increases blood flow and supplies the muscle with the necessary oxygen for oxidation of glucose (McComas, 1994). Results of several experimental studies are subsequently summarized to illustrate the extent and variability of responses to endurance

training. After an 18-week running protocol, mitochondrial protein content (which signifies increased aerobic capacity) in guinea pig muscle increased significantly over control animals (Barnard et al., 1970). In the same animals there also was an increase in the number of red fibers in the trained muscles reflecting an increase in Type I muscle fibers. The intensity of a running protocol dictates the types of biochemical adaptation that occur in specific muscle fiber types (Baldwin et al., 1977). For example, citrate synthase, a mitochondrial enzyme, increased with chronic running in rats but the response was dependent on muscle fiber type (Miller, 1992). Type IIA fibers showed the largest increase followed by Type IIB fibers. In contrast, intense interval training (short bursts of rapid running) had the greatest effect on Type IIB fibers, but the change was transient. Phosphofructokinase (PFK), the rate-limiting enzyme of glycolysis, increased by 20% in Type I but not Type II fibers compared to control levels in rats after high-speed interval training (Baldwin et al., 1977). However, a decrease in glycolytic enzyme activity in rats was observed with sprint training compared to control animals (Hickson et al., 1975), indicating that shifts in enzymatic activity are dependent on training intensity.

The data suggest that training protocols of different intensity result in different effects in the expression of enzymatic proteins. Terjung et al. (1973) set out to determine whether these differences were due to an increase in synthesis, a decrease in the degradation, or a combination of the two processes. An increase in the synthesis of cytochrome c and a decrease in its degradation rate were observed in rats exposed to endurance training (Terjung et al., 1973). Training durations that lasted longer than 2 hours/day did not further increase cytochrome c and citrate synthase levels in working

muscles (Terjung, 1976). There thus appears to be a time frame for maximum benefit of protein expression.

Edgerton et al. (1969) used swimming as a form of endurance exercise to evaluate changes in several aerobic enzymes in the soleus muscle (malate dehydrogenase, succinate dehydrogenase, nicotine adenine dinucleotide diaphorase, and α-glycerophosphate dehydrogenase). Chronic swim training altered the enzyme capabilities of a muscle, but as with running protocols, the response depended on the fiber composition of the muscle involved. Myosin ATPase (an enzyme that controls cross-bridge cycling) activity was not altered by swim training (Edgerton et al., 1969). Saltin et al. (1976) also failed to detect changes in muscle fiber myosin ATPase staining with either one-legged or two-legged bicycle ergometer training in humans.

Endurance training apparently alters protein expression at the neuromuscular junction. In rats running for 2-hours per day, the number of acetylcholine receptors in the postsynaptic membrane of the neuromuscular junction increased significantly by 20% over control animals (Desaulniers et al., 1998). A corresponding increase in endplate surface area was also seen. These two elements of the NMJ seem to be tightly coupled and would therefore imply no change in overall receptor density.

Endurance training also alters muscle fiber phenotype. Type IIB fibers readily transform to Type IIA fibers with endurance training (McComas, 1994). The following sequence underlies this change: IIB→IIA→IIC→I. Type IIC fibers have properties intermediate to Type IIA and I and have higher oxidative capacity than IIA or IIB fibers. It appears that fibers are more receptive to becoming more aerobic in their energy production capabilities than they are in becoming more anaerobic. Klitgaard et al. (1990)

used gel electrophoresis to show that endurance training may cause both Type I and IIA myosin heavy chain isoforms to exist in the same muscle. Fiber type conversion with endurance training needs further study because it is often difficult to partition between effects due to differences in genotype and training.

Functional changes in muscle after resistance training

Resistance training has different effects on skeletal muscle than endurance training. Resistance training is defined as muscle acting against a load to produce an increase in strength. The goal of resistance training is to increase strength and produce muscle hypertrophy or an increase in fiber size. The principle of overload is used to accomplish this goal. When a muscle is forced to move a load that is greater than it is accustomed to, it will adapt its physical structure to accommodate the additional load. The initial performance enhancement associated with a strength-training program does not involve hypertrophy. Rather, the changes include alterations in motor unit recruitment and a decrease in co-contraction of agonist and antagonist muscles (Carolan and Cafarelli, 1992; Sale, 1983). These initial changes are considered *neural* adaptations, which are discussed in more detail later in this chapter.

The study of motor unit recruitment is based on the seminal work of Henneman et al., (1965), who proposed that motor units are recruited in an orderly fashion based on intrinsic electrical characteristics of the motor neuron. This is referred to as Henneman's "size principle" and it is still a valid description of motor unit recruitment. The size principle states that motor units are recruited based on the ease at which they are brought to threshold. Small, fatigue-resistant motor units (Type S) are activated first, followed by

larger fast-fatigue-resistant units (Type FR) and then fast-fatigueable units (Type FF) (Bawa et al., 1984; Binder et al., 1983). The reverse occurs when the force requirements decrease.

Each muscle in the body has different recruitment characteristics. For example, recruitment in muscles of the hand seems to plateau at about 50% of maximal force, but recruitment in other muscles of the arm continues until 80% of maximum force is attained (DeLuca et al., 1982). One study that refutes the size principle showed recruitment order reverses during eccentric (lengthening) contraction where high threshold units (type F) are activated and low-threshold units are suppressed (Nardone et al., 1989). However, this particular study is not widely accepted because of the overwhelming electrophysiological evidence that supports the size principle of Henneman.

Several nerve pathways descend from the brainstem to control goal-directed limb movements, such as reaching and manipulation. The pathway that influences motor neurons controlling distal muscles of the extremities is the *rubrospinal tract.* The rubrospinal tract generates five times as much excitatory synaptic drive to type FF units than to type S units (Powers et al., 1993). This is important because fast-twitch muscle fibers need to be activated when fine-tuning a limb movement. A strong rubrospinal system input does not reverse the normal recruitment sequence; rather it produces near randomness in the recruitment pattern (Heckman and Binder, 1993). When muscle spindle Ia excitation was blocked complete reversal of the normal recruitment order occurred (Heckman and Binder, 1993). Thus, sensory input from Ia afferents is involved

in fiber type recruitment and the rubrospinal tract is one pathway that plays a role in recruitment.

Muscle hypertrophy does not occur in humans until after several months of consistent and progressive overloading has occurred. However, inter-individual variability is considerable, which suggests that some individuals may not have the genetic capacity for muscular hypertrophy even though the principles of overload are applied, whereas others can build muscle with less effort (Bouchard et al., 1997)

Adaptations associated with resistance training are highly specific to the muscle used in the task, range of motion, duration of exercise, and movement velocity. *Task specificity* implies that the muscles used in lifting the load will show the adaptations. *Length specificity* implies that the adaptations are limited to the range of motion of the task, e.g., a biceps curl through various ranges of motion; 30°, 45°, 90°, 120°, and 180°. A movement at 30° range of motion shows changes in a very small portion of the muscle length, whereas a movement across 180° produces changes along the whole length of the muscle fiber (Jones et al., 1989). Length specificity is influenced by the length-tension relationship between groups of muscles across a joint. Muscles that pass over complex joints, such as the knee or elbow, each have their own length-tension relationship. However, when the muscles that move these joints work together, they have an optimal angle-tension relationship, which implies that certain muscles are activated only at high tension and others at low tension. If training causes one or more of the muscles to hypertrophy, it alters the optimal angle-tension of the entire group of muscles (Jones et al., 1989). Thus, effective training must occur at an optimal length for all of the muscles

that are involved in the joint movement. Otherwise, one will have a lower production of maximum power and poor biomechanical efficiency of movement.

Velocity specificity is the speed at which a movement is performed to generate a specific force. The amount and type of adaptation seen in the muscle is based on how fast or slow the exercise is performed and the load being lifted. This specificity is measured on an isokinetic machine which measures the relationship between force and velocity. The underlying rationale is that if isometric strength is increased, a similar percentage increase in force sustained should occur over all velocities (Jones et al., 1989). Presently, available data are variable because each isokinetic machine measures the force-velocity relationship differently and torque is not always measured.

There is no clear evidence to suggest that fiber type conversion occurs with strength training (McComas, 1996). However, studies have not been of sufficient duration to clarify this issue. The longest hypertrophy study was 16-weeks (McComas, 1996). It is believed that a longer study period is needed to see hypertrophy changes.

Effects of resistance training on muscle biochemistry

The effects of strength training on skeletal muscle take longer to develop than corresponding changes in endurance properties with aerobic training. Strength training is an anaerobic activity, which by definition utilizes glycolysis for energy production. Creatine phosphate (CP) another energy storage system, is also used in the initial stages of exercise. Intense activity that lasts at least 20 seconds, but less than 3 to 4 minutes, utilizes the glycolytic and CP energy systems. Thorstensson et al. (1976) used a 5 second, repetitive sprint protocol for 8 weeks in humans. Myosin ATPase activity increased by

30% and creatine phosphokinase by 36%, whereas lactate dehydrogenase (a glycolytic enzyme) activity did not change. However, an earlier study by Thorstensson (1975) using a squatting protocol, 3 times per week, observed no change in myosin ATPase or creatine phosphokinase levels. Tesch et al. (1989) compared olympic weight lifters and body builders to sedentary controls in the context of several metabolic characteristics. Lactate dehydrogenase (LDH) activity was 62% higher in fast twitch fibers and 50% higher in slow twitch fibers in the olympic athletes and body builders. It is interesting to note that slow-twitch fibers responded to the strength training with an increase in LDH activity, a glycolytic enzyme. Slow twitch fibers normally have low glycolytic enzyme levels and rely heavily on aerobic oxidation to meet their energy demands.

Glycolytic enzyme adaptations, in general, are fiber-specific and response to resistance training occurs primarily in fast-twitch fibers (Thorstensson et al., 1976). This is due to the recruitment of fast-twitch fibers during intensive exercise because fast-twitch fibers produce more force, and creatine phosphate and glycolytic energy supplies can be activated immediately (Table 1). On the other hand, aerobic metabolism takes several minutes before it is capable of producing the required energy. Resistance training that produces a maximum effort for short duration increases the glycogen content of resting muscles and increases phosphocreatine storage to a lesser degree.

Effects of chronic resistance training on muscle size and composition

McComas (1996) reviewed several studies of the effects of intensive resistance training in adult males on muscle fiber structure. Based on needle biopsies of the vastus lateralis muscle, fiber cross-sectional area increased from 17 to 57% in Type II fibers and

from 3 to 15% in Type I fibers. This type of hypertrophy was apparent only with training periods of 10 to 26 weeks duration, and the greatest increase in area occurred in studies of the longest duration [where the cross-sectional area of the whole muscle was measured]. Caution must be used when interpreting needle biopsies because it is difficult to reliably obtain repeated measures at the same location. In this context, studies using animals are important because the whole muscle can be removed, weighed and measured in a standardized manner, and the muscle can be quickly frozen for subsequent structural and histochemical analyses.

The increase in muscle size associated with training can be due to an increase in contractile machinery and a loss of fat and connective tissue between muscle fibers. An increase in myofilaments has been observed in animal muscle after a period of heavy exercise (Jones et al., 1989), and an increase in the radiological density of muscle tissue after strength training in humans as measured by computerized tomography (CT) scans has also been observed (Jones, et al., 1989). This increase in image density could occur for a number of reasons: 1) a decrease in the muscle fat content, 2) an increase in contractile element packaging, and/or 3) an increase in connective tissue content. The first two possibilities would theoretically produce an increase in the force per unit area.

Several studies have reported that strength training increases fiber size (hypertrophy) and fiber number (hyperplasia) although the latter result is controversial. Gonyea and Bonde-Peterson (1978) used an established hypertrophy exercise protocol in cats and reported a 19% increase in the number of fibers in the flexor carpi radialis muscle (the prime mover used in the hypertrophy exercise) compared to control animals. Histological data suggested "splitting" of both slow and fast twitch muscle fibers.

Muscle fiber splitting was defined as an increase in fiber number when a muscle is exposed to strenuous exercise. Reitsma (1969) used a surgical model to investigate hypertrophy and later disputed the idea of hyperplasia. His surgical model severed all muscles but the one that performed a movement, producing a functional overload of the spared muscle (e.g., the rectus femoris muscle was the only muscle left intact during knee extension). The rectus femoris normally contributes to about 35% of knee extension in rats. The plantaris and soleus muscles were also studied; the former is involved in 15% of plantar flexion and the latter is involved in 6% of plantar flexion in rats. After exercising the animals for 3 weeks, 12 hours per day, there was only a 28% increase in rectus femoris weight whereas the weights of the plantaris and soleus muscles increased by 104% and 151%, respectively (Reitsma, 1969). Some split fibers were also noted in the muscles after this surgical manipulation and were classified as being external or internal (Reitsma, 1969). Depending on where along the fiber length the histological cross-section of the muscle was taken, the appearance of splitting appeared different (Figure 3). The splitting that was observed in the operated and non-operated leg was muscle and load-dependent. Histological analysis of the split fibers showed that hyperplasia did not occur.

Controversy has continued to surround the idea that the splitting of muscle fibers represents actual hyperplasia. Edgerton (1970) used swimming and running wheel protocols to demonstrate fiber splitting in rats. He reported that the sarcoplasm was continuous across all of the split fibers (subfibers), which implied that the fibers were of the same type and had the same neural innervation thus demonstrating that hyperplasia did not occur.

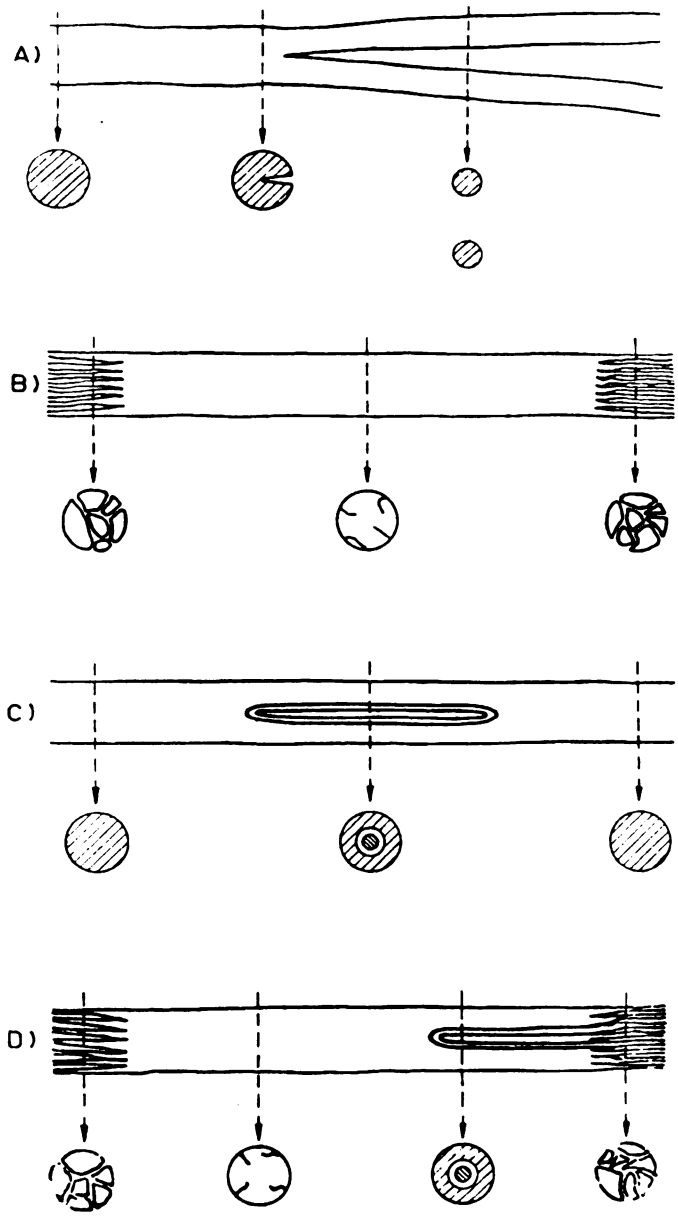


Figure 3. Diagrammatic patterns of muscle fiber splitting. **A** and **B** in the figure represent simple and extensive external fiber splitting. **C** demonstrates internal fiber splitting, while **D** shows a combination of external and internal splitting. From Reitsma (1969, p.248).

Splitting of muscle fibers is also observed after very intensive, damaging exercise. Eccentric exercise, which produces muscle lengthening under load, is a prime example of damaging exercise (Takekura et al, 2001; Proske et al., 2001). Under these conditions, splitting does not occur along the whole length of the fiber, but is confined to a specific region, usually at the point of maximal tension. Thus, fiber-splitting is not interpreted as a true increase in fiber number. Rather, it is viewed as reflecting fiber damage, which translates into inefficient performance and a decreased mechanical force that is proportional to the degree and severity of muscle damage (Takekura et al., 2001).

Fiber hyperplasia has been reported in avian species when a load is placed on the wing that results in stretching of the muscle fibers (McComas, 1996). That hyperplasia is observed in avian species, but not mammalian species, may reflect mechanical overload from stretch and not work performed by the muscle (Kelley, 1996). Results of a statistical meta-analysis of a multitude of studies on hypertrophy and hyperplasia suggest that changes in skeletal muscle fiber number associated with mechanical overloading are inconclusive, but that muscle fiber hyperplasia is in response to mechanical stretch not mechanical overload in birds (Kelley, 1996)

Effects of training on the motor axon

Changes in the motor axons that innervate skeletal muscle are considered alterations in the *peripheral* component of the motor unit. The axon emerges from the axon hillock region of the motor neuron cell body. It projects out of the spinal cord in a peripheral nerve, and terminates in synaptic endings at the neuromuscular junction. The

subsequent discussion considers only the motor axon. Effects of exercise on the motor neuron cell body are discussed in the next section.

The axon is essentially a cylindrical tube that conducts an electrical impulse from the cell body of the neuron to the muscle fiber. The axon also functions in the transport of protein and chemical messengers from the motor neuron to the synaptic endings. During fetal development, axons emerge from motor neurons in the medial and lateral motor column of the spinal cord. They migrate in groups and split away upon reaching their target muscles. The differentiation of fiber types begins at about the same time as the growing axons innervate the muscle fibers. Each motor neuron appears to influence how the muscle fibers that it innervates will differentiate (Grinnell, 1995). The motor axon, however, is not required for fiber differentiation. This is demonstrated by the fact that aneural limb myotubes (lacking neural innervation) still express proteins that are specific to fast or slow twitch fibers (Grinnell, 1995).

It had been hypothesized that increased muscle activity causes motor axons to hypertrophy in order to meet the demands of muscle activation (Key et al., 1984; Samorajski and Rolsten, 1975; Edds, 1950; Andersson and Edstrom, 1957; Roy et al., 1983; Moritani et al. 1979). In general, endurance activity, either voluntary or forced training below 80% of VO_2 max, did not alter axon diameter. When animals were forced to train at an intensity level, which they would not voluntarily perform, requiring aversive stimuli, a decrease in axon diameter actually resulted (Andersson and Edstrom, 1957). Andersson and Edstrom (1957) postulated that the hyperactivity of muscle was met by an increased rate of protein synthesis in motor neurons and the proteins were being used for cellular maintenance. However, they postulated that when the activity level was too

intense, protein synthesis did not keep pace with demand and a decrease in motor axon diameter resulted. Roy et al. (1983) subsequently reported that both motor axon diameter (10%) and myelin cross-sectional area (19%) decreased in intensely trained rats. When exercise was kept at moderate or low levels, the demands on muscle fibers are apparently met by an increased rate of synthesis in the neuronal cell body with no increase in axon diameter. The decrease in motor axon diameter associated with strenuous exercise may be explained by neural fatigue. The motor neuron simply could not keep up with the demands of protein production so that protein transport was slowed and structural maintenance was decreased (Fitts, 1994).

Tomanek and Tipton (1967) used a surgical procedure called a *tenectomy* to study exercise-induced changes in motor neurons. In this procedure, the tendon connecting the gastrocnemius to the calcaneus (tendo-calcaneous tendon) was severed. This provided the muscle with an intact blood supply, but it was unable to generate tension. Tenectomized rats showed a significant decrease in diameter of the medial gastrocnemius nerve and a corresponding reduction in the number of axons in the nerve. There was no significant difference in mean nerve fiber diameter between the control and exercise groups. The reduction in myelinated nerve fibers following tenectomy may be attributed to atrophy or demyelination resulting from lack of activation or reduced proprioceptive sensory feedback. The tenectomized muscles contained smaller muscle fibers and the muscle weighed significantly less than the muscle of control animals.

Eisen et al. (1973) used immobilization and sciatic nerve crush to investigate the role of activity in determining motor nerve diameter. With the crush protocol, the nerve was gently squeezed with forceps to produce a reversible conduction block and

subsequent decrease in voluntary muscle activation that recovers with time. The contralateral limb in the immobilized control and crushed nerve groups was the hyperactivity contrast. After six weeks, the soleus nerve, which lies below the crushed sciatic nerve, was extracted. Immobilization caused a significant reduction in mean fiber diameter in both intact and regenerating sciatic nerves of the soleus. The hyperactivity group, in contrast, showed an increase in mean axon diameter compared to immobilized control. The size of nerve fibers that were >5μm in diameter at the start of the protocol increased the most.

Eisen et al. (1973) used the soleus, a tonically active antigravity muscle, which is more dependent upon neurotrophic and/or neuroregulatory factors than phasic muscles. The greater tendency of axons of tonically activated muscles (soleus) to increase in diameter with hyperactivity and to decrease in diameter with hypoactivity could be explained by the greater presence of neurotrophic and neuroregulatory proteins.

Changes at the synaptic terminal in response to exercise

The neuromuscular junction (NMJ) is comprised of the synaptic terminal of the motor axon (presynaptic membrane), a synaptic space (cleft), and the postsynaptic muscle membrane. The NMJ undergoes constant remodeling to meet the activation requirements of the muscle. Wernig et al. (1991) used a treadmill running protocol with mice in an attempt to demonstrate a form of NMJ remodeling called *terminal sprouting*. They described two types of sprouting, *terminal* and *nodal*. Terminal sprouting was defined as thin-branches originating within the terminal arborization and extending at least 5um beyond the edges of the endplate, while nodal sprouting was defined as thin

outgrowths from the nodes of Ranvier. Sprouting of the NMJ creates more surface area for transmission to occur. The training protocol produced muscle injury (assessed by immunohistochemistry staining of contralateral leg muscle), which was evident within the first hour after exercise and observed up to two weeks later. Three to 21 days after a single bout of running, terminal and nodal sprouting increased twice as much as in control animals (Wernig et al., 1991). Sprouting increased further after repeated running episodes.

Rotshenker and McMahan (1976) described increased motor nerve sprouting in multiple muscles on the contralateral side of the body after unilaterally severing the nerve innervating the pectoris muscle in frogs. The growth on the opposite side of the body was interpreted as a response to a compensatory central signal responding to the loss of muscle innervation on the experimental side of the body, possibly involving one or more neurotrophic factors. Sprouting occurred even earlier if the denervation occurred closer to the spinal cord. However, Brown et al., (1979) conducted a similar experiment in the mouse by cutting the sciatic nerve in one leg and found no evidence of sprouting in the contralateral limb. Taking into account that a hypothetical central signal might not cross the spinal cord in mice the lower leg was completely denervated except the soleus muscle. Only minimal sprouting was observed. Thus, contralateral motor axon sprouting following contralateral limb denervation is more limited in mammals than in amphibians. Sprouting in mammals must be triggered by a local factor not a hypothetical central signal.

Deschenes et al. (1993) used a high- and low-intensity running protocol of 12-weeks duration in young adult rats to examine the effect of endurance training on

junctional area of the motor endplate region. The results suggested that running led to an increase in the size of the motor endplate. High-intensity-trained rats showed an increase in junction area and an irregular shape of the endplate compared to low-intensity-trained rats, in which synapse structure was more compact and symmetrical. The nerve terminal branching in high-intensity-trained rats also demonstrated greater length and complexity compared to low intensity trained animals (Deschenes et al., 1993). The increase in NMJ area would increase the surface area to which neurotransmitter vesicles could dock and thus lead to a greater probability of release and greater binding on the postsynaptic membrane. Increased transmitter release would increase the size of the endplate potential increasing the probability of evoking a contractile response in the muscle fiber and increasing it amplitude.

Application of tetrodotoxin (TTX), a neurotoxin that blocks sodium channels, was used to determine the effects of nerve block (hypoactivity) on acetylcholine receptor (AChR) clustering. TTX prevents motor nerve conduction distal to the site of application (Katz and Miledi, 1968). When rats were exposed to TTX-induced long-term nerve block, AChR clustering still occurred in the electrically silent motor endings (Pasino et al., 1996). It was postulated that an activity-independent factor must be present in the motor terminal that regulates postsynaptic AChR clustering, possibly agrin, acetylcholine receptor-inducing activity (ARIA), or calcitonin gene-related peptide (CGRP) (Pasino et al., 1996).

Chronic treatment of nerves with TTX and colchicine (which blocks axon transport) has been reported to increase the frequency of miniature end-plate potentials (MEPPs) recorded from muscle fibers (Siebler and Schmidt, 1986). MEPPs result from

the spontaneous release of individual ACh-containing synaptic vesicles. The nerve-block induced increase in MEPPs suggests presynaptic mechanisms may lead to a compensatory increase in the spontaneous release of ACh.

Effect of exercise on axonal transport and release

Axon transport is the process of delivering proteins assembled in the motor neuron cell body to the NMJ. There are two transport pathways, fast and slow. Fast axonal transport (400mm/day) is associated with the delivery of proteins essential for neurotransmission, while slow axonal transport (0.02-2.5 mm/day) is involved with maintenance of the neuronal cytoskeleton, a kind of support scaffolding. In order to increase release of transmitter, there must be an increase in transmitter production and packaging.

Jasmin et al. (1987) examined the effects of 8 weeks of running and swimming on fast-axonal transport of acetylcholinesterase (AChE) in the sciatic nerve of rats. On the last day of training, ablation of L_4 and L_5 spinal ganglia was performed on one side of all animals to eliminate possible contribution of AChE transport by afferent nerve fibers. Twenty-four hours following ganglionectomy, sciatic nerves of each animal were ligated bilaterally to block fast axonal transport. The ligatures were left in place for 4 hours, the sciatic nerve was removed, and proximal and distal accumulation of AChE was assessed around the ligatures. Swimming induced no significant change in AChE transport in either the intact or deafferated nerves. On the other hand running, significantly increased AChE transport in the deafferated nerve compared to control rats, suggesting that sensory neurons exert an inhibitory influence on AChE transport in motor axons, or that a

central compensatory mechanism which is masked by increased AChE transport (Jasmin et al., 1987). In another study using a similar running protocol, radiolabeled amino acids were injected into motor neurons that supply the sciatic nerve to examine fast orthograde axonal transport and to determine its velocity (Jasmin et al., 1988). After 8 weeks of training, transport of labeled protein increased in the motor axons. In contrast, exposing untrained animals to one session of exhaustive exercise (running once at the grade and speed of chronically trained animals until exhaustion) reduced total transport by 36%. Thus, increase in axonal transport is an adaptive change of motor neurons to the requirements of chronic sustained exercise, and not an immediate response to a single training episode.

Dorlochter et al. (1991) used a chronic voluntary wheel-running protocol in mice to study transmitter release at the neuromuscular junction. Exposure of the NMJ to high Mg^{2+} blocked Ca^{2+} channels on the presynaptic membrane, thus preventing transmitter release. The trained extensor digitorum longus muscle had, on average, a 30% higher transmitter quantal release content than the control muscle after 2-8 months of training.

Activity and impulse propagation in motor axons

Nerve conduction velocity is the speed at which impulses are propagated along an axon. In motor axons it is directly related to the speed at which the innervated muscle fibers contract (twitch contraction). Rapid conduction velocity is indicative of a short refractory period, meaning that the motor axon can generate action potentials at a high frequency. A decrease in refractory period increases the discharge frequency of the motor

neuron, thereby increasing the level of force production by the muscle. See Table 1 for the general characteristics of motor neurons.

Several studies investigated whether exercise training changes motor nerve conduction velocity (Buller et al., 1960, Czeh et al., 1978) but results are equivocal. Two methods were used to assess conduction velocity, 1) by transecting the spinal cord, which leaves the peripheral neuromuscular connections intact but allows the assessment of the effects of central nervous system on conduction, and 2) by pharmacologically blocking Na^+ channels on the postsynaptic muscle membrane and preventing muscle contraction. Czeh et al., (1978) reported a decrease in the duration of the afterhyperpolarizing potential (a.h.p.) of cat soleus motor neurons within 8 days after spinal transsection. The decrease in a.h.p. duration was reportedly prevented by daily nerve stimulation. Chronic application of TTX to muscle also produced a significant decrease in the duration of the a.h.p. 8-days after TTX blockade. However, maximum twitch tension and fast axoplasmic transport were unaffected. It was concluded that motoneuron properties depend on an intact innervated muscle and that trophic factors retrogradely transported by the motor axon could influence conduction velocity.

Another method to assess the effect of conduction velocity is cross-innervating a fast muscle nerve to a slow muscle and visa versa. Buller et al. (1960) used the cross-union technique, where the nerve to the soleus muscle was severed and attached to the flexor digitorum longus muscle (FDL), to assess speed of muscle contraction on the conduction velocity of the axon and the a.h.p. of the motor neuron. The FDL nerve was in turn attached to the soleus muscle. There was considerable acceleration in the contraction of the soleus muscle innervated by the FDL nerve, and considerable slowing of the FDL

muscle re-innervated by the soleus nerve. Contraction times, half-relaxation times and twitch tension were reversed in the crossed muscles. See Table 1 for muscle characteristics. The soleus muscle innervated by the FDL motor neurons required a considerably higher frequency of stimulation to achieve a fused contraction. The reverse scenario was observed in the FDL muscle. There was no change in the a.h.p. and conduction velocity of the soleus and FDL nerves. Thus, muscles apparently do not influence the physiological properties of motor axons that innervate them. On the other hand, muscle fibers changed their properties over the 4-week period. The soleus had a higher concentration of fast twitch fibers and the FDL gained a higher percentage of slow twitch fibers (Buller et al., 1960). The authors postulated that the neural influence on muscle contraction is not exerted by the nerve impulse per se; rather, factors must be passed to the muscle fibers from the motoneuron that causes the muscle to alter its characteristics.

Salmons and Sreter (1976) implanted a stimulator into the peritoneum of rabbits and attached electrodes to the peroneal nerve to see if the same affect Buller et al., (1960) saw occurred in muscle properties after cross-innervation. The study showed that the flexor digitorum longus (FDL) muscle, which is stimulated by the peroneal nerve, developed increased fatigue resistance and contracted slower when continuously stimulated at a low frequency. In addition, slow myosin light chains replaced fast myosin light chains (LC's), a characteristic of a slow muscle type (see Table 1). When the soleus nerve was cross-innervated with the extensor digitorum longus (EDL) nerve and an implantable stimulator (firing at a low frequency) was connected to the soleus nerve and allowed to recover for 8-weeks, the effects of cross-innervation disappeared (Salmons

and Sreter, 1967). Thus, in this study firing frequency was a factor in determining muscle fiber type, and appears to contradict the hypothesis of Buller et al. (1960) that nerve activity (firing frequency) does not in and of itself influence contractile properties of a muscle. However, the release of trophic factors in Salmons and Sreter (1976) experiments cannot be ruled out. Regardless of what mediators are involved, motor neurons appear to exert profound effects on muscle physiology.

Central adaptations to exercise

Central adaptation to exercise entails changes in the motor neuron cell body that is located in the spinal cord and changes that occur in various regions of the brain in response to exercise. A general description of the motor neuron was given earlier. Structural modifications that occur in motor neurons in response to exercise are subsequently considered.

Size of motor neurons and metabolic characteristics

Edstrom (1957) investigated the effects of endurance activities on the size of motor neuron cell bodies in guinea pigs. Animals were assigned to one of two protocols: treadmill running with the hindquarters suspended (to avoid possible effects of general fatigue as a measure of acute activity), and longer running trials employing all four limbs as a measure of chronic motor training. A single bout of acute exercise resulted in a 47% increase in cell body volume. However, after 4 weeks of chronic running, motor neuron size did not differ from control animals. The increase after an acute exercise bout, therefore, was probably due to a transient uptake of water and not hypertrophy. However,

nucleolar size in the chronically trained animals increased by 141% compared to controls, suggesting that an increase in RNA transcription may be activated by long-term physical exercise.

Gilliam et al. (1976) used two interval-training protocols of different intensities to study the effects of activity on the diameter of motor neuron cell bodies in rats. The distribution of motor neuron size for both sprint- and endurance- trained groups showed a "retardation effect" after 12 weeks of wheel running compared to control animals. The decrease was even greater in the more intensively interval-trained group. Differences between this study and Edstrom (1957) were attributed to the greater running intensity and differences in methodology in which comparisons were made between frequency distributions rather that sample means.

Nakano et al. (1996) used a 10-week treadmill running protocol (2h/day, 30m m/min, 5 days/week) and found a significant increase in the size of soleus motor neurons. Thus the enlargement of motor neurons may be associated with an increase in neuronal activity to meet the demands of physical training. The effect of exercise on motor neuron size thus appears dependent on the type of the duration of activity.

In addition to size and morphological features, metabolic differences between motor neurons also have been reported. Campa and Engel (1970) stained lumbar motor neurons of cats for phosphorylase and succinate dehydrogenase (SDH), enzymes involved in glycolytic and aerobic metabolism, respectively. Two pools of neurons in the ventrolateral area were reported, 1) those > 30μm in diameter stained high for phosphorylase, and 2) those < 30μm stained low for phosphorylase but high for succinate

dehydrogenase. On the other hand, motor neurons in the ventromedial, intermediate and posterior gray matter stained high for succinate dehydrogenase, regardless of size.

Sickles and McLenson (1983) also used histochemical techniques to differentiate subpopulation of motor neurons and to determine whether metabolic variation was correlated with cell size. High lactate dehydrogenase (LDH) staining was found in most motor neurons suggesting that *aerobic* metabolism predominates. However, staining for α-glucan phosphorylase, an enzyme used to assess glycogen utilization was more variable, and indicated that larger motor neurons have a greater capacity for glycogen catabolism. Smaller motor neurons exhibited relatively denser staining for succinate dehydrogenase and NADH-D consistent with higher aerobic capacity. Thus, this study and the results of Campa and Engel (1970) suggest metabolic variability in spinal α-motor neurons that parallel similar differences in skeletal muscle (see Table 1).

Gerchman et al. (1975) used a forced swimming protocol and voluntary running in rats to determine whether exercise training induces changes in motor neuron metabolism. Acid phosphatase staining of motor neurons, a marker for ATPase metabolism, was increased in the forced swimming group compared to sedentary controls, and a group subjected to both running and forced swimming. On the other hand, the aerobic marker malic acid dehydrogenase was higher in motor neurons of animals performing both running and swimming activities. These results, together with those of Sickles and McLenson (1983) suggest that motor neurons can adapt to the demands placed on them during exercise training by enhancing their oxidative capabilities.

Nakano et al. (1996) used a 10-week running protocol (2h/day, 30 m/min, 5 days/week) and concluded that succinate dehydrogenase activity (SDH) in motor neurons

innervating the soleus and EDL muscles were unchanged by training. However, the size of soleus motoneurons increased and there was significantly higher total SDH activity (SDH activity x cell body size) in soleus motor neurons than controls. This data suggests that chronic endurance activity has a stronger impact on cell body size and total oxidative enzyme activity of motor neurons innervating slow-twitch than those innervating fast-twitch muscles.

In summary, spinal motor neurons exhibit evidence of metabolic enzyme variation in immunohistochemistry staining. Larger motor neurons generally stain high for anaerobic enzymes and smaller motor neurons stain higher for aerobic enzymes. There is a shift to a more anaerobic metabolism in forced swimming and toward more aerobic metabolism in voluntary running. Although there is variability in the literature it appears that motor neuron metabolism adapts in response to the demands of exercise training.

Motor neuron firing frequency

One way that the spinal motor system adapts to exercise training is by increasing motor neuron output (firing frequency). However, recording from motor neurons is difficult in humans and behaving animals. Bigland-Ritchie et al. (1983) used tungsten microelectrodes to record activity from motor units in the human adductor pollicis muscle (an intrinsic hand muscle controlling the thumb). When maximal force was sustained for 40-120 seconds, a progressive decline in mean firing rate was observed, with a 50% decline within the first 60 seconds. When the data from different units were pooled, there was a minimal decline in the discharge rate at low firing frequencies. However, units with

an initially high firing frequency changed the most. Bigland-Ritchie et al. (1983) postulated that the reduction in motor unit firing frequency is not responsible for force loss, but may enable effective strength modulation by rate coding and may also enable continued contraction during fatigue. Sale et al. (1983) studied motor neuron excitability from the potentiation of reflexes during maximal voluntary contraction after 9-21 weeks of strength training of the thumb musculature. Motor neuron excitability was measured indirectly by the degree to which two reflex responses V1 and V2 (volitional wave 1 and wave 2) were potentiated (50% and 39%, respectively) by voluntary effort, interpreted as due to training. It was hypothesized that motor neuron excitability was increased by the training protocol, although the mechanism for this response was not clear. The authors postulated that either additional motor units were recruited, or the activated motor units discharged at a higher frequency. Both mechanisms would produce an increase in the reflex response.

Role of chemical messengers in activity-dependent motor system plasticity

Trophic factors, neuromodulators, neuropeptides, hormones and proteins play the role of chemical messengers in the central nervous system and function in turning "on" or turning "off" synthetic processes in the body. However, little is known about the function of these mediators with regard to responses of the spinal motor system to physical activity. Calcitonin gene-related peptide (CGRP) and serotonin (5-HT) are the only chemical messengers investigated to date.

Injection of 5-HT into the vicinity of spinal motoneurons leads to an increase in motoneuron excitability and facilitates several spinal reflexes (Hounsgaard et al., 1986).

Locally applied 5-HT produces a small depolarization in spinal motoneurons in vivo that is accompanied by an increase in membrane input resistance and a reduction in the spike afterhyperpolarization period. These actions would allow motor neurons to reach threshold more quickly, to produce a strong twitch force, and to recover from stimulation more rapidly. 5-HT also activates a depolarizing plateau potential in motor neurons by a voltage-dependent non-inactivating Ca^{+2} conductance (Hounsgaard et al., 1988) thus providing motoneurons with two stable firing states (Hounsgaard and Kiehn, 1989). 5-HT also reduces the afterhyperpolarization following an action potential. In essence 5-HT "turbo charges" the motor neuron, thereby increasing its discharge frequency in response to a given input, and augmenting the contractile force generated by the muscle.

Serotonergic neurons in the raphe nucleus project to many areas of the CNS, including ventral horn neurons, and are spontaneously active during the waking state. With treadmill running, there is an increase in firing rate of these neurons in cats (Veasey et al., 1995). When the body is resting (when asleep) there is a reduction in 5-HT levels in the raphe nucleus. Raphe neurons project onto motor neurons and tonically release 5-HT during repetitive motor activity such as when exercising (Jacobs and Fornal, 1993). An important effect of 5-HT is the enhancement of spinal and cranial motor neuron excitability. The mechanism of how 5-HT mediates these changes in motor neurons during activity is not clearly understood but likely occurs through one or more of the following mechanisms: 1) reduction in K^+ conductance would allow the cell to stay depolarized longer, 2) activation of an L-type Ca^{2+} currents creating a stronger depolarization, 3) reduction of after-hyperpolarization amplitudes allows the neuron to fire more rapidly, and 4) an increase in input resistance (Rekling et al., 2000). This

increased central serotonergic drive during activity likely increases motor neuron responsiveness, thereby facilitating recruitment of motor units during force production.

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide present in motor neurons (Homonko and Theriault, 1997) and also in many sensory neurons (Miki et al., 1998). It is stored in large, dense core vesicles and coexists with ACh in the motor terminal. CGRP induces acetylcholine receptor (AChR) synthesis, modulates AChR-channel properties, and prolongs AChR channel open time in cultured myotubules (Mulle et al., 1988) and in adult muscle fibers by an adenosine 3', 5'-cyclic monophosphate (cAMP) - mediated pathway (Sala et al., 1995). CGRP levels are high during neuromuscular development, but fall when the neuromuscular junction is mature. CGRP content in motor neurons and their terminals increases after nerve crush and functional denervation by TTX (blocks Na^+ channels so impulse cannot get through). The up-regulation of CGRP disappears once the nerve is repaired and neurotoxin is removed (Sala et al., 1995). Electrical stimulation of a nerve distal to the application of TTX blockade will deplete motor terminals of CGRP. Thus, nerve impulses are capable of stimulating CGRP release. CGRP increases ACh receptor production in muscle (Desaulniers et al., 1998). CGRP can also down-regulate the production of trophic factors (BDNF and NT-3) in the muscle by its inhibitory effect on motor nerve terminal sprouting (Sala et al., 1995).

Endurance-trained rats (16-weeks running) showed a 90% increase in the staining for CGRP in soleus motor neuron cell bodies and neighboring neurons (Gharakhanlou et al., 1999). The results suggest that aerobic training up-regulates synthesis, transport, release and subsequent actions of CGRP at the neuromuscular junction. This increase

could play a significant role in morphological and functional adaptations that have been reported at the neuromuscular junction following chronic training. It is not known whether resistance training, which produces significant changes in muscle fiber size and physiology has a similar effect on motor neuron CGRP expression.

In addition to chronic running, a single, 30-minute bout of eccentric exercise (downhill running resulting in muscle fiber tearing) in rats was reported to increase expression of CGRP in motor neurons of the medial gastrocnemius (Homonko and Theriault, 2000). The motor endplates of medial gastrocnemius fast-twitch glycolytic muscle fibers, had a larger degree of muscle fiber damage from the single bout of eccentric exercise than the flexor muscles which were not active during this protocol. Thus, the elevation in CGRP may be a response to tissue damage and involved in repair or remodeling of the neuromuscular junction.

Low intensity treadmill walking in rats (9 m/min, 135 minutes of walking interspersed with rest period, 1-2 days) resulted in a selective increase in the membrane-bound globular form of acetylcholinesterase (AChE) tetramer (G_4) in fast skeletal muscle (Fernandez and Hodges-Savola, 1996) after one day of treadmill activity. It is postulated that G_4 AChE prevents the accumulation of ACh during high frequency stimulation, thereby limiting acetylcholine receptor (AChR) desensitization (Fernandez and Hodge-Savola, 1996). When exogenous CGRP was given to the trained rats, G_4 AChE did not increase after treadmill training (Fernandez and Hodges-Savola, 1996). The actions of CGRP are selective for G_4AChE since other forms of AChE (G_1, G_2 and A_{12}) were not affected by exercise or exogenous CGRP. It was suggested that CGRP does not have a

direct influence on the catalytic activity of G_4 receptors (Fernandez and Hodges-Savola, 1996))

Choline-acetyltransferase (Chat) is an enzyme that binds choline and acetyl CoA together to form acetylcholine (ACh). ACh is the chemical neurotransmitter that is released by motor terminals in response to an action potential. Upon its release, ACh travels across the neuromuscular junction to activate ligand-gated ion channels in the muscle membrane. It is not known whether exercise training alters motor neuron Chat activity.

The peptide responsible for the packaging of newly synthesized acetylcholine in the neuronal cytoplasm into synaptic vesicles is vesicular acetylcholine transporter (Vacht) (Roghani et al., 1998). An increase in Vacht may underlie the increase in ACh release by motoneurons after exercise training (Dorlochter et al., 1991). The larger the vesicular supply, the greater the release response and the lower the probability of zero quantal release. One might expect that Vacht activity will be altered in response to increased levels of motor neuron activity associated with exercise training.

Many structures in the central nervous system express the peptide galanin in various quantities. It is found in the dorsal horn and dorsal root ganglion and it is thought to be involved in pain processing (Zhang et al., 1998; Wiesenfeld-Hallin and Xu, 1998). Galanin receptors in dorsal horn neurons are up-regulated in experimentally induced inflammatory reaction studies (Zhang et al., 1998). It is postulated that galanin may be involved in processing pain information by exerting an analgesic effect. Galanin is also co-localized with CGRP in motor neurons of the thoracic, lumbar and sacral regions of the ventral spinal cord (Johnson et al., 1992). But its function is not known.

Calcium (Ca^{+2}) acts as a second messenger in many neural events. A Ca^{+2} current prolonged by binding to calmodulin activates a Ca^{+2}/calmodulin protein kinase (CaM kinase) pathway (Hanson and Schulman, 1992). These authors also described how CaM kinase isoforms phosphorylate many different proteins; such as enzymes, cytoskeletal proteins, ion channels and transcription factors. In sciatic motor neurons, the expression of the two CaM kinase isoforms IIα and IIβ, are altered after axotomizing L4-5 spinal nerves. CaM kinase IIβ is down regulated in motor neuron cell bodies where CaM kinase IIα was upregulated (Lund et al., 1997). The upregulation of CaM IIα is consistent with a role in axon regeneration and synapse formation. The down- regulation of CaM IIβ could be related to the loss of electrical activity or loss of maintenance "factor" that is retrogradely transported from the periphery (Lund et al., 1997).

The enzymes responsible for catalyzing the addition and removal of phosphate from serine and threonine residues are referred to as phosphatases (PPs). There are 4 major classes; PP1, PP2A, PP2B, and PP2C (Ingebritson and Cohen, 1983). PP2B is also known as calcineurin (CaN). Calcineurin immunostaining has been demonstrated in the central nervous system. CaN functions include 1) mediating events involving elevation of intracellular Ca^{+2}, 2) affecting neurotransmitter release machinery and 3) using negative feedback to regulate NMDA receptor properties (Ingebritson and Cohen, 1983). FK506 inhibits CaN activity. When applied to nerve terminals it causes an increase in exocytosis of synaptic vesicles containing glutamate and an increase in Ca^{2+} influx (Shira et al., 1995). Strack et al. (1996) demonstrated CaN like immunostaining in 25% of α-motor neurons in the facial trigeminal nerve motor pool. This selective staining was thought to be specific for a group of motor neurons controlling the firing of fast-twitch muscles. Lin

and Lin-Shiau. (1999) used electrical stimulation of mice motor nerve terminals to find out if CaN plays a role in regulating acetylcholine release. The CaN inhibitor cyclosporin A (CsA) was applied into the nerve terminal, which resulted in increased MEPP frequency, and after brief tetanic stimulation CsA alone also caused an increase in MEPP frequency.

Morphological changes in Higher Order Structures in the brain

The brain controls the coordination and execution of motor activity. What is not well understood is how exercise training may influence the centers of the brain that coordinate movement. This section explores what has been studied as far as brain adaptations to exercise.

Cerebellum

The cerebellum (Latin " little brain") contains both sensory and motor components. The cerebellum regulates movement and posture indirectly by adjusting the output of descending motor systems from the brain. It was hypothesized to perform this task by acting as a comparator, fixing errors in movement by comparing intentions with performance (e.g. review by Kandel, Schwartz and Jessell, 2000).

Parallel fibers are the branched, unmyelinated axons of granule cells, which contact and excite the dendritic trees of Purkinje cells. Climbing fibers are a special group of incoming fibers from outside the cerebellum. They are derived from the inferior olivary nuclei in the medulla oblongata and contact the dendritic tree of Purkinje cells. The inferior olivary nucleus is important in task learning. If it is damaged, learning of

motor skills is not optimal in animals and humans. Anderson et al. (1996) used an obstacle course that required balance and coordination in rats and noted an increase in parallel fiber branching onto Purkinje cells in the cerebellar cortex. Rats that ran on a treadmill and performed voluntary wheel running also had more climbing fiber synapses than the control group.

Kleim et al. (1998) also used an obstacle course and a voluntary running protocol to investigate synapse number within the cerebellar cortex after training. The cerebellar cortex of "acrobatic" trained rats (obstacle course) showed greater synaptic density than the voluntary running and control groups. The authors postulated that cerebellar cortical activity might be represented differently in the cerebellar cortex of voluntary running verse those of the obstacle course group which required more skilled limb movement and coordination than was needed by the running rats. The greater density of synapses in the obstacle group was attributed to an increase in parallel fibers that followed skill acquisition.

Anderson et al. (1994) found an increase in capillary density in the cerebellar cortex of exercising rats but not an increase in synaptogenesis. Rats that were trained on an obstacle course showed significantly greater volume of glia per Purkinje cell compared to runners and control animals. Glial cells are involved in the synthesis and uptake of glutamate and GABA.

Developing rats that were exposed to an environment of physical activity (which included climbing a ladder, climbing through tunnels and wheel running), demonstrated greater dendritic branching of Purkinje cells (Pysh et al., 1979). There was a 15% lower

spine density (area where synapses bind on Purkinje cells) in controls compared to the group exposed to various types of activity.

Hippocampus

The dentate gyrus is located in the hippocampus and is important for memory function. Running increases cell proliferation in the dentate gyrus, but cell survival after training was greater in mice trained in an enriched environment (85%) compared to runners (56%) (van Praag et al., 1999a). An enriched environment gave the animals exposure to different types of social interactions, larger housing and learning (water maze platform locations) and resulted in a greater effect on cell survival than just running. The labeling of cells in the dentate gyrus showed runners' cell survival number was 201% of controls and the enriched environment mice had 175% of controls.

Long-term potentiation (LTP) is an electrical event that is thought to be involved with learning. van Praag et al. (1999b) demonstrated that wheel running in mice increased dentate gyrus LTP. N-methyl-D-aspartate (NMDA) receptors mediate LTP by the medial perforant pathway of the dentate gyrus. When APV was added to the NMDA receptor electrical recording bath, LTP disappeared. Reduction in serotonin diminishes dentate gyrus LTP.

Norepinephrine (NE) regulates cardiovascular and endocrine responses during stressful situations such as during exercise training. Treadmill running in rats caused an increase in the metabolic byproduct of NE breakdown, 3-methyl-4-hydroxyphenylglycol in the hippocampus compared to control animals (Dunn et al. 1996). This suggests that

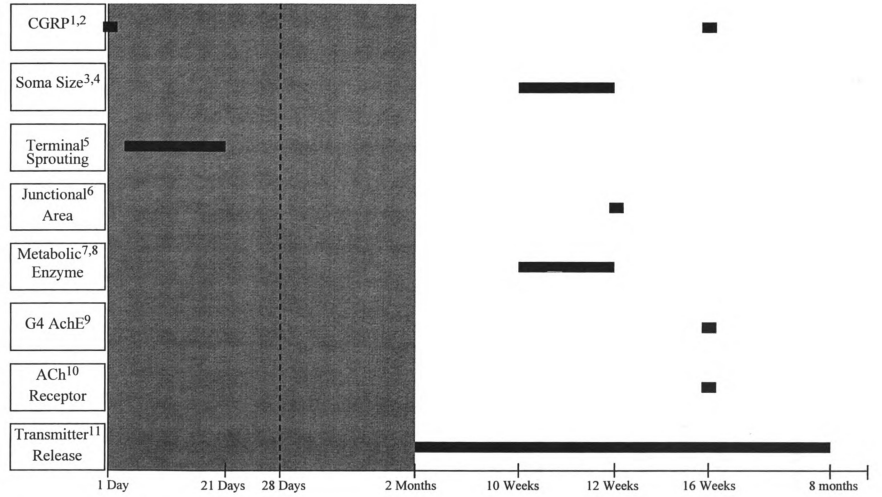
treadmill running may influence the functioning of noradrenergic metabolism in the brain.

Summary

Many changes occur within skeletal muscles and the central nervous system in response to systematic physical activity. The time course of adaptive changes varies between the two systems. Figure 4 summarizes all the adaptations that have been reported in motor neurons and the time point of each study. The gray area shows that little in known about the responses of motoneurons to exercise training of less then 2 months in duration. The proposed project attempts to fill this gap by investigating changes that occur within motor neurons after 28 days of static resistance and endurance training. In reviewing the studies on muscle and motor neuron metabolism, endurance-training protocols lasted a minimum of 8 weeks. Changes were evident in the metabolic enzyme systems that provide ATP to the working muscles, in fiber type composition, recruitment, and in the size and shape of the neuromuscular junction. Twelve weeks of resistance training in humans is associated with increases in the capacity to lift weights by about 200%, but there was only a 15% gain in maximum voluntary force in an isometric contraction (McComas, 1994). This gain in work capacity in light of a small gain in peak force may be accounted for, to some extent, by an increase in synergistic muscle activity, decrease in co-contraction of antagonists, better muscle length-tension relationships, and proper lifting technique. Yet, these factors do not account for all of the gain in strength and performance.

Figure 4. Summary of adaptations in motor neurons after exercise training. This diagram illustrates the variation in training lengths where adaptations have been observed in motor neurons. The shaded area indicates the lack of understanding about motor neuron adaptations in animals that exercise trained for less than 2-months. The dashed line at twenty-eight days is the time point chosen by this study to investigate protein expression in motor neurons following resistance and endurance exercise training.

[1] Homonko et al., 1997, [2] Gharakhanlou et al., 1999, [3] Gilliam et al., 1976, [4] Nakano et al., 1996, [5] Wernig et al., 1991, [6] Dueschenes et al., 1993, [7] Gerchman et al., 1975, 8 Nakano et al., 1996, [9] Fernandez et al., 1996, [10] Desaulniers et al., 1998, [11] Dorlochter et al., 1991.


Exercise Effect on
CGRP[1,2]
Soma Size[3,4]
Terminal[5] Sprouting
Junctional[6] Area
Metabolic[7,8] Enzyme
G4 AchE[9]
ACh[10] Receptor
Transmitter[11] Release
1 Day
21 Days
28 Days
2 Months
10 Weeks
12 Weeks
16 Weeks
8 months

The motor neuron is the final point of integration for generating voluntary movement. It is thus logical to look at the motor neuron as having the capability to modulate information that is being sent to the muscle. Motor neurons may have the potential, genetically and chemically, to modify their output characteristics under chronic stress conditions. It is proposed that motor neurons have this capacity and possibly carry this out by increasing the synthesis and packaging of synaptic neurotransmitters, and changing the architecture of the neuromuscular junction, which would lead to increased excitability.

The specific aim of this study is to determine whether the expression of CGRP, Chat, Vacht and CaN in ventral horn motor neurons change in response to endurance and resistance training.

Chapter 3

Experimental Design

Animal care and training protocols

The animal care protocol was consistent with NIH guidelines and approved by The All University Committee on Animal Care and Use of Michigan State University. Thirty-six, sexually mature female Sprague-Dawley rats (125-150g) were housed in individual cages. The light/dark cycle of the housing room was reversed (12hrs light; 12 hrs dark) so that the training occurred at the start of the dark period when rats are most active. Animals were pre-selected to be treadmill runners (runner), those that would not run during running trials were randomly assigned to: static resistance training (hangers) or control groups.

The running group ran twice daily, each session being no longer then 50 minute with a maximum duration of 100 minutes per day. An acclimatization period was needed to get the animals to run at a speed of 31 m/min at 8% grade, 6 days/week, [after Kiiskinen and Suominen, 1975; Dohm et al., 1977; Fitts and Holloszy, 1977; Hickson, 1981). A human treadmill (Roadmaster Corporation, Tyler, TX) was fitted with three running lanes (4 x 16 x 9 inches) for the animals to run in. An electrified grid was used to generate a mild aversive stimulus (1-1.5 mA/sec, pulsed) to keep the animals actively engaged in exercise. Treadmill speed was calibrated with a measuring wheel (Rolatape Measuring Systems, Spokane, WA). Time run per session and treadmill speed was recorded for each training session. Total distance run by each rat was calculated at the end of training.

The hanger group was trained to voluntarily support themselves as motionless as possible on an inclined grid (60° from horizontal) inside of a 3.5-inch tube (Exner et al., 1973). Weights were attached to the animals' tail, requiring the animals to support an additional 70-80% of body weight. As performance improved, additional weights were added. The rats were trained to hang for a maximum of 3 minutes per trial. The task was performed 3 times a day with 30 minutes rest between each trial, 5 days/week. Time hung for each set, weight held and total hang time per day (work performance measurement) was recorded. All animals received cereal treats (Cheerios) after each training session.

Control animals were confined to their cages, but were handled by the experimenter 5 days a week to get them used to human contact and to rule out a nurturing effect.

Body weight was recorded in grams with a dietetic scale the day before the study started and then weekly throughout the study. Animals had access to food and water ad libitum.

Force measurement

To assess whether the resistance protocol produced a "training effect" a force lever system (series 300B lever system, Cambridge Technology, Inc., MA) was used to measure resistance-trained and control animal's maximum pulling force. The force transducer was attached to the animal's tail in lieu of weights via a phosphorbronze guitar string held by a c-clamp. Animals were placed in the resistance training apparatus and were encouraged to give a maximal pulling effort by pinching their tail or applying a puff of compressed air. Measurements were taken before the exercise protocol started and 4

weeks after the last training session. The force signal produced by the animals was recorded on a chart recorder (Gould, TA 240) and calibrated with laboratory balance weights. Comparisons were made between pre- and post-training pulls within the control and hanger groups as well as between the control and hangers after training. All 12 rats from the control and hanger groups were measured.

Tissue collection

Muscle

Animals were sacrificed after 4 weeks of training, 24 hours after their last bout of exercise. Muscle enzyme analyses were performed to assess effects of the running protocol. Nine runners and controls were anesthetized with sodium pentobarbital (60mg/kg) 24 hours after the last training session. The following muscles were removed from both legs of all animals: extensor digitorum longus (EDL), tibialis anterior (TA), soleus (S), plantaris (P) and gastrocnemius (G). The muscles were snap-frozen in liquid nitrogen immediately upon removal.

Spinal cord

Immediately following muscle dissection the animals were decapitated and the spinal cord was removed by dorsal laminectomy. Based on results of retrograde tracing experiments the motor neuron pools of interest were located between T_{13}-L_4. A transverse knife cut was made through the spinal cord at the last rib (an anatomical marker for the rostral most extent of the lumbar enlargement) and a second cut made 3cm caudally. This procedure helped to insure that the same region of spinal cord (containing extensor and

flexor motor neuron pools of the hindlimb) was consistently removed from all rats. After the cord was harvested, a dry dissection was performed. The ventral and dorsal surfaces were identified with a dissecting microscope and the cord was cut into 2-3mm blocks. A horizontal cut was made through the blocks dividing the cord into dorsal and ventral halves (see Figure 5). The ventral half was placed in a buffer containing protease inhibitors [2 M TRIS (pH 7.5), 4 M NaCl, 200mM NaOVa, 400mM NaF, 250mM PMSF, Leupeptin, Aprotinin, Igepal CA-630], homogenized, sonicated and centrifuged at 14,000 rpm for 15 minutes at 4°C. Protein concentrations of the supernatant were determined by Bradford Assay (Bio-Rad). The dorsal half of the cord was put in a separate tube and the same procedure was applied.

Radioimmunoassay

Tissue for the anatomical analysis and RIA measurement of CGRP was performed on 24 sexually mature female Sprague-Dawley rats (Charles River) weighing between 125-150g. Animals were randomly assigned to one of four groups: treadmill running (runner), static resistance training (hanger), eccentric running and control. The runner, hanger and control groups followed the same protocol guidelines as used in the biochemical assay protocol except that the electrified grid was not installed on the treadmill. The eccentric running group ran continuously downhill (-20° decline) at a speed of 12m/min, for a single 30-minute exercise bout (Homonko et al., 1997). This procedure has been reported to elevate CGRP expression in motor neurons of tricep surea and anterior crural muscles after 72 hours. The purpose of the study was to validate these

results and to determine if 28 days of chronic resistance training and inclined treadmill running similarly up-regulate CGRP in hindlimb muscle motor neurons.

The six animals in each training group were anesthetized with sodium pentobarbital (Nembutal, 60 mg/kg i.p.) 72 hours after their last training session. The hind-limb muscles were harvested as described earlier. The spinal cord was removed and sectioned as described earlier, wrapped in tin foil and dropped into liquid nitrogen. Once frozen, all tissue was transferred to the -70 °C freezer and stored until RIA analysis could be performed.

Biochemical Assay Procedures

Muscle enzyme assay

Tissue dissected from the gastrocnemius (prime mover of the ankle used in locomotion) was removed from the -70°C freezer, weighed and pulverized into a fine powder over dry ice, removing all tendons. Citrate synthase was assayed according to standard procedures (Srere, 1960) and measured spectrophotometrically (Shimadzu UV-601). Student T-test was performed on numerical data and accepted level of P-value at 0.05.

Antibody specifications

The interpretation of antibody reaction product from immunohistochemical experiments is technique-specific and subjective. In this study antibodies were used in Western blot and immunohistochemical analysis to measure the expression of proteins in

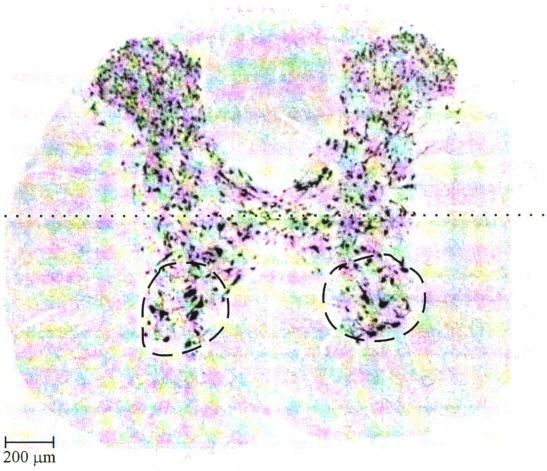


Figure 5. Cytoarchitecture of the rat spinal cord. This image shows a cross-section of the third lumbar spinal segment (L3). The butter fly-shaped central region is the *gray matter* that contains the cell bodies and dendrites of spinal neurons (stained here by antibodies to the neuronal nuclear-specific protein NeuN). The surrounding region is *white matter* composed of myelinated sensory axons and nerve fibers that interconnect the brain and spinal cord. Spinal tissue for Western blot analysis was divided into dorsal and ventral hemisections (dotted line). The ventral region contains the motor neurons and associated neural circuits that are the focus of this study. Dashed lines mark the location of hindlimb motor neuron pools (large, darkly-stained cells).

the spinal ventral horn. Antibodies are molecules that combine specifically with a second molecule, *antigen*. The site on the molecule that the antibody combines with is referred to as the *epitope*. The epitope is a sequence of amino acid residues that is species- and protein- specific. Two types of antibodies were used in this study: monoclonal and polyclonal. Monoclonal antibodies are serums that recognize a single antigenic epitope, whereas polyclonal antisera contain multiple antibodies with varying affinities and specificities against different epitopes on the same antibody. Thus, polyclonal antibodies may be more sensitive but less specific than monoclonal antibodies.

Antibodies for both Western blot and immunohistochemical experiments were obtained from the same commercial source. A rabbit polyclonal antibody to CaN (Chemicon;Temecula, CA) was used in western blot experiments (dilution 1:1000). It produced a single band at ~58 kDa. When used in immunohistochemistry, this antibody produced a weak reaction product with high background staining. For immunohistochemistry experiments a rabbit polyclonal antibody directed against residues 490-514 of CaN (Upstate; Lake Placid, NY) was used at a dilution of 1:50. A goat polyclonal antibody against Chat (Chemicon) was used for Western blot and immunohistochemistry experiments at a dilution of 1:200. The antibody produced a single band at ~56 kDa on Western blots. A goat polyclonal antibody to Vacht (Chemicon) was used for both Western blot (dilution of 1:1000) and immunohistochemistry (dilution of 1:500). This antibody produced 2-3 bands on Western blots with the primary band of interest at ~60 kDa. The additional bands fell below the 60 kDa band at ~ 20 and ~40 kDa. These bands could represent degraded subunits of the primary band. A mouse monoclonal antibody to β-actin (Sigma, St. Louis, MO) was used

in Western blots and slot blots to control for loading errors and run at a dilution of 1:25,000 and 1:5,000 respectively (peptide antigenic sequence: Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asp-Gly-Ser-Gly-Lys conjugated to keyhole limpet hemocyanin). A mouse monoclonal anti-NeuN antibody (Chemicon) was used for immunohistochemistry experiments at 1:500. Santa Cruz Biotechnology (Santa Cruz, CA) is the commercial supplier for the secondary antibodies used in Western blots. Immunoreactive sites were revealed by species-specific secondary antibodies coupled to horseradish peroxidase (HRP) at a dilution of 1:10,000.

Western blotting procedure

Sample concentrations were calculated and 20 μg of each specimen were loaded into an electrophoresis lane. Proteins were separated by molecular weight in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system. The voltage setting was 160V (Mini-Protean 3, Bio-Rad). To assess the molecular weight of the protein bands, a prestained molecular weight standard (Bio-Rad) was loaded into one lane.

Proteins were separated on 8% gels and transferred to membranes (Immobilon P, Millipore Corp.) at 90V for 120 minutes using the Mini-Protean 3 cell transfer system. Transfer time was dependent on the molecular weight of the protein. Binding sites on the membrane were blocked by incubating the membrane for 1 hour in 5% milk powder dissolved in 0.01M phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Tween-20 (TBST). The membrane was then incubated in primary antibody for 20 hours at 5°C. After repeated washing in TBST, the membrane was incubated for 90 minutes in the

appropriate species-specific secondary antibody. The secondary antibody was rinsed off with TBST and the membrane was washed in a 1:1 dilution of chemiluminescent substrate for 5 minutes (Supersignal west Pico Luminol enhancer solution plus Supersignal west Pico stable peroxide solution; Pierce). The membrane was placed in a clear sheet protector (Avery) and placed in a light-tight box on photographic film (OMAT, Kodak) and allowed to expose. The film was removed, developed (GBX developer and replenisher; Kodak) and fixed (General purpose fixer, Kodak). The developed image was scanned on a densitometer (GS-700, Bio-Rad) to quantify protein in each sample lane.

To minimize the possibility of errors when the samples were loaded into the electrophoresis lanes, a loading controller, ß-actin, was used. Samples from all treatment groups were probed with antibodies to ß-actin to insure loading and electrophoresis accuracy. The developed films were scanned on a densitometer to assess consistent protein loading. Adjusted fold changes were calculated and one-way ANOVA was performed and an adjusted density values was calculated.

Immunolabeling of membranes probed with antibodies to CaN demonstrated large variance on Western blot films that could mask small changes in measured protein expression. To control for sample variance between multiple repeats of Western blots, samples were loaded onto a Bio-Dot SF microfiltration apparatus (Bio-Rad). The Bio-Dot apparatus was assembled after presoaking the nitrocellulose membrane (Amersham Bioscience) and filters in deionized water. Slot wells were washed initially with 200μl of TBS under vacuum pressure. Samples (10μg) were added to 200μl of lysis buffer and loaded into each dot blot well. Vacuum was applied until the sample well content was

emptied onto the membrane, followed by another washing with TBS. The membrane was removed and placed in 5% dry milk blocking buffer for 60 minutes. The primary antibodies were diluted (CaN 1:1000, β-actin 1:10,00, GAPDH 1:500, NeuN 1:1000) in 5% dry milk solution and allowed to incubate overnight at 5° C. After incubation the membrane was washed with TBST then placed into the secondary antibody [CaN secondary is goat anti-rabbit 1:5000 (800 λ, IR dye, Rockland), β-actin secondary is rabbit anti-mouse 1:5000 (700 λ, Alexafluor 680, Molecular Probes)] for 60 minutes and followed by another TBST washing. The membrane was placed face down on the wetted glass surface of Odyssey infrared reader (Li-Cor, Lincoln, Nebraska). Intensity was adjusted until both fluorescent antibodies achieved optimal banding exposure. The digital images of CaN staining and the loading controls from the blots underwent analysis to derive intensity by the Li-Cor system. Fold change and adjusted fold changes were calculated and one-way ANOVA was run on the results.

Anatomical studies

The anatomical distribution of peptide markers in the ventral spinal cord was investigated by peroxidase-based immunohistochemistry. Twenty-four hours following the last training session, three rats selected randomly from each group were anesthetized with sodium pentobarbital (60mg/kg i.p.) and perfused transcardially with heparinized 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde in PB (pH 7.4). The lumbar spinal cord was removed by ventral laminectomy and post-fixed for 3 hours in 4% paraformaldehyde. The spinal cord was cleaned of spinal meninges and placed in PB with 20% sucrose overnight on a shaker table at 50 rpm in 5°C.

Peroxidase Immunohistochemistry

Forty-micron-thick, frozen transverse sections of spinal cord were processed to localize peptide markers of motor neurons using a technique that produces an optically dense reaction product. Initial test staining experiments produced an abnormally weak reaction product. The tissue was therefore exposed to a second post-fixation in a tissue processor (Tissue-Tek VIP, Sakura, CA) to insure uniform fixation. The procedure started with 2 x 30 minute washing of 4% formaldehyde in sodium phosphate buffer (pH 7.0) at 40 ^{0}C and was followed by a graded series of alcohol washings (70%, 80%, 95%, 100%) to dehydrate the tissue. Two washings in xylene were followed by 4 x 30 minute sessions of paraffin infusion at 60 ^{0}C. The total time for fixation, dehydration and paraffin infusion was 7.5 hours. Tissue was then embedded in paraffin blocks, and sectioned at a thickness of 4μm on a sliding microtome (Microm, Germany).

Slides were split into three rows and labeled "control"," runner" and "hanger" with the corresponding animal number. Twenty-five sections of the L3 spinal cord segment were cut at 4μm and discarded. A ribbon of 25 sections was then cut and three sections from the middle of the ribbon were placed on Superfrost Plus slides (Fisher) under the corresponding group and animal number. A twenty-five-section ribbon corresponds to 100 μm insuring that with each new slide we were sampling a different group of motor neurons. By placing controls and experimental groups on the same slide we kept staining conditions consistent for all tissue.

Immunohistochemical staining began by placing the slides into a drying oven for 16 hours at 56°C, insuring proper adhesion of the sections to the glass. All subsequent

steps were carried out at room temperature. After drying, the slides were placed in 2 x 10 minute xylene rinses, rehydrated by running through a series of alcohol washings (100%, 95%, 80%, 70% and distilled water) , and ending in 0.05 M trizma buffered saline (TBS, pH 7.4). Endogenous peroxidase activity was blocked by incubating the slides in peroxidase (3% peroxide/methanol) for 30 minutes. Initial staining attempts on the tissue indicated a weak epitope binding with the antibody, prompting the use of antigen retrieval. Antigen retrieval is a high-temperature heating method to recover the antigenicity of tissue sections that had been masked by formalin fixation (Shi et al., 2001). Slides were exposed to CitraRetrieval solution (Biogenex, San Ramon, CA) for 30 minutes using a vegetable steamer (Black & Decker) at 100°C. After cooling slides to room temperature they were run through a series of deionized water rinses and placed into TBS. Slides were incubated in normal serum from the host of the biotinylated secondary antibody for 15 minutes to block nonspecific binding. Endogenous tissue biotin activity was blocked by incubating slides for 15 minutes in Avidin D (Vector Labs, CA) and Biotin-D reagents (Sigma, MO). Slides were run through a series of TBS washes before applying the primary antibody (CaN 1:50 (Upstate), Chat 1:200, Vacht 1:1500, NeuN 1:1500 (Chemicon)) for 60 minutes. The tissue was then incubated with biotinylated anti-primary species host [biotinylated anti-rabbit made in goat and biotinylated anti-goat made in horse (Vector Labs, CA)] for 30 minutes. Another rinsing in TBS was followed by incubation in Vectastain Elite ABC reagent (Vector Labs, CA) for 30 minutes to conjugate the Avidin-Biotin complex. Another set of TBS washes was followed by incubation in Nova Red chromogen (Vector Labs, CA) solution until reaction product was optimized, usually about 15 minutes. Slides were dehydrated in a

series of alcohol and xylene washes and cover slipped with Flo Tex (Lerner Labs, PA) mounting media.

Tissue sections were viewed with a light microscope (Olympus, BX60) using 20x and 40x objectives and photographic images of the motor neurons were taken with SPOT camera imaging system (RT Slider Diagnostic Instruments, Inc.) at the same exposure settings. Images were imported into ImagePro Plus software (version 4.5 Media Cybernetics Inc., MD) and converted to 8-bit gray scale. The software defined reaction product-labeled motor neurons containing a nucleus as objects based on size and pixel intensity, and area and density were derived. One-way ANOVA tests were performed on the results with the accepted level of P-value set at 0.05.

Radioimmunoassay (RIA)

The sensitivity of Western blotting technique was insufficient to detect the low levels of CGRP expression in the spinal cord ventral horn (Wimalawansa et al., 1996) where levels were measured at 25 pmol/g wet weight, 16 fold lower than in the dorsal horn (400 pmol/g wet weight). Therefore, ventral horn CGRP levels were measured using radioimmunoassay (RIA).

Frozen spinal cord samples were removed from the freezer and placed on ice. A RIA assay kit (Pennisula Laboratories, Inc.) was used to measure spinal cord samples level of CGRP expression. The RIA buffer concentrate was reconstituted with distilled water and a small amount was used to dilute a standard peptide solution as well as the rabbit anti-peptide serum. Tissue samples were diluted with the RIA buffer and a standard peptide dilution series was prepared. Test tubes were labeled in duplicate with

sample identification as well as the following TC-1 (total counts 1), TC-2 (total counts 2), NSB-1 (non-specific binding 1), NSB-2 (non-specific binding 2), TB-1 (total binding 1), TB-2 (total binding 2) and 15 tubes labeled with our standard values. A volume of the standards and each tissue sample was pipetted into their assigned tube and the primary rabbit antibody was applied to all test tubes except TB-1, TB-2, NSB-1 and NSB-2. The test tubes were vortexed, covered and placed in the refrigerator at 5^0C for 20 hours. The following day the I-peptide was reconstituted with RIA buffer. The tracer was optimized in the scintillation gamma counter (Beckman Coulter LS6500, Fullerton, Ca). With the optimal I-peptide value determined, 75ml of RIA buffer was added and checked on the gamma counter to make sure it was in the 10,000-15,000 cpm (counts per million) unit range. The I-peptide tracer was added to each test tube covered, vortexed and incubated overnight in the refrigerator at 5°C. On the third day the goat anti-rabbit serum (GARGG) and normal rabbit serum (NRS) were reconstituted with RIA buffer and added to each test tube, vortexed and allowed to sit at room temperature for 90 minutes. The samples were then centrifuged at 3,000 rpm for 20 minutes at 4^0C. The supernatant was removed from all test tubes except TC tubes. All the tubes were placed in the scintillation gamma counter (LS6500 Beckman Coulter Inc., CA) and counts per million (cpm) recorded. Once the cpm values were determined the non-specific binding, total binding and bound value were calculated. A standard curve was generated and sample values were compared to this curve to derive the concentration of peptide (pg/100µl) in each sample. This number was multiplied by the dilution factor to get the value amount in the original sample

Statistical Methods

One-way ANOVA was used to assess significant differences between control and trained animals in the expression of peptide markers. Endurance training has been shown to decrease weight gained by runners relative to sedentary controls (Terjung, 1976). This rate of slow weight gain by runners is one indicator that the endurance training is having a training effect on the animals (analysis of muscle citrate synthase is another). Thus, animals body mass was monitored throughout the study. A P-value of 0.05 was accepted.

Hypothesis

It was hypothesized that there would be differences in expression of certain physiologically-relevant peptide markers by motor neurons in resistance and endurance-trained animals relative to controls. The adaptations should be dependent on the spinal motor output and response to the physical demands of the respective training protocols. This hypothesis makes the assumption that an increase in motor neuron output will be significant enough to increase ACh release at the motor endplate in response to the demands of training. One would predict that an increase in ACh release would likely require an increase in synthesis and subsequent vesicular packaging via active membrane transport. Thus, it was expected that exercise training would increase motor neuron expression of Chat and Vacht. Past evidence (Dorlochter et al., 1991) suggests that synaptic transmission at the neuromuscular muscular junction is enhanced in endurance-exercised animals. Therefore, it was expected that CGRP and CaN, two factors expressed in motor neurons that exert facilitative actions at motor terminals (e.g. Mulle et al., 1988,

Lin et al., 1999), will be up-regulated within motor neurons to enhance motor output in response to physical exercise.

Chapter 4

Results

Specific experiment

To investigate whether different training protocols, resistance and endurance, cause motor neurons to change the expression of specific protein markers compared to control animals.

Rationale

There are many sports that require different types of motor output. To master a sport an individual must train to become efficient in the motor demands of that activity. Different training protocols produce different physiological effects on the body. The motor neuron is the final point of integration (Sherrington, 1906) before motor output is generated and thus warrants systematic study in the context of specific protein markers that are impacted by different training protocols. Performance enhancement may be facilitated by an increase in the expression of motor neuron proteins.

Previous research suggests that motor neurons may adapt to increased demands placed on them by physical training of the muscles that they innervate. For example, fast axonal transport in motor axons is increased with chronic running (Jasmin et al., 1989), and an increase in quantal release of ACh (Dorlochter et al., 1991) and neuromuscular terminal area has been reported after repeated long duration running (Wernig et al., 1991). The increase in ACh release may necessitate increased synthesis of ACh, which is catalyzed by the enzyme choline-acetyltransferase (Chat), and may lead to an increase in loading of ACh into synaptic vesicles by vesicular acetylcholine transporter (Vacht).

CGRP has also been reported to increase ACh receptors at the motor endplate, regulate ACh receptor function and to induce sprouting of motor terminals (Mulle et al., 1988; Booj et al., 1989; Sala et al., 1995; Fernandez and Hodges-Savola, 1996). The expression of CGRP by motoneurons was reported to increase after one bout of damaging exercise (Homonko et al., 1997; 2000) and after 16-weeks of running (Gharakhanlou et al., 1999).

Expected Outcome

Differences in the expression of protein markers between the two exercise-trained and control groups were expected. More specifically, Chat, Vacht and CaN should increase in order to meet the increased motor demands of the systematic training in the two experimental groups. CGRP is expected to increase to mediate changes in the neuromuscular junction in response to training.

Rationale of preliminary studies

A review of the literature revealed a lack of information about central nervous system adaptations to exercise training. What few studies that were found used different training paradigms and most where conducted after only one bout of exercise or after an extended period involving several months. The focus of the experiment was on what happens early in a training protocol, before functional adaptations in muscle occurs. In reviewing endurance-training studies it was found that a variety of exercise paradigms were utilized, including activity wheels, swimming and motorized treadmill running, with running being the most common. A search of the literature on treadmill running revealed

that published protocols differed markedly with regard to treadmill speed, % incline, bout duration, number of daily bouts and number of days per week animals were exercised (Samorajski and Rolsten, 1975, Terjung, 1976, Gilliam et al., 1976, Jasmin et al., 1987, Bawa et al., 1984, Desauliniers et al., 1998, Kliitgaard et al., 1990, Deschenes et al., 1993). The principle goal of pilot studies was to determine training parameters for the running protocol that would produce a quantifiable increase in biochemical markers that are indicative of training effects. A second goal of pilot studies was to gain experience training the animals on a running treadmill and in the resistance hanging apparatus.

Preliminary experiments were conducted to define the parameters and produce measurable shifts in muscle CS activity, a common marker of elevated aerobic capacity in skeletal muscle indicative of a physiological response to exercise training. In the first pilot study a time line approach was used. Since adaptations that occur early in training were of primary interest animals were sacrificed after 7, 12 and 17 days of exercise. CS measurements were done at the 17 day mark. No significant difference in CS activity was seen between runner and control animals (Table 2). This was not surprising since it was very difficult to maintain consistent running performance (8% incline, 23m/min, 25 min/day, 5 days per week) from session-to-session using only innocuous aversive stimuli (brushing the treadmill and puffing compressed air at the animal's feet). The animals soon habituated to these stimuli and began sitting on the moving track which often produced injury.

These results prompted the installation of an electrified grid to the treadmill apparatus to motivate the animals with mild aversive stimuli. The training protocol was modified to increase the work load, gradually increasing the speed to 31m/min, running

the animals twice per day (2 x 50 minutes sessions), exercising the animals 6 days/ week and doubling the study duration from 17 days to 4-weeks (Fitts and Holloszy, 1977, Hickson, 1981, Morrison et al., 1989, Neufer and Dohm, 1993, Nakano et al., 1996). These modifications were made because it appeared the animals were not being sufficiently challenged to produce physiological adaptations in the muscle. The adjustments that were made to the running protocol produced a positive training effect as indicated by significant elevation in gastrocnemius and soleus CS activity (Table 2). With this result in hand the dissertation study proceeded using the modified running protocol.

Study results

The effects of physical exercise training on muscle metabolism and pulling force are summarized in Table 3. After 4-weeks of training there was a significant elevation in CS activity (22 ± 1.7 %) in the gastrocnemius muscle of runners ($P < 0.05$) compared to controls. This increase in CS activity indicates that the running protocol was sufficiently demanding to produce a physiological shift toward aerobic metabolism in response to the challenge of exercise. The increase in CS activity is within the range described by previous studies (Morrison et al., 1989; Fitts and Holloszy, 1975; Terjung, 1976; Neufer and Dohm, 1983). Another indicator that the running protocol was effective is that during 4 weeks of exercise the runners gained weight more slowly than the controls ($P < 0.05$). This finding is consistent with a study by Baldwin et al. (1977) that used both an interval and chronic running protocol and reported that the experimental running animals weighed significantly less than sedentary controls after training. There was no correlation between

Table 2. Summary of pilot study results. Pilot Study 1 shows the effects of 17 days of treadmill running (8% incline, 23m/min, 25 min/day, 5 days/wk) on gastrocnemius muscle citrate synthase (CS) activity. Pilot Study 2 shows the effect of 4 weeks of treadmill running (8% incline, up to 31m/min, 100 min/day, 6 days/wk) on hindlimb muscle citrate synthase activity

Pilot Study 1 (n = 6)	**Sedentary**	**Trained**	**% Change**	**P-value**
Body wt, (g)	231 ± 11.4	230 ± 10.4	NS	
Gastrocnemius wt,(g)	0.97 ± 0.04	1.04 ± 0.07	NS	
CS activity (μmole/g/min)	12.1 ± 2.5	11.4 ± 3.7	11 % ↑	P > 0.05
Pilot Study 2 (n = 6)				
Body wt, (g)	250 ± 18	244 ± 26	NS	
Gastocnemius wt, (g)	1.07 ± 0.19	1.05 ± 0.15	NS	
CS Activity (μmole/g/min)	23.5 ± 5.2	29.9 ± 4.22	28% ↑	P < 0.05
Soleus wt, (g)	0.12 ± 0.03	0.12 ± 0.02	NS	
CS activity (μmole/g/min)	31.9 ± 4.1	36.3 ± 10	45% ↑	P < 0.01

Values are mean ± SD.

CS activity and total distance run by each animal at the end of the training period. Dividing runners into three groups based on performance; "great" (ran > 50,000m), "intermediate" (ran between 30,000 and 40,000 m) and "poor "(ran < 20,000m) did not reveal a significant correlation between performance and either CS activity or weight gain. Thus, although total distance run by an animal was not a good predictor of the magnitude of physiological adaptation it does indicate that training parameters were sufficient overall to cause a shift in muscle oxidative capacity.

The resistance-trained exercise group showed a 43% increase (1575 ± 98 to 2252 ± 59) in pulling force over the 4-week training period ($P < 0.05$). During the same time, control animals only demonstrated a 13% increase (1802 ± 67 to 2037 ± 88) in strength (Table 3). The performance gain of the resistance group during the training protocol is illustrated in figure 6. The relationship between pulling force and time trained in the resistance apparatus (measurement of performance) was not significant ($P > 0.05$). It was thought that heavier animals might be stronger but that was not found. Since the increase in force generated by the experimental animals was unrelated to weight gain and work performance some other biological adaptation must be responsible.

It was hypothesized that exercise training would alter expression of proteins involved in motoneuron synaptic function. Since the training protocols produced measurable effects on muscle metabolic capacity and force production, any change in protein expression can be interpreted as a result of training.

Table 3. Summary of animal performance by experimental group as a result of exercise training.

		Aerobic Trained Animals			Resistance Trained Animals		
Group	**Body wt. (g)**	**Gastroc. Wt. (g)**	**CS activity** (μmole/g/min)	**Percent increase CS Activity**	**F_0 (Kg)**	**F_f (Kg)**	**Percent increase Force Diff.**
Control (n = 9)	252 ± 20	1.13 ± .07	22.3 ± 1.7		1802 ± 67	2037 ± 88	13 ± 13 %
Runner (n = 8)	231 ± 22	1.05 ± .11	27.2 ± 1.52	22 ± 0.7%*			
Hanger (n = 9)	236 ± 14				1575 ± 98	2252 ± 59	43 ± 14%*

Values are given as mean ± SE. * Significant at $P < 0.05$. Formula for % force difference is $(F_f - F_{0)} / F_0 * 100$. $F_{0,}$ is force generated at start of protocol; F_f final force developed after training

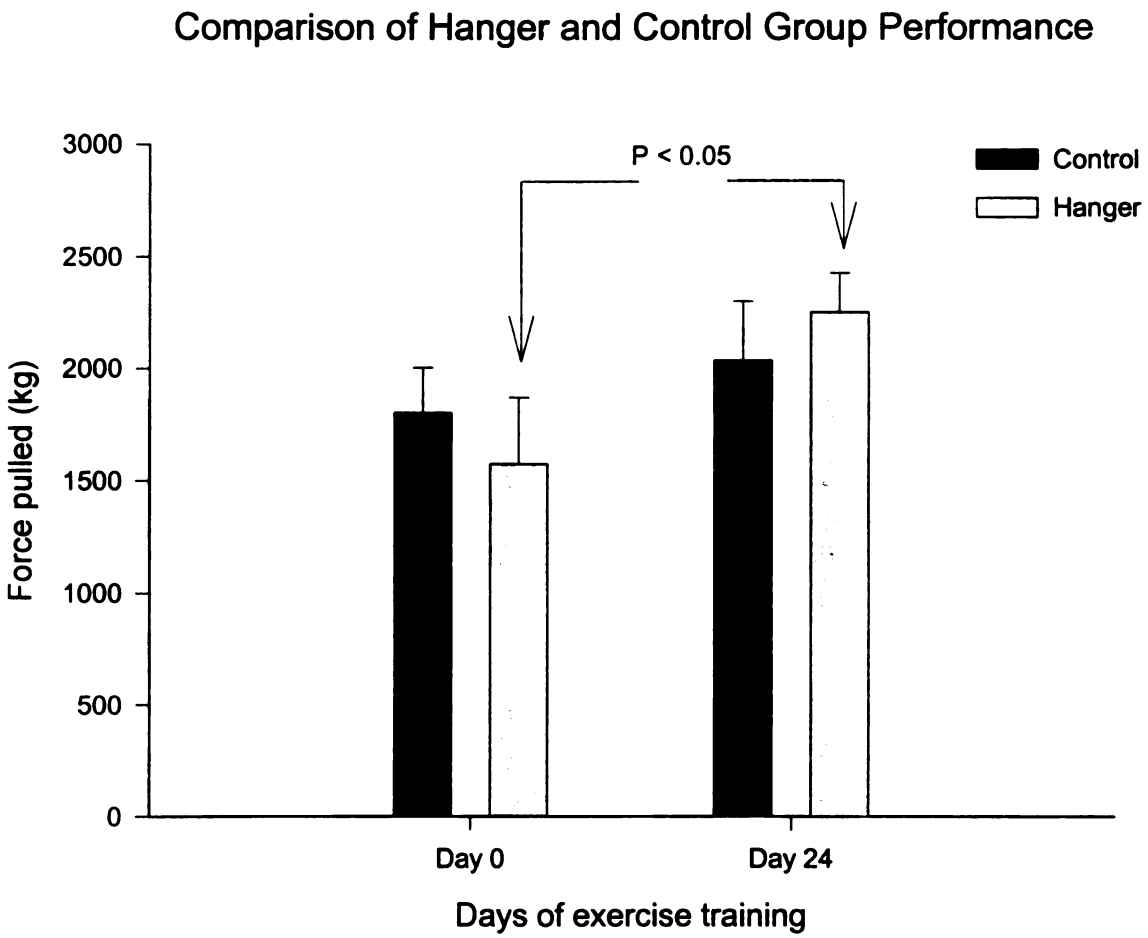


Figure 6. Performance of resistance-trained animals as a result of exercise training. Graph represents the comparisons between Controls and Hangers before (day zero) and after 24 days of exercise training. Exercised rats showed a significant increase in strength (force developed) during the study, whereas the sedentary control animals did not.

Effect of exercise on motoneuron CGRP expression

Ventral horn expression of i-CGRP ("i" signifies the radio-labeled isotope) in exercise trained animals were all within four units of each other and standard deviations were equal. Dorsal horn samples from exercise-trained animals demonstrated small but insignificant difference from controls. Thus, radioimmunoassay results indicated exercise training had no measurable effect on ventral and dorsal spinal cord levels of CGRP ($P > 0.05$, Table 4). Wimalawansa (1987) reported the distribution of i-CGRP in the nervous system of rats and reported that the dorsal horn of the spinal cord contains 370.0 ± 10.2 pmol/g wet weight while the ventral horn contains 35.5 ± 3.1 pmol/g wet weight. This equates to a 9.6 fold higher expression of i-CGRP in the dorsal horn compared to the ventral horn of the spinal cord.

Expression of Chat, Vacht and CaN: Western blot analysis

The architecture of the spinal cord and location of motor neurons are illustrated by the photomicrograph in figure 5. Analysis of Chat, Vacht and CaN expression by Western blot showed no measurable differences ($P > 0.05$, Table 5) after 4 weeks of exercise training (Chat expression in runners, 1.02 ± 0.47; in hangers 1.03 ± 0.51; Vacht expression in runners 1.23 ± 0.47, in hangers 0.98 ± 0.39; CaN expression in runners 1.26 ± 1.28, in hangers 1.02 ± 0.96 with control values equal to one). The finding that Chat and Vacht expression in exercise trained rats was not significantly different from controls was not surprising since protein level assays done in pilot study 2 (see table 2) showed no measurable differences (Chat expression in runners $1.01 \pm .38$, in hangers $.95 \pm .56$;

Table 4. iCGRP (pg/ug) expression in ventral horn and dorsal horn of control and exercise trained animals.

Groups	Dorsal Horn	Ventral Horn
Control (n= 6)	26.2 ± 3.7	0.49 ± .09
Runner (n=6)	27.2 ± 5.2	0.52 ± .19
Hanger (n=6)	30.5 ± 4.3	0.52 ± .12
Eccentric (n=6)	25.5 ± 4.4	0.48 ± .09

Data values are given as mean (pg/ug) ± standard deviation.

Vacht expression in runners 1.14 ± .17, in hangers .93 ± .25 with controls values equal to one).

However, Western blot analysis of ventral horn CaN expression in pilot study 2 had shown a significant increase in CaN expression in runners over controls (fold change runner 2.62 ± 0.43). This earlier result from six rats was not confirmed by experiments performed in a larger population of animals. Table 5 suggests that the lack of significant difference of CaN in the ventral horn samples may have resulted from sample variability produced by multiple assays on each of the samples. Figure 7 shows photomicrographs of the proteins probed by Western blot. Running multiple assays on many samples increased the contribution of interexperiment measurement error. Thus, all samples were run on a single slot-blot filtration apparatus to measure the variability seen on repeat runs of CaN by Western blot.

Slot-blot density measurements were done on Odyssey infrared imaging system (Licor, Lincoln, Nebraska). Figure 8 shows images of CaN and β-actin blots analyzed on the Odyssey system. When loading is controlled, the adjusted CaN fold change (density of group/ β-actin value of group) / control adjusted density) is significant for runners (1.68 ± 0.37, $P < 0.05$, see Table 6). This is consistent with the results in pilot study 2 (loading was uncontrolled) in which Western blots had previously shown a significant elevation of ventral horn CaN in runners relative to control animals (CaN expression in runners 2.62 ± 0.43, in hangers 0.45 ± 0.65). The slot-blot results also showed a surprising increase in ventral horn β-actin expression in resistance-trained after exercise (0.086 ± 0.029, $P < 0.05$). The alteration of a control protein in response to resistance-

Table 5. Mean fold values obtained from Western blots on ventral horn samples from exercise trained animals. Values are adjusted for loading by the control protein ß-actin.

Protein	**Runner**	**Hanger**
Vacht (n = 9)	1.23 ± 0.47	0.98 ± 0.39
Chat (n = 9)	1.02 ± 0.31	1.03 ± 0.51
CaN (n = 9)	1.26 ± 1.28	1.02 ± 0.96

Data values are given as mean fold change ± standard deviation.

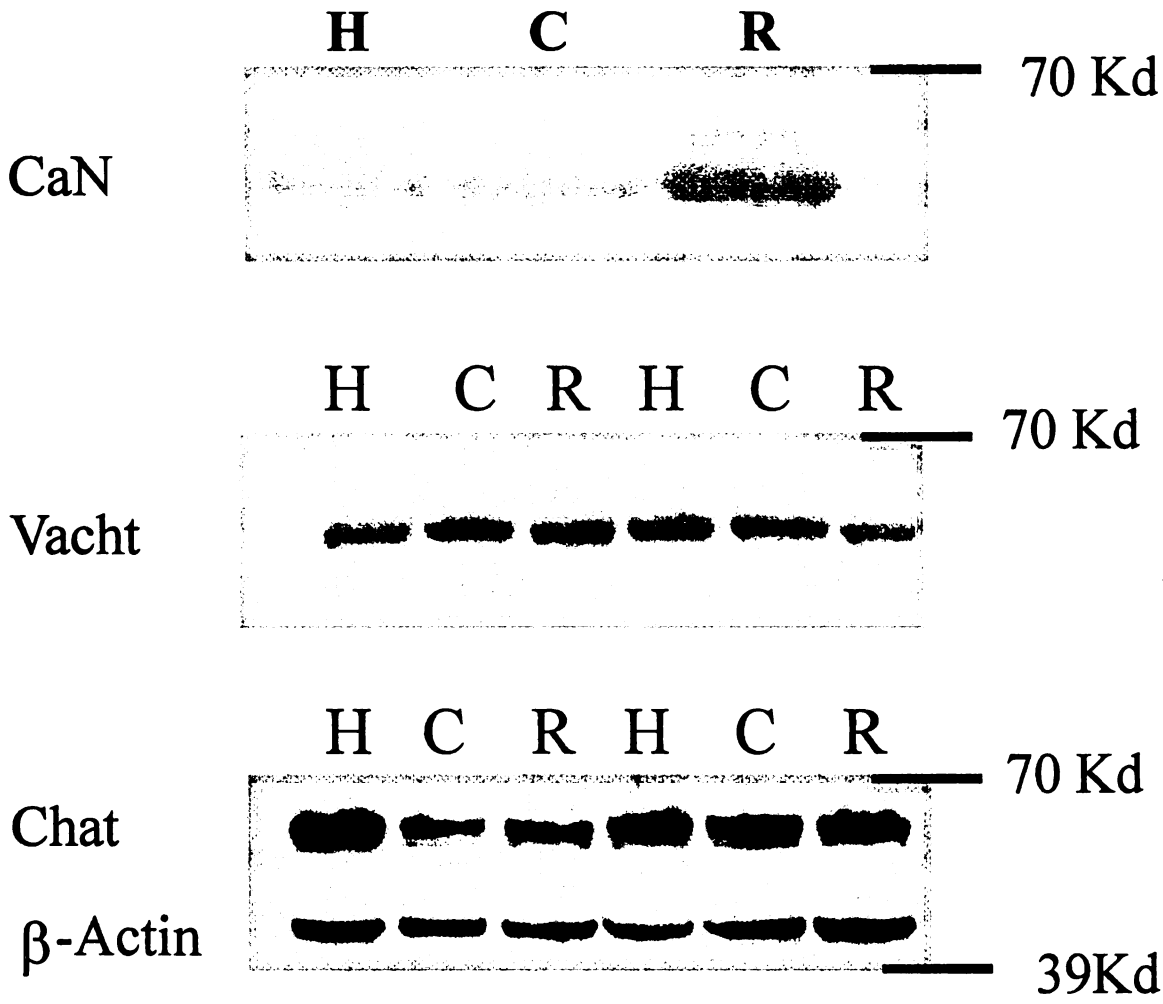


Figure 7. Western blots of proteins expressed in ventral horn samples of control and exercise-trained animals. **C** equals control band, **R** equals runner band and **H** equals hanger band.

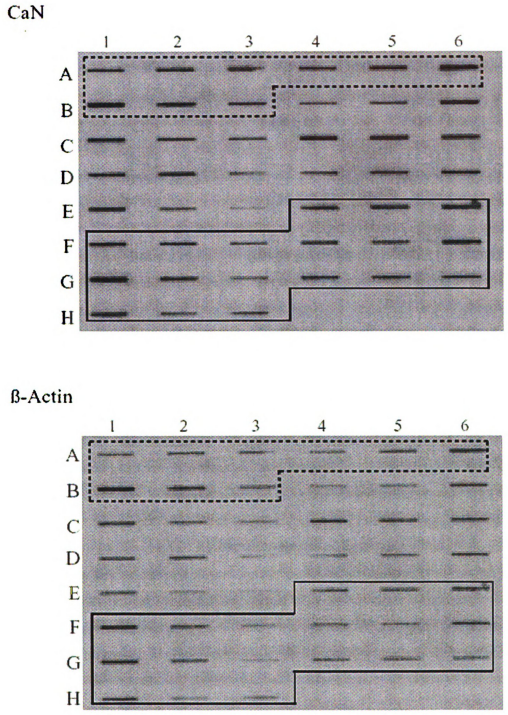


Figure 8. Slot-blots of CaN and ß-actin loading control. A:1 to B:3 equals control samples (dashed line above). B:4 through E:3 equals runner samples (no outline). E:4 though H:3 equals hanger samples (solid line above)

Table 6. Optical density values of CaN and loading controls using slot-blot filtration apparatus. Adjusted fold is the fold change value after controlling for loading variation using control proteins β-actin, GAPDH and NeuN. Control values are expressed as mean optical density, no fold calculations are done because it is the reference.

Calculation	Control	Runner	Hanger
CaN	0.16 ± 0.04	0.20 ± 0.08	0.31 ± 0.06
β-actin	0.039 ± 0.02	0.029 ± 0.018	0.086 ± 0.029 *
CaN Adjusted Fold		1.68 ± 0.37*	0.87 ± 0.73
CaN	582 ± 189	502.55 ± 164.5	657.36 ± 148.92
GAPDH	7005 ± 2830	5057.21 ± 3170.46	6865.9 ± 769.81
CaN Adjusted Fold		1.36 ± 0.45	1.27 ± 0.45
CaN	17.5 ± 8.6	18.73 ± 4.55	20.35 ± 7.2
NeuN	594 ± 275	630.81 ± 208.89	657.11 ± 230.52
CaN Adjusted Fold		1.02 ± 0.28	0.97 ± 0.16

*Data vales are given as mean ± standard deviation. * Denotes significant $P < 0.05$. Note: differences in CaN density values are seen between the three experiments. This difference is due to differences in antibody binding between experiments, film development, and interactions between the antibodies (experiments were done on membranes with a cocktail containing two antibodies to detect the experimental and control peptides).*

training was unexpected, indicating that its expression could be activity dependent. Alternative loading control proteins were investigated that had been utilized in other Western blotting applications. One control used is glyceraldehydes phosphate dehydrogenase (GAPDH), an anaerobic enzyme of glycolysis, which is found in all cells. Another control protein, unproven in similar application is neuronal nuclear specific protein (NeuN), a protein of unknown function that is expressed only in post-mitotic neurons. Neither the use of GAPDH or NeuN as loading controls produced the same significant result ($P > 0.05$) in CaN expression as ß-actin. Table 6 shows the corrected values of calcineurin for all three loading controls.

Expression of Chat, Vacht and CaN: Immunohistochemical analysis

To analyze the anatomical distribution of proteins in the ventral horn, immunohistochemistry was performed. Figure 5 shows the anatomical structure of the lumbar spinal cord. The motor neurons of interest are located in the ventral lateral and medial motor neuron pools as circled in Figure 5. Western blot images of the proteins probed by immunohistochemistry are shown in Figure 7.

Interpretation of immunohistochemical staining assumes specific recognition of the desired protein by the antibody. Thus to determine the specificity of the primary and secondary antibodies several control experiments were performed. Two experiments were performed to demonstrate staining specificity. The first tests whether the staining system (all reagents excluding the primary antibody) produced a non-specific reaction product. This procedure determines any staining that is not specific to binding of the primary antibody to the antigenic epitope (i.e. nonspecific labeling). The second control involves

replacing the primary antibody with the species-specific immunoglobin (IgG) fraction to determine the specificity of the primary antibody. This method reveals whether the secondary antibody produces labeling in the absence of the primary (i.e. false nonspecific labeling not involving antigenic site recognition). See Figure 9 for images of system controls for each of the four antibodies investigated (no IgG controls were available for Chat and Vacht antibodies).

The reaction product for CaN is seen in the motor neuron cytoplasm and has a clumped appearance like rough sandpaper. The runner's reaction product in the cytoplasm has a rough sand paper texture whereas the control and resistance trained animals have a finer grade of sand paper texture. Visual inspection of the photomicrographs in figure 10 suggests that the reaction product is heavier (darker) in motor neurons of the runner group than in control and resistance-trained animals. In addition, filamentous extensions of the motoneuron cell bodies of running animals are more abundant than in control and resistance-trained animals. The mean density measurements of CaN-like immunoreactivity showed a significant difference between runners and controls and runners and hangers ($P < 0.001$). There is a significant difference in motor neuron size ($P < 0.001$) with motor neurons of exercise-trained animals larger than controls. The cross-sectional area (major axis in runners 45.5 ± 13.5, in controls 40 ± 7.7, in hangers 44.8 ± 8.8; minor axis in runners 26.1 ± 4.8, in controls 23.7 ± 4.3, in hangers 27.9 ± 11) of motor neurons in both the runners and hangers is greater than the controls ($P< 0.001$, figure 12). The area of motor neuron nuclei was not measured in this analysis. See figures 10, 11 and 12 for images of CaN reaction product and graphical visualization of the results.

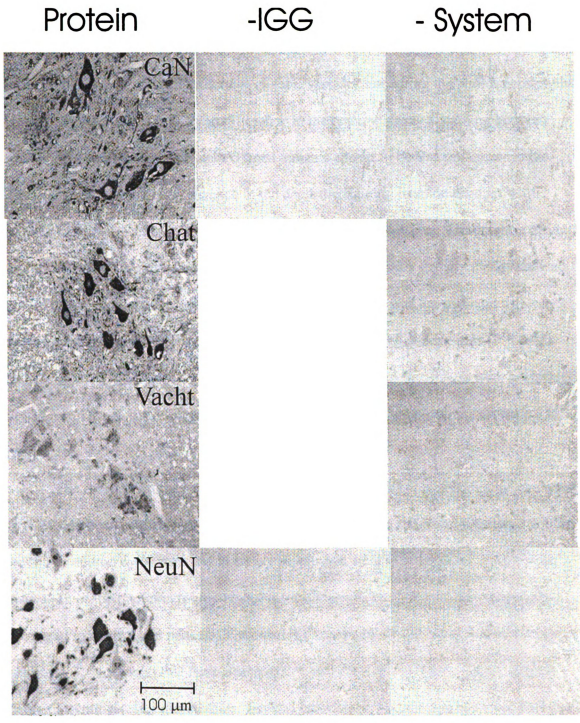


Figure 9. Antibody staining controls. Images are at 40x magnification. As can be seen in the control images there is little or no reaction product created by the secondary antibody (-IGG control) or the solutions used in the staining procedure (-system control), ndicating that the secondary antibody and staining solutions are not contributing to the reaction product seen in immunohistochemical experiments.

Figure 13 shows the reaction product for Chat immunohistochemistry. Chat immunoreactivity can be seen in the motor neuron cytoplasm and the reaction product has a clumped appearance like cottage cheese. Visually inspecting the photomicrographs (figure 13) there appears to be no difference in the reaction product staining between runners and controls but resistance trained animals appear to be less immunoreactive. There does appear to be a difference in the dendritic staining reaction product with runners expressing more in their dendrites than control and hangers. The reaction product in the dendrites was not quantified. Chat immunohistochemistry density results showed no difference between runners and control ($P > 0.05$). There was a significant difference between runners and hangers ($P < 0.001$) and between control and hangers ($P < 0.01$). Data is normally distributed and Tukey comparisons between the groups were performed. See Figure 13 and 14 for images of the reaction product and graphical visualization of results.

The reaction product for Vacht immunohistochemistry was found to be associated with two distinctly different structures. Figure 15 shows that the reaction product within the motor neuron cytoplasm and is evenly distributed within the motor neuron. Visual inspection of the photomicrographs shows greater reaction product in the runner group compared to control and hangers. The second Vacht reaction product is in the cholinergic terminals (C-terminals) that have a perisomatic distribution around the motor neuron. These C-terminals are referred to as "puncta" and they are influencing the excitability of motor neurons. Vesicular acetylcholine transporter immunohistochemistry density results demonstrated that runner's motor neuron expression was greater than control and hangers ($P < 0.001$, figure 16). Controls Vacht motor neurons expression was greater than hangers

($P < 0.001$). Running caused a significant elevation in Vacht immunoreactivity in the cholinergic terminals surrounding the motor neuron compared to control and resistance trained animals ($P < 0.001$, figure 18). The number of cholinergic terminals stained was greater in the exercise-trained animals than in controls (runner n = 3919, control n = 2206, resistance trained n = 3811, figure 17). See Figure 15,16, 17 and 18 for images of the reaction product and graphical visualization of results.

NeuN immunoreactivity

Immunohistochemistry was performed on NeuN because in working out the antibody dilution for slot-blot analysis a difference in staining intensity was seen between the exercise-trained animals and control. Figure 19 shows the NeuN immunohistochemical reaction product in ventral horn neurons. Visually inspecting the images there appears to be heavier reaction product in the cytoplasm of motor neurons in exercise trained animals compared to controls. The 100x magnification images show what appears to be a darker reaction product in the control animal nucleus, this is misleading because the reaction product in control animals cytoplasm is weaker than that of runner and resistance trained animals which have a smooth, heavy reaction product in both their cytoplasm and nucleus. Nuclear density measurements were not performed in this study. There also appears to be an increase in dendritic staining in the runner group. NeuN immunohistochemistry density results showed a significant difference between exercise-trained animals and controls ($P < 0.001$). Expression of NeuN is significantly greater in motor neurons of runners than control and resistance-trained animals. See Figure 19 and 20 for images of reaction product and graphical visualization of results.

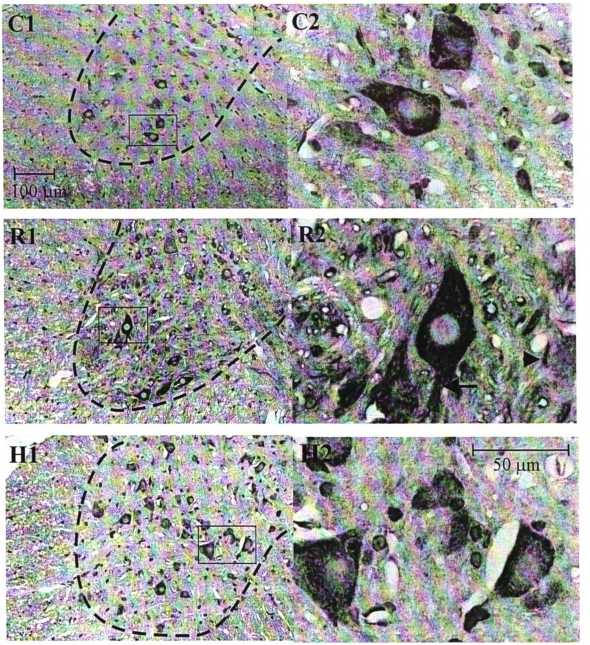


Figure 10. Calcineurin immunohistochemistry. **C** means Control group. **R** means Runner group. **H** means Hanger (resistance) group. The two columns are sequential enlargements of the ventral horn of the spinal cord. The number **1** means image was taken at 20x magnification. The number **2** means image was taken at 100x magnification. The dashed line indicates the boundary between white and gray matter in the ventral horn. The rectangle in image 1 indicates the cells seen in image 2. Arrows are pointing out dendritic staining and arrow heads point out filamentous tubular staining in the ventral horn motor pools.

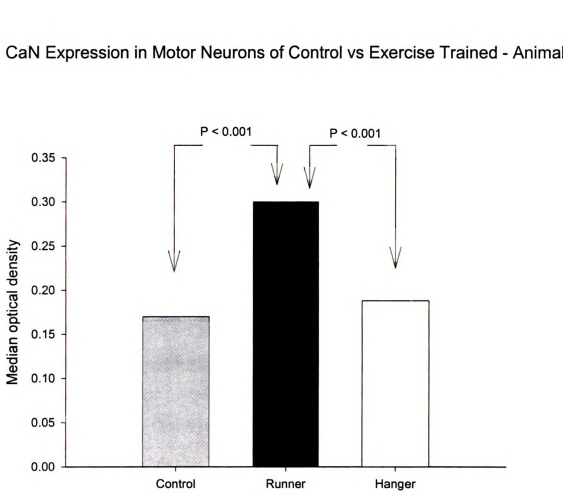


Figure 11. CaN immunohistochemistry density measurements. Measurement values depart significantly from a Gaussian distribution. Mean rank differences between control and exercise-trained animals are significant. (Kruskal – Wallis)

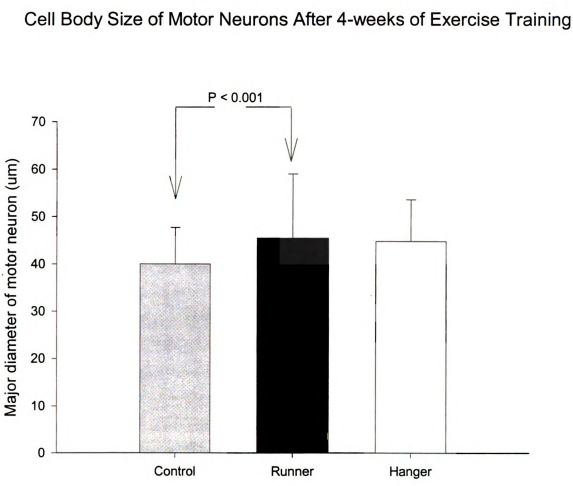


Figure 12. Effect of 4-weeks of exercise training on size of motor neuron cell bodies. Motor neurons of exercise-trained animals are significantly larger than in control. Values are represented as mean ± standard deviation.

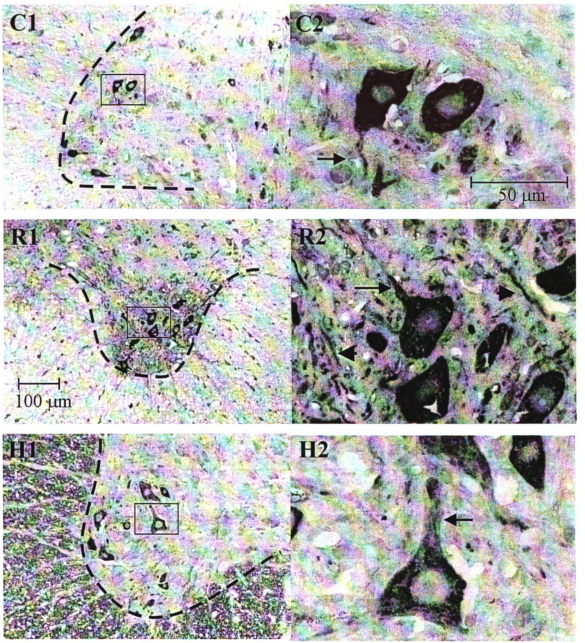


Figure 13. Choline acetyltransferase (Chat) immunohistochemistry. See Figure 10 for legend key.

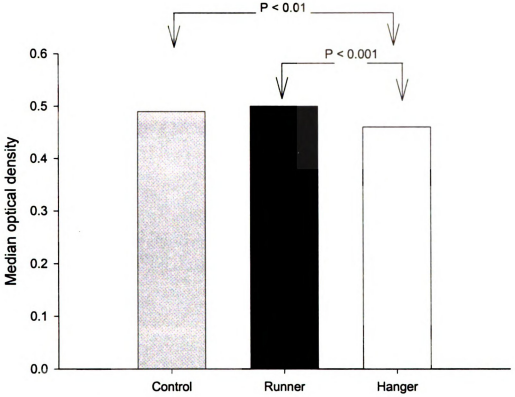


Figure 14. Chat immunohistochemistry density measurements. Measured values depart significantly from a Gaussian distribution. Mean rank differences were made between control and exercise-trained animals are significant. (Kruskal-Wallis)

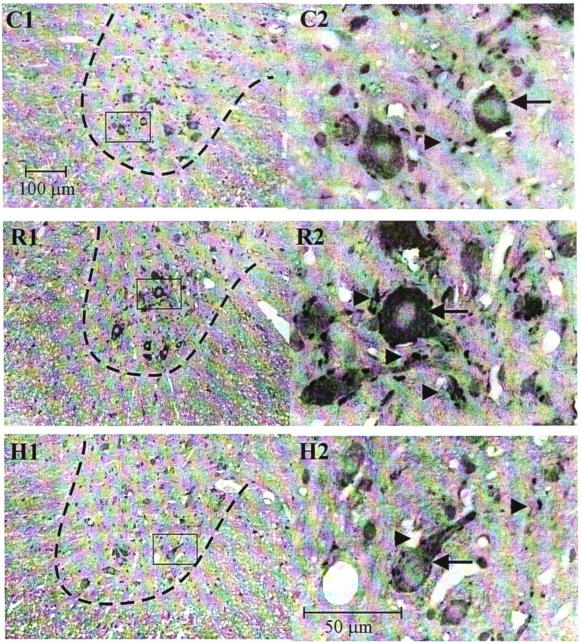


Figure 15. Vesicular acetylcholine transporter (Vacht) immunohistochemistry. See figure 10 for legend key. Arrows are demonstrating the two types of reaction product seen with Vacht staining. Arrows point to the staining seen within the motor neuron and arrow heads point to bar shaped structures, referred to as "puncta", which are synapsing cholinergic terminals of other spinal cord neurons.

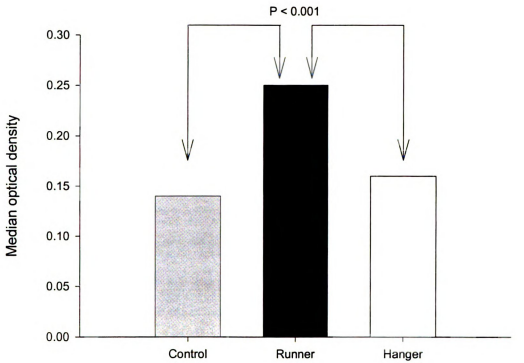


Figure 16. Vacht immunohistochemistry: motor neuron cell body density measurement. Measured values significantly depart from Gaussian distribution. Mean rank differences between control and exercise-trained animals are significant ($P < 0.05$).

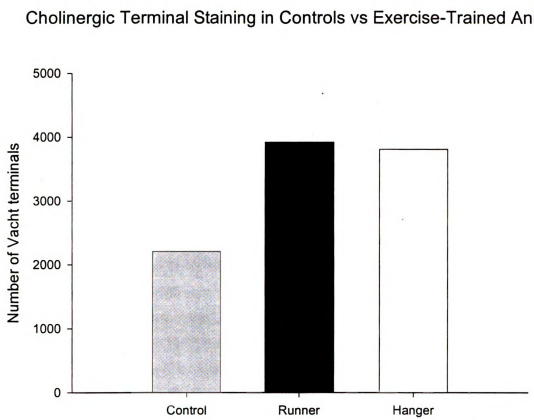


Figure 17. Number of Vacht-stained cholinergic terminals in controls and exercise-trained animals. The graph shows that exercise-trained animals have more cholinergic terminal staining than control animals.

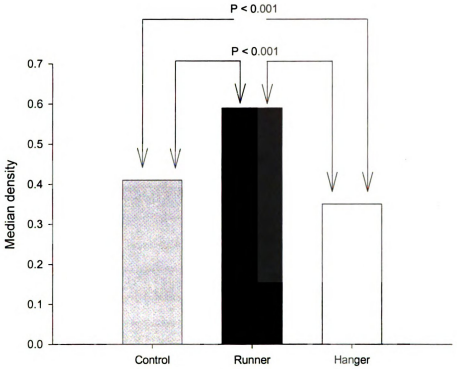


Figure 18. Vacht immunohistochemistry: density measurement of synaptic terminals. Measured values significantly depart from Gaussian distribution. Mean rank differences between control and exercise-trained animals are significant ($P < 0.05$).

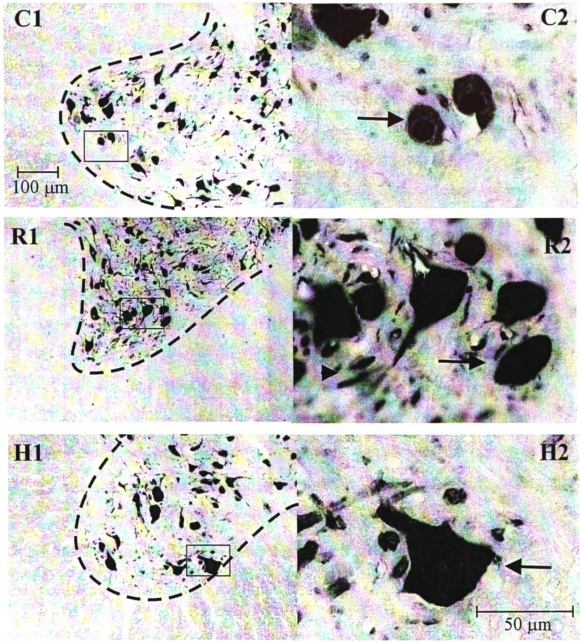


Figure 19. Neuronal nuclear–specific protein (NeuN) immunohistochemistry. See Figure 10 for legend key. Arrows are pointing out the difference in the cytoplasm staining in control animals which has less cytoplasmic staining than the exercise-trained animals which have heavier cytoplasmic and nuclear staining.

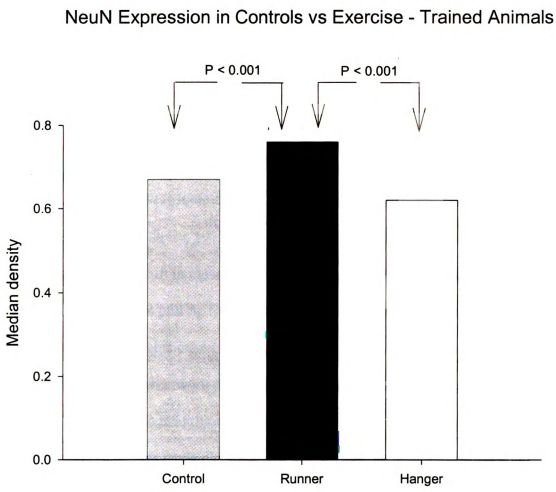


Figure 20. NeuN immunohistochemistry density measurement. Measured values significantly depart from Gaussian distribution. Mean rank differences between control and exercise-trained animals are significant ($P < 0.05$).

Chapter 5

Discussion

Performance Improvement

The finding that muscle CS activity increased in the running group is evidence that the endurance-training protocol produced a physiological effect after 4-weeks. The magnitude of increase (22%) is comparable to that reported by other studies (Morrison et al, 1989, Fitts and Holloszy, 1977, Terjung, 1976), using a similar running schedule and reflects an expected shift in muscle oxidative enzyme in response to aerobic training.

The increase in pulling force by the resistance-trained group was also evidence that 4-weeks of anaerobic exercise caused a significant increase in muscle strength. Since neither weight gained nor work performed by the hangers could account for the increase in pulling force a biological (biochemical or neural adaptation) must have taken place during this period. This study did not assay the effect of anaerobic training on muscle metabolic enzymes. However, Exner et al. (1973) reported increases in glycogen phosphorylase, creatine kinase and triosephosphate dehydrogenase in rectus femoris following similar isometric training. Thus, it can be assumed that similar changes in glycolytic enzymes occurred in the hindlimb muscles of the resistance-trained group and contributed to increased pulling force demonstrated by the present study. Another factor that may have contributed to the increase in strength is a decrease in co-activation of agonist and antagonist muscles (Carolan and Cafarelli, 1992; Sale, 1988). As reviewed in Chapter 2, this mechanism is thought to represent a change in "motor" outflow from the

central nervous system, producing an increase in force by increasing the efficiency of muscle activation.

General considerations

Since an animal model was used to investigate central nervous system adaptations to exercise training, inferences to humans may be limited. Although the structure of the rat nervous system is similar to that of humans, extrapolations from the rat to the human may not be warranted because rats are quadrupeds and humans are bipedal, thus mechanical stress and function of the extremity muscles differ. Differences in diet may also influence adaptations.

Female rats were used in this study because they have a better disposition for the demands of physical training compared to male rats, where androgens can become a confounding influence on adaptations. Getting the rats to perform at a intensity sufficient to stimulate adaptive changes in protein markers can be difficult to accomplish, especially in training protocols such as resistance training that require the rat to perform behaviors that are not natural. This pitfall was managed by training the animals to perform the task in a non-threatening environment by slowly acquainting them to the repetitive tasks and by rewarding them with food treats when the tasks were performed correctly. Animals do not start out as runners or resistance lifters; they have to be trained. In the process of learning these tasks, they can be injured. To avoid injury the animals were observed while performing the training tasks and their physical development was monitored throughout the 4-week study period. The performance of individual rats in

each training protocol was expected to be variable. This variability likely affected the magnitude of peptide expression by motor neurons.

Another potential pitfall of the study is consistency in harvesting spinal tissue, specifically removal time, volume and anatomical location. This problem was complicated by the fact that no two animals are exactly the same behaviorally and physically. Utilizing anatomical landmarks to guide dissection and skill in the technique of laminectomy helped to limit variation among samples.

Effects of exercise on expression of CGRP in the spinal cord

Calcitonin gene related peptide (CGRP) has been linked to ACh receptor clustering at the NMJ (Desaulniers et al., 1998), synthesis of myotubules (Mulle et al., 1988), AChE production (Fernandez and Hodges-Savola, 1996), increase in MEPP (Van der Kloot et al., 1998), and changes in ion channel kinetics (Tokuda and Hatase, 1998, Piehl et al., 1991). Homonko and Theriault (1997) reported an increase in CGRP expression in tricep surae motor neurons after a single bout of "damaging" eccentric exercise to the muscle fiber arrangement. This finding was not repeated in the eccentric exercise trained group in this study. The present study found no change in CGRP expression in the ventral horn or dorsal horn spinal cord samples. One reason simply could be differences in assays used by the two studies. Wimalawansa (1987) reported that ventral horn i-CGRP content is extremely low (35.5 ± 3.1 pmol/g wet weight) whereas dorsal horn content is high (370 ± 10.2 pmol/g wet weight). This is a 9.6 fold difference in ventral horn compared to dorsal horn expression of i-CGRP. Radioimmunoassay is a highly sensitive, widely used method to measure tissue distribution of peptides but

requires diluting the specimen whereas immunohistochemistry does not. Thus it could be postulated that immunohistochemistry was more sensitive at detecting changes in CGRP than RIA because immunohistochemistry measured CGRP anatomically in-situ whereas the RIA method measured CGRP expression in a small aliquot of the ventral spinal cord homogenate. The absence of a CGRP increase in the runner and hanger groups was not surprising. It is unlikely that the running and resistance protocol produced damage to the muscle fiber arrangement or the NMJ similar to eccentric exercise (Homonko and Theriault, 1997, 2000; Smith et al., 1996) although one may not have seen it even if it had occurred for the above reason. CGRP increases have been associated with repair of either the NMJ or muscle fibers after eccentric exercise (Homonko and Therialut, 1997). Another possibility that CGRP elevation was not detected in the runners and hangers could be due to the fact that animals were sacrificed 72 hours after their last training session. That time was chosen because it was previously reported that peak elevation of CGRP occurred 48-72 hours after one bout of eccentric running (Homonko and Theiault, 1997). Running and resistance training may have produced a brief, transient increase in CGRP that was not detected at this time point. On the other hand CGRP was reported to increase in soleus muscle motor neurons after 16 weeks of running (Gharakhanlou et al., 1999). Technical considerations not withstanding, it is possible that 28 days of running or resistance training were not sufficient to stimulate an elevation of CGRP expression in motor neurons.

Validation of density measurements

Two methods were employed in this study to assay protein expression associated with ventral horn motor neurons. Both Western blot and immunohistochemistry have rarely been used in the same study to measure protein expression. In Western blots aliquots of diluted homogenized tissue are subjected to SDS-PAGE electrophoresis. The issue of diluting out the protein of interest is a potential problem as well as replicating results. The chance of pipetting the same amount of protein in repeated western blot experiments is variable. Immunohistochemistry was performed on fixed tissue where antibodies were directly applied to 4μm thick sections of spinal cord tissue. Thus the measurement of the reaction product of the specific protein antibody occurs in situ minimizing dilution effects. Therefore, immunohistochemistry is likely to be more sensitive than Western blots when the peptide of interest is confined to a small number of cells or when its expression in the tissue is low.

Comparison of Western blot to immunohistochemistry

The measurement of CaN expression by Western blot was problematic. Data from Pilot study 2 showed a 2.6-fold increase in ventral horn CaN expression in runners relative to sedentary controls and a 55 % (0.45 ± 0.65) decrease in hangers. However, CaN expression showed considerable variance on Western blots when loading errors were controlled. All samples were loaded into a slot-blot and analysis showed there was a mean fold increase of 1.68, (68%) in CaN expression in the runners relative to controls, after adjustment for loading errors. This was consistent with the pilot study findings. There are sources of error in using both Western blot and slot-blot techniques. One of the

biggest errors is pipetting. To control for pipetting errors a loading controller (protein expressed in the tissue that is not believed to change as a result of treatment) was run in parallel with the experimental markers. The fold change (experimental density /control density) was adjusted for the amount of protein loaded into the sample blot by dividing all groups by optical density of the loading control density before making the fold change calculation. β-actin, a structural protein found in all cells was initially used as a loading control with Western blots. Surprisingly, slot-blot results showed that ventral horn β-actin was elevated in animals that performed resistance training. This result suggests that motoneuron β-actin expression may be activity-dependent. This possibility is consistent with the involvement of β-actin in axoplasmic transport (Bearers and Reese, 1999) and restructuring of motor terminals, which have been shown to undergo sprouting in response to chronic running (Wernig et al., 1991). If remodeling of motor terminals occurred in response to resistance training then β-actin expression would be expected to increase. β-actin did not show a similar increase in ventral horn samples from the treadmill-trained animals. Thus, the suitability of β-actin as a loading control for the resistance group could be called into question. Another loading control used in many studies is glyceraldehyde phosphate dehydrogenase (GAPDH), an anaerobic enzyme of glycolysis, which is found in all cells. Gerchman et al, (1975) reported that GAPDH expression in motor neurons decreased after 52 days of stressful swimming but increased when animals ran voluntarily and were forced to swim twice daily. The increase in GAPDH under the more demanding exercise protocol may indicate that GAPDH expression is time-dependent or exercise-specific. In this study ventral horn GAPDH expression in exercise-trained animals was no different from controls (Table 6). Thus,

suggesting that GAPDH expression is not activity-sensitive. However, Nakano et al., (1997) reported that the activity of an aerobic enzyme succinate dehydrogenase did not increase in motoneurons after 10 weeks of running. They concluded that total SDH activity (SDH activity x soma size) was greater in runners than controls because motor neuron cell body size was significantly increased. The current study observed an increase in motor neuron cell body size after 4-weeks of exercise training. Thus, using any metabolic enzyme as a loading control maybe problematic. Another protein used as a loading control, neuronal nuclear specific protein (NeuN) is widely distributed through out the central nervous system (Mullen et al., 1992) and specifically stains post-mitotic neurons, although its function is unknown. NeuN was evaluated for use as a loading control and showed no difference in expression between exercise-trained and control animals on slot blot. There is still uncertainty about what the best loading control is and this uncertainty will not be answered until more is understood about central nervous system plasticity in response to exercise training. Traditional loading controls used in other tissue many not be applicable when interpreting spinal cord adaptations to exercise training.

The overall finding of the Western and slot blot analyses is that CaN expression in runners is elevated. This conclusion is based on the use of β-actin as a loading control, which did exhibit differences in expression between experimental groups. However, this finding when the data are corrected for loading using GAPDH and NeuN did not exist. These results suggest that there are difficulties in using Western blots to measure optimal protein expression of cellular and transport peptides in the spinal cord after 4-weeks of exercise training.

Immunohistochemistry also showed that CaN was significantly elevated in motoneurons of runner rats relative to controls and hangers. The difference in CaN expression by immunohistochemistry and Western blot suggests that immunohistochemistry is a more sensitive and reliable technique for detecting protein expression changes under these conditions. Immunohistochemistry may be a more precise indicator of CaN expression since the detection of the protein was done in situ.

Functional considerations

How could an increase in motoneuron CaN expression affect exercise performance? CaN is a serine/threonine phosphatase that is regulated by protein kinase C (PKC). It has been reported that inhibition of CaN by cyclosporine A (CsA) leads to a marked increase in MEPP frequency at the motor endplate (Lin et al., 1999), an increase in voltage dependent Ca^{+2} influx at the motor terminal (Shira et al., 1995) and prolongation of NMDA receptor channel opening in the brain (Tokuda and Hatase, 1998). CaN also plays a role in nerve terminal transmitter release by 1) phosphorylating Na^+ and K^+ channels, 2) phosphorylation of Ca^+ channels resulting in alteration of Ca^+ influx and 3) phosphorylation of proteins that control synaptic vesicles binding for exocytosis, thus affecting release (Shira et al., 1995). Strack et al. (1996) demonstrated CaN staining in a population of large bodied motor neurons. They postulated that CaN positive α-motoneurons in the cervical spinal cord innervate fast-fatigue resistant muscle fibers. The CaN-staining pattern in the immunohistochemistry results showed diffuse antibody reaction product throughout the cytoplasm of motor neurons and smaller cells that could be small motor neurons or interneurons. The population of cells stained in this

study is different from what Strack et al. (1996) observed in the cervical spinal cord. The increase in CaN expression in the runner group suggests that its role in runners is greater than that of controls and hangers. The increase in CaN expression in runners could be explained as follows. It was previously reported that voluntary wheel running for 5 days produced a significant increase in total motor neuron dendritic area (μm) relative to sedentary controls (Gazula, et al., 2004). This increase in area would permit the establishment of new synapses, thus increasing synaptic drive. An increase in synaptic drive could increase motor neuron CaN expression. Elevated CaN may function as a damping mechanism against the increased ACh release brought about by the increased motor neuron activity. This negative feedback effect may help prevent ACh receptor sensitization and depletion of neurotransmitter stores. Thus, an increase in CaN insures that the communication pathway between the motor neuron and muscle remains efficient and functionally strong. There may be other roles that CaN plays during exercise and would have to be investigated in future experiments.

Chat is the rate-limiting enzyme of ACh synthesis, the neurotransmitter that initiates muscle contraction. The effect of exercise training on Chat expression in the spinal cord had not been investigated prior to this study. The finding that Chat immunostaining in motor neurons was unchanged in rats undergoing aerobic training but not resistance training suggests that Chat expression is exercise-specific. One could postulate that the increased demands of long term running (6 days/ week) were insufficient to upregulate Chat expression. The motor neuron cholinergic pathway is not the only cholinergic pathway in the spinal cord. Another cholinergic pathway are neurons that form C-terminals onto motor neurons, so named because of their cistern shaped

terminal (Nagy et al., 1993)) and are believed to be cholinergic because their terminals are immunoreactive for Vacht, Chat and AChE (Hellstom et al., 1999; Li et al., 1993; Nagy et al., 1993). Thus, another reason why Chat expression did not change is that an increase in Vacht expression in C-terminals, which led to increased motor neuron excitability through m2 receptors and thus, produced an increase in ACh release from the motor terminal to meet the demands of exercise. Thus, an increase in Chat expression was not necessary because the increase in motor neuron excitability and ACh storage was great enough to meet demands. Why motor neuron Chat expression in hangers decreased relative to runner and controls is not clear. Future experiments will be needed to clarify the relationship if any, between Chat and CaN.

Vacht functions in transporting the newly synthesized ACh into the synaptic vesicle. Results showed that runners had a higher expression of Vacht in C-terminals that synapse onto motor neuron cell bodies than controls or hangers. Vacht expression in motor neuron cell bodies was also elevated in runners compared to controls and hangers. There was no difference in Vacht motor neuron immunoreactivity between controls and hangers. The increased expression of Vacht in runner motor neurons could be explained by an increase in the recycling of ACh brought on by increased C-terminal sprouting which caused a change in motor neuron excitability. Thus, an increase in Vacht was necessary to recycle the increased release of ACh by the motor terminal due to the increased synaptic drive. Another possibility is that the increase in synaptic drive brought on by increased C-terminal sprouting in the ventral horn caused an elevation in Vacht with greater sprouting in the runner group than control and hangers. The increase in number of C-terminals in both runners and resistance-trained animals suggests that

terminal sprouting and remodeling of synaptic input onto ventral horn motor neurons had occurred. The neurons from which these C- terminals originate are in question. They belong to axon collaterals from other motor neurons or neurons in Rexed's lamina VII and X (Hellstrom et al., 1999). However, Brownstone et al. (2004) recently used patch clamp recordings of motor neurons and neurobiotin labeling and reported that neurons in lamina X are a source of C-terminals. Now that a source for C-terminals has been reported understanding C-terminals importance to motor neurons function needs to be clarified. Chevallier et al. (2004) used patch clamp recordings in salamanders and demonstrated that ACh applied in the bath solution increased firing frequency of motor neurons, reduced the after-hyperpolarization period and activated cation current. The increase in the number of C-terminals in exercise-trained animals could have occurred to modify the firing pattern of motor neurons through a muscarinic (m2) ACh receptor (Welton et al., 1999). Muscarinic receptors are G-protein coupled receptors and control the excitability of motor neurons by modulation of N- and P-type calcium channels by increasing their open time (Stewart et al., 1999). This close association of the m2 receptor on the motor neuron cell body and C- terminals in the ventral horn of the exercise trained animals could be an adaptation that fine tunes the firing rate of motor neurons by reducing the after-hyperpolarization period and possibly enhancing motoneuron excitability by prolonging Ca^{+2} channel opening (Hellstrom et al., 2003). The increased neural activity in response to exercise training resulted in an increased Vacht expression in the C-terminals. This increased sprouting and the possibility of an increase in m2 receptor number resulted in increased motor neuron excitability and could explain how

limb movement is coordinated in both fictive locomotion (e.g. running) or in synchronizing muscle contraction when lifting weights (e.g. resistance training).

NeuN specifically stains neurons that are post mitotic. Analysis showed that NeuN expression is elevated in runners over controls and hangers. In cultured cerebellar neurons it was shown that chronic depolarization of these neurons lead to a decrease in the expression of NeuN (Weyer and Schilling, 2003) an effect opposite of that reported here. The increase in expression of NeuN in the running group cell body does not support this finding. Thus, there must be another explanation as to why NeuN expression is elevated in the running group compared to the controls and hangers. Until the function of NeuN is better understood its role in exercise training will have to wait.

Summary

Figure 21 is a model that summarizes the performance adaptations seen after 4-weeks of exercise training and how these adaptations could affect performance. The adaptations seen in runners in this study are an increase in motor neuron size, increase in C-terminal sprouting, increase in C-terminal Vacht expression and an increase in Vacht and CaN expression in the motor neuron. So, increased C-terminal number and Vacht activity suggests increased excitatory cholinergic input assuming m2 receptors also increased. This may increase motor neuron firing, which could result in increased force generation during muscle contraction. Although ACh recycling may increase (increased Vacht) there is no change in quantal content. Future research will have to determine whether the postulated performance adaptations actually did occur. One specific experiment that needs to be done is to measure if the spontaneous release of ACh in

runners increased. This would help support CaN's role as a negative feedback enforcer in the face of increased motor neuron excitability. Another experiment would be to see if there is a change in the architecture of the motor terminal by measuring motor terminal branching. A third experiment would be to see if there is an increase in the association of m2 muscarinic receptors with C- terminals in the ventral horn. An increase in this association would further support muscarinic receptors role in increased motor neuron excitability. Resistance trained animals in this study had decreased expression of all the proteins investigated except Vacht in the motor neuron. Does resistance training cause another neural pathway to increase its expression of proteins that were not investigated in this study? Measuring the expression of anaerobic enzymes may help to answer this question. Learning more about exercise specific adaptations will fill in the gap of knowledge about how the central nervous system changes in response to physical activity.

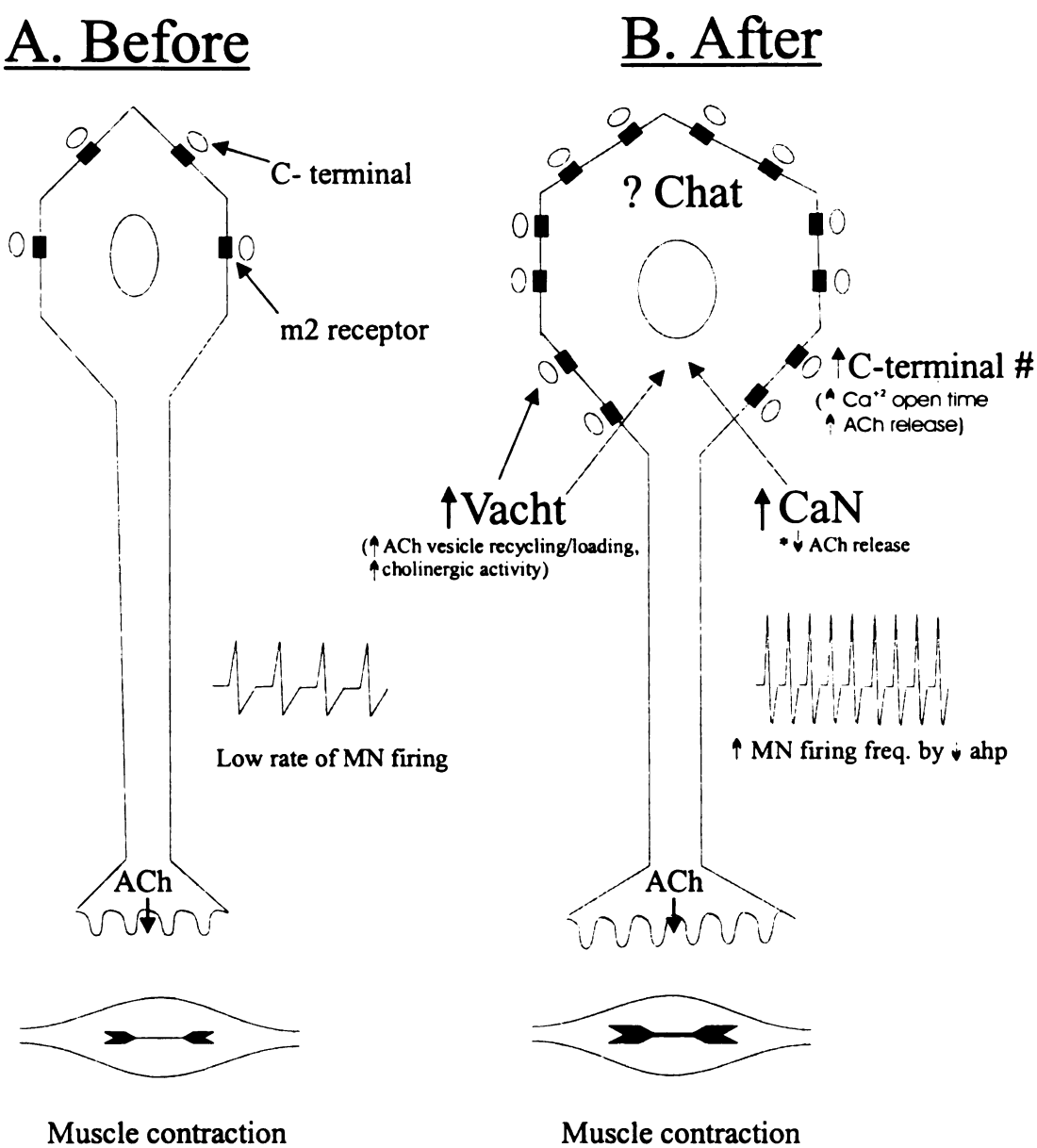


Figure 21. Model of adaptations that occurred in motor neuron after 4-weeks of exercise training. Drawing **A** is the motor neuron before training while drawing **B** is after. Adaptations include an increase in motor neuron size, increase in Vacht and CaN expression, increase in C-terminal Vacht immunostaining and increase in C- terminal sprouting. It is postulated that an increase in m2 receptor number and ACh release occurred in concurrence with the observed adaptations.

Cited Literature

Anderson B, Alcantara A, Greenough W. 1996. Motor-skill learning: Changes in synaptic organization of the rat cerebellar cortex. Neur Learning Memory 66: 221-229.

Andersson R, Edstrom J. 1957. Motor hyperactivity resulting in diameter decrease of peripheral nerves. Act Physiol Scand 39: 240-245

Baldwin K, Cooke D, Cheadle W. 1977. Time course adaptations in cardiac and skeletal muscle to different running programs. J Appl Physiol 42: 267-272.

Barnard R, Edgerton R, Peter J. 1970. Effect of exercise on skeletal muscle I. Biochemical and Histochemical properties. J Appl Physiol 726-766.

Bawa P, Binder M, Ruenzel P, Henneman E. 1984. Recruitment order of motoneurons in stretch reflexes is highly correlated with their axonal conduction velocity. J Neurophysiol 52: 410-420.

Bearer E, Reese T. 1999. Association of actin filaments with axonal microtubule tracts. J Neurocytology 28: 85-98.

Berne R, Levy M. 1998. Physiology 4th Ed.. New York, NY: Mosby.

Bigland-Ritchie B, Johansson R, Lippold O, smith S, Woods J. 1983. Changes in motoneuron firing rates during sustained maximal voluntary contractions. J Physiol 340: 335-346.

Binder M, Bawa P, Ruenzel P, Henneman E. 1983. Does orderly recruitment of motoneurons depend on the existence of different types of motor units? Neuro Lett 36: 55-58.

Booj S, Goldstein R, Fischer-Colbries, Dahlstrom A. 1989. Calcitonin gene-related peptide and chromogranin A: presence and intra-axonal transport in lumbar motor neurons in the rat, a comparison with synaptic vesicle antigens in immunohistochemical studies. Neuroscience 30: 479-501.

Bouchard C, Malina R, Perusse L. 1997. Genetics of fitness and physical performance. Champagne, IL: Human Kinetics.

Brown M, Holland R, Ironton R. 1979. Evidence against an intraspinal signal for motoneurone sprouting in mice. J Physiol London 291: 35-36P.

Brown M, Holland R, Hopkins W. 1981. Motor nerve sprouting. Ann Rev Neurosci 4: 17-42.

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

Burke R. 1999. Revisiting the notion of 'motor unit types'. Progress in Brain Research 123: 167-175.

Cajal S. 1995. Histology of the nervous system of man and vertebrate. Vol. 1. New York, NY: Oxford University Press. Translated by Swanson and Swanson.

Campa J, Engel W. 1970. Histochemistry of motor neurons and interneurons in the cat lumbar spinal cord. Neurology 20: 559-568.

Carolan B, Cafarelli E. 1992. Adaptations in coactivation after isometric resistance training. J Appl Physiol 73: 911-917.

Czeh G, Gallego R, Kudo N, Kuno M. 1978. Evidence for the maintenance of motoneurone properties by muscle activity. J Physiol 281: 239-252.

DeLuca C, LeFever R, McCue M, Xenakis A. 1982. Behaviour of human motor units in different muscles during linearly varying contractions. J Physiol 329: 113-128.

Desauliners P, Lavoie P, Gardiner P. 1998. Endurance training increases acetylcholine receptor quantity at neuromuscular junctions of adult rat skeletal muscle. Neuroreport 9: 3549-3553.

Deschenes M, Maresh C, Crivello J, Armstrong L, Kraemer W, Covault J. 1993. The effects of exercise training of different intensities on neuromuscular junction morphology. J Neurocyt 22 603-615.

Dohm G, Beecher G, Stephenson T, Womack M. 1977. Adaptations to endurance training at three intensities of exercise. Exer Physiol 42: 753-757.

Dorlochter M, Irintchev A, Brinkers M, Wernig A. 1991. Effects of enhanced activity on synaptic transmission in mouse extensor digitorum longus muscle. J Physiol 436: 283-292.

Dunn A, Reigle T, Youngstedt S, Armstrong R, Dishman R. 1996. Brain norepinephrine and metabolites after treadmill training and wheel running in rats. Med Sci Sports Exerc 28: 204-209.

Eccles J, Eccles R, Lundberg A. 1957. The convergence of monosynaptic excitatory afferents onto many different species of alpha motoneurones. J Physiol 137: 22-50.

Edds M. 1950. Hypertrophy of nerve fibers to functionally overloaded muscles. J Comp Neurol 93: 259-274.

Edgerton V. 1970. Morphology and histochemistry of the soleus muscle from normal and exercised rats. Am J Anat 127: 81-88.

Edgerton V, Gerchman L, Carrow R. 1969. Histochemical changes in rat skeletal muscle after exercise. Exp Neur 24: 110-123.

Edstrom J. 1957. Effects of increase motor activity on the dimensions and the staining properties of the neuron soma. J Comp Neur 107: 295-304.

Edstrom L, Grimby L. 1986. Effect of exercise on the motor unit. Muscle & Nerve 9: 104-126.

Eisen A, Carpenter S, Karpati G, Bellavance A. 1973. The effects of muscle hyper- and hypoactivity upon fiber diameters of intact and regenerating nerves. J Neuroscience 20: 457-469.

Exner G, Staudte H, Pette D. 1973. Isometric training of rats: Effects upon fast and slow muscle and modification by anabolic hormone (nandrolone decanoate) in female rats. Pflugers Arch 345: 1-14.

Fernandez H, Hodges-Savola C. 1996. Physiological regulation of G4 Ache in fast-twitch muscle: Effects of exercise and CGRP. J Appl Physiol 80: 357-362.

Fitts R, Holloszy J. 1977. Contractile properties of rat soleus muscle: effects of training and fatigue. Amer J Physiol 233: C86-C91.

Fitts R. 1994. Cellular mechanisms of muscle fatigue. Physiol Rev 74: 49-94.

Gardiner P. 2001. Neuromuscular Aspects of Physical Activity. Champaign, IL: Human Kinetics.

Gazula V, Roberts M, Luzzio C, Jawad A, Kalb R. 2004. Effects of limb exercise after spinal cord injury on motor neuron dendrite structure. J Comp Neurol 476: 130-145.

Gerchman L, Edgerton V, Carrow R. 1975. Effects of physical training on the histochemistry and morphology of ventral motor neurons. Exp Neur 49: 790-801.

Gharakhanlou R, Chadan S, Gardiner P. 1999. Increased activity in the form of endurance training increases calcitonin gene-related peptide content in lumbar motoneuron cell bodies and in sciatic nerve in the rat. Neuroscience 89:1229-1239.

Gilliam T, Roy R, Taylor J, Huesner W, Van Huss W. 1976. Ventral motor neuron alterations in rat spinal cord after chronic exercise. Experientia 33: 665-667.

Gonyea W, Ericson G, Bonde-Petersen F. 1976. Skeletal muscle fiber splitting induced by weight-lifting exercise in cats. Acta Physiol Scand 99: 105-109.

Gonyea W, Bonde-Petersen F. 1978. Alterations in muscle contractile properties and fiber composition after weight-lifting exercise in cats. Exp Neuro 59: 75-84.

Grimby L, Hannerz J, Hedman B. 1979. Contraction time and voluntary discharge properties of individual short toe extensor motor units in man. J Physiol 289: 191-201

Grinnell A. 1995. Dynamics of nerve-muscle interaction in developing and mature neuromuscular junctions. Physiol Rev 75: 789-834.

Hansen P. Schulman H. 1992. Neuronal Ca2+/calmodulin-dependent protein kinases. Annu Rev Biochem 61: 559-601.

Heckman C, Binder M. 1993. Computer simulations of the effects of different synaptic input systems on motor unit recruitment. J Neurophysiol 70: 1827-1840.

Hellstrom J, Oliveira A, Meister B, Cullheim S. 2003. Large cholinergic nerve terminals on subsets of motoneurons and their relation to muscarinic receptor type 2. J Comp Neur 460: 476-486.

Hellstrom J, Arvidsson U, Eldi R, Cullheim S, Meister B. 1999. Differential expression of nerve terminal protein isoforms in VACHT-containing varicosities of the spinal cord ventral horn. J Comp Neur 411: 578-590.

Henneman E, Somjen G, Carpenter D. 1965. Excitability and inhibitibility of motoneurons of different sizes. J Neurophysiol 28: 599-620.

Henneman E. 1991. The size principle and its relation to transmission failure in Ia projections to spinal motoneurons. Ann New York Acad Sci 627: 165-168.

Hickson R, Heusner W, Van Huss W. 1975. Skeletal muscle enzyme alterations after sprint and endurance training. J Appl Physiol 40: 868-872.

Hickson R. 1981. Skeletal muscle cytochrome c and myoglobin, endurance, and frequency of training. J Appl Physiol 51: 746-749.

Homonko D, Theriault E. 2000. Downhill running preferentially increases CGRP in fast glycolytic muscle fibers. J Appl Physiol 89: 1928-1936.

Homonko D, Theriault E. 1997. Calcitonin gene-related peptide is increased in hindlimb motoneurons after exercise. Sports Med 18: 503-509.

Hounsgaard J, Hultborn H, Jespersen B, Kiehn O. 1984. Intrinsic membrane properties causing a bistable behaviour of α-motoneurones. Exp Brain Res 55: 391-394.

Hounsgaard J, Hultborn H, Kiehn O. 1986. Transmitter-controlled properties of α-motoneurons causing long-lasting motor discharge to brief excitatory inputs. In Progress Brain res 64: 39-49. ed. Freund H, Buttner U, Cohen B, and Noth J. Amsterdam: Elsevier.

Hounsgaard J, Hultborn H, Jespersen B, Kiehn O. 1988. Bistability of α-motoneurones in the decerebrate cat and in the acute spinal cat after intravenous 5-hydroxytryptophan. J Physiol 405: 345-367.

Hounsgaard J, Kiehn O.1989. Serotonin-induced bistability of turtle motoneurones caused by a nifedipine-sensitive calcium plateau potential. J Physiol 414: 265-282.

Ingebritson T, Cohen P. 1983. The protein phosphatases involved in cellular regulation. 1 classification and substrate specificities. Eur J Biochem 132: 255-261.

Jacobs B, Fornal C. 1993. 5-HT and motor control: a hypothesis. Trends Neurosci 16: 346-352.

Jasmin B, Lavoie P, Gardiner P. 1987. Fast axonal transport of acetylcholinesterase in rat sciatic motoneurons is enhanced following prolonged daily running, but not following swimming. Neurosci Lett 78: 156-160.

Jasmin B, Lavoie P, Gardiner P. 1988. Axonal transport of labeled proteins in motoneurons of exercise-trained rats. Am J Physiol 255: C731-C736.

Johnson H, Hokfelt T, Ulfhakes B. 1992. Galanin and CGRP-like immunoreactivity coexist in rat spinal motoneurons. Neuroreport 3: 303-306.

Jones D, Rutherford O, Parker D. 1989. Physiological changes in skeletal muscle as a result of strength training. Quar J Exp Physiol 74: 233-256.

Kandel E, Schwartz J, Jessell T. Eds. 2000. Principles of Neural Science, 4th Ed. New York, NY: McGraw-Hill.

Katz B, Miledi R. 1968. The effect of local blockage of motor nerve terminals. J Physiol 199: 729-741.

Kelley G. 1996. Mechanical overload and skeletal muscle fiber hyperplasia: a meta-analysis. J Appl Physiol 81: 1584-1588.

Key B, Parker A, Giorgi P. 1984. Endurance exercise does not modify nerve fiber morphology in the rat soleus nerve. Brain Research 287: 137-144.

Kiiskinen A, Suominen H. 1975. Blood circulation of long bones in trained growing rats and mice. Eur J Appl Physiol 34: 303-309.

Kleim J, Swain R, Armstrong K, Napper R, Jones T, Greenough W. 1998. Selective synaptic plasticity within the cerebellar cortex following complex motor skill learning. Neur Learn Mem 69: 273-289.

Klitgaard H, Bergman R, Betto R, Salviato G, Schiaffino S, Clausen T, Saltin B. 1990. Co-existence of myosin heavy chain I and IIa isoforms in human skeletal muscle fibers with endurance training. Pfugers Arch 416: 470-472.

Kurz E, Sengelaub D, Arnold A. 1986. Androgens regulate the dendrite length of mammalian motoneurons in adulthood. Science 232: 395-398.

Lin M, Lin-Shiau S. (1999). Enhanced spontaneous transmitter release at murine motor nerve terminals with cyclosporine. Neuropharm 38: 195-198.

Lund L, McQuarrie G. 1997. Calcium/calmodulin-dependent protein kinase II expression in motor neurons: effect of axotomy. J Neurobiol 33: 796-810.

Magherini P, Precht W, Schwindt P. 1976. Electrical properties of frog motoneurons in the in situ spinal cord. J Neurophysiol 39: 459-473.

McPhail L, McBride C, McGraw J, Steeves J, Tetzlaff W. 2003. Axotomy abolishes NeuN expression in facial but not rubrospinal neurons. Exp Neuro 185: 182-190.

McArdle W, Katch F, Katch V. 1996. Exercise physiology: energy, nutrition, and human performance 4th Ed. Baltimore, MD: Williams & Wilkens.

McComas A. 1994. Human neuromuscular adaptations that accompany changes in activity. Med Sci Sports Exer 26: 1498-1509.

McComas A. Ed. 1996. Skeletal muscle form and function. Champagne, IL: Human Kinetics.

Miki K, Fukuoka A, Tokunaga A, Noguchi K. 1998. Calcitonin gene-related peptide increase in the rat spinal dorsal horn and dorsal column nucleus following peripheral nerve injury: Up-regulation in a subpopulation of primary afferent sensory neurons. Neuroscience 82: 1243-1252.

Miller W. 1992. The Biochemistry of Exercise and Metabolic Adaptations. Dubuque, IA: Brown and Benchmark.

Moritani T, Devries M, Devries H. 1979. Neural factors versus hypertrophy in the time course of muscle strength gain. Amer J Phy Med 58:115-130.

Morrison P, Biggs R, Booth F. 1989. Daily running for 2-weeks and mRNAs for cytochrome c and alpha-actin in rat skeletal muscle. Am J Physiol 257 (5 Pt 1): C936-9.

Motorina M. 1993. The structural organization of the synaptic connections of the motor neuron in the mammalian spinal cord. Morgolofilia 105: 9-36.

Mulle C, Benoit P, Pinset C, Roa M, Changeux J. 1988. Calcitonin gene-related peptide enhances the rate of desensitization of the nicotinic acetylcholine receptor in cultured mouse muscle cells. Proc Natl Acad Sci 85: 5728-5732.

Mullen R, Buck C, Smith A. 1992. NeuN, a neuronal specific nuclear protein in vertebrates. Development 116: 201-211.

Nagy J, Yamanoto T, Jordon L. 1993. Evidence for the cholinergic nature of C-terminals associated with subsurface cisterns in α-motorneurons of rats. Synapse 15: 17-32.

Nakano H, Masusa K, Sasaki S, Katsuta S. 1996. Oxidative enzyme activity and soma size in motoneurons innervating the rat slow-twitch and fast-twitch muscles after chronic activity. Brain Res Bull 43: 149-154.

Nardone A, Romano C, Schieppati M. 1989. Selective recruitment of high-threshold human motor units during voluntary isotonic lengthening of active muscles. J Physiol 409: 451-471.

Neufer P, and Dohm G. 1993. Exercise induces a transient increase in transcription of the GLUT-4 gene in skeletal muscle. Am J Physiol 265: 597-603.

Pasino E, Buffelli M, Arancio O, Busetto G, Salviati A, Cangiano A. 1996. Effects of long-term conduction block on membrane properties of reinnervated and normally innervated rat skeletal muscle. J Physiol 497: 457-472.

Perrier J, Mejia-Gervacio S, Hounsgaard J. 2000. Facilitation of plateau potentials in turtle motoneurones by a pathway dependent on calcium and calmodulin. J Physiol 528: 107-113.

Piehl F, Arvidsson U, Johnson H, Cullhelm S, Villar M, Dagerlind A, Terenius L, Hokfelt T, Ulfhake B. 1991. Calcitonin gene-related peptide (CGRP)-like immunoreactivity and CGRP mRNA in rat spinal cord motoneurons after different types of lesions. Eur J Neurosci 3: 737-757.

Powers R, Robinson F, Konodi M, Binder M. 1993. Distribution of rubrospinal synaptic input to cat triceps surae motoneurons. J Neurophysiol 70: 1460-1468.

Proske U, Morgan D. 2001. Muscle damage from eccentric exercise: mechanisms, mechanical signs, adaptation and clinical applications. J Physiol 537: 333-345.

Pysh J, Weiss G. 1979. Exercise during development induces an increase in Purkinje cell dendritic tree size. Science 206: 230-232.

Ranvier M. 1873. Properties et structures differentes des muscles rouges et des muscles blancs, chez les lapins (French: Differences in the properties and structure of the red and white muscles of rabbits). Comptes Rendus Academie des Sciences 77: 1030-1034.

Reitsma W. 1969. Skeletal muscle hypertrophy after heavy exercise in rats with surgically reduced muscle function. Am J Phys Med 48: 237-257.

Rekling J, Funk G, Bayliss D, Dong Z, Feldman J. 2000. Synaptic control of motoneuronal excitability. Physiol Rev 80: 767-852.

Renshaw B. 1946. Central effects of centripetal impulses in axons of spinal ventral roots. J Neurophysiol 9: 191-204.

Renshaw B. 1941. Influence of discharge of motoneurons upon excitation of neighboring motoneurons. J Neurophysiol 4: 167-183.

Roghani A, Shirzadi A, Butcher L, Edwards R. 1998. Distribution of the vesicular transporter for acetylcholine in the rat central nervous system. Neuroscience 82: 1195-1212.

Ross A, Leveritt M, Rick S. 2001. Neural influences of sprint training: training adaptations and acute responses. Sports Med 31: 409-425.

Rotshenker S, McMahan U. 1976. Altered patterns of innervation in frog muscle after denervation. J Neurocytol 5: 719-730.

Roy R, Gilliam T, Taylor J, Heusner W. 1983. Activity-induced morphologic changes in rat soleus nerve. Exp Neur 80: 622-632.

Sala C, Andreose J, Fumagalli G, Lomo T. 1995. Calcitonin gene-related peptide: Possible role in formation and maintenance of neuromuscular junctions. J Neurosci 15: 520-528.

Sale D, MacDougall J, Upton A, McComas A. 1983. Effect of strength training upon motoneuron excitability in man. Med Sci Sports Exerc 15: 57-62.

Salmons S, Sreter F. 1976. Significance of impulse activity in the transformation of skeletal muscle type. Nature 263: 30-34.

Saltin B, Nazar K, Costill D, Stein E, Jansson E, Essen B, Gollnick P. 1976. The nature of the training response; peripheral and central adaptations to one-legged exercise. Acta Physiol Scand 96: 289-305.

Saltin B, Rowell L. 1980. Functional adaptations to physical activity and inactivity. Fed Proc 39: 1506-1513.

Samorajski T, Rolsten C. 1975. Nerve fiber hypertrophy in posterior tibial nerves of mice in response to voluntary running activity during aging. J Comp Neurol 159: 553-558.

Srere P. 1969. Citrate synthase. Methods Enzymology 13: 3-5.

Sherrington C. 1906. The integrative action of the nervous system. New Haven, CT: Yale University Press.

Shi S, Cote R, Taylor C. 2001. Antigen retrieval techniques: Current perspectives. J Histo Cyto 49: 931-937.

Shira T, Nairn A, Kloppenburg P, Lin Z, Pouzat C. 1995. A role for calcineurin (protein phosphatase-2B) in the regulation of glutamate release. Biochem Biophy Res Comm 212: 609- 616.

Sickles D, McLendon R. 1983. Metabolic variation among rat lumbosacral α-motoneurons. Histochem 79: 205-217.

Siebler M, Schmidt H. 1986. Induction of the action potential in innervated slow muscle fibers of the frog. Effects of tetrodotoxin, vincristine and colchicines. Brain Res 362: 299-307.

Smith H, Plyley M, Rodgers C, McKee N. 1997. Skeletal muscle damage in the rat hindlimb following single or repeated daily bouts of downhill exercise. Int J Sports Med 18: 94-100.

Staron R. 1997. Human skeletal muscle fiber types: delineation, development and distribution. Can J Appl Physiol 22: 307-327.

Stewart A, Yan Z, Surmeier D, Foehring R. 1999. Muscarine modulates Ca^{2+} channel currents in rat sensorimotor pyramidal cells via two distinct pathways. J Neurphysiol 81: 72-84.

Strack S, Wadzinski B, Ebner F. 1996. Localization of the calcium/calmodulin-dependent protein phosphatase, calcineurin, in the hindbrain and spinal cord of the rat. J Comp Neurol 375: 66-76.

Takekura H, Fujinami N, Nishizawa T, Ogasawara H, Kasuga N. 2001. Eccentric exercise-induce morphological changes in the membrane systems in excitation-contraction coupling in rat skeletal muscle. J Physiol 533: 571-583.

Terjung R, Winder W, Baldwin K, Holloszy J. 1973. Effect of exercise on the turnover of cytochrome c in skeletal muscle. J Bio Chem 248: 7404-7406.

Terjung R. 1976. Muscle fiber involvement during training of different intensities and durations. Am J Physiol 230: 946-950.

Tesch P, Thorsson A, Essen-Gustavsson B. 1989. Enzyme activities of FT and ST muscle fibers in heavy-resistance trained athletes. J Appl Physiol 67: 83-87.

Thorstensson A, Hulten B, von Dobeln W, Karlsson J. 1976. Effect of strength training on enzyme activities and fiber characteristics in human skeletal muscle. Acta Physiol Scand 96: 392-398.

Thorstensson A, Sjodin B, Karlsson J. 1975. Enzyme activities and muscle strength after "sprint training" in man. Acta Physiol Scand 94: 313-318.

Tokuda M, Hatase O. 1998. Regulation of neuronal plasticity in the central nervous system by phosphorylation and dephoshporylation. Molecular Neurobiology 17: 137-156.

Tomanek R, Tipton C. 1967. Influence of exercise and tenectomy on the morphology of a muscle nerve. Anat Rec 159: 105-114.

Van der Kloot W, Benjamin W, Balezina O. 1998. Calcitonin gene-related peptide acts presynaptically to increase quantal size and output at frog neuromuscular junctions. J Physiol 507: 689-695.

Van Praag H, Kempermann G, Gage F. 1999a. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nature Neurosci 2: 266-270.

Van Praag H, Christie B, Sejnowski T, Gage F. 1999b. Running enhances neurogenesis, learning and long-term potentitation in mice. PNAS 96: 13427-13431.

Veasey S, Fornal C, Metzler C, Jacobs B. 1995. Response of serotonergic causal raphe neurons in relation to specific motor activities in freely moving cats. J Neurosci 15: 5346-5359.

Welton J, Stewart W, Kerr R, Maxwell D. 1999. Differential expression of the muscarinic m2 acetylcholine receptor by small and large motoneurons of the rat spinal cord. Brain Research 817: 215-219.

Wernig A, Salvini T, Irintchev A. 1991. Axonal sprouting and changes in fiber types after running-induced muscle damage. J Neurocyt 20: 903-913.

Weyer A, Schilling K. 2003. Developmental and cell type-specific expression of the neuronal marker NeuN in the murine cerebellum. J Neuro Res 73: 400-409.

Wiesenfeld-Hallin Z, Xu X. 1998. Galanin in somatosensory function. Ann NY Acad Sci 863: 383-389.

Wimalawansa S. 1996. Calcitonin gene-related peptide and its receptors: molecular genetics, physiology, pathophysiology, and therapeutic potentials. Endocrine Reviews 17 (5): 1-52.

Wimalawansa S, Emson P, MacIntyre I. 1987. Regional distribution of calcitonin gene-related peptide and its specific binding sites in rats with particular reference to the nervous system. Neuroendocrinology 46: 131-136

Xu I, Grass S, Xu X, Wiesenfeld-Hallin Z. 1998. On the role of galanin in mediating spinal flexor reflex excitability in inflammation. Neuroscience 85: 827-835.

Zou D, Cline H. 1996. Expression of constitutively active CamKII in target tissue modifies presynaptic axon arbor growth. Neuron 16: 529-539.

Zhang X, Xu Z, Shi T, Landry M, Holmberg K, Ju G, Tong Y, Bao L, Cheng X, Wiesenfeld-Hallin Z, Lozano A, Dostrovsku J, Hokfelt T. 1998. Regulation of expression of galanin and galanin receptors in dorsal root ganglia and spinal cord after axotomy and inflammation. Ann New York Acd Sci 863: 402-412.