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Changes in Fiber Size and Fiber Type Proportions in the Extensor Digitorum Longus (EDL) and Soleus (SOL) Muscles of the Rat After Reinnervation Following Sciatic Nerve Crush

Sumera Ackbarali

BARRY UNIVERSITY

Changes in fiber size and fiber type proportions in the extensor digitorum longus (EDL) and soleus (SOL) muscles of the rat after reinnervation following sciatic nerve crush

Sumera Ackbarali

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The undersigned certify that they have read, and recommend to the Faculty of the School of Podiatric Medicine for acceptance, a thesis entitled Changes in fiber size and fiber type proportions in the extensor digitorum longus (EDL) and soleus (SOL) muscles of the rat after reinnervation following sciatic nerve crush submitted by Sumera Ackbarali in partial fulfillment of the requirements for the degree of Master of Science in Anatomy.

Approved by:

Dr. Ramjeet Pemsingh,
Chair of Thesis Committee

Dr. Sanjay Sesodia,
Thesis Supervisor

Dr. Allen Smith,
Member of Thesis Committee

Dr. Gilbert Ellis,
Member of Thesis Committee

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Abstract

Sciatic nerve crush is followed by a short period of denervation which results in muscle fiber atrophy and changes in parameters, such as fiber size and myosin heavy chain (MHC) expression. Reinnervation interrupts and may sometimes reverse the pattern of atrophy and other altered parameters induced by the short period of denervation. The purpose of this study was to determine the changes in fiber size and muscle fiber proportion in the extensor digitorum longus (EDL) and soleus (SOL) following reinnervation. The left sciatic nerve of female Wistar rats was crushed for 20 seconds and the wound was closed with 6-0 nylon sutures. After 21 days, the EDL and SOL muscles were collected from each rat and frozen. Sections (10μm) were cut for immunohistochemical staining for type 1, 2A, 2B, and 2X MHC isoforms, myosin ATPase staining, and H&E staining. The cross-sectional area (CSA) of each muscle fiber was measured using Scion Image Software 1.63 and the fiber type was determined by visual inspection. The differences in mean cross-sectional area (CSA) and mean fiber type proportions for reinnervated and control muscles were compared using an ANOVA test (Predictive Analytics Software [PASW] Statistics 17.0). In the fast-twitch EDL, there were significant changes in muscle fiber type proportions and evidence of transition towards a slower MHC profile throughout the muscle. Significant changes in fiber size but not proportion suggest that the SOL maintained its function as a slow-twitch muscle despite the atrophy of all fiber types observed 21 days after sciatic nerve crush.

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Introduction

Organization of skeletal muscle

Skeletal muscle is organized into subdivisions by connective tissue. The muscle is surrounded by the epimysium, a tough layer of collagen fibers ranging from 600 to 1800 nm in diameter which attaches the muscle to the skeleton. The epimysium connects to the perimysium, another layer of collagen fibers that surround the bundles, or fascicles, of individual muscle fibers. Major nerves and blood vessels that supply the muscle are found within the perimysium. The arterioles, venules, and nerve branches are located in regions where collagen fibers of the perimysium connect with the endomysium, the connective tissue layer surrounding each muscle fiber (MacIntosh et al., 2006).

Muscle fibers are formed by the fusion of mononucleate precursor cells called myoblasts (Hughes et al., 1993). Myofibrils are bundles of thick and thin protein filaments that run along the axis of each muscle fiber and are responsible for contraction and relaxation of the fiber. The striated appearance of skeletal muscle is the result of a repeating, regular arrangement of these filaments throughout the fiber, as well as the alignment of adjacent myofibrils. The thin filament comprises a double-stranded helical arrangement of actin. It is also composed of other regulatory and structural proteins, such as troponin, tropomyosin, and α-actinin. The thick filament comprises myosin, a large protein made of six different polypeptides, as well as myomesin and creatine kinase which participate in the thick filament's structural scaffolding and metabolism. The contraction of muscle depends on the formation of cross-bridges between actin and myosin (Berne et al., 2004).

Myosin Heavy Chains

Myosin, a large molecule of approximately 150nm long, constitutes almost 25% of the protein content found in striated (skeletal and cardiac) muscle. The native myosin molecule has two globular heads, each called subfragment 1 (S-1), and an α-helical tail which participates in formation of the thick filament of the sarcomere (Spudich, 1989). Each globular S1 head contains a portion of the myosin heavy chain (MHC) (220 kD) and a pair of nonidentical myosin light chains (George et al., 1989, Sartorius et al., 1998, MacIntosh et al., 2006).

Myosin serves as a backbone for the structure of each sarcomere, the functional units of the muscle. The myosin heavy chain has both enzymatic and structural domains (George et al., 1989). Its ATPase activity converts the chemical energy of ATP into mechanical energy necessary to effect strong binding of actin and myosin and the power-stroke that allows sliding of these filaments past each other (Hughes et al., 1993, Baldwin and Haddad 2001). Since the myosin heavy chain is responsible for hydrolyzing ATP for cross-bridge formation with actin that brings about shortening of the sarcomere and the resultant muscle contraction, it is a major determinant of the muscle's speed of contraction (Baldwin and Haddad, 2001).

Myosin Heavy Chain Isoforms

There are many isoforms of MHC which are expressed at certain developmental times in specific locations (Hughes et al., 1993). Extensive literature exists on MHC isoforms and their usefulness in classifying muscle fiber types. The fiber type of a muscle fiber corresponds to the MHC isoform it expresses.

Current research indicates that there are at least nine different MHC isoforms expressed within mammalian striated muscle: two developmental (embryonic and neonatal), an α-cardiac, a slow isoform (β-cardiac or type 1), three fast isoforms (type 2A, 2B, and 2X), an extraocular isoform, and a masticatory isoform (m-MHC) (Rinaldi et al., 2008, Baldwin and Haddad, 2001). Each isoform is the product of a specific gene belonging to the MHC gene family. In the rat, the skeletal MHC genes are found in a cluster on chromosome 10 whereas they are located on chromosome 17 in humans. These genes are linked in a head-to-tail arrangement in the following sequence: embryonic, 2A, 2X, 2B, neonatal, extraocular. The cardiac MHC genes, which include type 1 MHC, are also arranged in a cluster on chromosome 15 in rats and 14 in humans (Rinaldi et al., 2008).

The skeletal muscle of small mammals and adult rodents are known to express type 1, 2A, 2X, and 2B isoforms. Although the genes for these four MHC isoforms are also present in human skeletal muscle, studies have shown that type 2B is detectable at the mRNA level but not at the protein level (Weiss et al., 1999, Horton et al., 2001). The type 2B fibers in humans, identified by Brook and Kaiser's myosin ATPase staining method, actually express the type 2X MHC isoform (Pette et al., 1999, Horton et al., 2001, Schiaffino et al., 2007).

There is consensus that skeletal muscle is heterogeneous, i.e. it is composed of a mixture of different fiber types with varying physiological and histochemical properties (Wang and Kernell, 2000). These fiber types are present in varying proportions and give the muscle its characteristic speed of contraction, fatigue resistance, and power output (Rinaldi et al., 2008).

Muscle Fiber Types

In the present study, only four of the nine aforementioned muscle fiber types were examined: Types 1, 2A, 2X, and 2B fibers.

Type 1 fibers contain the type 1 MHC isoform. These are slow twitch, oxidative fibers which have a high capacity for generating ATP via oxidative phosphorylation. They hydrolyze ATP at a slow rate which causes them to contract slowly after stimulation. In addition to this, the numerous mitochondria, high levels of oxygen-binding myoglobin, and rich capillary supply observed in type 1 fibers enable them to sustain contraction for a longer period of time. Type 1 fibers are therefore resistant to fatigue and are suitable for muscles involved in maintaining posture and endurance tasks, such as running a marathon (Berne et al., 2004, Spangenburg and Booth, 2003).

Type 2A fibers are fast twitch fibers which contain the type 2A MHC isoform. These fibers exhibit both oxidative and glycolytic capacities (Berne et al., 2004). They have the highest oxidative capacity of all type 2 fibers which makes them relatively fatigue resistant and suited to sustained contraction (Spangenburg and Booth, 2003, Hämäläinen and Pette, 1993). Type 2A fibers, like all other type 2 fibers, contract at a faster rate than type 1 fibers. A study by Delp and Duan (1996) showed that type 2A fibers constitute 5% of rat skeletal muscle mass in adult male rats. This percentage increases with the size of the animals (Sartorius et al., 1998).

Type 2X fibers express the type 2X MHC isoform. Type 2X fibers exhibit more oxidative activity but lower glycolytic capacity than type 2B fibers. Both the cross-sectional area and mitochondrial content of these fibers are larger than type 2A but smaller than type 2B fibers (Hämäläinen and Pette, 1993). The rate of contraction, and

therefore, the myosin ATPase activity found in these fibers is intermediate between type 2A and type 2B fibers. Type 2X fibers are more abundant than type 2A in rat skeletal muscle as well as larger mammals (Sartorius et al., 1998).

Type 2B fibers are fast twitch, glycolytic fibers that express the type 2B MHC isoform. They contract very quickly following stimulation owing to their high myosin ATPase activity. They contain very few mitochondria and low oxidative activity, but high glycolytic activity. They fatigue easily owing to their inability to replenish ATP and creatine phosphate during contraction (Berne et al., 2004). Of all the fiber types currently known, type 2B fibers have the fastest rate of contraction and largest cross-sectional area. It is the most abundant fiber type in rat skeletal muscle, constituting 71% of muscle mass, but decreases in proportion in larger animals. In humans, no type 2B MHC isoforms have been detected except at the genetic level (Weiss et al., 1999, Horton et al., 2001). Therefore, type 2X MHC is the fastest MHC isoform present in humans. Harridge et al. (1993) demonstrated that sprinting, which activates type 2B fibers in smaller mammals, does not produce significant changes in fast MHC isoform expression but results in an increase in overall strength of the muscle.

The various MHC isoforms possess ATPase properties that are distinct from each other. As a result, the speed of contraction of fibers depends upon the type of MHC isoform it expresses (Barany, 1967). The calcium ATPase staining method designed by Brook and Kaiser (1970) operates on the principle of pH lability of different myosin ATPase enzymes. Since the myosin ATPase enzymes are very sensitive to pH, they can be inactivated depending on the pH of the solution in which the muscle fibers are incubated. The enzymes that remain active in the muscle hydrolyze ATP to produce

phosphate which binds to calcium, forming a compound that precipitates at pH 9.4. Cobalt then displaces calcium and reacts with ammonium sulfide to produce black cobalt sulfide (Brook and Kaiser, 1974).

The myosin ATPase of type 1 fibers is acid-stable but alkali-labile (Gorza, 1990). In the calcium ATPase staining method, these fibers in rat appear very light after incubation in an alkaline solution, not more than pH 10.2, but dark in acidic solutions. Type 2 fibers comprise an acid-labile but alkali-stable myosin ATPase enzyme. Both type 2A and 2B myosin ATPase enzymes are deactivated at a pH of 4.3 but type 2B fibers will show intermediate staining at a pH of 4.6. Therefore, incubation in a solution at pH 4.5 to 4.6 shows light type 2A fibers and darker type 2B fibers (Samaha et al., 1970, Pierobon-Bormioli et al., 1981, Gorza, 1990). By comparing sections incubated at these particular pHs, type 1, 2A, and 2B fibers can be identified, as well as type 2X fibers by the process of elimination.

Hybrid Fibers

Hybrid fibers are individual muscle fibers that co-express more than one MHC isoform (Baldwin and Haddad, 2001, Stephenson, 2001). It was previously thought that only two MHC isoforms are expressed in these fibers in the following specific combinations: I/2A, 2A/2X, and 2X/2B (DeNardi et al., 1993, Sartorius et al., 1998). However, many studies have demonstrated that hybrid muscle fibers can express between two and four MHC isoforms in various combinations (Stephenson, 2001, Čebašek et al., 2007). The contractile properties of these hybrid fibers are a mixture of the contractile characteristics of their contributing pure MHC isoforms.

Hybrid fibers have been observed in muscles that are subjected to changing conditions, such as abnormal neural and hormonal stimuli. These transforming muscles were originally thought to be the only scenario in which hybrid fibers are found (Stephenson, 2001). However, hybrid fibers exist in normal skeletal muscle. A possible function of these fibers is to enhance the capabilities of the muscle. For example, the laryngeal, mylohyoid, and stapedius muscles exhibit high proportions of hybrid fibers which may enable the performance of highly specialized mechanical tasks (Stephenson, 2006). Hybrid fibers are also present as a temporary state during postnatal development, before the fibers differentiate to a pure MHC composition (Agbulut et al., 2003).

Development of Muscle Fibers

The formation of multinucleated muscle fibers begins with fusion of mononucleate myoblasts and is accompanied by the expression of different MHC isoforms in the fiber (Ontell et al., 1988, Hughes et al., 1993). Two generations of muscle fibers (primary and secondary) contribute to the formation of skeletal muscle in utero. Prior to innervation and the formation of distinct muscles, primary muscle fibers arise in the embryonic limb (Gunning and Hardeman, 1991, Stockdale, 1997). These fibers express embryonic and slow MHC concurrently, indicating that the expression of type 1 MHC in these fibers is independent of innervation (Gunning and Hardeman, 1991, Agbulut et al., 2003).

For muscle formation to continue during fetal development, innervation of primary muscle fibers must occur, followed by the formation of secondary muscle fibers (Stockdale, 1997). These fibers express embryonic and neonatal MHC and will

eventually transition to fast MHC (Gunning and Hardeman, 1991). The development of type 1 MHC in secondary muscle fibers depends on innervation (Agbulut et al., 2003).

Postnatal development is divided into two phases, the first being a downregulation of developmental MHC isoforms (embryonic and neonatal) and the second being an upregulation and stabilization of the adult MHC isoforms (Agbulut et al., 2003). Establishment of the adult MHC profile of all muscles is controlled by extrinsic factors, such as motor innervation and hormonal control.

Neural Influence on Fiber Type Determination

Immature muscle fibers are usually innervated by more than one motor axon (polyneuronal innervation) (Brown et al., 1976). The axon terminals are initially bulbous but one eventually develops terminal branches while the others withdraw from the end plate (O'Brien et al., 1978). Maturation of the muscle fiber is marked by the elimination of polyneuronal innervation to be replaced by 1:1 muscle fiber: motor axon innervation ratio. This occurs much earlier in fast-twitch muscles than in slow-twitch muscles (Brown et al., 1976). In addition, the findings of Agbulut et al. (2003) demonstrated that developmental isoforms persist longer in the slow-twitch muscles than in the fast-twitch muscles.

The nervous system exerts a strong influence on the size, gene expression, and activity of muscles. The muscle does not attain its adult MHC profile until four to five weeks after birth. Experiments by Adams et al. (1999) demonstrated that neural input was essential for optimal expression of type 1 MHC in a slow-twitch muscle and type 2B MHC in a fast-twitch muscle. Elimination of neural stimulation resulted in retention of

the neonatal MHC isoform, increased expression of type 2A and 2X MHC in muscles that did not typically exhibit these isoforms, and a dramatic decrease in type 2B fibers (Germinario et al., 2002). However, Russell et al. (1993) have shown that the transition from neonatal MHC to type 2A MHC also requires neural input. Therefore, neural stimulation appears to be a critical factor in MHC gene expression and MHC phenotype of all muscle fibers.

Cross-reinnervation studies have demonstrated that muscles can change their MHC profile based on the type of neural stimulation it receives. The experiments of Buller at al. (1960) showed that subjecting a fast muscle to low frequency discharges from a slow motor neuron resulted in a slower contraction speed, whereas slow muscles exhibited faster contraction speeds when exposed to the higher discharge frequencies of fast motor neurons. Fast motor neurons emit intermittent high-frequency bursts whereas slow motor neurons emit prolonged electrical stimulation (Harridge, 2007). The different discharge patterns of these neurons have been implicated in the up-regulation or down-regulation of MHC genes via changes in intracellular calcium concentrations and activation of pathways, such as the calcineurin pathway, which influence transcription (Harridge, 2007).

Skeletal Muscle Plasticity

Skeletal muscle shows a certain degree of plasticity in that it can alter its MHC profile in response to a certain stimulus, be it electrical, mechanical, hormonal, or neuronal. During such an alteration, the number of hybrid fibers increases since the muscle is transitioning to an MHC composition that better serves its functional demands

(Rinaldi et al., 2008, Sartorius et al., 1998, Agbulut et al., 2003). With changing conditions in the muscle, the MHC isoforms transition from very fast to slow or vice versa takes place as described by the following: type 2B ↔ type 2X ↔ type 2A ↔ type 1. The transition is often incomplete, and is accompanied by changes in muscle fiber diameter as well as fatigue resistance (Čebašek et al., 2007).

The various combinations of MHC isoforms observed in hybrid fibers suggest that the previously described isoform transition does not only occur in that particular sequence. Transforming factors trigger a series of transcriptional events in transitioning skeletal muscle. In the case of altered electrical activity, individual myonuclei within a muscle fiber may be responding differently to MHC gene regulators by expressing uncharacteristic MHC isoforms (Stephenson, 2001).

Sciatic Nerve Crush: a nerve injury paradigm

Sciatic nerve crush is frequently used as a model of nerve injury to test many neuroregenerative therapeutic methods (Magill et al., 2007). A nerve crush is followed by a short period of denervation during which there is a loss of nerve-stimulated contractility in the muscles supplied by the sciatic nerve and its branches. The lack of neural transmitter substances and electrical activity caused by denervation induces muscle fiber atrophy (Patterson et al., 2006, Mendler et al., 2008) and the transformation of contractile elements in the sequence of type 2B ↔ type 2X ↔ type 2A ↔ type 1, with a higher proportion of hybrid fibers (Čebašek et al., 2007). There is an overall decrease in weight of the whole muscle, as well as a marked reduction in cytoplasm of individual muscle fibers (Patterson et al., 2006). It has been suggested that the generation of a smaller

maximum force following denervation is due not only to a reduced whole muscle cross-sectional area but also to the reduced ability of each individual fiber to generate its maximum force (Patterson et al., 2006)

The 1:1 ratio of muscle fiber: motor axon is disrupted after sciatic nerve crush. At one week, there are areas of discontinuity between endplates and axons as well as axons that make distal contact with the endplate but have no proximal continuity. This indicates that complete denervation has occurred at the one week time point following sciatic nerve crush (Magill et al., 2007). By two weeks, partial innervation returns to the muscle, as shown by the presence of some continuous axons. By week 3, complete reinnervation has occurred with some hyperinnervation. Restoration of the normal ratio of innervation begins by week 4 (Brown et al., 1976, Magill et al., 2007). Reinnervation interrupts and sometimes reverses the pattern of atrophy induced by the short period of denervation (Kugelberg et al., 1970, Lowrie and Vrbová, 1984, Mendler et al., 2008).

The soleus (SOL), a slow-twitch plantar flexor of the posterior compartment of the hindlimb, is innervated by the tibial nerve which branches from the sciatic nerve. Reinnervation of the soleus occurs by the second week following sciatic nerve crush (Jaweed et al., 1975). The extensor digitorum longus (EDL) is a fast-twitch extensor of the lateral four toes and is situated in the anterior compartment of the hindlimb. It is innervated by the deep peroneal nerve, a branch of the common peroneal nerve which arises from the sciatic nerve. Motor end plate reinnervation begins by day 12 with small nerve endings starting to make contact with the denervated end plates. This process is completed by day 21 following sciatic nerve crush (Ribarič et al., 1991).

Reinnervation is made possible by the fact that the endoneurial sheaths around nerve fibers remain intact in a nerve crush. The endoneurial sheaths' innermost layer comprises the basal lamina which surrounds Schwann cells and continues across nodes of Ranvier, forming an ensheathment around the nerve fibers. Axon sprouts begin to bud from the proximal end of the nerve crush and grow towards the crush site inside the basal laminal tube (Hall, 2005). Some of the basal laminal tubes in the region of the crush may remain intact and provide the architecture for the regenerating axons and Schwann cells to find their way back to the appropriate endoneurial tubes on the others side of the lesion (Thomas, 1989). Compression injuries, such as nerve crush, reduce the possibility of axonal misrouting (Hall, 2005). However, recent studies show that damage to basal laminal tubes in the region of the nerve crush results in surprisingly low accuracy in regenerating axon direction (DeRuiter et al., 2008).

In the present study, the objectives were: (1) to evaluate the changes in muscle fiber size in the extensor digitorum longus (EDL) and soleus (SOL) muscles following reinnervation, and (2) to determine the proportions of muscle fiber types (type 1, 2A, 2B, 2X, and hybrid) in the reinnervated EDL and SOL.

The comparison of fiber size and fiber type proportions between reinnervated skeletal muscles and their control muscles may offer insight into the response of each fiber type to injury and reinnervation. Since this study investigates reinnervation-induced changes in a fast-twitch and a slow-twitch muscle, it may indicate if the changes in each fiber type are the same in a fast-twitch muscle as they are in a slow-twitch muscle.

Hypotheses

H1: Reinnervation would produce similar changes in fiber size in the fast-twitch EDL as in the slow-twitch SOL

H2: Reinnervation would produce similar changes in fiber type proportion in the fast-twitch EDL as in the slow-twitch SOL

Method

Surgical Procedure

Eight female Wistar rats (Charles River Laboratories, Raleigh, NC) were randomly divided into three groups: 0 day naive (n = 2), 21 day naive (n = 2), and 21 day reinnervated (n = 4). The rats of the 0 day naïve and 21 day naïve groups were unoperated and were euthanized at the times indicated with ketamine (180 mg/kg) and xylazine (20 mg/kg) along with cervical dislocation. The entire extensor digitorum longus (EDL) and soleus (SOL) muscles were collected.

The rats that were to comprise the 21 day reinnervated group were anesthetized by an intraperitoneal injection of ketamine (90 mg/kg) and a subcutaneous injection of xylazine (20 mg/kg). The left hindlimb was carefully shaved in the region of the sciatic notch and wiped with an alcohol prep pad (Moore Medical Corp., New Britain, CT). When each animal was areflexic (no movement of limb upon pinching of the foot), an incision was made in the skin posterior to the femur, just distal to the sciatic notch. The layers of connective tissue and overlying muscle were blunt dissected to expose the sciatic nerve which was crushed for 20 seconds with a pair of flat No. 5 forceps (Hamilton Bell, Montvale, NJ). The cut ends of the overlying muscle were apposed and the wound was closed with 6-0 nylon sutures (Cincinnati Surgical Co., Cincinnati, Ohio). After 21 days, each rat was euthanized using the same procedure described above for the 0 day naïve and 21 day naïve groups, and the EDL and SOL muscles of these rats were collected.

The EDL and SOL muscles from each rat were weighed. Each muscle was oriented diagonally on a labeled piece of cork using TBS Tissue Freezing Medium

(Triangle Biomedical Sciences, Durham, NC) with the proximal tendon at the top right corner and the distal tendon at the bottom left corner. The muscles were frozen in isopentane (2-methyl butane) (Fisher Scientific, Pittsburgh, PA) chilled to -78.5°C by adding dry ice chips and then transferred to the cryostat. Positioning a sharp razor blade at the center point between the two tendons (belly of the muscle), at 90° to the muscle's long axis, a cut was made from the surface of the muscle to the cork. A parallel cut was made about 3 mm proximally from the first cut. This block of muscle was gently removed and oriented on another labeled piece of cork with the medial surface exposed and the proximal surface against the cork. It was mounted in TBS Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) and sprayed with Flash Freeze Rapid Freezing Spray (Decon Laboratories, Inc., New Oxford, PA) to ensure firm adhesion. These blocks of muscle were stored in a -70°C freezer.

Immunohistochemistry

Cryostat sections (10μm thick) were cut from the blocks of EDL and SOL muscles and collected on charged microscope slides for immunohistochemical staining with monoclonal antibodies against slow and fast MHC isoforms. Sections were surrounded with a PAP pen and allowed to dry for 45 minutes. The Vectastain ABC system for immunoperoxidase staining (Vector Laboratories, Burlingame, CA) was used for visualization of the MHC isoforms. All primary antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). (See Appendix 1)

Myosin ATPase Staining

Cryostat sections (10μm thick) of EDL and SOL muscles were collected on coverslips and allowed to dry for 45 minutes. Sections were stained as described in Appendix 2 at pre-incubation pHs of 4.2, 4.6, and 10.2.

Hematoxylin and Eosin (H&E) Staining

Hematoxylin is a basic compound that binds to acidic cell structures such as DNA. Eosin is an acidic compound that binds to the basic structures in the cell, such as the proteins in the cytoplasm. This stain is used to reveal general morphology of the sections. Cryostat sections (10μm thick) of EDL and SOL muscles were collected on coverslips and stained with hematoxylin and eosin. (See Appendix 3)

Morphometry of fibers

An Olympus BH-2 microscope equipped with an Infinity 1 digital camera (Lumenera Corp, Ontario, Canada) was used to take 40X images of each section. Since the section was too large to be captured in one picture, multiple images were taken and then reassembled in Adobe Photoshop Elements 3.0 (Adobe Systems Inc., San Jose, CA) to create the whole section. This image was then imported into a Microsoft Word 2004 (for Mac) (Microsoft Corportation, Redmond, WA) grid. The horizontal and vertical axes were labeled with letters and numbers respectively. Each grid square measured 1.3cm × 1.3cm (0.5" × 0.5"). The first 10 out of 100 grid coordinates produced by a random number generator were used to identify 10 random areas of the section for sampling. 100X images were taken of those specific areas and the cross-sectional area (CSA) of 10

fibers per square, for a total of 100 fibers, was measured using Scion Image Software 1.63 (Scion Corporation, Frederick, MD). The fiber type of each fiber was determined by visual inspection. The differences in mean cross-sectional area (CSA) and mean fiber type proportions for reinnervated, and control muscles were compared using an ANOVA test (Predictive Analytics Software [PASW] Statistics 17.0, SPSS Inc, Chicago, IL).

Results

The three experimental groups in this study were the 0 day naïve group, 21 day naïve group, and the 21 day reinnervated group. The rats of the 0 day naïve group and 21 day naïve group were unoperated. Fewer than 50% of the fibers in one set of the right and left EDL muscles of these groups were stained. As a result, this data had to be discarded, leaving only one animal in the 0 day and 21 day naïve experimental groups for the EDL muscle. In the 21 day reinnervated group, sciatic nerve crush was performed only on the left sciatic nerve. Therefore, the muscles of the right hindlimb will be referred to as the controls and those of the left side will be referred to as the reinnervated muscles. The weight of each muscle is expressed as a percentage of the total body weight of the rat because the normal EDL and SOL each constitute a certain proportion of the body weight in all rats, regardless of body weight.

Weight of EDL and SOL

Muscle	Mean Muscle Weight (% of Body Weight)		
	0 day naïve	21 day naïve	21 day reinnervated
R. EDL	**0.037%**	**0.045%**	**0.047%**
	-	-	0.002%
L. EDL	**0.034%**	**0.036%**	**0.034%***
	-	-	0.001%

Table 1: Mean weight of right and left EDL muscles (expressed as % of total body weight of rat) for the three experimental groups. Values are ± S.E. Values of naïve groups are from a single muscle as explained in Results. For the reinnervated group, n = 4. * indicates a significant difference between control and reinnervated means, $p < 0.05$.

The data in Table 1 above show that the right and left EDL muscles of the 0 day naïve group had similar weights. In the muscles of the 21 day naïve groups, the weight of the right EDL was larger than the left EDL by 0.009% but it is unclear if this was a

significant difference since there was only one EDL per side in this group. The weight of the reinnervated EDL, 0.034% of the body weight, was significantly smaller ($p < 0.05$) than that of the control muscle which has a weight of 0.047% (Table 1).

The right SOL muscles of the 0 day naïve group and 21 day naïve group were not significantly different in weight ($p > 0.05$) from the left SOL muscles of those respective groups. The weight of the reinnervated SOL was significantly smaller ($p < 0.05$) than that of the control muscle, as shown in Table 2 below.

Muscle		Muscle Weight (% of Body Weight)		
		0 day naïve	21 day naïve	21 day reinnervated
R. SOL	**Mean**	**0.040%**	**0.049%**	**0.052%**
	S.E.	0.002%	0.000%	0.002%
L. SOL	**Mean**	**0.035%**	**0.046%**	**0.036%***
	S.E.	0.000%	0.005%	0.003%

Table 2: Mean weight of right and left SOL muscles (expressed as % of total body weight of rat) for the three experimental groups. Values are ± S.E. n = 2 in naïve groups, n = 4 in reinnervated group. * indicates a significant difference between control and reinnervated means, $p < 0.05$.

Cross-sectional Area (CSA) of EDL Fibers

0 Day and 21 Day Naïve Groups

In the 0 day naïve experimental group, there were four pure fiber types present as well as hybrid fibers. Table 3 shows that in both right and left EDL muscles, the type 2B fibers were the largest with a CSA of 1215.95 μm^2 in the right EDL and 957.62 μm^2 in the left EDL, followed in order by the type 2X, type 1, and type 2A fibers. The hybrid fibers, which had CSAs of 623.81 μm^2 and 464.80 μm^2 in the right and left respectively, exhibited the smallest CSA of all fibers within this group. Although the data suggested that the CSA of fibers in the right EDL was larger than the left EDL, these differences could not be tested for significance since there was only one EDL per side in this group.

Muscle	Fiber CSA (μm^2)				
	Type 1	Type 2A	Type 2B	Type 2X	Hybrid
R. EDL	712.47	666.49	1215.95	762.67	623.81
L. EDL	611.31	528.05	957.62	638.20	464.80

Table 3: Cross-sectional area (CSA; μm^2) of pure and hybrid fibers within the right and left EDL muscles of the 0 day naïve experimental group; n = 1.

In the 21 day naïve experimental group, there were four pure fiber types and hybrid fibers. As seen in Table 4, the type 2B fibers of both muscles were the largest with a CSA of 2552.27 μm^2 and 1804.95 μm^2 in the right and left EDL muscles, respectively. This was followed in size by the type 2X fibers, as in the 0 day naïve group. However, unlike the 0 day naïve group (Table 3), the hybrid fibers were not the smallest fibers; the type 1 and the type 2A fibers had the lowest CSA in the right and left EDL muscles respectively.

It is unclear if the differences in CSA between right and left EDL muscles were significant since there was only one right and left muscle in this group. In comparison to the CSAs of the fibers in the 0 day naïve group, all fiber types were greater in size after 21 days.

Muscle	Fiber CSA (μm^2)				
	Type 1	Type 2A	Type 2B	Type 2X	Hybrid
R. EDL	899.30	1001.76	2552.27	1431.77	1082.65
L. EDL	974.48	613.45	1804.95	1096.85	744.98

Table 4: Cross-sectional area (μm^2) of pure and hybrid fibers within the right and left EDL muscles of the 21 day naïve experimental group; n = 1.

21 Day Reinnervated Group

The EDL muscles within this experimental group were found to contain the same fiber types as in the naïve groups: Type 1, type 2A, type 2B, type 2X, and hybrid fibers (shown in Table 5). The differences in CSA observed between the control and reinnervated muscles were not significant ($p > 0.05$). In the reinnervated EDL, only two of the four EDL muscles contained type 2B fibers. The smaller sample size, which was half that of the control EDL, may be the reason for a large numerical difference but not a significant difference between the CSA of the control EDL and the reinnervated EDL.

In both control and reinnervated muscles, the type 2B fibers were the largest of all the fiber types, with a CSA of 2029.96 ± 170.94 μm^2 and 1430.82 ± 38.88 μm^2 in the right and left EDL muscles respectively. This was followed by the 2X fibers. This trend was the same as that of the 0 day and 21 day naïve groups (Table 3 and 4). The type 2A fibers of the control EDL exhibited a much lower CSA of 784.45 ± 43.02 μm^2 than that of the type 2A fibers of the right EDL in the 21 day naïve group (CSA of 1001.76 μm^2).

Muscle		**Fiber CSA (μm^2)**				
		Type 1	**Type 2A**	**Type 2B**	**Type 2X**	**Hybrid**
Control EDL	**Mean**	**989.54**	**784.45**	**2029.96**	**1195.78**	**850.79**
	S.E.	164.43	43.02	170.94	59.44	60.89
Reinn. EDL	**Mean**	**824.47**	**983.41**	**1430.82**	**1178.49**	**945.81**
	S.E.	84.53	96.72	38.88	199.11	88.10

Table 5: Mean cross-sectional area (μm^2) of pure and hybrid fibers within the control and reinnervated EDL muscles of the 21 day reinnervated experimental group; n = 4. $p > 0.05$ indicating no significant difference between control and reinnervated means.

Cross-sectional Area (CSA) of SOL Fibers

0 Day and 21 Day Naïve Groups

In the 0 day naïve group, there were 2 pure fiber types (type 1 and 2A) and hybrid fibers. As shown in Table 6 below, the type 1 fibers exhibited the largest CSA in both the right and left SOL muscles, with a CSA of 1481.85 ± 193.84 μm^2 and 1283.19 ± 143.29 μm^2 respectively. The hybrid fibers and the type 2A fibers were similar in size and smaller than the type 1 fibers. The difference in CSA between right and left SOL muscles was not significant ($p > 0.05$).

Muscle		**Fiber CSA (μm^2)**		
		Type 1	**Type 2A**	**Hybrid**
R. SOL	**Mean**	**1481.85**	**910.16**	**844.85**
	S.E.	193.84	132.99	73.32
L. SOL	**Mean**	**1283.19**	**819.76**	**909.82**
	S.E.	143.29	41.04	105.13

Table 6: Mean cross-sectional area (μm^2) of pure and hybrid fibers within the right and left SOL muscles of the 0 day naïve experimental group, n = 2. $p > 0.05$ indicating no significant difference between right and left means.

As in the 0 day naïve group, there were no type 2B and type 2X fibers present in the 21 day naïve group. The type 1 fibers were the largest fibers of the 21 day naïve SOL (Table 7), as they were in the 0 day naïve SOL (Table 6). In the 21 day naïve group, the CSA of the fibers in the right SOL were similar ($p > 0.05$) to that of the corresponding fiber types in the left SOL. All fiber types of the 21 day naïve group exhibited larger CSAs than those of the 0 day naïve group.

Muscle		Fiber CSA (μm^2)		
		Type 1	Type 2A	Hybrid
R. SOL	**Mean**	**2604.68**	**1419.04**	**1392.96**
	S.E.	133.71	84.11	3.33
L. SOL	**Mean**	**2567.75**	**1505.98**	**1511.00**
	S.E.	4.83	291.06	150.79

Table 7: Mean cross-sectional area (μm^2) of pure and hybrid fibers within the right and left SOL muscles of the 21 day naïve experimental group; n = 2. $p > 0.05$ indicating no significant difference between right and left means.

21 Day Reinnervated Group

Type 1, type 2A, and hybrid fibers were present in the control and reinnervated SOL muscles of the 21 day reinnervated group. In both the control and reinnervated muscles, the type 1 fibers were the biggest fibers compared with the other fiber types of the same muscles. The hybrid fibers were the second largest, followed by the type 2A fibers. All fiber types of the reinnervated SOL were significantly smaller ($p < 0.05$) than their counterparts in the control SOL. As shown in Table 8, the mean fiber CSA of the reinnervated type 1 fibers was1363.51 ± 109.07 μm^2, less than 50% of the CSA of the control SOL (2844.01 ± 96.71 μm^2). Similarly, the type 2A and hybrid fibers were smaller in the reinnervated SOL. Only type 1 and type 2A MHC isoforms were co-expressed in all of the hybrid fibers. The CSA of the fibers in the control SOL were very similar to those of the 21 day naïve group (Table 7). However, the CSA of the fibers in the reinnervated SOL were more comparable to those of the 0 day naïve group (Table 6).

Muscle		Fiber CSA (μm^2)		
		Type 1	Type 2A	Hybrid
Control SOL	**Mean**	**2844.01**	**1453.57**	**1568.18**
	S.E.	96.71	62.46	126.54
Reinn. SOL	**Mean**	**1363.51***	**767.58***	**839.36***
	S.E.	109.07	89.43	74.18

Table 8: Mean cross-sectional area (μm^2) of pure and hybrid fibers within the control and reinnervated SOL muscles of the 21 day reinnervated experimental group; n = 4. * indicates a significant difference between control and reinnervated means, $p < 0.05$.

Fiber Type Proportion of EDL Fibers

0 Day and 21 Day Naïve Groups

In both right and left EDL muscles of the 0 day naïve group, the type 2B fibers were the most abundant of all fiber types, making up 40% and 34% of all fiber types. The type 2X was present in the second highest proportion, followed by type 2A in the right EDL and hybrid fibers in the left EDL. The type 1 fibers were present in far lower proportions in both muscles, with 2% in the right EDL and 3% in the left EDL, as seen in Table 9. It cannot be determined if the differences in proportion observed between the right and left EDL muscles are significant since there was only one right EDL and one left EDL.

Muscle	Fiber Type Proportion (%)				
	Type 1	Type 2A	Type 2B	Type 2X	Hybrid
R. EDL	**2.0**	**19.0**	**40.0**	**24.0**	**15.0**
L. EDL	**3.0**	**14.0**	**34.0**	**26.0**	**23.0**

Table 9: Fiber type proportion (expressed as % of 100 sampled fibers) of pure and hybrid fibers within the right and left EDL muscles of the 0 day naïve experimental group; n = 1.

The type 1 fibers of the EDL muscles in the 21 day naïve group were present in equal proportions and also the lowest proportion of all fiber types. Table 10 shows that type 2B and 2X fibers were the most abundant fibers in the group. Compared with the 0 day naïve group (Table 8), there appeared to be a higher percentage of type 2X fibers in the EDL muscles after 21 days. As in the 0 day naïve group, it is unclear if the differences in proportion between right and left EDL muscles were significant since there was only one right and left muscle in this group.

Muscle	Fiber Type Proportion (%)				
	Type 1	Type 2A	Type 2B	Type 2X	Hybrid
R. EDL	1.0	13.0	37.0	36.0	13.0
L. EDL	1.0	20.0	28.0	37.0	10.0

Table 10: Fiber type proportion (expressed as % of 100 sampled fibers) of pure and hybrid fibers within the right and left EDL muscles of the 21 day naïve experimental group; n = 1.

21 Day Reinnervated Group

In the 21 day reinnervated group, type 2B and 2X fibers of the reinnervated EDL showed significant decreases in proportion from 38.3% to 5.0% for type 2B and 29.5% to 3.8% for type 2X (Table 11). These two fiber types were present in the highest proportion within the control EDL of the 21 day reinnervated group as well as the naïve groups. As in the control EDL, the reinnervated EDL exhibited a similarly low proportion of type 1 fibers which was only slightly higher than the type 1 proportions of the 21 day naïve group (Table 10). The proportion of type 2A and hybrid fibers in the reinnervated EDL tripled compared with the control, going from 17.0% and 12.3% to 51.5% and 40.3%, respectively. The differences in mean fiber type proportions between the control and

reinnervated EDL muscles of the 21 day reinnervated group were significant for all type 2 fibers and hybrid fibers ($p < 0.05$). However, type 1 fibers did not show any significant difference in proportion between control and reinnervated muscles ($p > 0.05$).

In the control EDL, type 2X MHC was co-expressed in 92% (34 out of 37) of hybrid fibers, and type 2A MHC in 78% (29 out of 37) of hybrid fibers. Twenty-six of those thirty-seven hybrid fibers expressed a combination of type 2X and 2A MHC only. In the reinnervated EDL, all hybrid fibers expressed type 2A MHC along with either one or two other MHC isoforms. Approximately 50% (48 out of 94) of these hybrid fibers co-expressed type 1 MHC together with type 2A MHC. Type 2B and 2X MHC were also expressed in conjunction with other MHC isoforms but in fewer fibers.

Muscle		Fiber Type Proportion (%)				
		Type 1	Type 2A	Type 2B	Type 2X	Hybrid
Control EDL	**Mean**	**2.8**	**17.0**	**38.3**	**29.5**	**12.3**
	S.E.	1.2	4.0	2.9	3.2	2.9
Reinn. EDL	**Mean**	**2.0**	**51.5***	**5.0***	**3.8***	**40.3***
	S.E.	0.7	11.9	2.8	1.2	11.0

Table 11: Mean fiber type proportion (expressed as % of 100 sampled fibers) of pure and hybrid fibers within the control and reinnervated EDL muscles of the 21 day reinnervated experimental group; n = 4. * indicates significant difference between control and reinnervated means, $p < 0.05$.

Fiber Type Proportion of SOL Fibers

0 Day and 21 Day Naïve Groups

In the 0 day naïve group (Table 12), the SOL muscles were made up predominantly of type 1 fibers and type 2A fibers. The proportion of hybrid fibers was similar and low in both right and left SOL. The slight differences in mean fiber type

proportions between right and left SOL muscles of this group were not significant ($p > 0.05$).

Muscle		Fiber Type Proportion (%)		
		Type 1	Type 2A	Hybrid
R.SOL	**Mean**	**63.5**	**31.0**	**5.5**
	S.E.	6.5	2.0	4.5
L.SOL	**Mean**	**54.5**	**39.0**	**6.5**
	S.E.	1.5	5.0	3.5

Table 12: Mean fiber type proportion (expressed as % of 100 sampled fibers) of pure and hybrid fibers within the right and left SOL muscles of the 0 day naïve experimental group; n = 2. $p > 0.05$ indicating no significant difference between right and left means.

In the 21 day naïve SOL (Table 13), the type 1 fibers were present in an even greater proportion than at 0 days. The proportion of type 2A fibers showed a markedly lower value from the 0 day value while the proportion of hybrid fibers was still low and similar to the 0 day naïve group. The right and left SOL muscles exhibited similar proportions of each fiber type ($p > 0.05$).

Muscle		Fiber Type Proportion (%)		
		Type 1	Type 2A	Hybrid
R.SOL	**Mean**	**80.0**	**13.0**	**7.0**
	S.E.	2.0	2.0	0.0
L.SOL	**Mean**	**83.5**	**9.0**	**7.5**
	S.E.	8.5	6.0	2.5

Table 13: Mean fiber type proportion (expressed as % of 100 sampled fibers) of pure and hybrid fibers within the right and left SOL muscles of the 21 day naïve experimental group; n = 2. $p > 0.05$ indicating no significant difference between right and left means.

21 Day Reinnervated Group

There was no significant difference ($p > 0.05$) in the proportion of fiber types between the reinnervated SOL and the control SOL (Table 14). As in the 0 day naïve and 21 day naïve groups, the SOL muscles of the 21 day reinnervated group contained a markedly greater proportion of type 1 fibers than type 2A and hybrid fibers.

Muscle		**Fiber Type Proportion (%)**		
		Type 1	**Type 2A**	**Hybrid**
Control	**Mean**	**75.0**	**4.0**	**23.0**
SOL	S.E.	4.9	0.7	4.9
Reinn.	**Mean**	**69.0**	**7.5**	**27.3**
SOL	S.E.	5.8	3.2	7.4

Table 14: Mean fiber type proportion (expressed as %) of pure and hybrid fibers within the control and reinnervated SOL muscles of the 21 day reinnervated experimental group; n = 4. $p > 0.05$ indicating no significant difference between control and reinnervated means.

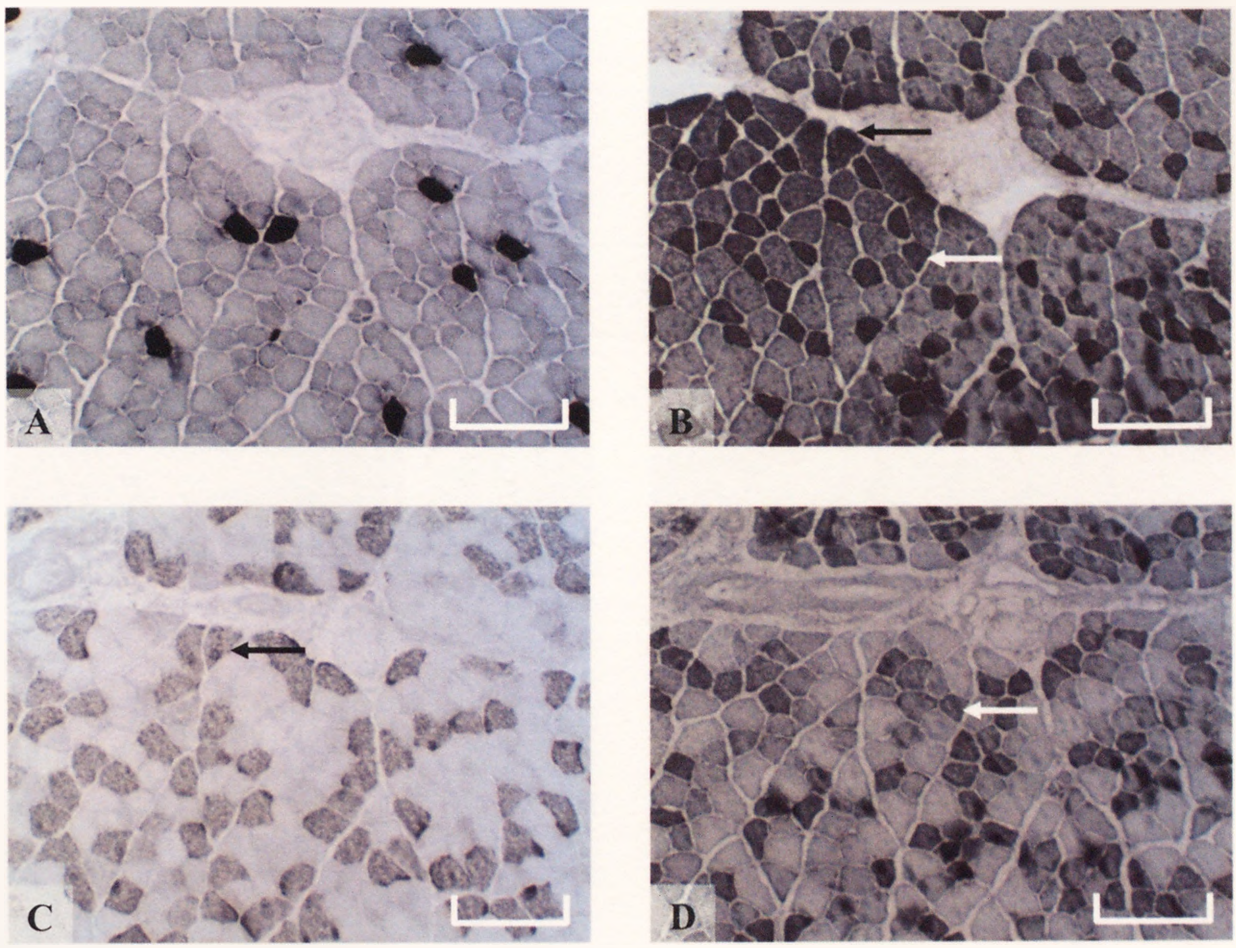

Figure 1: Immunohistochemical staining for MHC isoforms in the 0 day naïve EDL muscle. A: fibers stained with monoclonal Ab against type 1 MHC, B: fibers stained with monoclonal Ab against type 2A MHC, C: fibers stained with monoclonal Ab against type 2B MHC, D: fibers stained with monoclonal Ab against type 2X MHC. Arrows indicate hybrid fibers. Scale bar = 100 μm in sections A to D.

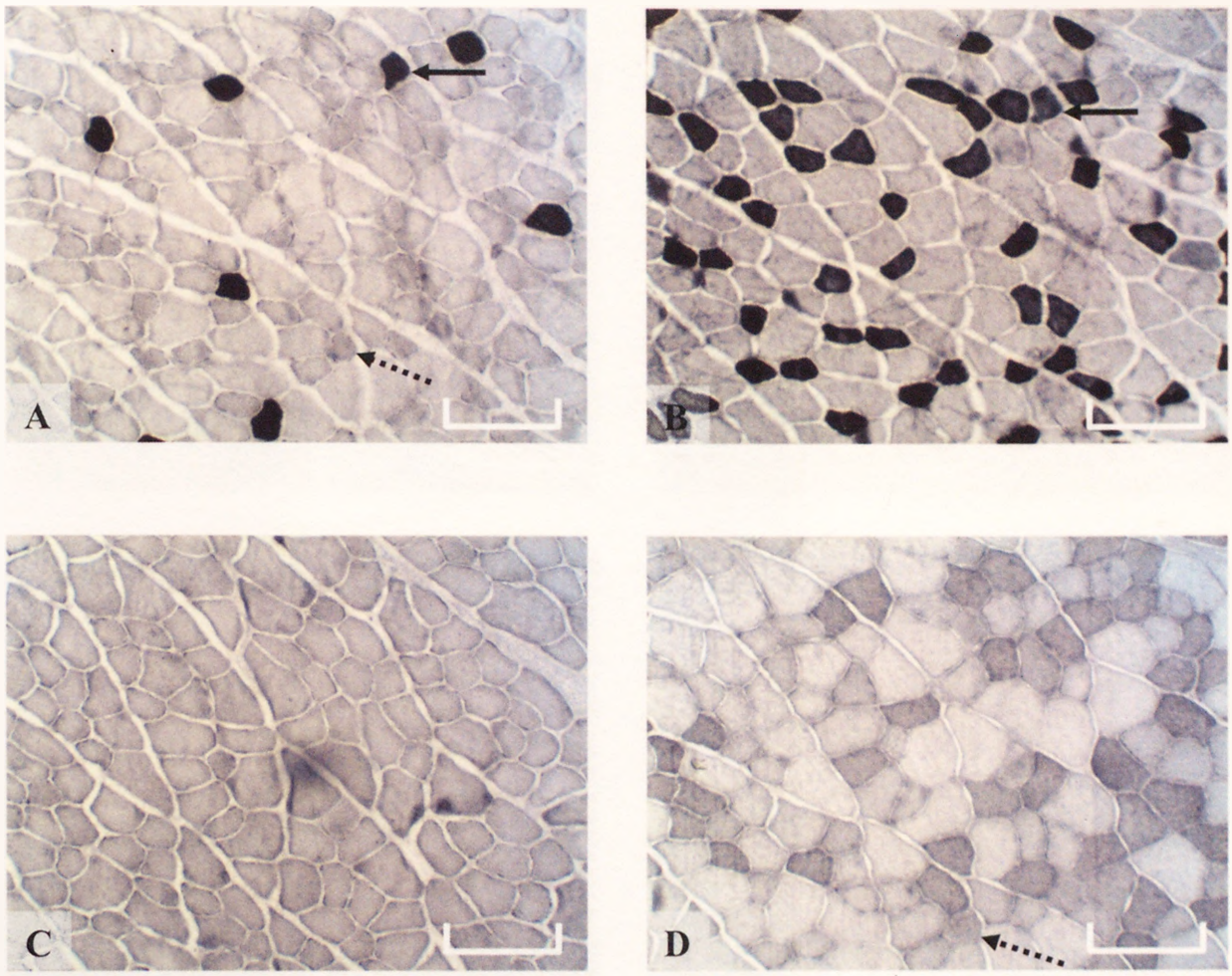

Figure 2: Immunohistochemical staining for MHC isoforms in the 21 day naïve EDL muscle. A: fibers stained with monoclonal Ab against type 1 MHC, B: fibers stained with monoclonal Ab against type 2A MHC, C: fibers stained with monoclonal Ab against type 2B MHC, D: fibers stained with monoclonal Ab against type 2X MHC. Arrows indicate hybrid fibers. Scale bar = 100 μm in sections A to D.

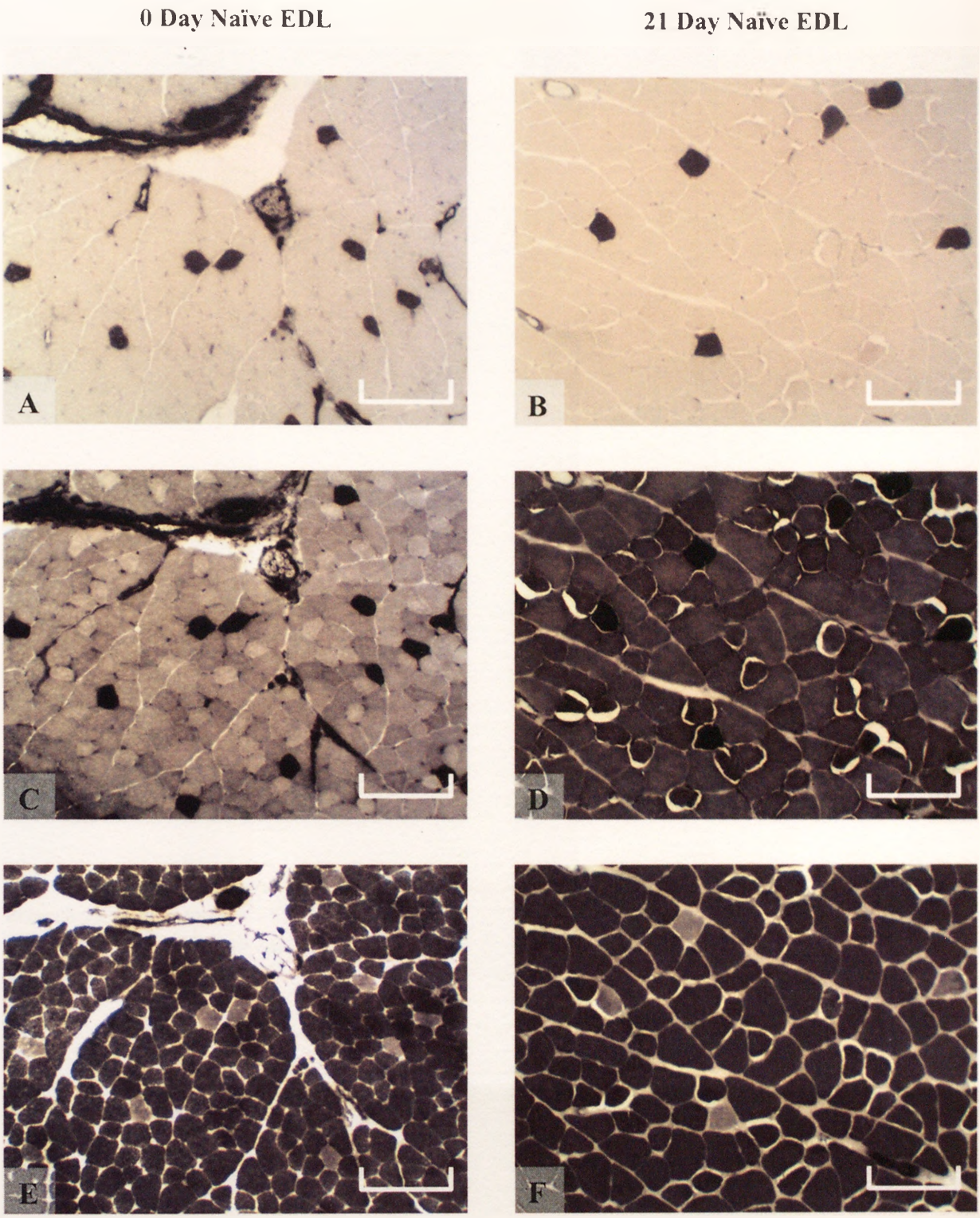

Figure 3: Myosin ATPase staining of the 0 day naïve and 21 day naïve EDL muscles. A and B: ATPase staining at pH 4.2 showing dark type 1 fibers and light type 2 fibers; C and D: ATPase staining at pH 4.6 showing dark type 1 fibers, intermediate type 2B fibers, and light type 2A fibers; E and F: ATPase staining at pH 10.2 showing light type 1 fibers and dark type 2 fibers. Scale bar = 100 μm in sections A to F.

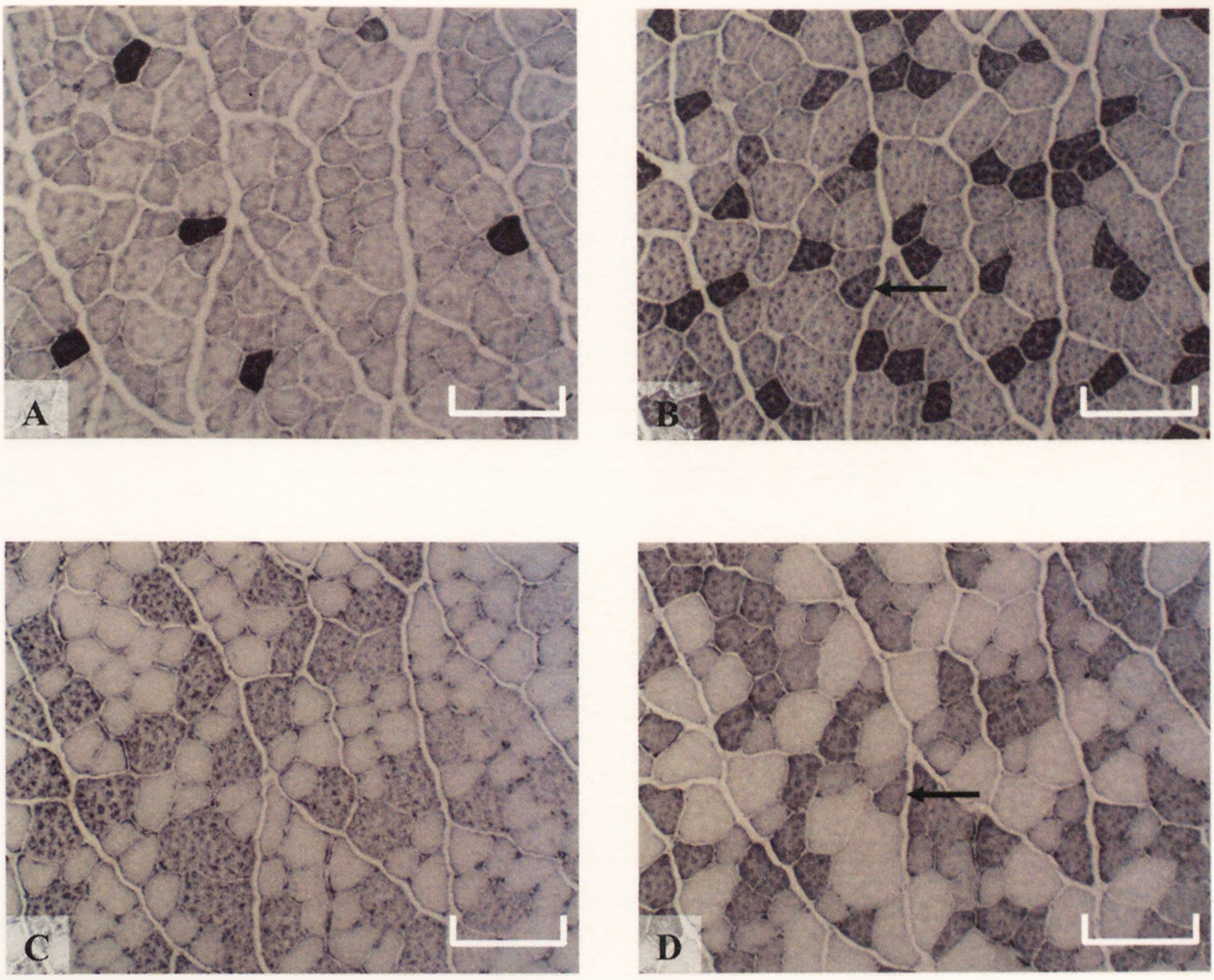

Figure 4: Immunohistochemical staining for MHC isoforms in the control EDL muscle. A: fibers stained with monoclonal Ab against type 1 MHC, B: fibers stained with monoclonal Ab against type 2A MHC, C: fibers stained with monoclonal Ab against type 2B MHC, D: fibers stained with monoclonal Ab against type 2X MHC. Arrows indicate hybrid fibers. Scale bar = 100 μm in sections A to D.

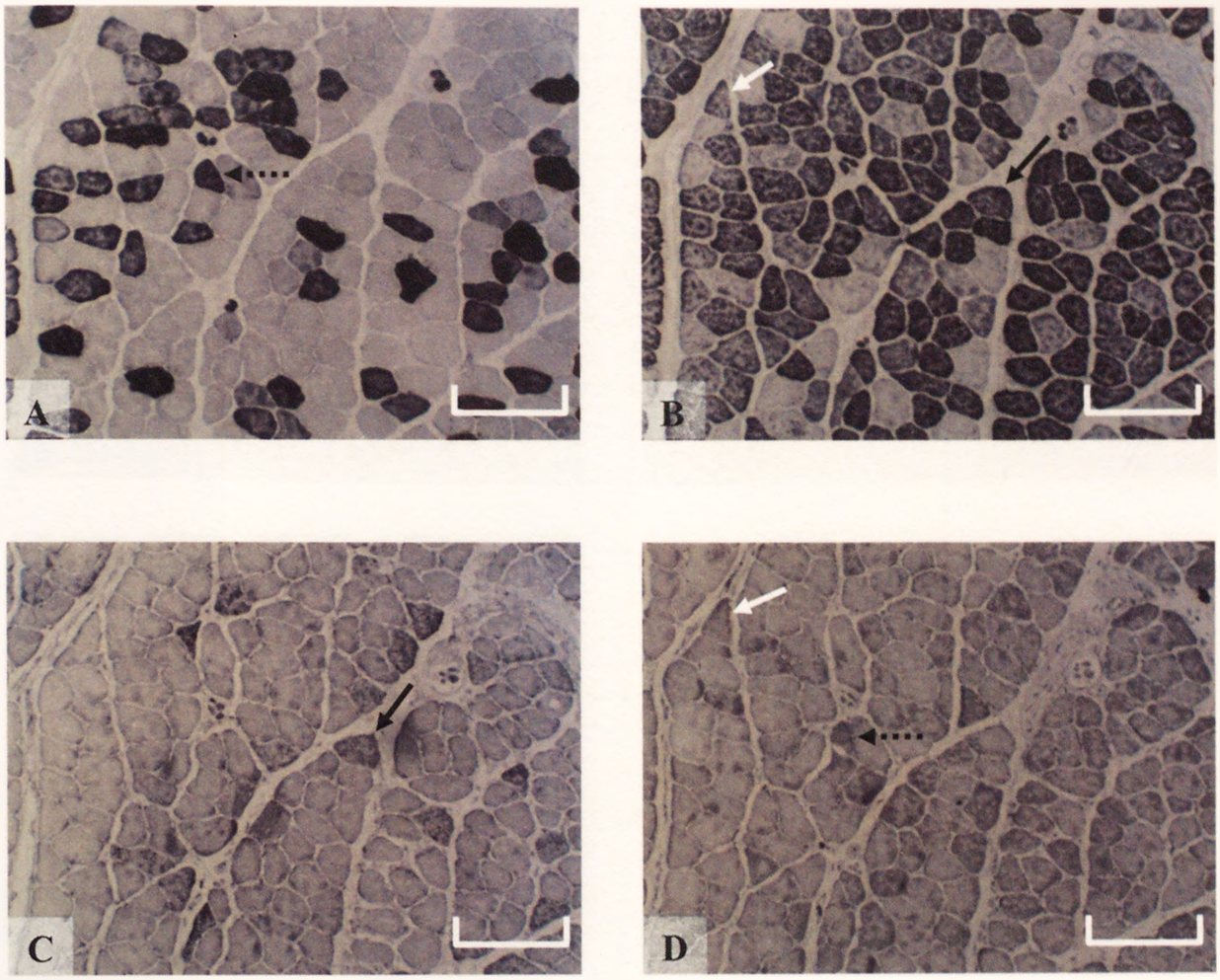

Figure 5: Immunohistochemical staining for MHC isoforms in the reinnervated EDL muscle. A: fibers stained with monoclonal Ab against type 1 MHC, B: fibers stained with monoclonal Ab against type 2A MHC, C: fibers stained with monoclonal Ab against type 2B MHC, D: fibers stained with monoclonal Ab against type 2X MHC. Arrows indicate hybrid fibers. Scale bar = 100 μm in sections A to D.

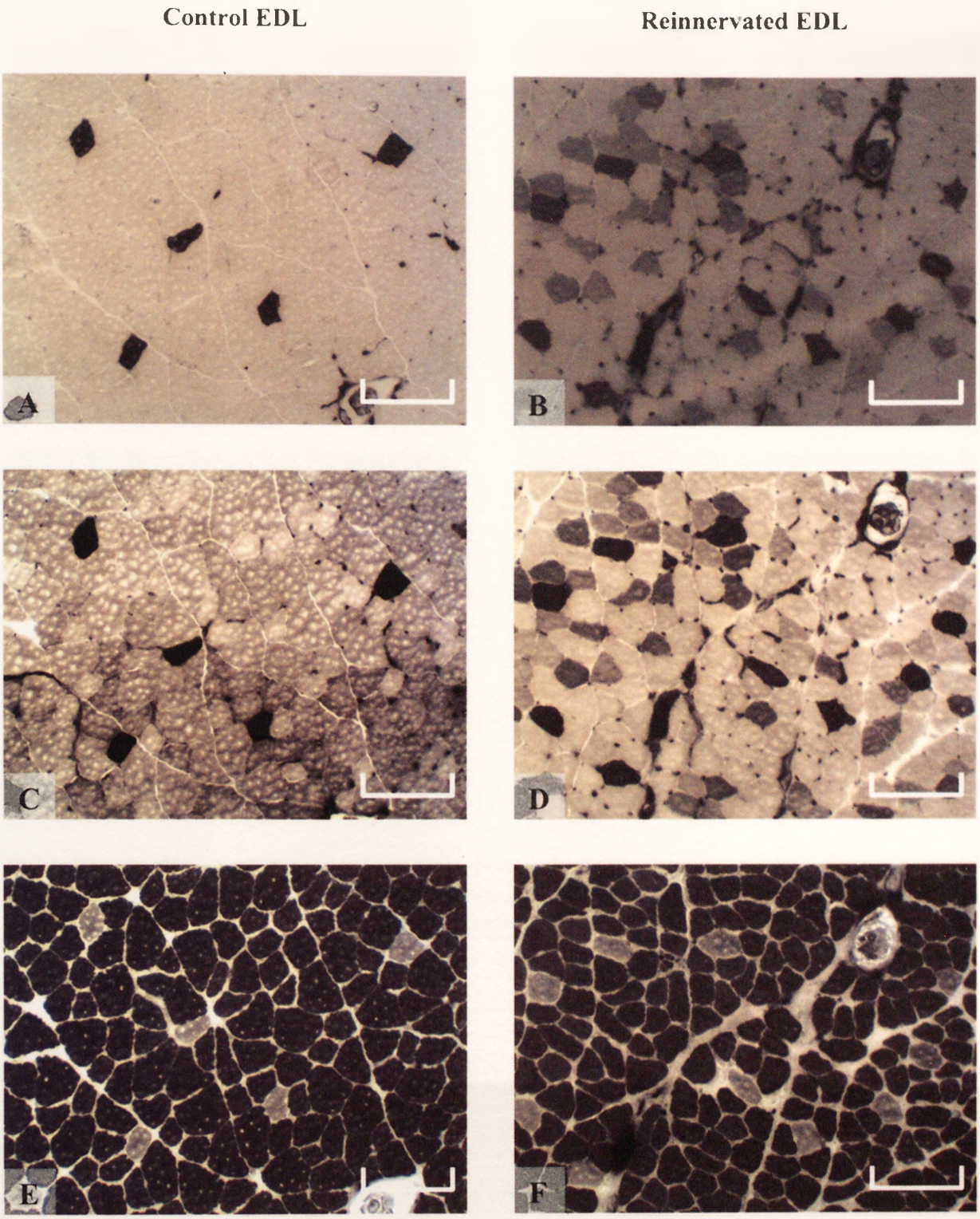

Figure 6: Myosin ATPase staining of the control and reinnervated EDL muscles. A and B: ATPase staining at pH 4.2 showing dark type 1 fibers and light type 2 fibers; C and D: ATPase staining at pH 4.6 showing dark type 1 fibers, intermediate type 2B fibers, and light type 2A fibers; E and F: ATPase staining at pH 10.2 showing light type 1 fibers and dark type 2 fibers. Scale bar = 100 μm in sections A to F.

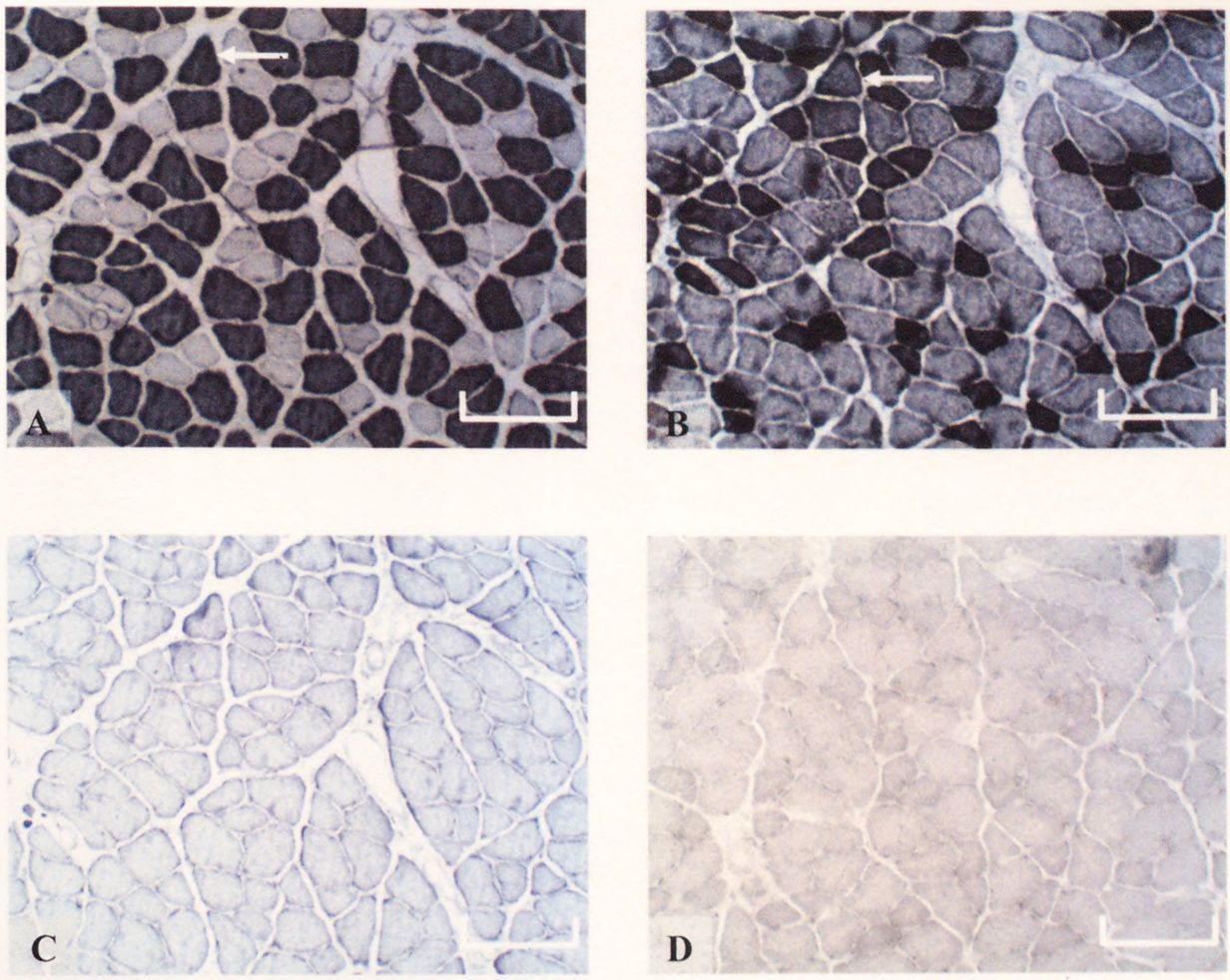

Figure 7: Immunohistochemical staining for MHC isoforms in the 0 day naïve SOL muscle. A: fibers stained with monoclonal Ab against type 1 MHC, B: fibers stained with monoclonal Ab against type 2A MHC, C: no Ab staining against type 2B MHC, D: no Ab staining against type 2X MHC. Arrows indicate hybrid fibers. Scale bar = 100 μm in sections A to D.

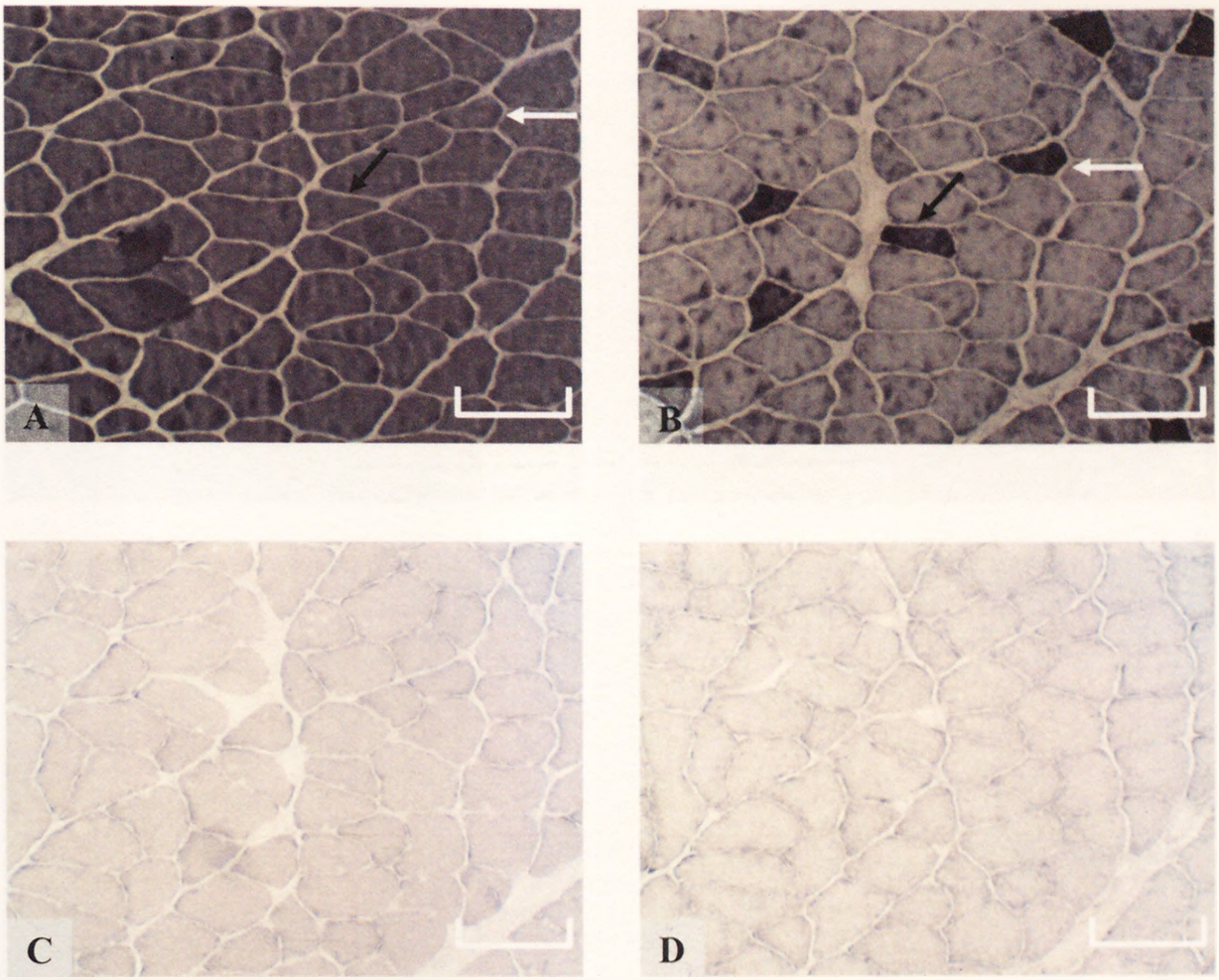

Figure 8: Immunohistochemical staining for MHC isoforms in the 21 day naïve SOL muscle. A: fibers stained with monoclonal Ab against type 1 MHC, B: fibers stained with monoclonal Ab against type 2A MHC, C: no Ab staining against type 2B MHC, D: no Ab staining against type 2X MHC. Arrows indicate hybrid fibers. Scale bar = 100 μm in sections A to D.

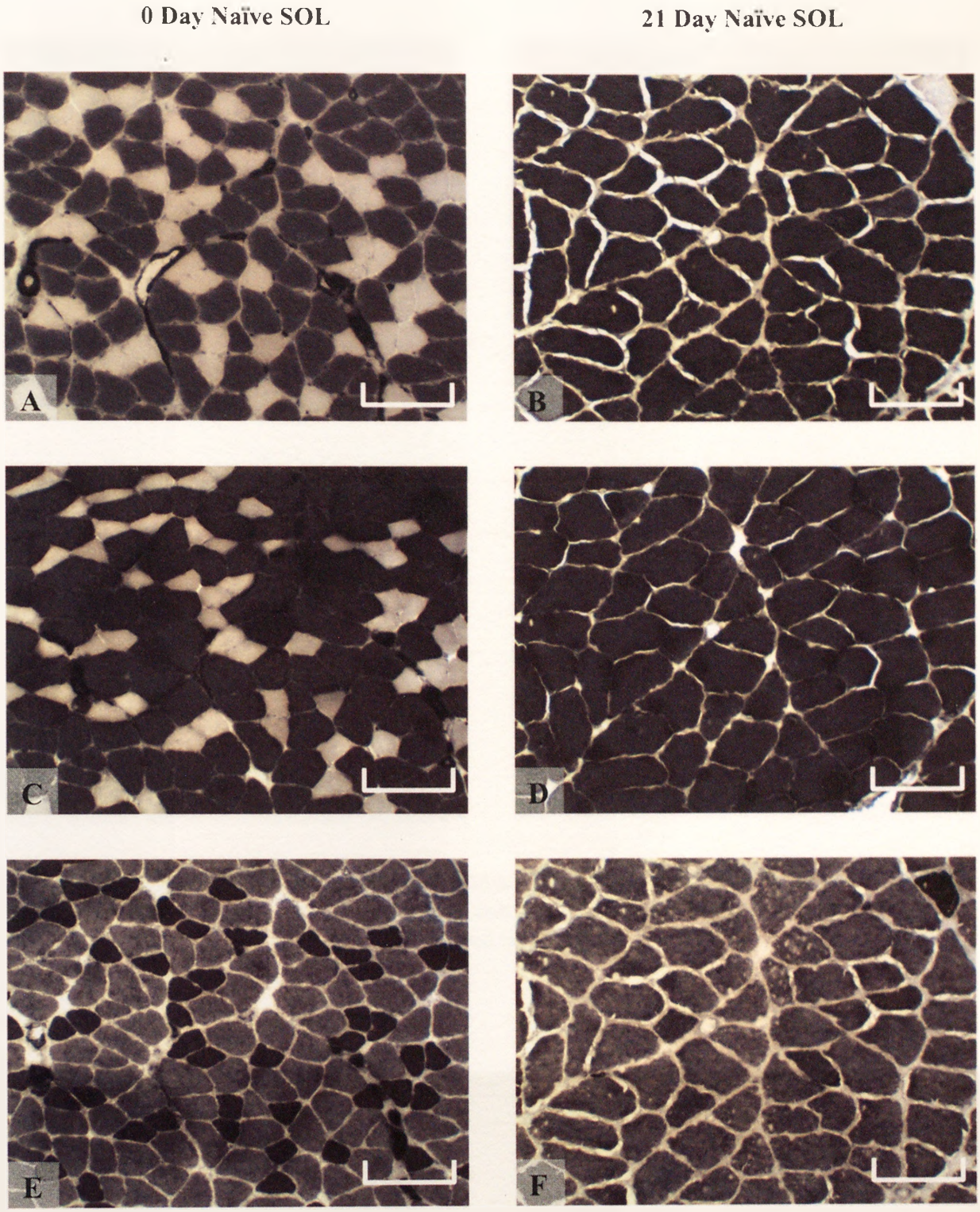

Figure 9: Myosin ATPase staining of the 0 day naïve and 21 day naïve SOL muscles. A and B: ATPase staining at pH 4.2 showing dark type 1 fibers and light type 2 fibers; C and D: ATPase staining at pH 4.6 showing dark type 1 fibers and light type 2A fibers; E and F: ATPase staining at pH 10.2 showing light type 1 fibers and dark type 2 fibers. Scale bar = 100 μm in sections A to F.

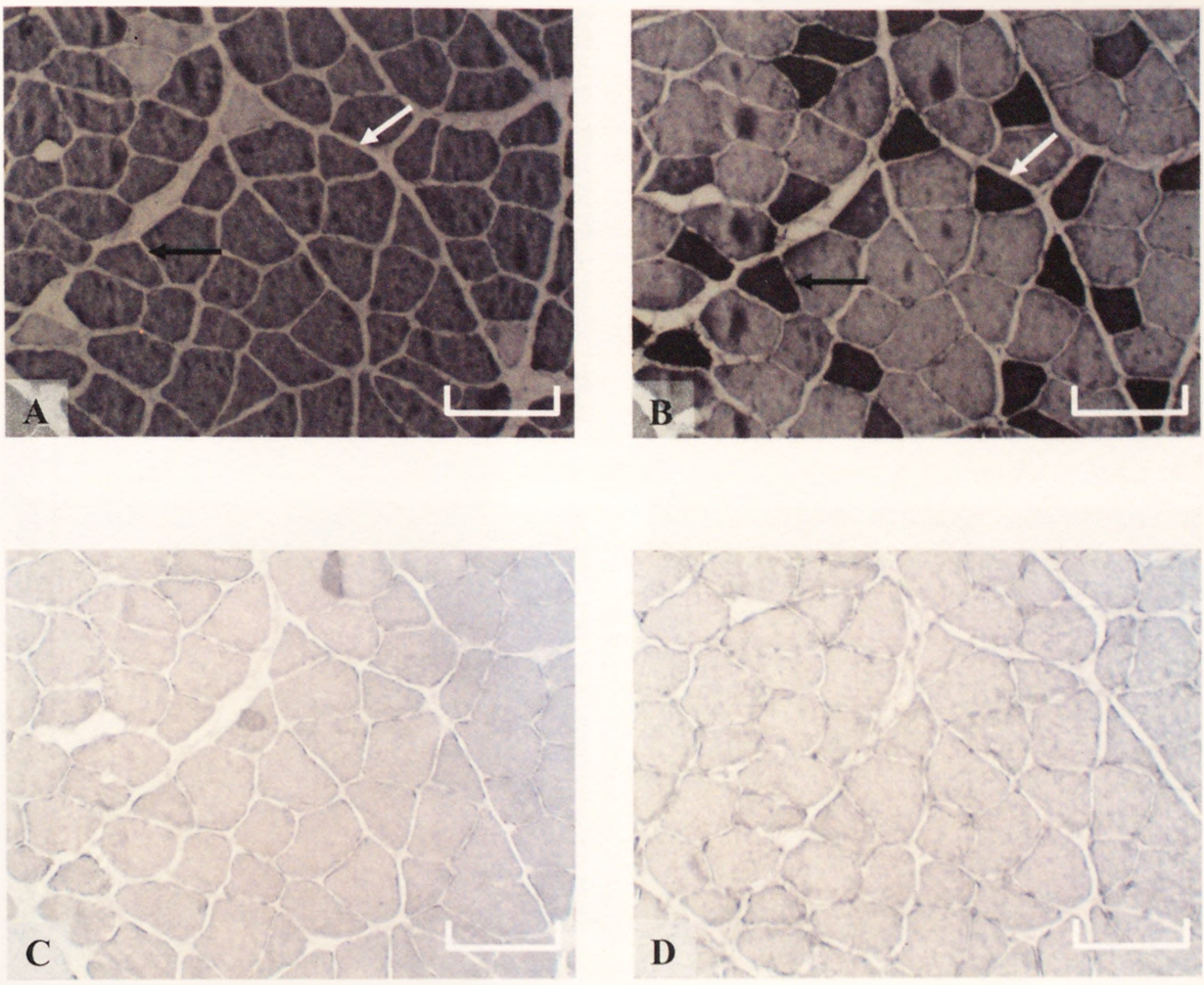

Figure 10: Immunohistochemical staining for MHC isoforms in the control SOL muscle. A: fibers stained with monoclonal Ab against type 1 MHC, B: fibers stained with monoclonal Ab against type 2A MHC, C: no Ab staining against type 2B MHC, D: no Ab staining against type 2X MHC. Arrows indicate hybrid fibers. Scale bar = 100 μm in sections A to D.

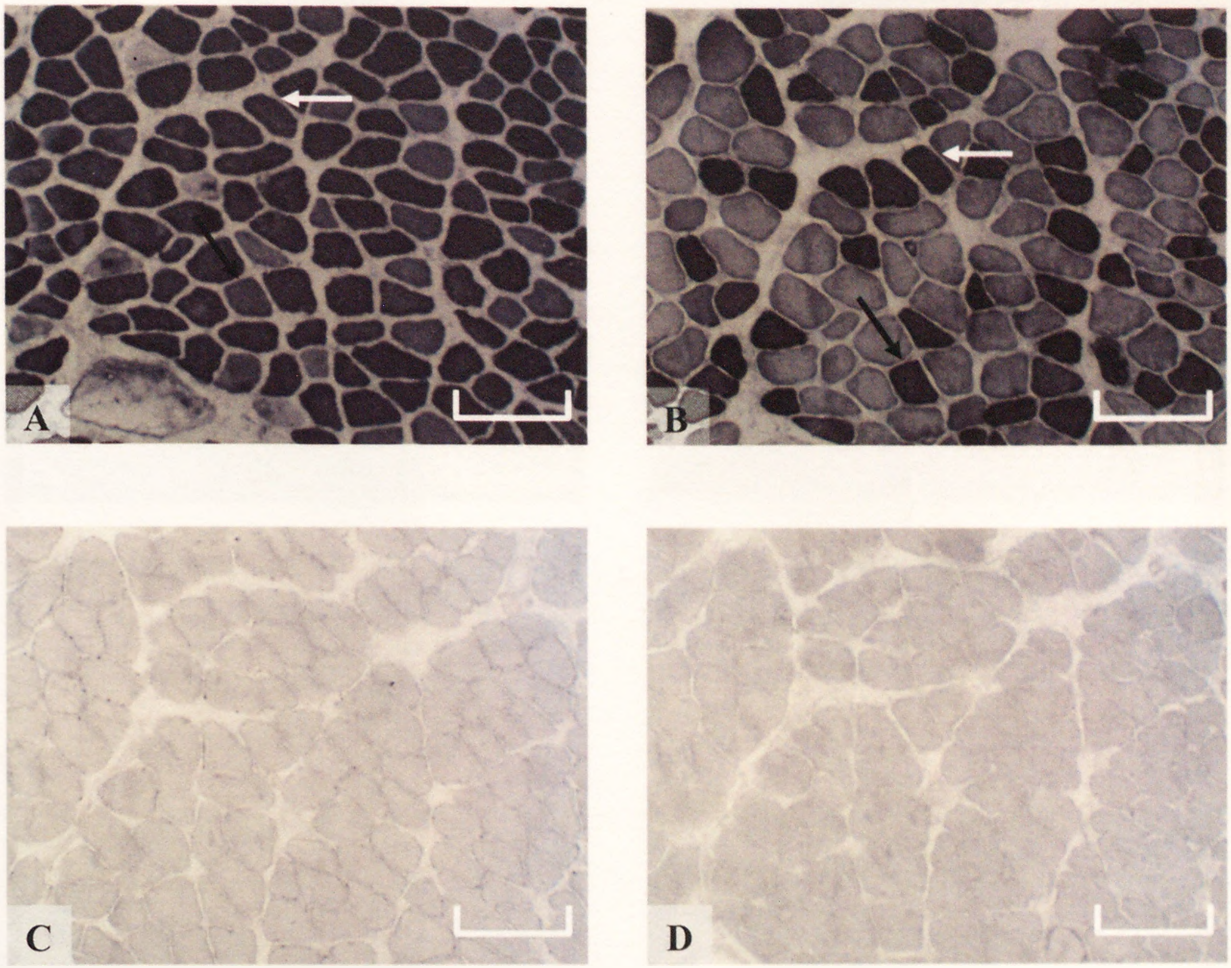

Figure 11: Immunohistochemical staining for MHC isoforms in reinnervated SOL muscle. A: fibers stained with monoclonal Ab against type 1 MHC, B: fibers stained with monoclonal Ab against type 2A MHC, C: no Ab staining against type 2B MHC, D: no Ab staining against type 2X MHC. Arrows indicate hybrid fibers. Scale bar = 100 μm in sections A to D.

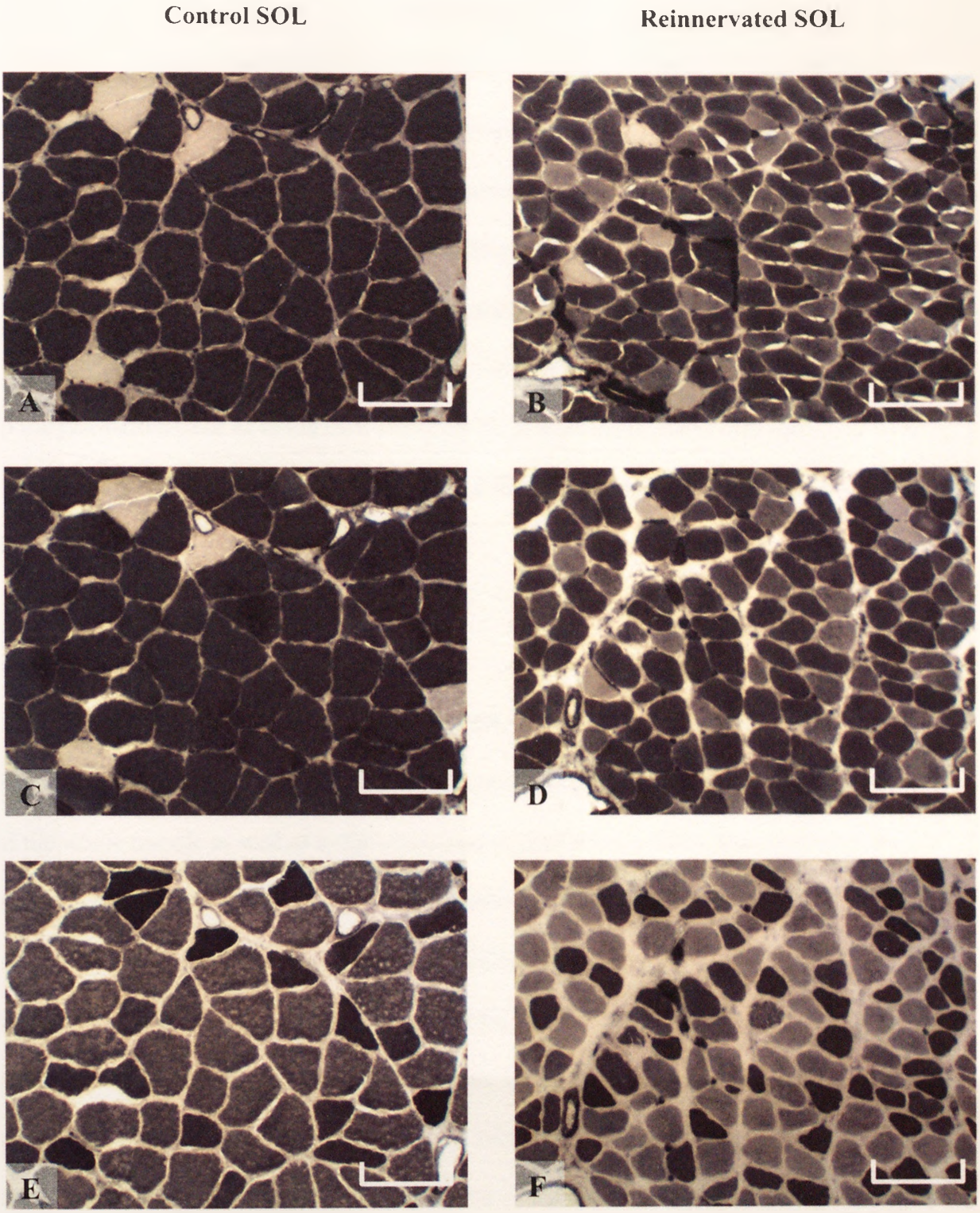

Figure 12: Myosin ATPase staining in control and reinnervated SOL muscles. A and B: ATPase staining at pH 4.2 showing dark type 1 fibers and light type 2 fibers; C and D: ATPase staining at pH 4.6 showing dark type 1 fibers and light type 2A fibers; E and F: ATPase staining at pH 10.2 showing light type 1 fibers and dark type 2 fibers. Scale bar = 100 μm in sections A to F.

Discussion

The purpose of this study was to examine the changes in fiber size and fiber type distribution in the fast-twitch EDL and the slow-twitch SOL following reinnervation, and to evaluate if each fiber type responded to reinnervation in the same manner in both types of muscles. The model of nerve injury was sciatic nerve crush which involved a short period of denervation followed by reinnervation of muscle fibers.

It was hypothesized that reinnervation would produce similar changes in fiber size in the fast-twitch EDL as in the slow-twitch SOL. It was also hypothesized that reinnervation would produce similar changes in fiber type proportion in the fast-twitch EDL as in the slow-twitch SOL

Changes in EDL after Reinnervation

The size of fibers in the reinnervated EDL remained similar to those of the control EDL. According to Patterson et al. (2006), denervation resulted in a reduction in weight of the whole muscle as well as in the cytoplasm of each muscle fiber. The results of this study showed that there was a significant decrease in the weight of the reinnervated EDL which indicated that some atrophy occurred as a result of the nerve crush. However, no change in fiber size may mean that the re-establishment of neural stimulation enabled the restoration of protein synthesis within the fibers. This would have helped to recover the sizes of the fibers to values similar to those of normal muscle. Another possible explanation for the maintenance of fiber size but a decrease in weight in the reinnervated EDL was that, during dissection, a smaller portion of the tendon may have been removed with the reinnervated muscle than in the control muscle.

This study demonstrated that sciatic nerve crush brings about significant changes in fiber type proportion in the EDL. As a fast-twitch muscle of the lower extremity, the EDL would be expected to have low type 1 fiber proportions and higher proportions of the type 2 fibers (Soukup et al., 2002, Germinario et al., 2002). As shown in Table 11, this pattern of fiber type distribution existed in both the control EDL and the reinnervated EDL. However, there were considerable differences within the type 2 fiber population: Increases in the proportion of type 2A fibers and hybrid fibers of the reinnervated EDL, and decreases in types 2B and 2X fibers. The decrease in the type 2B proportion has previously been reported by Čebašek et al. (2007).

Since type 2B fibers were the largest and the fastest fibers, the lower type 2B proportion in the reinnervated muscle compared with the control muscle suggested that the speed of contraction should be slower at 21 days post-crush. Type 2X fibers, which have a contraction speed second only to 2B fibers (Sartorius et al., 1998), were also fewer which supported the presumption that the muscle may have a lower contraction speed. Type 2A fiber proportions in the reinnervated muscle were three times higher than in the control muscle. Thus, reinnervation of the EDL was accompanied by an increase in the slowest of the type 2 fibers at the expense of faster varieties, type 2B and type 2X fibers. This observation would suggest that the overall speed of contraction of the reinnervated EDL was slower than that of the normal EDL.

The hybrid fiber population showed an increase similar to that of the type 2A population. Hybrid fibers exist naturally in muscle (Stephenson, 2001, Stephenson, 2006) and were observed in the present study to be between 10% and 13% within the 21 day naïve group as well as the control EDL of the 21 day reinnervated group. A rise to 40.3%

in the reinnervated EDL showed that the difference in hybrid fiber numbers reflect transitions in MHC profile of individual EDL fibers.

The typical pattern of MHC transition in hybrid fibers is as follows: 1↔ 1/2A ↔ 2A ↔2A/2X ↔ 2X ↔ 2X/2B ↔ 2B (Stephenson, 2006, Schiaffino et al., 2007), but the hybrid fibers observed in this study express a combination of either two or three MHC isoforms. Four MHC isoforms were never co-expressed in a single fiber. In both the control and reinnervated EDL muscles, type 1 MHC was co-expressed with all type 2 MHC isoforms except type 2B. Types 2A and 2X MHC was found in combination with all MHC isoforms. Type 2B is only co-expressed with other type 2 MHC isoforms. In the control EDL, the hybrid fibers continued to express mostly fast MHC isoforms (types 2B and 2X) whereas all hybrid fibers of the reinnervated EDL expressed 2A MHC, half of which co-expressed type 1 MHC.

It has been suggested that switching of MHC expression between type 2 MHC isoforms results from communication between the genes of the type 2 MHC cluster which are adjacent to each other (Rinaldi et al., 2008). The gene encoding type I MHC is located on a different chromosome from the type 2 MHC cluster, in both rats and humans. Therefore, it would seem that co-expression of type 1 in hybrid fibers cannot be a result of communication between genes. However, past studies have demonstrated that unusual combinations of MHC isoforms, such as 1/2X and 1/2A/2X observed in this study, do occur (Stephenson, 2006). MicroRNAs, strands of ~22 nucleotides that regulate mRNA translation and degradation, have been implicated in muscle development and stress-induced changes, particularly in the α-cardiac and β-cardiac (type 1) MHC genes

(Van Rooij et al., 2008). These molecules may play a role in the expression of type 1 MHC in the reinnervated muscle.

With the marked decline in type 2X and type 2B proportions, it appeared that some of these pure fibers were in a state of transition to a different MHC profile, accounting for the rise in hybrid fiber percentages. The increase in type 2A pure fibers would seem to suggest that many 2X and 2B fibers have undergone the transition to the slower MHC isoform profile. Type 1 MHC is co-expressed in more hybrid fibers of the reinnervated EDL than the control EDL, implying that some of the type 2 fibers will eventually transition to type 1 fibers. It is also possible that these hybrid fibers are innervated by more than one axon, one of which may be stimulating the expression of the type 1 MHC gene. These data indicated that the reinnervated EDL may have switched to a more oxidative metabolism (Čebašek et al., 2007), and therefore a slower MHC profile at 21 days following sciatic nerve crush. Since oxidative metabolic staining was not used in this study, this presumption can only be made based on the fact that type 1 and 2A fibers rely more heavily upon oxidative metabolism than other muscle fiber types (Spangenburg and Booth, 2003, MacIntosh et al., 2006).

Changes in the MHC content of certain fibers in the reinnervated EDL may demonstrate that functionality as a fast twitch muscle has not been preserved. The typical transition sequence of MHC isoforms is from type 2B ↔ type 2X ↔ type 2A ↔ type I (Čebašek et al., 2007). However, this transition is incomplete since there was no significant change in the type 1 fiber population.

Many studies have established that innervation and the pattern of neuromotor impulses are necessary for maintenance of proper function in a muscle (Buller at al.,

1960, Germinario et al., 2002, Harridge, 2007). Fast motor neurons emit intermittent high-frequency bursts that up-regulate fast MHC genes whereas slow motor neurons emit prolonged electrical stimulation that up-regulate slow MHC genes (Harridge, 2007). The experiments of DeRuiter et al. (2008) showed that not all axons innervating the EDL fibers returned to their corresponding endoneurial sheaths on the other side of the nerve crush. This would imply that some fibers, formerly innervated by an axon transmitting electrical impulses at a specific frequency, were reinnervated by a different axon that emitted a different frequency and stimulated the expression of a different MHC isoform (Mendler et al., 2008).

It has been suggested that hyperinnervation is part of the reinnervation process particularly around three weeks following nerve crush (Magill et al 2007). If this was true in the case of the reinnervated EDL, it may offer an explanation for the co-expression of two or more MHC isoforms within one fiber since the axons may be firing at different nerve impulse frequencies. Hyperinnervation is transient however and disappears after reestablishment of a 1:1 muscle fiber: motor axon ratio (Magill et al., 2007, Brown et al., 1976).

Changes in SOL after Reinnervation

In contrast to the reinnervated EDL, the reinnervated SOL exhibited significant decreases in cross-sectional area across all fiber types present. As shown in Table 8, the reinnervated SOL fibers were approximately half the size of the fibers in the control SOL, which would suggest that the return of neural stimulation after nerve crush could not restore the fibers of the SOL to their original size by three weeks. The areas of the

fiber types in the reinnervated SOL are closer to those of the 0 day naïve group whereas the areas in the control SOL are typical of the 21 day naïve group. This showed that the acute denervation that immediately followed sciatic nerve crush had a marked atrophic effect on SOL muscle to the extent that the fiber sizes were comparable to the sizes of a three week younger group. Patterson et al. (2006) demonstrated that, as early as seven days after denervation, the weight of the SOL decreased by approximately 49%. In the present study, the weight of the reinnervated SOL was 31% smaller than the control SOL at 21 days after nerve crush which indicated that some recovery of fiber size had occurred after reinnervation.

Previous studies on the response of SOL to reinnervation have indicated that the type 1 fibers of both normal and reinnervated SOL are larger than the type 2 fibers (Mendler et al., 2008). The findings of the present study, which showed that the type 1 fibers were larger than other fibers within the same muscle in both control SOL and reinnervated SOL, were consistent with those observations.

As expected, the type 1 fiber proportion was the highest followed by a much smaller proportion of type 2A fibers and hybrid fibers throughout the three experimental groups (Soukup et al., 2002). This would seem to suggest that the metabolic profile of the reinnervated SOL was mainly oxidative based on the preponderance of a fiber type (type 1) which depends on oxidative metabolic processes for energy production (MacIntosh et al., 2006).

Type 1 and type 2A MHC isoforms were co-expressed in all of the hybrid fibers within the three experimental groups. These fibers were three times more numerous in the 21 day reinnervated group than in the 21 day naïve group. A possible explanation for the

unusually high proportion of hybrid fibers in both the control and reinnervated SOL muscles could be that the control SOL was compensating for the higher hybrid population in the contralateral side by also increasing its hybrid fiber population.

Despite these changes, the proportions of pure type 1 and type 2A fibers were comparable within both the 21 day naïve and the 21 day reinnervated groups. Mendler et al. (2008) have suggested that the SOL muscle transitions to a faster phenotype by three months following reinnervation, but this observation was accompanied by changes in the size and proportion of pure fiber types. The results of this study showed changing fiber sizes but stable proportions of pure fiber types in the reinnervated SOL. In addition, the hybrid fibers are similar in size to the type 2A fibers in both the 21 day naïve and the 21 day reinnervated groups so fiber size cannot be used as an indication of which fibers, type 1 or type 2A, are undergoing a transition. Therefore, the data do not necessarily negate the findings of Mendler et al. (2008) but indicate that the direction of the MHC transition, if any, is unclear. The presence of these hybrid fibers could simply be the result of hyperinnervation which was observed at three weeks following sciatic nerve crush but disappeared by week four (Brown et al., 1976, Magill et al., 2007).

Hyatt et al. (2006) demonstrated that slow muscles, which are involved in posture, are activated a greater number of times per day than fast muscles, and show a greater dependence on neural activity. This may provide an explanation for the findings of this study since repeated activation of the SOL created a steady stimulus for gene expression of the intrinsic MHC isoforms within fibers. Unlike the EDL, the SOL did not contain any pure type 2B or 2X fibers (Soukup et al., 2002). In addition, these faster MHC isoforms were not detected in any of the hybrid fibers. This may indicate that, despite the

overall atrophy of the fibers, the soleus maintained its MHC profile and slow-twitch contractility.

Comparison of the response of EDL and SOL to Reinnervation

The reinnervated EDL and SOL muscles both showed a significant decrease in weight compared with their control muscles which suggested that both muscles undergo atrophy after sciatic nerve crush. Reinnervation counteracted this atrophic effect in the fast-twitch EDL since the fiber sizes of the reinnervated EDL were similar to control values. However, as shown by the smaller sizes of reinnervated SOL fibers compared with control SOL fibers, reinnervation did not have the same effect on fiber size in the slow-twitch SOL after three weeks. This would seem to suggest that the slow-twitch SOL does not recover fiber size as quickly as the EDL following reinnervation.

In the reinnervated EDL, four pure fiber types and hybrid fibers were observed while only type 1, type 2A, and hybrid fibers were present in the reinnervated SOL. The hybrid fibers of the reinnervated EDL exhibited a greater variety of MHC isoform combinations than those of the reinnervated SOL in which only the type 1/2A MHC combination was detected.

The proportions of the type 2 fibers in the reinnervated EDL differed significantly from the control EDL. There was a marked decrease in proportion of the fastest fiber types (type 2B and type 2X) of the reinnervated EDL while type 2A and hybrid fibers increased in proportion. In contrast, the reinnervated SOL muscle maintained its normal fiber type proportions. Many of the SOL fibers may have been reinnervated by the original axons which stimulated expression of the MHC genes active in the fibers prior to

nerve crush. The type 1 fibers of the reinnervated EDL behaved similarly to the type 1 fibers of the reinnervated SOL since these also maintained normal proportions. This may suggest that reinnervation does not readily alter MHC gene expression of type 1 fibers as it does in type 2 fibers.

In the reinnervated EDL, all of the hybrid fibers co-expressed type 2A MHC and the majority of these also expressed type 1 MHC. These observations lead to the presumption that the reinnervated EDL had become a slower muscle since the hybrid fibers of the control EDL contained mostly type 2B and 2X MHC. It is also possible that many EDL fibers were not reinnervated by their original axons which affected the MHC isoform expressed in each fiber. In the SOL of the 21 day reinnervated group, all of the hybrid fibers co-expressed type 1 and type 2A MHC. Since these were the only two isoforms found in this muscle, and not the faster type 2B and type 2X isoforms, the SOL preserves its MHC profile as a slow-twitch muscle.

These findings imply that the original function of the fast-twitch EDL was not as well preserved following reinnervation as it was in the slow-twitch SOL. In hypothesis 1, it was postulated that similar changes in fiber size in both the EDL and SOL would be observed at 21 days following sciatic nerve crush. In hypothesis 2, it was postulated that similar changes in fiber type proportions would occur within these two muscles. Based on the results of this study, neither of these hypotheses can be accepted as stated. The results demonstrate that, over a three week period, the EDL and SOL muscles respond differently to nerve injury and reinnervation. In the EDL, reinnervation brought about changes in fiber type proportion but not fiber size, with an overall transition to a slower MHC profile. In the SOL, reinnervation caused atrophy of muscle fibers but not changes

in fiber type proportion which indicated that the SOL maintained its slow MHC profile and functionality as a slow-twitch muscle.

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Appendix 1

Immunohistochemistry

The Vectastain ABC system for immunoperoxidase staining (Vector Laboratories, Burlingame, CA) was used for visualization of the MHC isoforms. All primary antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA).

Reagents:

- 0.01M PBS (Phosphate Buffered Normal Saline)
    - sodium chloride 3.950 g
    - sodium phosphate monohydrate (monobasic) 0.325 g
    - sodium phosphate heptahydrate (dibasic) 2.013 g
    - distilled water → 500 g

Adjusted to pH 7.4 with 1N HCl and 1N NaOH

- 0.01M PBS (Phosphate Buffered Normal Saline) with Tween 20
    - 0.01M PBS (as described above) 500 g
    - Tween 20 0.5 ml

- 0.3% Hydrogen Peroxide (H_2O_2)
    - hydrogen peroxide 100 µl
    - 0.01M PBS → 1000 µl

- Blocking Serum
    - Normal Horse Serum (Vectatstain *Elite* ABC Kit) 150 µl
    - 0.01M PBS → 1000 µl

- Biotinylated Secondary Antibody
    - Normal Horse Serum (Vectatstain *Elite* ABC Kit) 15 µl
    - Biotinylated secondary antibody (Vectatstain *Elite* ABC Kit) 5 µl
    - 0.01M PBS → 1000 µl

- Vectastain Elite ABC Reagent
    - 0.01M PBS 2.5 ml
    - Reagent A (Vectatstain *Elite* ABC Kit) 1 drop
    - Reagent B (Vectatstain *Elite* ABC Kit) 1 drop

Reagent allowed to stand for 30 minutes

- Diaminobenzidine (DAB) Substrate (DAB Substrate Kit)
    - distilled water 2.5 ml
    - buffer stock solution 1 drop
    - DAB stock solution 2 drops
    - hydrogen peroxide 1 drop
    - Nickel solution 1 drop

Solution made just prior to use to prevent precipitation

Procedure:

1. Cryostat sections (10μm thick) were cut from the blocks of EDL and SOL muscles and collected on charged microscope slides.
2. Sections were surrounded with a PAP pen (Kiyota International, Inc., Elk Grove Village, IL) and allowed to dry for 45 minutes.
3. Sections were fixed in cold acetone for 10 minutes and washed in 0.01M PBS with Tween 20 for 5 minutes.
4. Section were then placed in 0.3% hydrogen peroxide for 5 minutes to eliminate endogenous peroxidase activity and washed in 0.01M PBS with Tween 20 for 5 minutes.
5. Sections were incubated in normal blocking serum for 20 minutes to reduce non-specific binding.
6. Excess blocking serum was blotted from the sections which were then incubated overnight at 4°C in primary mouse monoclonal antibodies against MHC isoforms (Developmental Studies Hybridoma Bank, Iowa City, IA). The four primary antibodies used were: A4.840 (MHC 1), 2F7 (MHC 2A), 10F5 (MHC 2B), and 6H1 (MHC 2X).
    a. All antibodies were used neat except 2F7 which was diluted to 1:50 with a solution of PBS/BSA and 0.1% sodium azide.
7. On the following day, sections were incubated in the biotinylated secondary antibody for 30 minutes and washed in 0.01M PBS with Tween 20 for 5 minutes.
8. The Vectatstain *Elite* ABC reagent was then added to the sections for 30 minutes, followed by a wash in 0.01M PBS with Tween 20 for 5 minutes.

9. Incubation in diaminobenzidine (DAB) substrate for 10 minutes to allowed development of the desired stain intensity, followed by a rinse in tap water for 1 minute.
10. Sections were then dehydrated for 1 minute in each of the following solutions: 70% alcohol, 95% alcohol, 95% alcohol, 100% alcohol, 100% alcohol.
11. After the sections were cleared in xylene, the slides were mounted in Permount (Fisher Scientific, Fair Lawn, NJ).

Appendix 2

Myosin ATPase Staining

The calcium method for ATPase staining used in this study was developed by Brooke and Kaiser (1970).

Reagents:

- 0.1M Sodium Barbital
    - barbital powder 5.15 g
    - distilled water → 250 ml

- 0.005M Sodium Barbital
    - 0.1M sodium barbital 5.0 ml
    - distilled water → 100 ml

- 0.18M Calcium Chloride
    - calcium chloride 2.65 g
    - distilled water → 100 ml

- 1% Calcium Chloride
    - calcium chloride 5 g
    - distilled water → 500 ml

- 2% Cobalt Chloride
  - cobalt chloride 4 g
  - distilled water → 200 ml

- Barbital Acetate
  - sodium barbital 1.47 g
  - sodium acetate 0.97 g
  - distilled water → 50 ml

- Pre-incubating Solutions – pH 4.2 and 4.6
  - barbital acetate 2.5 ml
  - 0.1N HCl, 5.0 ml
  - distilled water 2.0 ml

Adjusted to appropriate pH (4.2 or 4.6) with 1N HCl

- Pre-incubating Solutions – pH 10.2
  - 0.1M sodium barbital 2.0 ml
  - 0.18M calcium chloride 2.0 ml
  - distilled water 6.0 ml

Adjusted to pH 10.2 with 0.1N NaOH

- ATP Incubating Solution
  - ATP powder (Sigma-Aldrich Co., St. Louis, MO) 60 mg

- 0.1M sodium barbital 6.0 ml
- distilled water 21.0 ml
- 0.18M calcium chloride* 3.0 ml

**Added last to prevent precipitation; adjusted to pH 9.4 with 0.1N NaOH*

Procedure:

1. Cryostat sections (10μm thick) of EDL and SOL muscles were collected on coverslips and allowed to dry for 45 minutes.
2. Sections were incubated at room temperature for 5 minutes in pre-incubating solutions of pH 4.2 to inactivate type 2 fibers or 4.6 to inactivated type 2A fibers Pre-incubation in solution of pH 10.2 for 15 minutes inactivated type 1 fibers.
3. After the pre-incubation periods, sections were rinsed once with distilled water.
4. Sections were then incubated in the ATP solution for the following time periods: 25 minutes for pH 4.2 and 4.6, 15 minutes for pH 10.2. This allowed ATP hydrolysis and subsequent release of phosphate.
5. Three changes of 1% calcium chloride, for a total of ten minutes, were used to wash the sections, forming calcium phosphate.
6. One change of cobalt chloride for ten minutes was used to displace the calcium, forming cobalt phosphate.
7. Sections were then washed with 5 changes of 0.005M sodium barbital, 5 changes of distilled water, and placed in 2% ammonium sulfide for 30 seconds to produce black cobalt sulfide.

8. After this, the sections were rinsed 10 times with tap water and dehydrated for 1 minute in each of the following solutions: 70% alcohol, 95% alcohol, 95% alcohol, 100% alcohol, 100% alcohol.
9. They were then cleared in xylene and mounted with Canada Balsam (Carolina Biological Supply Company, Burlington, NC).

Appendix 3

Hematoxylin and Eosin (H&E) Staining

Reagents:

- Harris Modified Hematoxylin (Fisher Scientific, Pittsburgh, PA)
- Eosin Yellowish (Fisher Scientific, Pittsburgh, PA)

Procedure:

1. Cryostat sections (10μm thick) of EDL and SOL muscles were collected on coverslips and allowed to dry for 45 minutes.
2. Sections were placed in Harris Modified Hematoxylin for 5 minutes, then rinsed in tap water.
3. Sections were placed in Eosin Yellowish solution for 5 minutes, and rinsed again in tap water.
4. After this, the sections were dehydrated for 1 minute in each of the following solutions: 70% alcohol, 95% alcohol, 95% alcohol, 100% alcohol, 100% alcohol.
5. Sections were then cleared in xylene and mounted with Canada Balsam (Carolina Biological Supply Company, Burlington, NC).