IMMUNOLOCALIZATION OF DYSTROPHIN AND NEUROFILAMENT PROTEIN IN MUSCLE SPINDLES OF NORMAL, MDX-DYSTROPHIC, AND DENERVATED MICE

By

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THESIS ABSTRACT

Dystrophin is a high molecular weight protein localized under the sarcolemma of normal extrafusal muscle fibers but absent in skeletal muscles of Duchenne muscular dystrophy patients and in the mdx mouse. Muscle spindles in the soleus of 32-week-old normal and age-matched mdx mice were examined by immunocytochemical methods to determine the localization of dystrophin in polar and equatorial regions of the intrafusal fibers. Spindles were serially-sectioned in transverse and longitudinal planes, and they were double-labelled with an antibody to dystrophin and with a 200 kD neurofilament protein antibody which revealed their sensory innervation. By fluorescence microscopy, intrafusal fibers in the soleus of mdx mice were deficient in dystrophin throughout their lengths, whereas their sensory nerve-terminals stained intensely with the nerve-specific antibody and appeared unaltered in dystrophy. In the normal soleus, polar regions of bag and chain fibers exhibited a peripheral rim of sarcolemmal staining equivalent to that seen in the neighboring extrafusal fibers. Dystrophin labelling in equatorial regions of normal intrafusal fibers, however, showed dystrophin-deficient segments alternating in a spiral fashion with positive-staining domains along the sarcolemma. Double-labelling for dystrophin and neurofilament protein showed that these dystrophin-deficient sites were subjacent to the annulospiral sensory-nerve wrappings terminating on the intrafusal fibers. Additionally, it was found that chronic denervation of muscle spindles in normal mice did not affect the expression of dystrophin either at these sites or at the non-sensory regions of the sarcolemma. The results of this study suggest that dystrophin is not an integral part of the subsynaptic sensory-membrane in equatorial regions of normal intrafusal fibers, and, that the neurotrophic effect of sensory innervation is not the principle cause of this unique arrangement of dystrophin in equatorial regions. In dystrophy, intrafusal fibers display the same primary defect in muscular dystrophy as seen in the extrafusal fibers. However, because of their small-diameters, capsular investment, and relatively low tension outputs, dystrophic intrafusal fibers may be less prone to the sarcolemmal membrane disruption that is characteristic of extrafusal fibers in this disorder.

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INTRODUCTION

A. Muscular Dystrophy

Muscular dystrophy is a term used to describe the heterogeneous group of genetically-linked disorders that cause progressive weakness and wasting of the skeletal muscles. The most common and devastating of these disorders, Duchenne muscular dystrophy, was first described in the mid-1800's by Meryon (1852) and later by Duchenne (1868). The incidence of this disease in North America has been estimated to be one in 3500 boys, and in approximately one-third of cases the disease is caused by a mutation in a gene located on the X chromosome. In the remaining two-thirds of cases the defective gene is inherited on the X chromosome from a carrier mother (Worton, 1992).

This genetic disorder exhibits no obvious clinical manifestation until the age of three to five years, when proximal muscle weakness is first observed. The ensuing progressive loss of muscle strength usually leaves affected individuals wheelchair-bound by the age of 11, and results in early death due to respiratory failure. To date there is no cure and no effective treatment although prednisone, a catabolic steroid, has, paradoxically, been shown to stabilize muscle strength for a period of up to three years (Brooke, 1989).

The Becker type of muscular dystrophy (Becker and Kiener, 1955) is a milder form of the disease with an incidence rate of about one-tenth of that of the severe Duchenne form (Becker, 1964). It is characterized by a much later onset with loss of ambulation after the age of 16 years and a relatively normal lifespan (Becker, 1965). Although thought for years to be a distinct disease, it is now recognized that it is caused by mutations in the same gene, and therefore a milder version of the same disease (Baumbach et al., 1989; Koenig et al., 1989). Boys who lose ambulation between the ages of 12 and 16 years are said to have an intermediate phenotype.

The basis of this disorder stemmed from molecular genetic studies using a strategy based on identification of sequences that mapped into a region of the X chromosome. This region had previously been shown by Kunkel and colleagues (1985) to be deleted in a boy with several X-linked disorders, including Duchenne muscular dystrophy. The identification of a clone from this part of the X chromosome that detected deletions in 6-7% of Duchenne patients suggested that this was the site of the Duchenne gene. Conserved sequences from this region of the X chromosome were used to identify cDNA (complementary DNA) clones made by reverse-transcribing mRNA (messenger RNA). The sequences encoded in the cDNA were shown to be deleted in a number of boys with Duchenne muscular dystrophy. This provided evidence that the isolated cDNA clones were indeed from the gene that is mutated in muscular dystrophy. The gene, known as the Duchenne muscular dystrophy gene, was shown to encode a protein that Hoffman and Kunkel's laboratory (Hoffman et al., 1987) named dystrophin, and as expected, this protein was missing from the skeletal muscle tissue of boys with Duchenne muscular dystrophy (Monaco et al., 1988).

The mdx mutant of the C57BL/10SnJ strain of genetically dystrophic mouse has recently been introduced as an animal model applicable to the study of X-linked human muscular dystrophies (Bulfield et al., 1984). In several of the earliest pathological studies of the mdx mouse, it was recognized that some of the histopathological features resembled quite closely those seen in Duchenne muscular dystrophy (Bulfield et al., 1984; Anderson et al., 1987, 1988; Carnwath and Shotton, 1987; Torres et al., 1987; Coulton et al., 1988) while others did not fit at all well. More importantly, however, was the fact that the same biochemical defect was shown to be present in mdx skeletal muscle, in that it lacked the protein dystrophin (Hoffman et al., 1987). Analysis of the dystrophin gene in the mdx mouse revealed that the genetic defect was a point mutation involving a single base change within an exon (Sicinski et al., 1989). This feature of the mdx mouse made it a reliable model for

characterizing the primary pathological processes linking the absence of dystrophin to necrosis in skeletal muscle fibers.

Proposed Structure and Function of Dystrophin

Once the cDNA had been isolated and sequenced, the deduced amino acid sequence of dystrophin was compared to those sequences already present in protein databases. By comparison with such known protein sequences it was found that certain domains of the protein were very similar to cytoskeletal proteins such as alpha-actinin and spectrin, suggesting that dystrophin is itself a cytoskeletal protein (Koenig et al., 1988). The amino acid sequence predicted a rod-shape protein approximately 150 nm in length that could be divided into four different domains: an N-terminal "actin-binding" domain (Hammond, 1987), a middle domain formed by 25 triple helical segments, a cystein-rich domain, and a less characterized C-terminal domain (Koenig et al., 1988). The first three domains show significant similarity to the three domains of alpha-actinin whereas amino acid sequences of spectrin are similar to the succession of 25 triple-helical segments in the second domain. The C-terminal of dystrophin, however, does not show any similarities to other known proteins but is thought to be the domain that mediates the attachment to an integral membrane glycoprotein in the sarcolemma (Campbell and Kahl, 1989).

Dystrophin has been shown to be associated with a complex of sarcolemmal glycoproteins that are believed to provide a linkage to the extracellular matrix protein, laminin (Campbell and Kahl, 1989; Ervasti et al., 1990; Ohlendieck and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992). The absence of dystrophin leads to a dramatic reduction in these dystrophin-associated glycoproteins in the sarcolemma of patients with Duchenne muscular dystrophy and mdx mice (Ervasti et al., 1990; Ohlendieck and Campbell, 1991). More recently, it has been demonstrated that a dystrophin-related protein named utrophin

(Matsumura et al., 1992; Tinsley et al., 1992), an autosomal homologue of dystrophin, is associated with an identical or antigenically similar complex of sarcolemmal proteins in skeletal and cardiac muscle of both normal and mdx mice.

Synthesis of an Antibody to Dystrophin

Additional information about dystrophin came from the synthesis of fusion proteins in bacteria from chimeric genes in which a portion of the dystrophin cDNA was joined with a bacterial gene (Hoffman et al., 1987). The resulting fusion protein was injected into rabbits or sheep to make an antibody against the dystrophin part of the protein. Alternatively, synthetic peptides of approximately 13 amino acids were created utilizing the sequence information, and these peptides were conjugated to larger molecules and injected into animals, again with the purpose of generating antibodies. The antibodies directed against dystrophin then became important molecular tools for the analysis of dystrophin structure and function. In particular, Western blot analysis of muscle and other tissues demonstrated that the antibodies recognized a protein of 427 kiloDalton (kD) molecular weight. The protein was present in skeletal muscle, cardiac muscle, smooth muscle, and brain of normal patients and absent in those tissues of patients with Duchenne muscular dystrophy (Hoffman et al., 1987).

Using these antibodies, immunocytochemical studies of dystrophin on tissue sections revealed that, in skeletal muscle, dystrophin is localized at the sarcolemmal membrane of extrafusal muscle fibers (Hoffman et al., 1987; Zubrzycka-Gaarn et al., 1988). Transmission electron microscopic studies coupled with immunogold labelling techniques have further shown that this protein is situated on the cytoplasmic side of the sarcolemma (Watkins et al., 1988; Carpenter et al., 1990; Cullen et al., 1990; Squarzoni et al., 1992). Observations from these

studies suggested that the dystrophin molecules are linked in a form of a lattice immediately beneath the plasma membrane.

Immunocytochemical studies have demonstrated elevated levels of dystrophin at the motor end-plate regions of extrafusal fibers in normal muscle (Miike et al., 1989; Shimuzu et al., 1989; Byers et al., 1991; Huard et al., 1991, 1992). In the study by Shimuzu and coworkers (1989), the accumulation of dystrophin at the neuromuscular junction was confirmed by revealing acetylcholinesterase activity by light microscopy, whereas Huard's group (1991) used alpha-bungarotoxin coupled to cascade blue to identify cholinergic receptors in skeletal muscle. Their observations emphasize a relationship between dystrophin expression and the site of innervation of skeletal muscle fibers. Additionally, studies of the central nervous system (Lidov et al., 1990) have revealed detectable amounts of dystrophin in postsynaptic regions of cerebral cortical neurons and cerebellar Purkinje cells, suggesting a possible role for dystrophin in synaptic transmission.

Recent work on dystrophin localization in smooth muscle has provided evidence that dystrophin is associated with specific regions of the smooth muscle sarcolemma (North et al., 1993). By immunofluorescence and immunoelectron microscopy, it was shown that two structurally distinct smooth muscle sarcolemmal domains exist in an alternating pattern parallel to the long axis of the smooth muscle cell. Dense plaque regions, known to be involved in actin anchorage and characterized by a specific set of junctional proteins (Geiger and Ginsborg, 1991), typically expressed a marked diminution of dystrophin whereas the intervening caveolae-rich areas of the sarcolemma showed relatively normal levels of sarcolemmal labelling. This mutually exclusive colocalization of specific membrane-associated proteins in smooth muscle suggests that dystrophin complements other cytoskeletal proteins that function in maintaining the integrity of the smooth muscle cell and its sarcolemma.

Since the discovery of the DMD gene, the literature on dystrophin localization in the extrafusal fibers of skeletal muscle has escalated at a rapid rate. In spite of this, however,

very little has been published on the localization of dystrophin in the specialized intrafusal muscle fibers of muscle spindles. The following section will review some of the characteristic features of the mammalian muscle spindle with the aim of providing an appreciation for its highly complex nature.

B. The Mammalian Muscle Spindle

Muscle spindles are specialized sensory stretch-receptors embedded within most vertebrate skeletal muscles. They were first described by Hassall (1849) and later by Kühne (1864) who gave them the name *Muskelspindeln* in describing their spindle shape. It was Cajal (1888) who recognized this structure as a sense organ and also suggested the existence of a specific motor innervation. Its sensory nature was elegantly confirmed by Sherrington (1894), and the first detailed studies of the anatomy of mammalian muscle spindles by Ruffini (1898) have provided the basis of our present understanding of this receptor.

The mammalian muscle spindle consists of discrete bundles of encapsulated skeletal muscle fibers, termed intrafusal fibers, that stand out by the comparative smallness of their size to the surrounding extrafusal fibers. These small-diameter muscle fibers are associated with sensory and motor nerve endings that terminate on specific regions along their lengths. Lying in close proximity, nerve axon bundles, extracapsular capillaries, and mast cells are also often encountered.

The microscopic appearance of these receptors can be described in terms of the level of section through each receptor (Sherrington, 1894). In a series of cross-sections, three regions or zones can be identified according to the overall diameter of the receptor and extent of capsular investment. The equatorial zone represents the central or widest portion of the receptor. In this region, both an inner and an outer spindle capsule are present, separated by a large fluid-filled periaxial space. The so-called polar regions represent the two

tapered ends of the spindle. In these regions, only the outer capsule persists and in some cases the intrafusal fibers extend beyond the capsular limits and either terminate in the endomysium of neighboring extrafusal fibers or insert into tendon. Midway between these two regions, the so-called juxtaequatorial zone is present.

Muscle spindles can be described as consisting of four basic elements: A capsule, intrafusal fibers, and their sensory and motor nerves. Each of these will be addressed in the following paragraphs.

Capsule

The muscle spindle capsule is composed of a multilayered outer capsule and a thin inner capsule that differ from each other both in structure and location (Merrillees, 1960; Landon, 1966; Corvaja et al., 1969; Ovalle and Dow, 1983). The outer capsule consists of several concentric layers of flattened epithelial-like cells and connective tissue elements. It is continuous with both the extrafusal and the intrafusal endomysium as well as with the perineurium which surrounds the small bundles of nerve fibers supplying the receptor organs. A dilatation of the capsule is generally found at the central, equatorial region of the spindle giving the receptor its characteristic fusiform shape (Kühne, 1864).

The inner capsule or axial sheath (Barker, 1974) is most prominent in the equatorial region where it may form an elaborate sheath around individual intrafusal fibers, or groups of fibers and their sensory nerve endings. It consists of flattened cells that resemble the endoneurial cells of peripheral nerve and unlike the outer capsule cells, cells of the inner capsule lack an external lamina although collagen and elastic fibrils are known to occupy the paracellular regions (Cooper and Gladden, 1974).

In the equatorial region, a space is present between the outer and inner capsules. This so-called periaxial space contains a jelly-like material rich in acid mucopolysaccharides and hyaluronic acid (Brzezinski, 1961a, b; James, 1971; Fukami, 1982, 1986; Ovalle and Dow, 1988). This substance bears some resemblance to vitreous humor and it is thought to insulate as well as mechanically protect and lubricate the internal components of the receptor in equatorial regions (Barker and Banks, 1986).

It should also be mentioned that the outer capsule presents a significant barrier to the passage of the exogenous protein tracer, horseradish peroxidase, and bears some resemblance to the permeability barrier formed by the perineurium of peripheral nerves (Dow et al., 1980). The horseradish peroxidase tracer was found to freely enter the spindle at its polar regions, but in the equatorial zone, the outer capsule cells were effective in preventing the indiscriminate penetration of this protein tracer into the periaxial space. Thus, a blood-muscle spindle barrier appears to exist in central equatorial zones, but not in more distal polar regions.

Intrafusal Fibers

The specialized skeletal muscle fibers in a muscle spindle are unique in that they vary morphologically not only from the surrounding extrafusal fibers but also show variations amongst themselves. Two distinctly different intrafusal fiber types can be identified on the basis of their appearance and according to the arrangement of nuclei in their central equatorial regions (Barker, 1948; Boyd, 1962; Cooper and Daniel, 1963). The nuclear bag fibers derive their name from the dilatation produced by the numerous tightly-packed, vesicular nuclei in the equatorial region. The nuclei are surrounded by a thin peripheral layer of sarcoplasm containing few myofibrils. At either end of this central portion there are two juxtaequatorial regions in which the nuclei continue as a single central row surrounded by

sarcoplasm and a peripheral layer of contractile material. These fibers have the widest diameter of the two, and are also the longest, often extending beyond the capsular investment of the spindle. Nuclear chain (chain) fibers, in contrast, contain in their equatorial region a more conspicuous amount of contractile material which surrounds a single, central row of elongated nuclei. They are smaller in diameter and shorter in length, often ending within the capsule. There are, however, some that extend beyond the limits of the capsule. These are termed *long chain* fibers and were first described by Harker and coworkers (1977). In polar regions of both types of fibers the nuclei assume a peripheral location underneath the sarcolemma and resemble more closely the architecture of extrafusal muscle fibers.

It is now known that the nuclear bag fibers are themselves of two types, which differ in their histochemistry, ultrastructure, and mechanical properties. They are designated as nuclear bag_1 (bag_1) and nuclear bag_2 (bag_2) fibers (Ovalle and Smith, 1972) according to their histochemical staining properties. Another important difference between bag_1 and bag_2 fibers is that the latter are surrounded by prominent elastic fibers in their polar regions whereas the former are not (Gladden, 1976). In terms of their differing mechanical properties, the nuclear bag fibers have been described as either dynamic nuclear bag (bag_1) or static nuclear bag fibers (bag_2) (Boyd et al., 1975).

The usual methods by which the three mammalian intrafusal fiber types are identified in light microscopy are the histochemical reactions for actomyosin adenosine triphosphatase (mATPase) and nicotinamide adenine dinucleotide-tetrazolamine reductase (NADH-TR). The differential staining observed with these reactions in bag_1, bag_2, and chain fibers suggests the presence in each of a different myosin profile and energy requirements. The bag_1 fiber exhibits low mATPase activity following alkaline preincubation and has a relatively low glycogen content. In contrast, the bag_2 fiber exhibits medium to high ATPase activity and has a medium

glycogen content. The chain fibers exhibit high ATPase activity and have a high glycogen content (Banks et al., 1977).

Recent immunofluorescence work with newly developed monoclonal antibodies has proved to be a more elaborate and comprehensive technique in understanding the myosin profiles (isoforms) that constitute each intrafusal fiber type. Studies of spindle fibers in capsular regions have revealed that antibodies specific for fast fiber myosins label predominantly the chain fibers and those specific for slow fiber myosins show a strong reaction in bag fibers (Rowlerson et al., 1985). Tonic myosin antibodies were shown to label bag_1 fibers, and when used at higher concentrations exhibited a weak reaction with chain fibers. In some occasions it was noted that this antibody reacted with bag_2 fibers. Antibodies raised against embryonic and neonatal myosins (Sartore et al., 1982) do not bind to any adult extrafusal fibers and have little reaction with bag_1 fibers. However, this antibody reacts very intensely with chain fibers and moderately with bag_2 fibers. Therefore, chain fibers appear to contain a mixture of either embryonic, neonatal and fast fiber myosins, or a myosin isoform that is recognized by both types of antibodies.

Kucera and Walro (1989) have demonstrated that regional differences occur in the myosin heavy chain expression in the intrafusal fibers. In capsular regions of intrafusal fibers, myosin heavy chains are significantly decreased whereas extracapsular regions exhibit a strong reaction similar to that of the type I (slow) extrafusal fibers. Moreover, specific regional differences were noted between the three intrafusal fiber types. The high correlation between the various antibody-specific myosin isoforms and the contractile and electrophysiological properties of intrafusal fibers have demonstrated that immunocytochemical techniques are reliable in the typing of intrafusal fibers.

In other immunocytochemical studies, the expression of certain structural muscle fiber proteins was shown to be distributed in a non-uniform arrangement in the different regions of the intrafusal fibers. Maier and Zak (1990) showed that actin, tropomyosin, desmin, and

myosin heavy chains take on a striated appearance in polar regions, while the equator appears non-striated. These observed immunofluorescent patterns suggest that, at the equator, these sarcoplasmic proteins are assembled into looser arrays than in the sarcomeres of the pole. This supports the concept that the equatorial zones are structurally more flexible, an appropriate substrate for distorting the affixed sensory endings during an applied stretch.

Innervation

Several histological methods are commonly used to study motor and sensory innervation of mammalian muscle spindles (Barker and Ip, 1963; Gladden, 1970; Namba et al., 1967; Nahirney and Ovalle, 1992a, b, 1993). Some of these procedures are normally performed on muscle pieces by block impregnation with silver salt solutions followed by teasing apart the impregnated tissue, while others are perfomed on histological sections.

The most commonly used procedure to study innervation patterns is the silver impregnation technique (Barker and Ip, 1963) which enables the innervation pattern of entire muscle spindles to be visualized more or less completely.

There are, however, other techniques that allow for the identification of nerve fibers in skeletal muscle. Roden and coworkers (1991) have shown by immunocytochemistry that the 200 kD neurofilament protein, a component of the intermediate filaments found in neuronal tissue, is expressed at high concentrations in both the axons and terminal endings (motor end-plates) of nerves. This immunocytochemical staining method may be useful in studies of muscle innervation since it can be used in conjunction with other antibody labelling procedures (Nahirney and Ovalle, 1992a, b, 1993).

The intrafusal fibers of muscle spindles are separately innervated by motor and sensory nerves which terminate on different regions along these fibers (Ruffini, 1898). While large myelinated sensory nerve fibers pass through the capsular sleeve to enter the equatorial space, the motor supply may enter the spindle either with afferent nerve fibers or independently through the polar regions.

The sensory innervation of mammalian muscle spindles comprises both primary and secondary nerve endings, the latter being less abundant or in some cases absent. The primary sensory endings consist of spiral or annular terminations, each of which encircles the densely nucleated equatorial regions of the intrafusal fibers (Ruffini, 1898). These endings can be found on each of the three intrafusal fiber types and all are connected to the same group Ia afferent nerve fiber leaving the spindle. The group Ia afferent nerve fibers can range in diameter from 7 μm to 15 μm. In the cat, the primary endings cover 33-37 % of the bag_1 fiber surface, 25% of the bag_2 fiber surface, and 5-12 % of the surface of individual chain fibers (Banks et al., 1982; Banks, 1986). Boyd (1962) has shown that the region of the intrafusal fibers surrounded by the primary endings in cat muscle spindles extends for about 300 μm in the equatorial zone.

Secondary endings can be found adjacent to the primary endings in the juxtaequatorial regions of the muscle spindle. They consist of spiral terminations around each nuclear chain fiber and less extensive flower-spray terminations on the nuclear bag fibers (Boyd and Smith, 1984). The spiral and spray terminations are connected to the same group II afferent axon, averaging 8 μm in diameter. Some spindles have been shown to have no secondary endings at all, whereas the most complex can have four or five such endings (Boyd and Smith, 1984). The most common arrangement is for a spindle to have one primary ending and one secondary ending (Boyd and Smith, 1984). In the cat tenuissimus, the total terminal contact area of secondary endings has been estimated to be 16-22% on individual chain fibers, 17% on bag_2 fibers, and 8% on bag_1 fibers (Banks et al., 1982).

The ultrastructure of both types of sensory endings is essentially identical (Adal, 1969; Landon, 1972). Both contain numerous mitochondria, neurofilaments, microtubules, and axoplasmic vesicles. The endings lie in close apposition to the sarcolemma of the intrafusal fiber and, in the case of the primary endings, sit in troughs indented on the surface of the intrafusal fiber (Landon, 1966). In both types of ending, the sensory terminals are separated from the sarcolemma of the adjacent muscle fiber only by the plasma membrane of the nerve terminal itself. That is, the external lamina of intrafusal fibers does not extend through the gap between the two plasma membranes of nerve and muscle, but continues over the surface of the sensory endings (Merrillees, 1960; Landon, 1966; Corvaja et al., 1969; Hennig, 1969). An intercellular gap of about 15-20 nm separates the adjacent cell membranes of the intrafusal fiber and sensory ending (Corvaja et al., 1969) and, in some primary endings, the presence of finger-like prolongations which penetrate from the internal surface of the primary sensory ending into the depth of the intrafusal fiber have been observed (Corvaja et al., 1969). Additionally, Schwann cells are not usually present on the sensory terminals (Merrillees, 1960), a feature that distinguishes sensory endings from motor terminals.

The complex nature of motor innervation to muscle spindles has been a topic of interest and one of controversy since it was first described by Cajal in 1888. Since a single spindle receives from eight to twenty-five branches of sensory or motor axons it has taken many years for the patterns of innervation to be elucidated. To date, many detailed reviews of the motor innervation to spindles have been published (Boyd, 1981; Boyd and Smith, 1984; Barker and Banks, 1986).

The motor endings are distributed in the juxtaequatorial and polar regions of mammalian intrafusal fibers. In general, two forms of motor innervation to intrafusal fibers have been described. The first type arises from gamma efferents, a group of axons with smaller diameters and slower conduction velocities than those of alpha efferents which supply the extrafusal fibers (Leksell, 1945). These nerve axons innervate all types of intrafusal

fibers and are sometimes referred to as *fusimotor* axons (Barker, 1974). They have been estimated to account for 30% of the nerve fibers in the ventral roots (Boyd and Smith, 1984). The second type are termed beta efferents or *skeleto-fusimotor* axons (Barker, 1974). They commonly innervate both extrafusal and intrafusal fibers by way of collateral branches. These nerve fibers are generally low in number and, when seen, are located in the distal polar regions of intrafusal fibers (Barker, 1974).

Both the gamma and beta efferents have been subdivided into static and dynamic subgroups based upon the intrafusal fibers they innervate and how they influence the response of the primary and secondary endings (Matthews, 1962; Boyd et al., 1975; Boyd, 1981). Generally speaking, gamma-static axons can innervate bag_2 fibers, chain fibers, or both. Motor axons supplying bag_1 fibers are often of the gamma-dynamic and beta-dynamic variety. The beta-static axons are usually restricted to the extracapsular regions of long chain fibers (Kucera, 1980).

The gamma axons exhibit two types of terminal endings. The gamma-static axons that terminate on either bag_2 or chain fibers or both are called "trail endings" (Barker et al., 1970). They are typically found in the juxtaequatorial regions of these fibers and almost all lie superficially on the muscle fiber with little or no postsynaptic folding. The other terminal endings that are usually found in the midpolar zone correspond to the gamma-dynamic axons and are termed "$plate_2$ endings" or "P_2 plates" (Barker et al., 1970). These axon terminals are nearly all clearly indented into the surface of the muscle fiber, however, little or no folding of the postsynaptic membrane is evident. "$Plate_1$ endings" or "P_1 plates" are thought to be the terminations of beta axons (Barker et al., 1970). They are smaller than P_2 plates, and, in fact, resemble the plates of alpha motor axons on extrafusal fibers, however, show a lesser degree of postsynaptic membrane folding. Beta-dynamic and beta-static axon endings have been shown to be found on bag_1 and long chain fibers respectively, the majority of which are present in the extracapsular regions (Kucera, 1980).

Thus, it is evident that the complex innervation, both sensory and motor, of intrafusal fibers is in contrast to the innervation of extrafusal fibers which, as a rule, are innervated by only one motoneuron, have a single motor end plate, and are not innervated by sensory nerves.

Pathology

Despite considerable progress in the basic knowledge of muscle spindles there is still no disease that can be directly attributed to defective or abnormal muscle spindle function. In most diseases, pathological changes in spindles appear to be nonspecific and do not parallel those in the surrounding extrafusal fibers (Lapresle and Milhaud, 1964; Patel et al., 1968). Nevertheless, numerous pathological changes have been recorded and, for the most part, have been consequences of either denervation experiments or neuropathies.

Studies of spindle morphology after denervation have been undertaken by Onanoff (1890), Tower (1932, 1939), Boyd (1962), Barker et al. (1970), and Schröder (1974a, b). Tower's studies (1932, 1939) revealed an increased thickness of the spindle capsules and of the fibrous tissue in the periaxial space after sensorimotor denervation and after ventral root section of prolonged duration, but not after sensory root section or dorsal ganglionectomy. In man, sensorimotor denervation, whether due to mononeuropathy, diabetes mellitus, or drug-induced neuropathy, results in striking capsular thickening consisting of increased numbers of lamellae of perineurial capsular cells and increased amounts of collagen (De Reuck, 1974; Swash and Fox, 1974). In sensory denervation due to tabes dorsalis, or to carcinomatous sensory neuropathy (Croft et al., 1965) deposition of collagen in the periaxial space is prominent only in the equatorial and juxta-equatorial regions, whereas in motor denervation, capsular thickening is marked only in polar regions (Swash and Fox, 1974).

In both sensory and motor denervation, it has been reported that the nuclear chain fibers undergo earlier and more severe degenerative changes than the nuclear bag fibers (Swash and Fox, 1974) and, after a year or more, all the intrafusal muscle fibers show degenerative changes, becoming clumped together in a poorly defined mass in the center of the periaxial space. After prolonged motor denervation, however, polar regions show similar atrophy of the intrafusal fibers, whereas equatorial regions show normal primary and secondary sensory endings on nuclear bag fibers of only slightly reduced diameter (Swash and Fox, 1974).

Morphological studies of muscle spindles in patients with Duchenne muscular dystrophy (Lapresle and Milhaud, 1964; Cazzato and Walton, 1968) have shown swelling of periaxial spaces of spindle capsules, and degeneration of intrafusal fibers. In a quantitative study of 230 muscle spindles by Swash and Fox (1976), the capsular thickness found in Duchenne dystrophy was not as great as that found in denervated spindles and only a slight decrease in diameter and in number of intrafusal muscle fibers was observed. However, they noted that in some spindles the intrafusal fibers consisted of a degenerate mass of centrally placed amorphous material, especially in areas of marked extrafusal fiber degeneration and fibrotic tissue accumulation whereas in regions of relatively well-preserved extrafusal fibers, the muscle spindles were usually less abnormal. In silver-impregnated longitudinal sections, the pattern of motor and sensory innervation appeared normal (Swash and Fox, 1976). These abnormalities described in spindles in Duchenne muscular dystrophy have been proposed to contribute to the relatively early loss of tendon reflexes often found in this condition (Swash and Fox, 1976).

C. Immunofluorescence Microscopy

Immunofluorescence is a special field of fluorescence microscopy that involves the detection of antigens by way of immune reactions. The original technique was developed by Coons and Kaplan (1950), and modifications of this technique are currently the most widely used microscopic fluorescence method in research. Originally restricted to fluorescein isothiocyanate (FITC), immunolocalization techniques now utilize other, newly developed fluorochromes such as rhodamine B 200, tetramethyl-rhodamine-isothiocyanate (TRITC), and Texas Red.

The basic principle of immunofluorescence is based on the reactions of specific antigens to specific antibodies. Antibodies are submicroscopic protein structures that attach themselves to specific recognition sites on the surface of antigens. They are proteins of the globulin group (immunoglobulins) that appear in plasma and tissue fluids after antigen injection to the host organism. The immunoglobulins that are produced by the host react specifically and bind strongly to the antigen.

Immunofluorescence is the coupling of immunoglobulins to substances that fluoresce (fluorochromes) rendering them visible in the microscope without causing loss of the antibody's biologic activity. Two basic methods are used for antigen localization in immunofluorescence (Coons and Kaplan, 1950). The direct method is a simple detection system that uses a solution containing a prelabelled antibody (i.e. conjugated to a fluorochrome) on sections of tissue. The excess antibody is washed off, and the tissue is observed under the fluorescence microscope. The second method is known as the indirect or "sandwich" method. To avoid the preparation and storing of specifically labelled antibodies for each antigen, the indirect method utilizes anti-antibodies that are produced in an intermediate host of a different species. These anti-antibodies, or so-called secondary antibodies, will react with every original (primary) antibody complex as if it were an antigen. The secondary antibody can be visualized by directly attaching a fluorochrome, or by

conjugating it to biotin which is then linked to a fluorochrome via the bacterial protein streptavidin. The biotin-streptavidin system offers the advantage of an amplification step and is therefore useful in the detection of antigens at low concentrations in tissues (Hsu et al., 1981a, b).

Until recently the use of transmitted-light fluorescence microscopy was the only method of observing fluorochromes. The few available exciter filters with their wide transmittance ranges did not allow the use of specific fluorescence techniques and was not a very reliable method for routine diagnosis. However, the situation has changed with the increasing impact of the fluorochrome labelling techniques. Epifluorescence microscopy, a system that uses incident-light excitation, has essentially replaced transmitted-light darkfield illumination. In this type of microscope, illumination and observation can be made from the same direction.

In summary, the use of immunofluorescence can reveal microscopic details of specific antigen localization not obtainable by other histochemical methodologies. It is a powerful technique that can provide a better understanding of the organization of specific components such as proteins within tissues. Furthermore, simultaneous localization of two specific antigens (double-labelling) using two monospecific antibodies and two different colored fluorochromes is possible and is an effective way to study the relationship of one antigen to another (Vandesande, 1983). In this respect, it would be advantageous to apply this method to the study of the localization of specific proteins in skeletal muscle and muscle spindles.

D. Thesis Objectives

Although there is extensive literature on dystrophin in normal and dystrophic extrafusal muscle fibers (Bonilla et al., 1988; Hoffman et al., 1988; Carpenter et al., 1990; Cullen et al., 1990; Byers et al., 1991), detailed information concerning the localization of dystrophin in either normal or diseased intrafusal muscle fibers of muscle spindles is lacking. To more clearly understand the role this protein plays in skeletal muscle it is necessary to determine its precise localization in the different types of muscle fibers that constitute a skeletal muscle. The intrafusal fibers of muscle spindles present a unique array of characteristics that differentiate them in both structure and function from the larger and more numerous extrafusal fibers.

Therefore, the present investigation was undertaken with the following aims:

(1) To determine the localization of dystrophin along the lengths of intrafusal fibers in normal and mdx-dystrophic skeletal muscle.

(2) In view of the elevated levels of dystrophin reported to occur at extrafusal fiber motor-end-plates of normal skeletal muscle, it was of interest to determine the relationship between sensory nerve innervation and dystrophin expression in the intrafusal fibers.

(3) To determine the effects of chronic denervation on dystrophin expression in skeletal muscles and muscle spindles of normal mice.

The following hypotheses were tested in this study. First of all, that intrafusal fibers of mdx-dystrophic mice exhibit a similar deficiency of dystrophin as that seen in the extrafusal fiber population. Secondly, in normal skeletal muscle, the sensory innervation of intrafusal fibers plays a role in modulating the subsarcolemmal expression of dystrophin. And thirdly, chronic denervation of normal skeletal muscle does not affect the expression of

dystrophin at synaptic and non-synaptic sarcolemmal membranes of extrafusal and intrafusal muscle fibers.

This study will attempt to bring together the current knowledge of dystrophin localization in skeletal muscle and will also provide new information about its distribution in the intrafusal fibers of muscle spindles in both normal and pathological states.

METHODS

Eighteen normal (C57Bl/10SnJ) and 8 dystrophic (C57Bl/10mdx) male mice were used in this study. Breeding pairs were originally obtained from Jackson Laboratories (Bar Harbor, ME) and were raised and maintained in our animal facility.

The soleus muscles of both normal and mdx mice at 32 weeks of age were used. A total of 24 muscles from 12 normal mice and 16 muscles from 8 dystrophic mice were examined in this study. Animals were first killed with an overdose of halothane. The soleus muscles from both hindlimbs were rapidly excised and pinned at resting length on small wooden blocks between two thinly sliced pieces of liver (for transverse sectioning), or adhered with a small drop of O.C.T. embedding medium (Miles Laboratories, Naperville, Ill.) to the wooden block for longitudinal sectioning. Specimens were quickly frozen by plunge immersion in isopentane cooled to -196° C in liquid nitrogen for 30 seconds and then transferred to a Forma Scientific Bio Freezer maintained at -63° C for storage until sectioning and subsequent staining. Before sectioning, specimens were mounted on metal chucks in either transverse or longitudinal orientations to the long axis of the muscle fibers and allowed to equilibrate to -20° C in a Bright Instruments 5030 series cryostat microtome (Huntington, England). Serial sections of 5-μm thickness were cut with a metal knife and collected on polylysine-coated glass slides or coverslips. The slides were stored in a -20° C freezer for up to 2 weeks until staining procedures were performed.

Histochemistry

Sections were stained with either hematoxylin and eosin (H & E) or with a modified procedure to detect myosin adenosine-triphosphatase (mATPase) at acid (pH 4.6) preincubation (Johnson and Ovalle, 1986). This ATPase method was adopted because it has

the advantages of being less time consuming than previously published protocols, is relatively safe since the ammonium sulfide step is eliminated, and can detect all three extrafusal fiber types simultaneously using the same pH (Johnson and Ovalle, 1986).

The following steps in this procedure are outlined as follows. Cryostat sections (5-μm thickness) were collected on coverslips and allowed to air dry at room temperature. The coverslips were preincubated for 3 min in barbitol acetate buffer (pH 4.6), washed twice for 60 sec in basic medium (pH 9.4), and then incubated in an adenosine triphosphate (ATP) basic medium (pH 9.4) at 37°C for 20 min. Following incubation, the sections were stained with a 1% aqueous toluidine blue solution for 8 sec and then rinsed well with distilled water. The coverslips were then processed through an ascending series of ethanols (50, 70, 90, 100%), cleared in xylene, and mounted with Histoclad (Clay Adams, Parsippany, NJ).

Intrafusal fibers were typed according to the bag1, bag2, and chain classification of Ovalle and Smith (1972). The histochemical profiles along the lengths of individual intrafusal fibers were determined by examining serial sections of the receptor at intervals of 70 μm. Selected transverse profiles of spindles were photographed on T-Max 100 Kodak film using a Leitz Orthoplan photomicroscope equipped with brightfield optics and a tungsten light source.

Immunofluorescence

Transverse and longitudinal sections were immunostained at room temperature in a humidified chamber with the original polyclonal antibody (made in sheep) directed toward the 60 kD fusion protein of dystrophin (kindly donated by Dr. Eric Hoffman from the University of Pittsburgh) diluted 1:1,000 in phosphate-buffered saline with bovine serum albumin (PBS BSA) at pH 7.3 for 2 hr. Control sections were incubated with pooled normal sheep serum. After repeated washes (3 x 15 min) with PBS BSA, a secondary biotinylated anti-sheep IgG (Amersham, Oakville, ONT) diluted 1:200 was applied for 1 hr, washed (3 x 15 min) and

decorated with a streptavidin-Texas red conjugate (Amersham, Oakville, ONT) (45-min incubation). Sections were washed in PBS BSA (3 x 15 min), coverslips were applied temporarily, and dystrophin immunolabelling was examined with a Zeiss axiophot epi-fluorescence photomicroscope using a G 365 exciter filter. Muscle spindles were located in both transverse and longitudinal planes and photographed on T-Max 400 Kodak (35 mm) black and white print film pushed to 1600 ASA.

Innervation patterns of extrafusal and intrafusal fibers were revealed by concommittant use of a nerve-specific antibody (Roden et al., 1991) that recognizes a 200 kD neurofilament protein. The coverslips of previously dystrophin-labelled slides were removed and sections were incubated for 1 hr with the anti-neurofilament (200 kD) antibody (Sigma, St. Louis, MO) diluted at 1:32, washed in PBS BSA (3 x 15 min), and then immunoreacted with an anti-rabbit-FITC conjugate (Sigma, St. Louis, MO) diluted 1:32 in PBS BSA for 30 min. After a final rinse in PBS BSA, coverslips were mounted with a 1:1 PBS BSA/glycerol medium. Previously photographed dystrophin-stained spindles were relocated and their innervation patterns were then photographed using epifluorescence and a BP 450-490 exciter filter.

Denervation

Six male C57Bl/10SnJ mice were used to study the effects of denervation by sectioning the sciatic nerve on dystrophin and neurofilament expression in normal skeletal muscle. Denervation was performed on mature (32 wk) mice under halothane anesthesia. An incision was made in the region just below the greater trochanter of the right femur and the sciatic nerve was located and teased apart from its surrounding connective tissue. A large section (5 mm) of the nerve was removed to prevent the possibility of reinnervation. The cutaneous incision was then closed and sutured. Upon regaining consciousness, the denervated leg of the mouse was flaccid and lack of motor control in the leg was evident by

the failure to maintain a flexed or extended position. At 21 and 42 days post-denervation intervals, the animals were killed with an overdose of halothane. The soleus muscle from the denervated limb was rapidly excised and placed between two thinly-sliced pieces of liver. Unoperated muscles from age-matched normal mice were placed alongside the denervated muscles to serve as controls for immunofluorescent and morphological evaluation.

To determine if the animal was chronically denervated, the site of the nerve lesion was relocated. In all cases the two cut ends of the sciatic nerve had not reunited. Additionally, immunostaining with the neurofilament protein antibody was utilized to confirm whether reinnervation had occurred. An absence of staining in axons and motor and sensory nerve terminals indicated non-reinnervation.

Serial transverse cryostat sections were immunolabeled with the dystrophin antibody (60 kD) using the previously described labelling procedure and were subsequently observed under epifluorescence. Denervated intrafusal fibers were examined throughout the different regions of the receptors for dystrophin labelling. Sections of muscle spindles were also post-stained with H & E to provide morphological details.

OBSERVATIONS

General Morphology

The mouse soleus muscle has been studied extensively by morphologists, histochemists, and physiologists (Jasch et al., 1982; Bressler et al., 1983; Ovalle et al., 1983; Johnson and Ovalle, 1986; Ovalle and Dow, 1986) due to its small size, ease of accessibility, unique fiber type population and functional properties, and the relative abundance of muscle spindles. It is classified as a slow-contracting muscle and, consequently, exhibits a proportionally high number of slow-twitch extrafusal fibers to other muscles in the leg. Located in the posterior compartment of the hindlimb just anterior to the large gastrocnemius, the soleus serves an important role in the maintenance of posture (Barker, 1974).

When viewed in transverse-section by light microscopy, the normal soleus muscle contained profiles of polygonally-shaped extrafusal fibers arranged in discrete pleomorphic fascicles (Fig. 1). Intramuscular nerve fascicles and blood vessels were also observed and coursed through the perimysial component of the muscle. The extrafusal fibers were characterized by peripherally located nuclei and exhibited overall diameters ranging from 35 to 70 μm. Very little intervening endomysial tissue was present between the muscle fibers.

In H & E stained sections of the mdx soleus, the extrafusal fibers exhibited obvious signs of pathological change including central nucleation, degeneration, and extreme variation in size and shape (Fig. 2). Extrafusal muscle fibers ranging from 20 μm to 90 μm in diameter were encountered and evidence of fiber splitting was seen in a relatively high number of the extrafusal fibers. Additionally, the endomysial and perimysial connective-tissue showed high levels of mononuclear cell infiltration and an overall increase in paracellular elements (Fig. 2).

Muscle spindles in the normal soleus typically contained 4-5 intrafusal fibers, two large-diameter bag fibers and two or three small-diameter chain fibers (Fig. 3a, b, c). Bag

fibers usually extended well beyond the limits of the capsular sleeve, while the chain fibers terminated within capsular regions or shortly thereafter. In equatorial regions, the intrafusal fibers were encased by a delicate inner capsule that separated them from a large periaxial space (Fig. 3a). The outer capsule formed a prominent layer around the receptor and was often flanked by nerves and blood vessels within the surrounding perimysial tissue.

In contrast to the extrafusal fibers, intrafusal fibers in the mdx soleus displayed a relatively minor degree of histological change in dystrophy. In sections stained with H & E (Fig. 4a, b, c), the total number and overall appearance of the intrafusal fibers was similar to those in the age-matched normal soleus. The number of intrafusal fibers ranged from 3 to 6 and the lengths of the fibers did not show a significant difference from that of their normal counterparts. Capsular thickening, however, was particularly evident in equatorial regions of mdx spindles (Fig. 4c). It has been suggested that this may either be due to the relative increase in overall endomysial connective tissue within the muscle or a morphological adaptation of the receptor to protect itself from the disease process (Ovalle and Dow, 1986).

Histochemistry

The histochemical profiles of extrafusal fibers from sections of the normal (Fig. 5) and the mdx (Fig. 6) soleus muscle are illustrated. Acid preincubation (pH 4.6) of the sections revealed two distinct extrafusal fiber types based on their staining properties. At this pH, darkly staining fibers were presumed to be of the type I (slow-oxidative) variety and pale-staining fibers were presumed to be of the type IIA (fast-glycolytic) variety. Very few intermediate staining fibers were observed. In this study, quantitative measurements of fiber type distribution were not determined, however, it was noted that the mdx soleus appeared

to contain a proportionally high number of type I fibers in comparison to its normal counterpart.

Three intrafusal fiber types were clearly distinguished in polar regions of all spindles using the mATPase reaction at an acid (pH 4.6) preincubation (Johnson and Ovalle, 1986). In equatorial regions, however, all intrafusal fibers of both normal and mdx spindles were poorly stained with this technique (Figs. 7a, 8a). Outside of this region, bag_2 fibers were darkly stained along their remaining lengths whereas chain fibers exhibited little reaction and were pale staining (Figs. 7b, 8b). The staining pattern of bag_1 fibers was variable. Within the juxtaequatorial zone, bag_1 fibers were observed to be lightly stained while in the more distal polar regions these fibers characteristically stained darkly (Figs. 7c, 8c). These staining profiles were noted in both the normal and dystrophic intrafusal fibers with little variation in either the intensity or distribution of mATPase staining between corresponding intrafusal fiber types.

Immunofluorescence microscopy

Frozen sections of the normal mouse soleus immunolabelled with the dystrophin antibody consistently exhibited a strong reaction at the sarcolemmal domains of the extrafusal fibers (Fig. 9a). These muscle fibers were homogeneous in size and appearance and were typically polygonal in shape with little intervening endomysial connective tissue. Additionally, a more intense staining reaction for dystrophin was particularly evident at the motor end-plate regions of these fibers (Fig. 9a) which were confirmed as such by colocalization in double-labelled sections with the 200 kD neurofilament-protein antibody (Fig. 9b). Presynaptic terminals of these neuromuscular junctions closely abutted the extrafusal fibers and were typically crescent-shaped in transverse section (Fig. 9b). Intramuscular nerve fascicles and sensory terminals in muscle spindles also displayed a high reactivity with the neurofilament-protein antibody (Fig. 9b).

Extrafusal fibers of the mdx soleus, in contrast, displayed little or no immunostaining for dystrophin indicating the specificity of the antibody (Fig. 10a). There were, however, a few extrafusal fibers in the mdx muscle (about 0.01 %) that exhibited either partial or complete dystrophin staining at their sarcolemmal membranes. These dystrophin-positive extrafusal fibres, previously reported by Karpati et al. (1990) in mdx mice, may reflect a somatic back-mutation in the extrafusal fiber nuclei.

Concommitant immunolabelling with the neurofilament-protein antibody revealed strong reactions in intramuscular nerve fascicles, individual axons, and sensory terminals of muscle spindles in the mdx soleus (Fig. 10b). Terminal arborizations contacting extrafusal fibers were distributed throughout the muscle and were assumed to be motor-nerve terminals (Fig. 10b). No dystrophin reactivity, however, was seen in the sarcolemmal membranes in contact with these terminals.

In muscle spindles of the normal soleus, the polar regions of intrafusal fibers displayed a thin homogeneous peripheral rim of dystrophin immunolabelling similar to that observed at the non-synaptic sarcolemmal membranes of neighbouring extrafusal fibers (Fig. 11a). Equatorial and juxtaequatorial regions of intrafusal fibers, in contrast, displayed an inconsistent dystrophin staining. Portions of the sarcolemma exhibited either decreased or deficient dystrophin expression, whereas other areas retained a relatively normal labelling intensity (Fig. 11b). Subsequent labelling of the same sections with the neurofilament-protein antibody revealed sensory nerve-terminals of the annulospiral type encircling the perimeters of the intrafusal fibers (Fig. 11c). Those areas deficient in dystrophin (Fig. 11b) consistenly corresponded to the contact sites of the sensory nerve terminals (Fig. 11c).

Serial cross-sections (70 μm intervals) of spindles in the normal soleus were cut to determine the extent to which these dystrophin-deficient sites are found along the lengths of the intrafusal fibers (Figs. 13a-f). Alternate serial-sections, in addition, were treated to detect mATPase (Figs. 13a'-f') making it possible to histochemically differentiate the

intrafusal fibers into bag_1 (pale), bag_2 (dark staining), and chain (pale) fibers (Ovalle and Smith, 1972). These sections were used, subsequently, to reconstruct a muscle spindle along its entire length (Fig. 13). Deficiencies of dystrophin at the intrafusal fiber sarcolemma were observed in all three fiber types in the dilated intracapsular zones (equatorial and juxtaequatorial regions) of the muscle spindle, occupying a region approximately 280-320 μm in length. The centralized portion of equatorial regions exhibited a relatively high frequency of dystrophin-staining deficiencies (Figs. 13c, d) whereas more distal juxtaequatorial regions displayed fewer focal deficiencies (Figs. 13b, e). Outside of this region and towards each pole, the sarcolemmal dystrophin expression of the intrafusal fibers was homogeneous and resembled that of the surrounding extrafusal fibers (Figs. 13a, f). No differences in dystrophin labelling between nuclear bag and chain fibers or between bag_1 and bag_2 fibers were evident in polar regions.

In longitudinal sections of normal intrafusal fibers (Fig. 14a) a more clearly demarcated staining pattern for dystrophin was observed. At low magnification, a distinct transition from a continuous sarcolemmal labelling in polar regions to a discontinuous pattern in the sensory equatorial regions was evident (Fig. 14a). The sarcolemmal membranes of both nuclear bag and nuclear chain fibers were characterized by alternating dystrophin-positive and dystrophin-deficient segments (Figs. 15a, 16a). These dystrophin-negative sarcolemmal segments measured 5 - 7 μm in length and were found in the highly-nucleated areas of the muscle fibers (Fig. 15b). In double-labelled longitudinal sections, an intensely-labelled network of nerves arranged in an annulospiral fashion (Fig. 16b) were revealed immunofluorescently by the 200 kD neurofilament-protein antibody. The areas of sarcolemma subjacent to these nerve-terminal wrappings (primary sensory endings) corresponded to the dystrophin-deficient segments seen previously. Secondary endings (flower-spray endings) were difficult to identify with certainty due to the limitations of the neurofilament staining method and, subsequently, could not be resolved as distinct terminations on the intrafusal fibers.

When immunolabelled with the dystrophin antibody (Fig. 12b) and counterstained with H & E (Fig. 12a), fluorescence microscopy showed a complete absence of dystrophin labelling in the intrafusal fibers of muscle spindles in the mdx soleus. This finding is in agreement with other immunocytochemical studies of skeletal muscle (Miike et al., 1989; Samitt and Bonilla, 1990; Tanaka et al., 1990) where dystrophin staining was briefly noted in muscle spindles. In the sections labelled with the nerve-specific antibody, the sensory nerve-terminals in mdx muscle spindles appeared unaltered morphologically and terminated in a similar fashion to that observed in the age-matched normal soleus (Fig. 12c).

Denervation

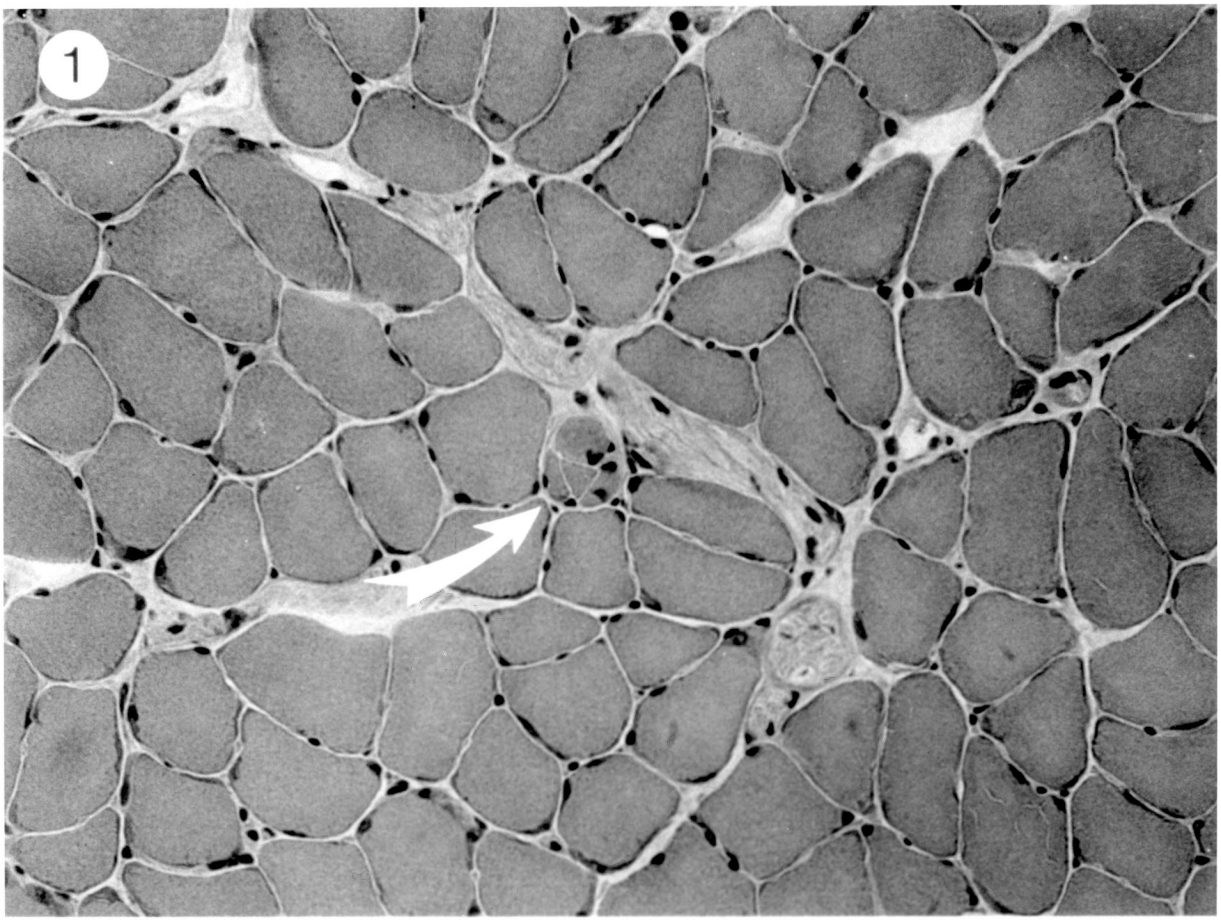
Following sciatic denervation of normal muscle, obvious morphological alterations in the muscle architecture of the soleus were observed. The extrafusal fibers exhibited a progressive decrease in size and an increase in the number of centrally located nuclei. No normal nerves were observed in either of the two denervation periods and the absence of neurofilament protein labelling in both nerves and nerve terminals confirmed that the muscle was not reinnervated. Although a decrease in extrafusal fiber size was observed, no changes in either the dystrophin labelling pattern or its intensity was seen in either the 21-day (Fig. 17) or 42-day (Fig. 18) denervations. Additionally, elevated levels of dystrophin seen at motor end-plates in normal muscle were also apparent in denervated muscle (Figs. 17, 18), however, morphological confirmation of the the motor endings was not possible since neurofilament protein labelling was absent.

In muscle spindles, several morphological characteristics were altered as a result of denervation. Capsular thickening in equatorial regions was evident in both groups (Figs. 19a, 20a) with a marked increase seen in the 42-day denervated spindles (Fig. 20a). An overall increase in the cellular components of the spindle capsule and the surrounding endomysial and perimysial connective tissues was also noted (Figs. 19a, 20a). Intrafusal fibers only showed a slight decrease in cross-sectional size and did not appear to parallel the dramatic decrease in

size of the extrafusal fibers. Dystrophin labelling was homogeneously distributed at the sarcolemma in polar regions, whereas equatorial regions displayed a heterogeneous expression of dystrophin at their sarcolemmal membranes. Deficient zones of dystrophin labelling were presumed to be sites of degenerating or necrotic sensory nerve terminals (Figs. 19b, 20b) and were consistent with those seen in intrafusal fibers of muscle spindles in the normal soleus (Figs. 12b, 13b-e). This was evident in all intrafusal fiber types examined in this study. In addition, neurofilament protein expression was consistently absent in sensory-terminal regions of the muscle spindles in the denervated soleus at both the 21-day and 42-day denervation periods (Fig. 19c, 20c).

Fig. 1. Transverse frozen section of a normal 32-week-old mouse soleus muscle stained with H & E. Regularly-sized extrafusal fibers predominate in the field and appear polygonal in shape with relatively little intervening connective tissue. A muscle spindle is indicated (curved arrow). x325. Bar = 50 μm.

Fig. 2. Comparative view of the soleus from a 32-week-old mdx-dystrophic mouse stained with H & E. Large variations in extrafusal fiber size and central nucleation are prominent features. A marked increase in connective tissue can be seen in the interstitium. A muscle spindle is indicated in the center of the field (curved arrow). x325. Bar = 50 μm.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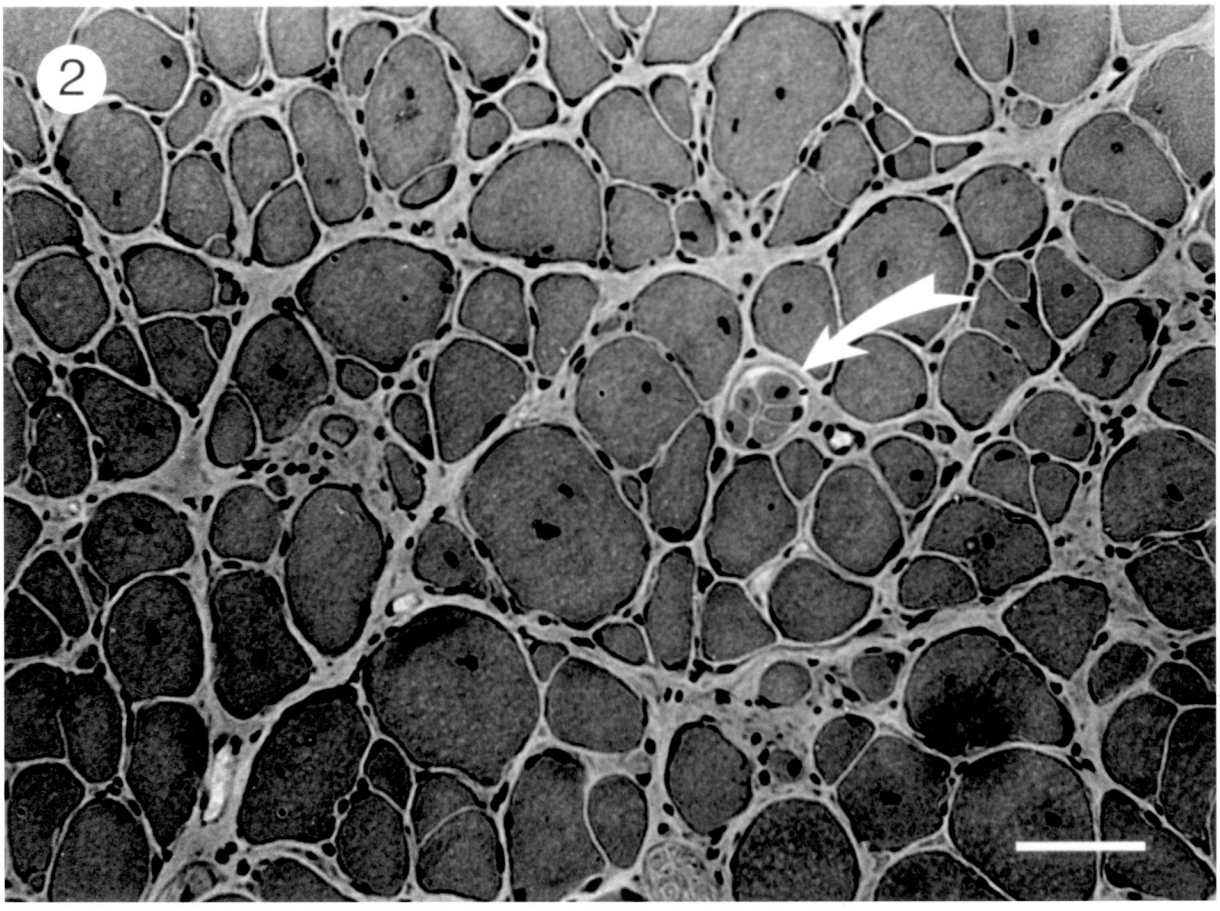
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Fig. 3. Higher magnification views of a muscle spindle in the normal soleus stained with H & E. The same muscle spindle is seen sectioned through its equatorial (3a), proximal polar (3b), and distal polar (3c) regions. x820. Bar = 10μm.

Fig. 4. Serial sections of a muscle spindle in the mdx-dystrophic soleus stained with H & E. The spindle has been sectioned through its equatorial (4a), proximal polar (4b), and distal polar (4c) regions. x820. Bar = 10μm.

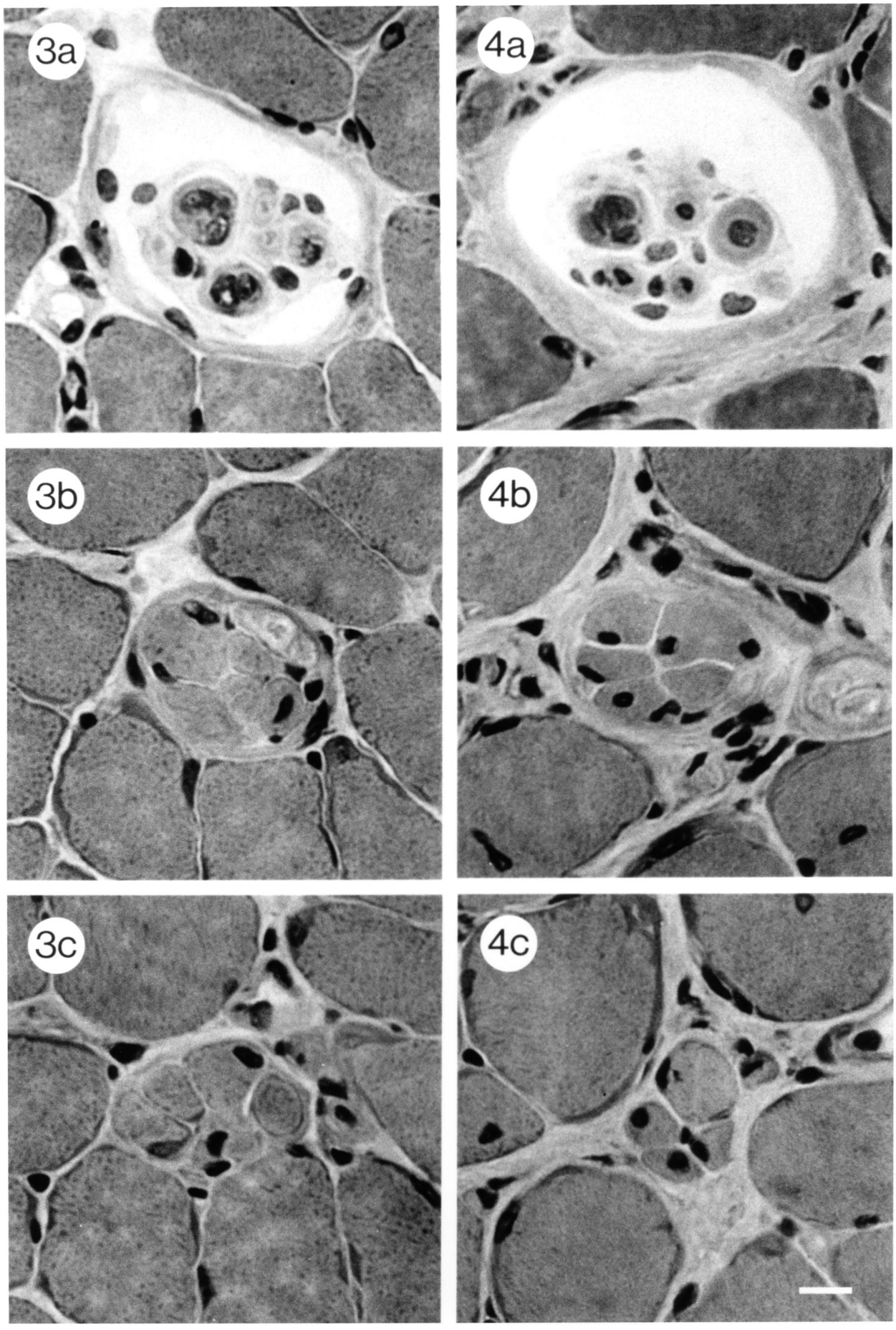
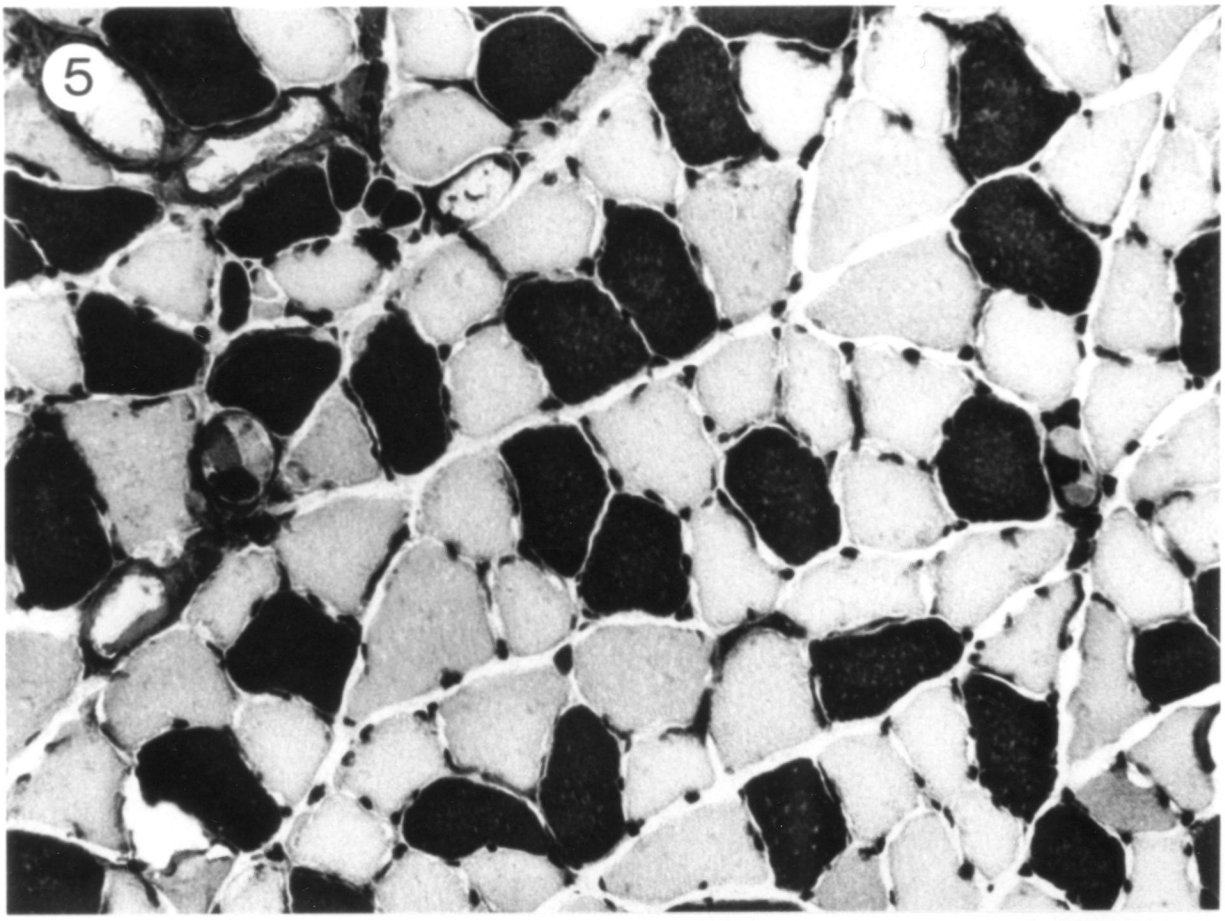

3a
4a
3b
4b
3c
4c

Fig. 5. Low magnification view of a portion of the normal soleus stained for myosin ATPase at pH 4.6. Note the mosaic-like appearance of the slow-twitch (dark staining) and fast-twitch (pale staining) extrafusal fiber types. x325. Bar = 50μm.

Fig. 6. Comparative view of a portion of the mdx-dystrophic soleus stained for myosin ATPase at pH 4.6. Groups of slow-twitch (dark staining) extrafusal fibers separated by relatively low numbers of fast-twitch (pale staining) extrafusal fibers are seen. x325. Bar = 50μm.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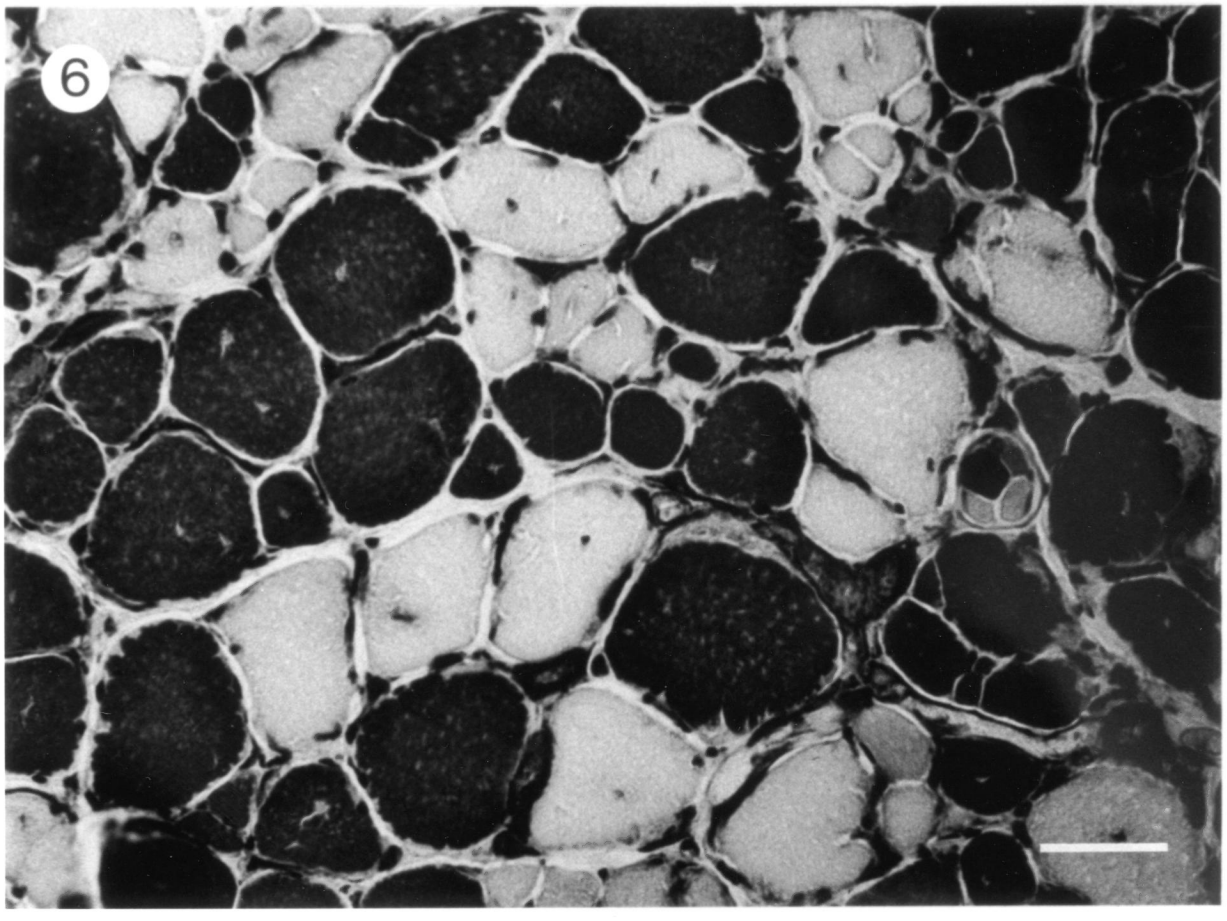
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Fig. 7. High magnification views of a muscle spindle in the normal soleus stained for myosin ATPAse at pH 4.6. Sections have been taken from equatorial (7a), proximal polar (7b), and distal polar (7c) regions. Note the change in staining properties along the lengths of the nuclear bag fibers. x820. Bar = 10μm.

Fig. 8. Serial sections of a muscle spindle in the mdx-dystrophic soleus muscle stained for myosin ATPase at pH 4.6. The spindle has been sectioned through equatorial (8a), proximal polar (8b), and distal polar (8c) regions. Note the change in staining properties along the lengths of the nuclear bag fibers. x820. Bar = 10μm.

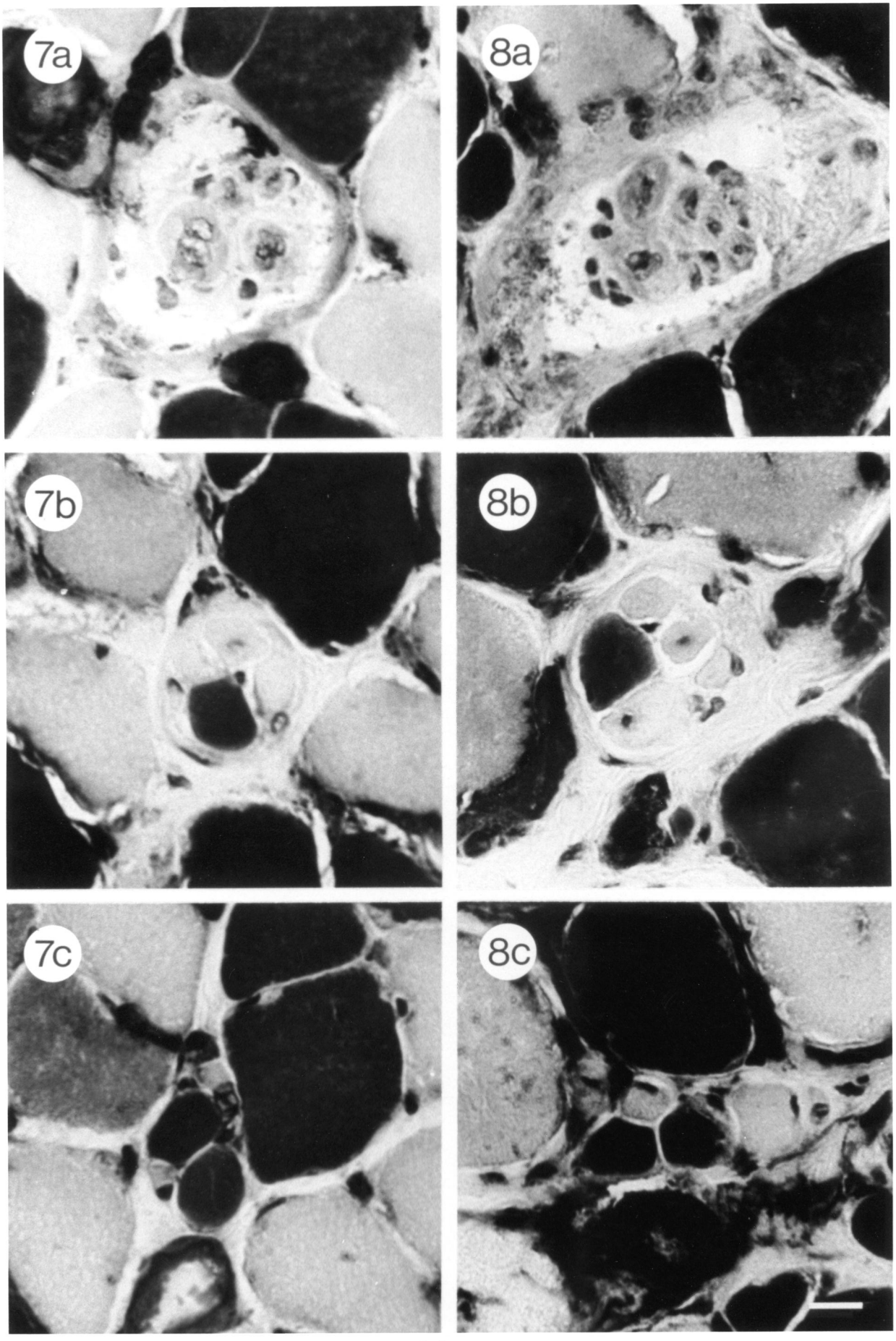

7a
8a
7b
8b
7c
8c

Fig. 9. Pair of fluorescence micrographs of the same transverse section of the normal soleus double-labelled with anti-dystrophin (a) and anti-neurofilament protein (b). The equator of a spindle with four intrafusal fibers (curved arrows), a neuromuscular junction on an extrafusal fiber (straight arrows), and a nerve fascicle (asterisks) are indicated. x300. Bar = 30 μm.

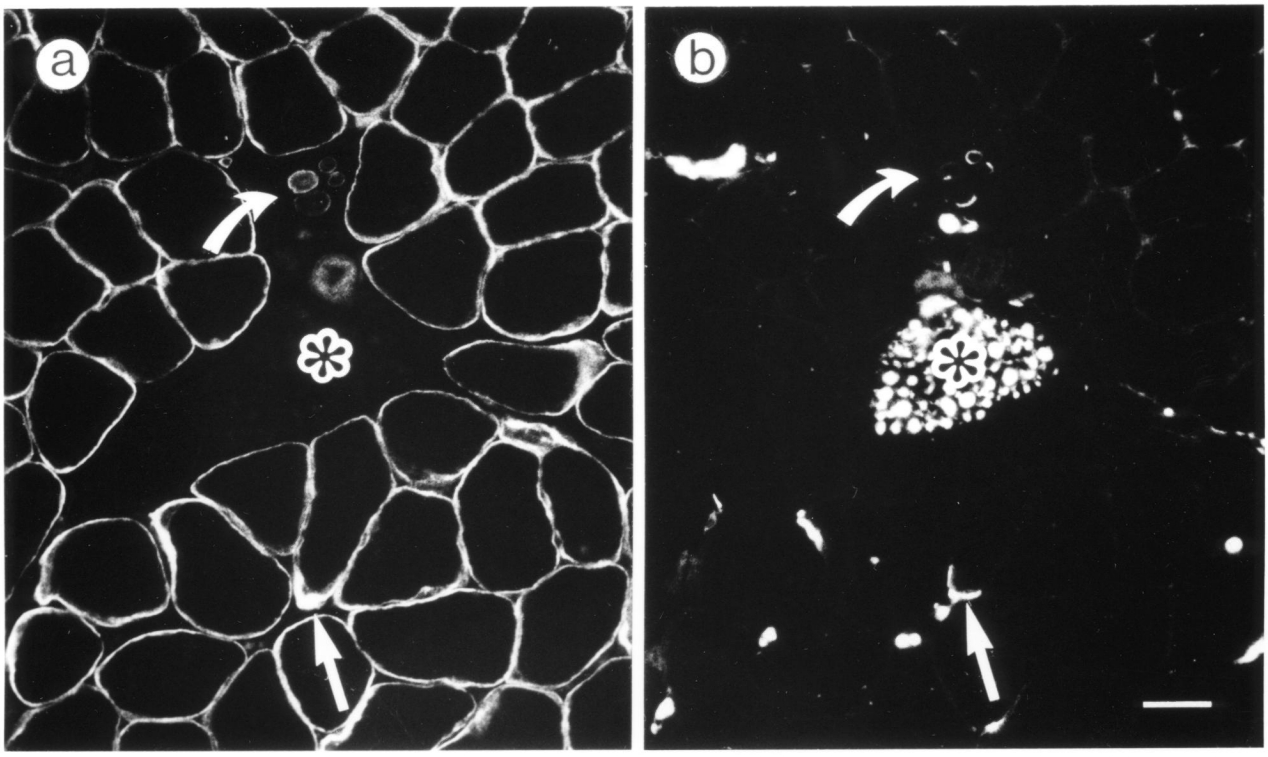

a
b

Fig. 10. Micrographs of the same section of the mdx soleus labelled with anti-dystrophin (a), anti-neurofilament protein (b), and post-stained with H & E (c). The equator of a spindle (curved arrows), a neuromuscular junction on an extrafusal fiber (straight arrows), and a nerve fascicle (asterisks) are indicated. Note the absence of dystrophin in both the extrafusal and the intrafusal fibers (a). x300. Bar = 30μm.

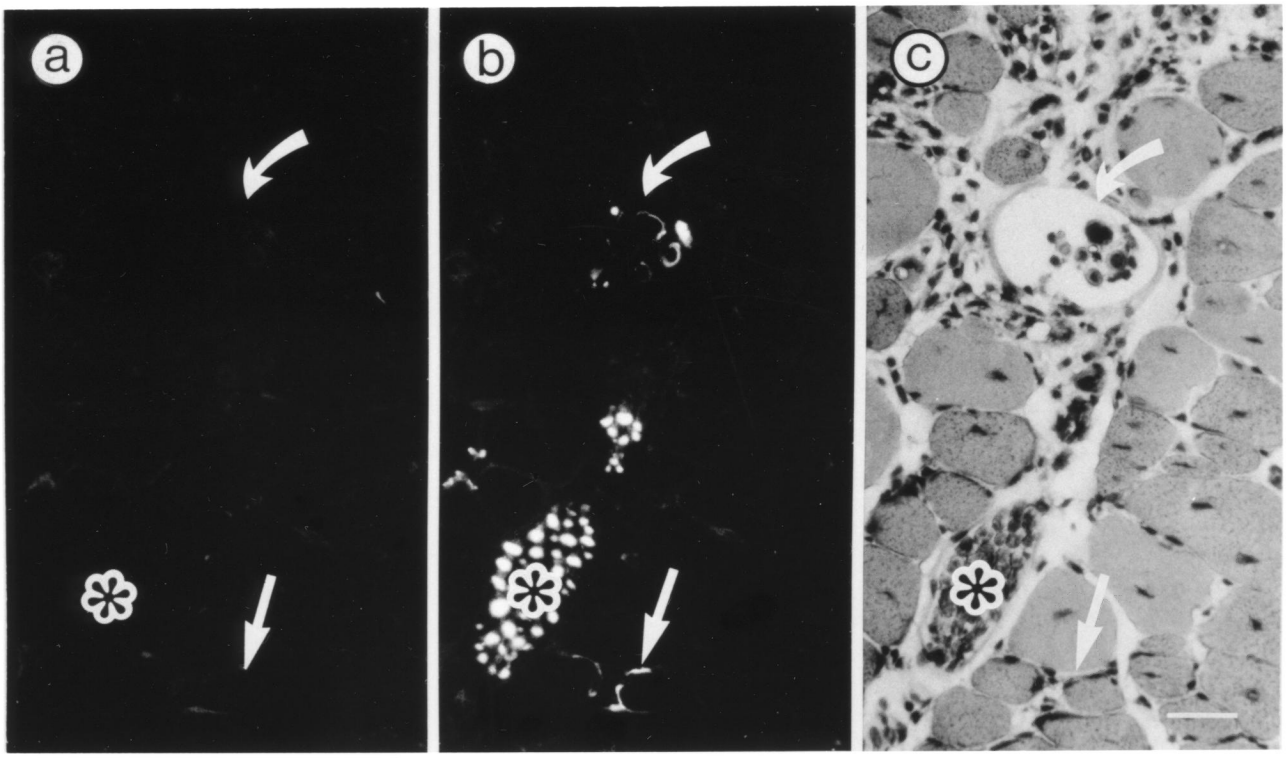

a
b
c

Fig. 11. Polar (a) and equatorial (b) regions of a normal spindle stained with anti-dystrophin show homogeneous staining at the periphery of polar intrafusal fibers (IF) and dystrophin-deficient areas (arrows) in the equator (b). Double-labelling of the equator with anti-neurofilament protein (c) shows sensory nerve terminals. x900. Bar = 10μm.

Fig. 12. Transverse section of an mdx spindle stained with H & E (a), anti-dystrophin (b), and anti-neurofilament protein (c). Intrafusal fibers (IF) show no dystrophin labelling at their periphery (b), whereas sensory terminals (curved arrows) appear normal (c). x900. Bar = 10μm.

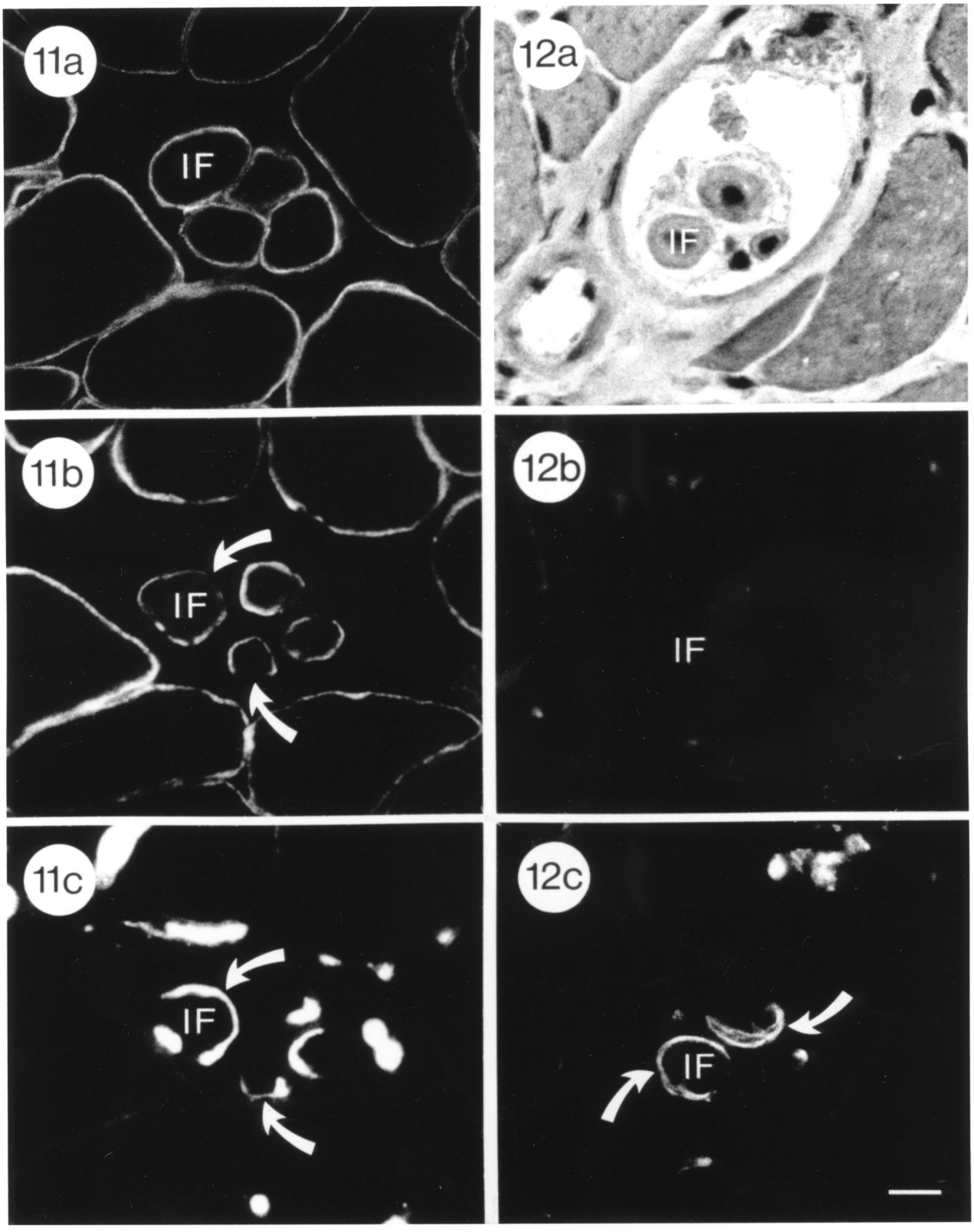

11a
IF
12a
IF
11b
IF
12b
IF
11c
IF
12c
IF

Fig. 13. Transverse serial sections (70μm intervals) of a normal spindle immunolabelled for dystrophin (a-f). Alternate sections are stained for myosin ATPase (a'-f'). Dystrophin in intrafusal fibers is reduced periodically in the equator (arrowheads). The three-dimensional reconstruction on the right shows polar (Pole) and equatorial (Eq) regions of the muscle spindle. x360. Bar = 30μm.

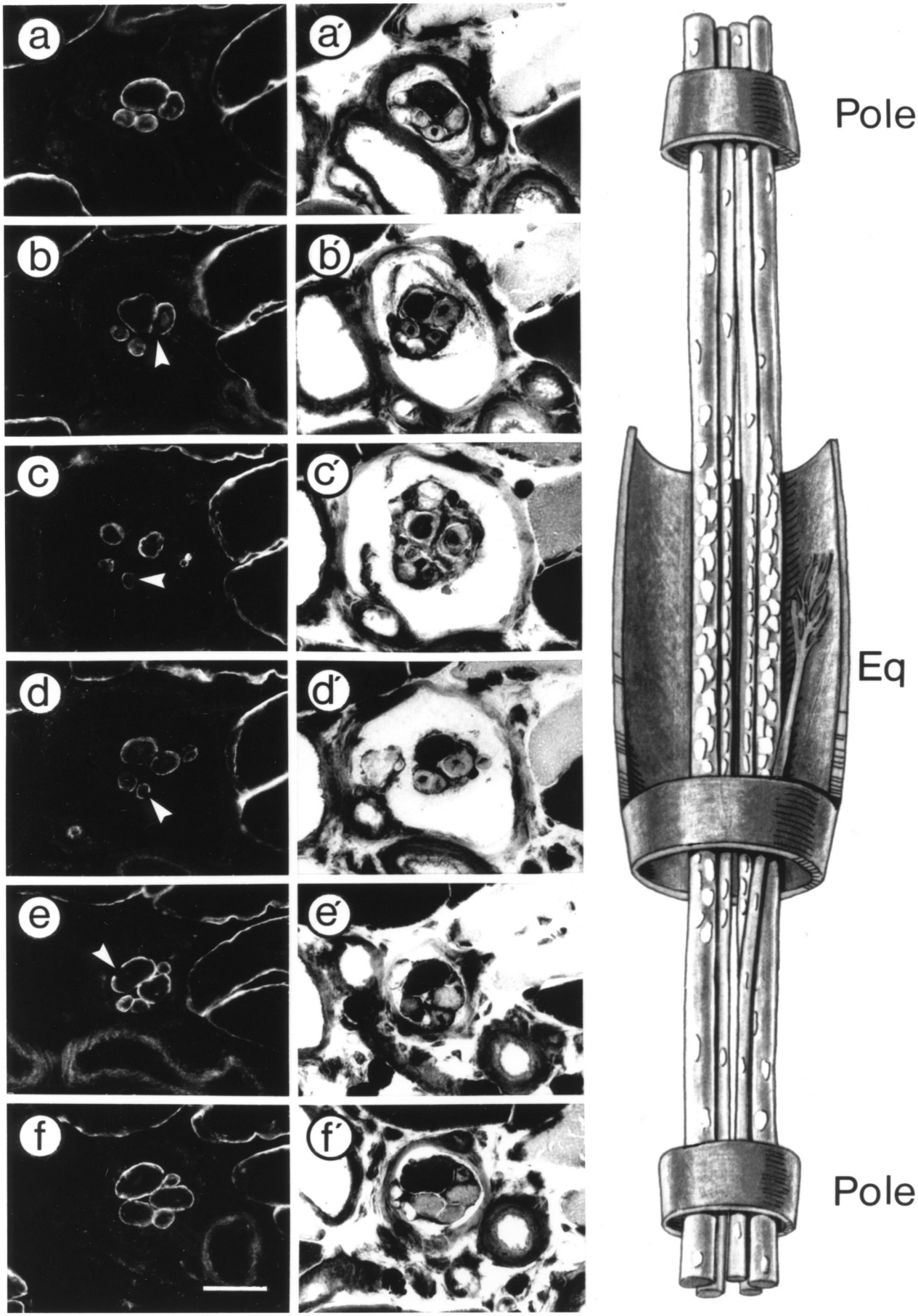

a
a'
b
b'
c
c'
d
d'
e
e'
f
f'
Pole
Eq
Pole

Fig. 14. Low magnification view of a longitudinal section of a muscle spindle in the normal soleus immunolabelled for dystrophin (a) and post-stained with H & E (b). Note the change in intrafusal fiber sarcolemmal staining from continuous in the polar region (Pole) to discontinuous in the equator (Eq). x425. Bar = 30μm.

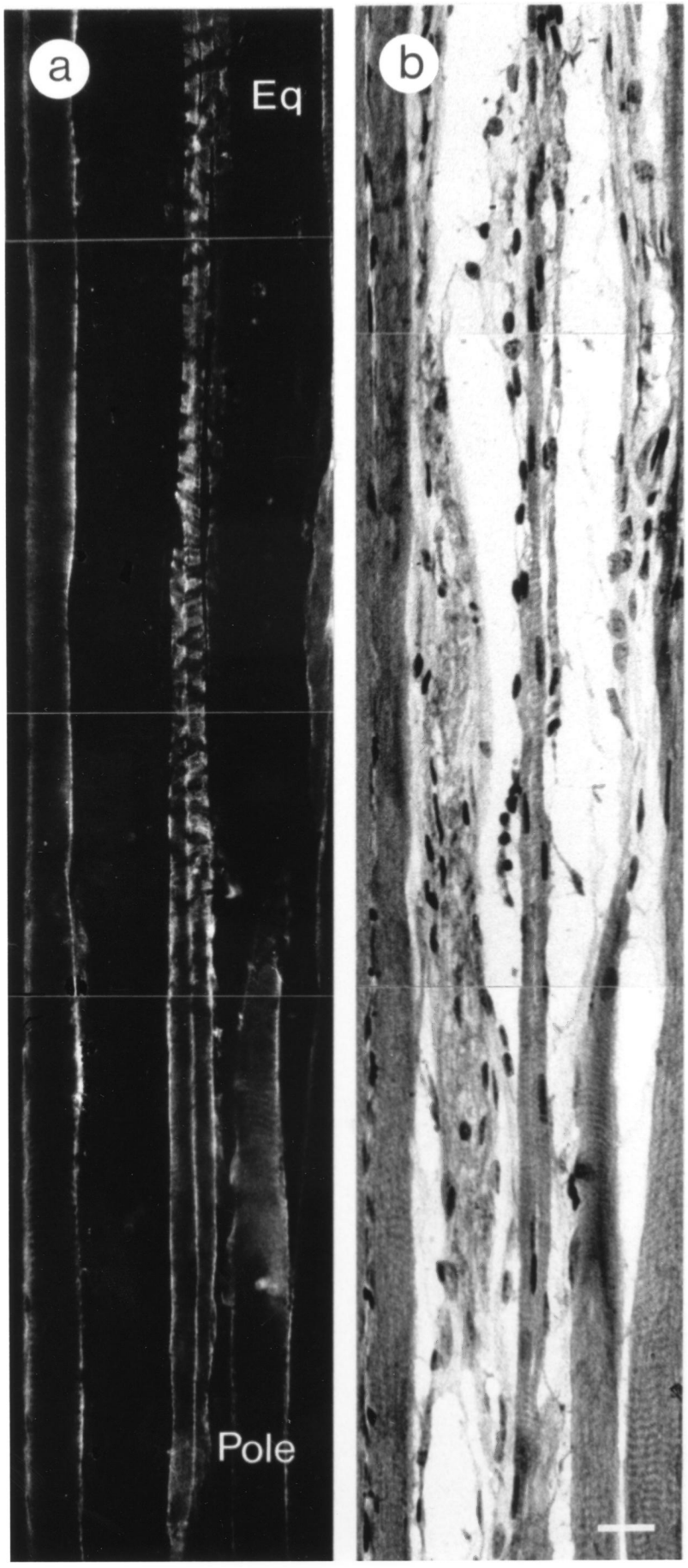

a
Eq
Pole
b

Fig. 15. Longitudinal section a a normal nuclear-bag fiber in the equatorial region viewed with fluorescence (a) and phase-contrast (b) microscopy. Note the periodic pattern of dystrophin expression along the sarcolemma (thin arrows) of the muscle fiber. Intervening regions (white arrowheads) are deficient in dystrophin. The phase-contrast image reveals punctate elevations (black arrowheads) corresponding to sensory nerve-terminals where dystrophin is absent. x1,630. Bar = 10μm.

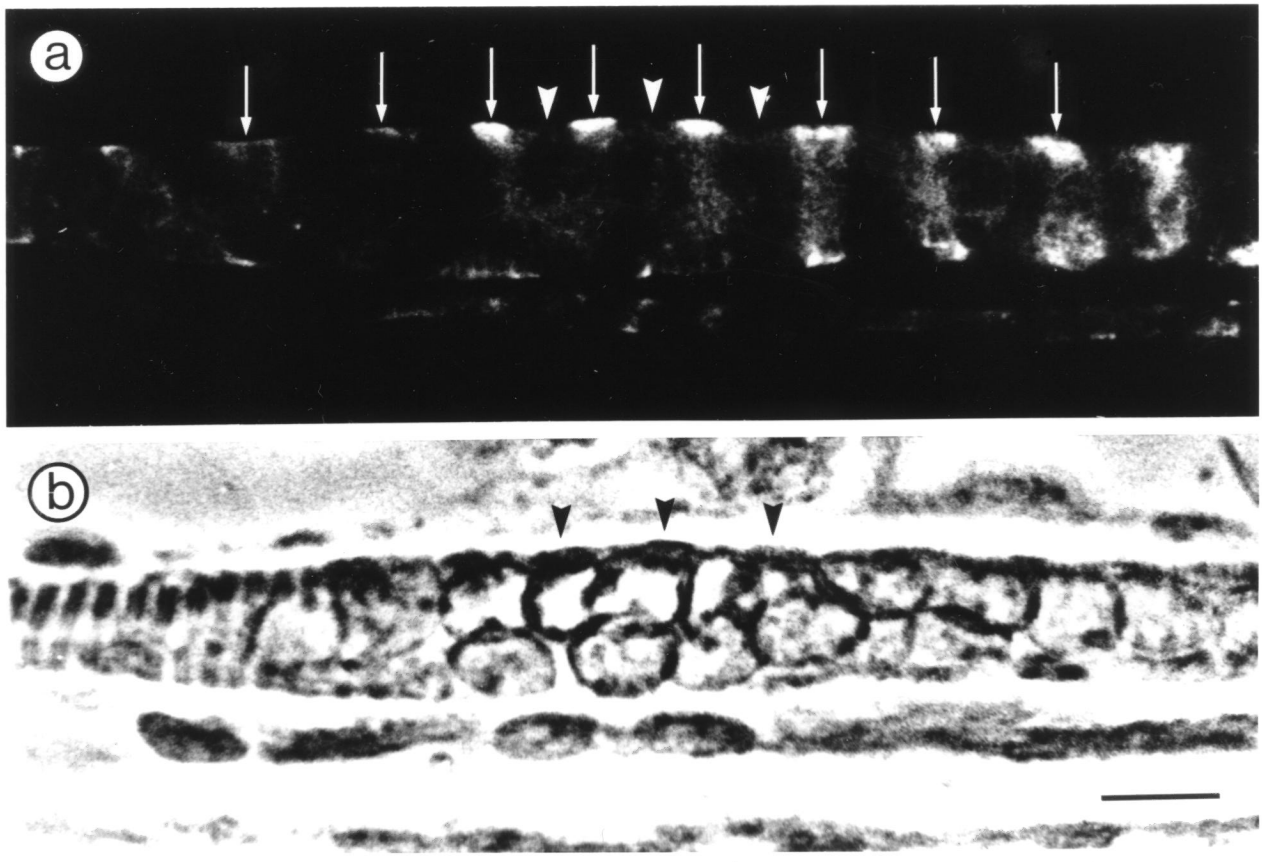

a
b

Fig. 16. Longitudinal views of two intrafusal fibers in the normal soleus double-labelled for dystrophin (a) and neurofilament protein (b). Dystrophin-deficient regions of the sarcolemma (arrows in a) are consistent with those areas in contact with the annulospiral sensory endings (arrows in b). The phase contrast image (c) reveals a nuclear bag (nb) and a nuclear chain (nc) fiber. x1330. Bar = 10μm.

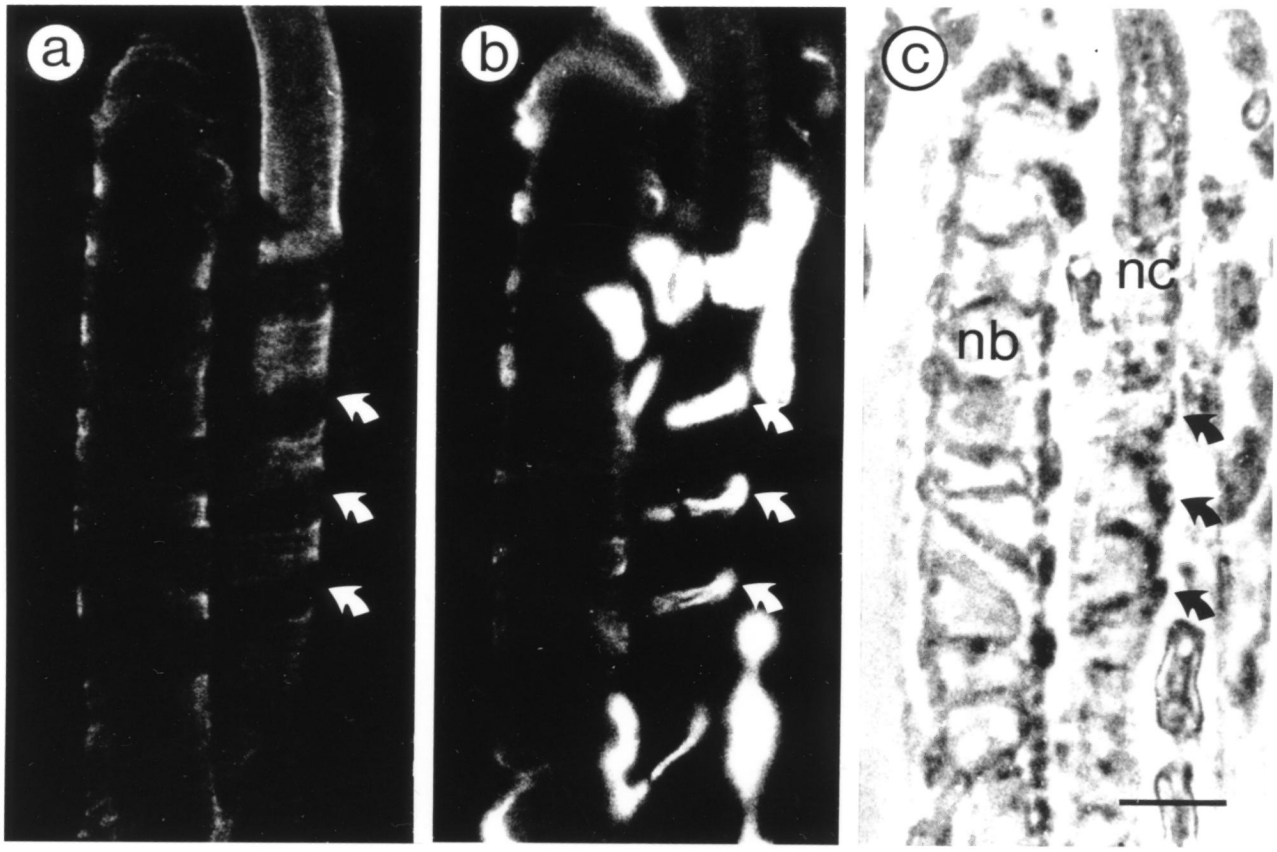

a
b
c
nc
nb

Fig. 17. Transverse sections of 21-day (a) and 42-day (b) denervated normal soleus muscles immunolabelled for dystrophin. Note the persistence of dystrophin in all the extrafusal fibers. Elevated levels of dystrophin are seen at the sarcolemmal membranes of some of the extrafusal fibers (arrows). Small portions of age-matched control soleus muscles (left) are included for comparison. x300. Bar = 50μm.

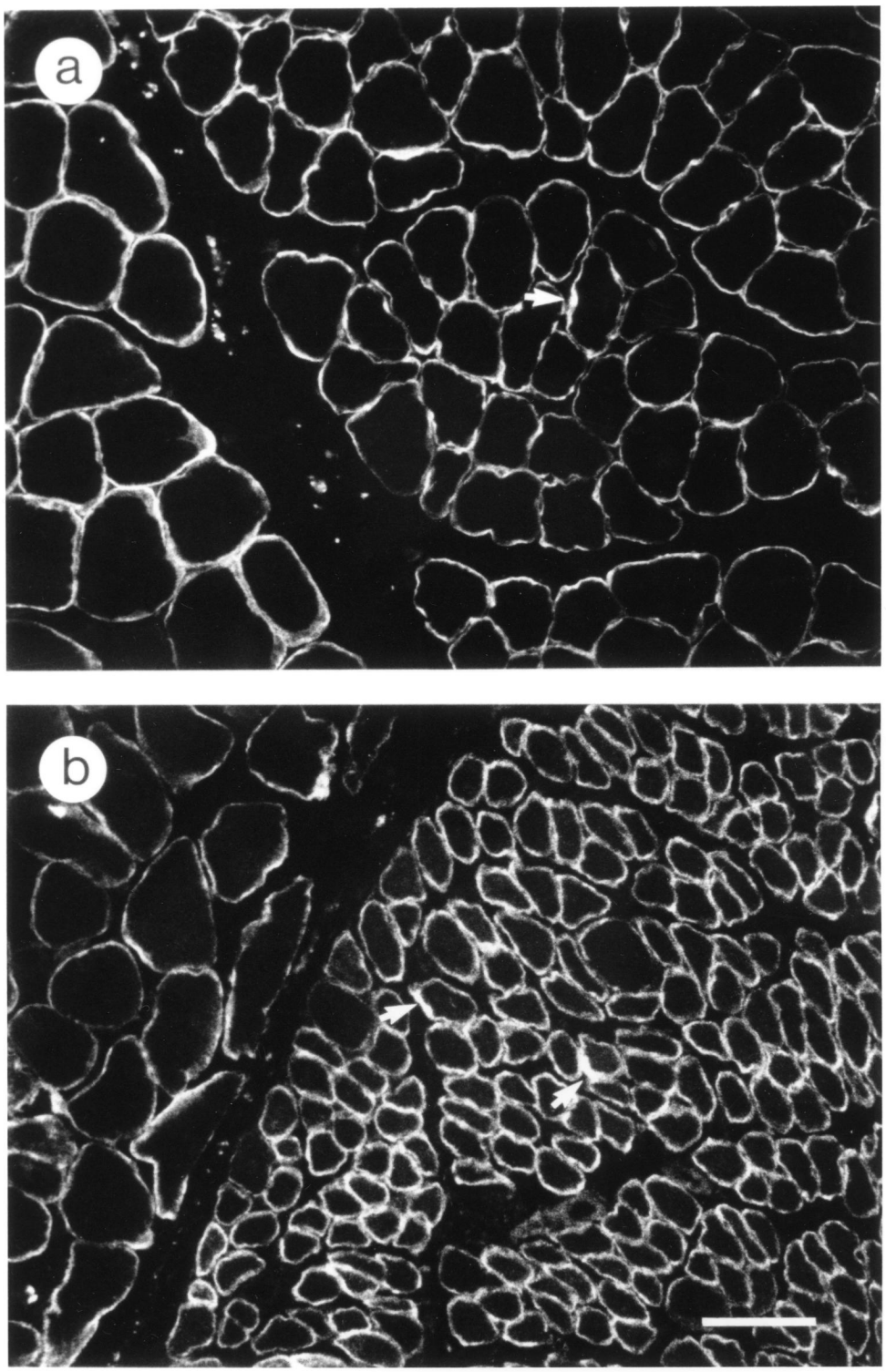

a
b

Fig. 18. Equatorial region of a 21-day denervated muscle spindle stained with H & E (a), anti-dystrophin (b), and anti-neurofilament protein (c). Dystrophin is deficient on portions of the intrafusal fiber sarcolemma (arrowhead). Note the complete absence of neurofilament protein labelling (c) in the sensory regions of the muscle spindle. x625. Bar = 20μm.

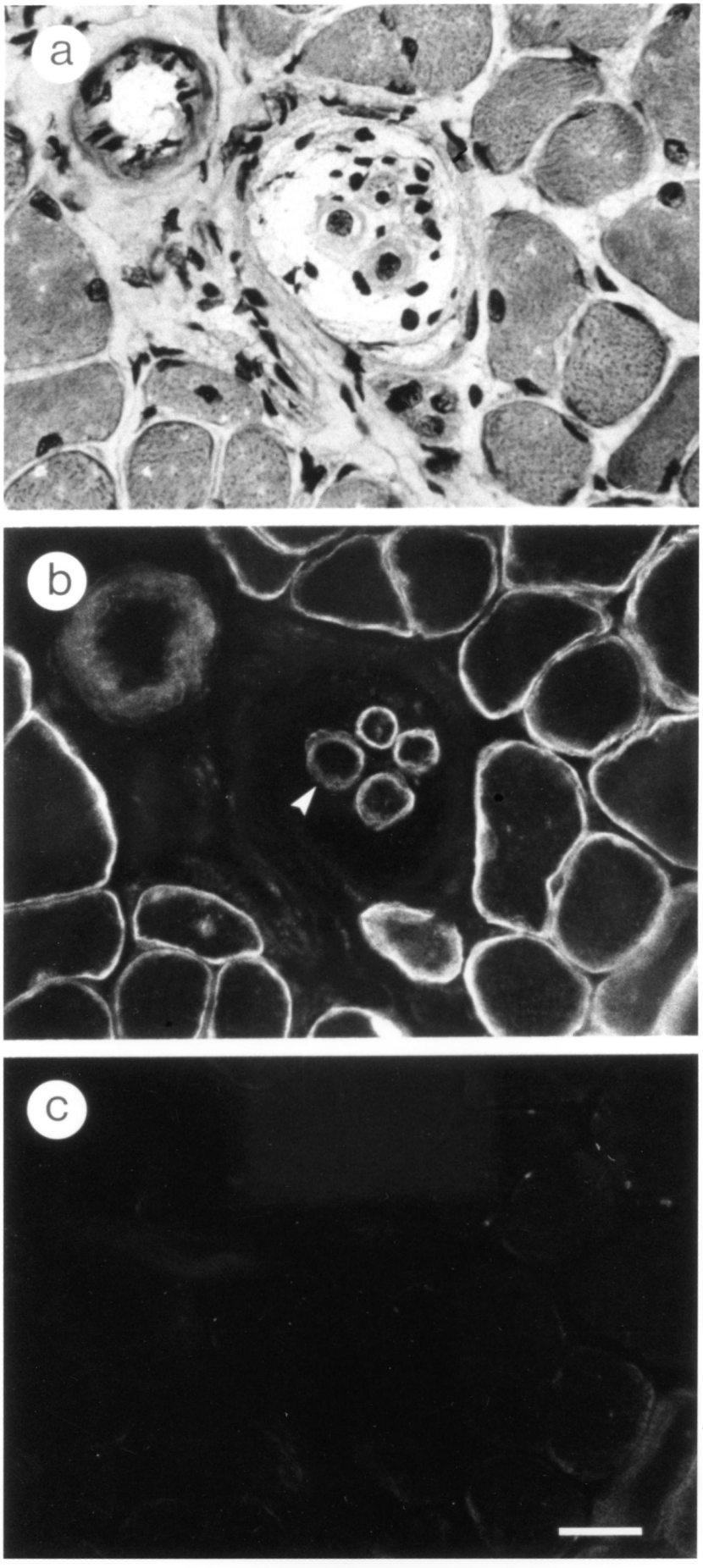
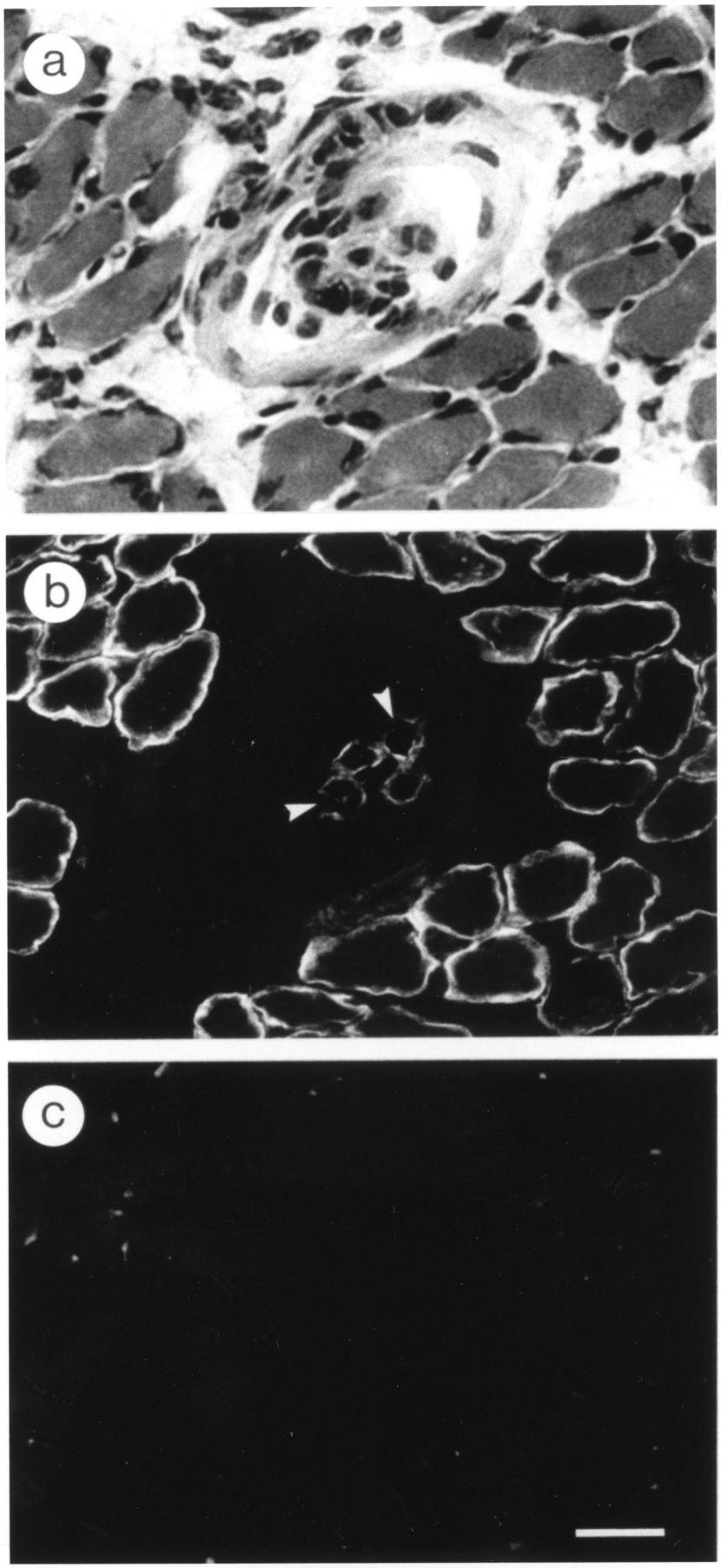

a
b
c

Fig. 19. Equatorial region of a 42-day denervated muscle spindle stained with H & E (a), anti-dystrophin (b), and anti-neurofilament protein (c). Several focal deficiencies in dystrophin labelling can be seen at the sarcolemmal membranes of the intrafusal fibers (arrowheads). Note the overall increase in paracellular and cellular elements in the connective-tissue capsule (a). x625. Bar = 20μm.


a
b
c

DISCUSSION

This study has shown for the first time how dystrophin is localized along the lengths of intrafusal fibers in both normal and mdx-dystrophic skeletal muscle, and these observations have recently been published (Nahirney and Ovalle, 1993). Although there have been many studies over the past five years on dystrophin and its localization in skeletal muscle, only a few have briefly alluded to the dystrophin staining characteristics of these fibers in muscle spindles (Miike at al., 1989; Samitt and Bonilla, 1990; Tanaka et al., 1990; Zhao et al., 1992). Use of the present methodologies has provided a more detailed description of the muscle spindle in normal and dystrophic states and, in addition, has revealed important features about dystrophin that directly contribute to our understanding of the functional role this protein plays in normal skeletal muscle fibers.

Histochemistry

Three intrafusal fiber types in muscle spindles of the normal and mdx-dystrophic soleus could be distinguished on the basis of mATPase staining characteristics. Moreover, regional variations in staining intensity were observed, similar to those noted in the study by Johnson and Ovalle (1986), and also to those seen in rat (Soukup, 1976; Banks et al., 1977; Khan and Soukup, 1979), cat (Banks et al., 1977; Bakker and Richmond, 1981; Kucera, 1981), monkey (Ovalle and Smith, 1972), and human (Kucera and Dorivini-Zis, 1979).

At acid preincubation (pH 4.6), equatorial regions of all intrafusal fiber types showed little or no histochemical reactivity. In juxtaequatorial regions, the bag_1 fibers were acid-labile whereas in distal polar (extracapsular) regions they were acid-stable. Thus, a staining pattern that gradually changed in a sequential fashion from dark to light in the bag_1 fiber was observed as serial sections of a spindle were followed from the polar end toward the equator.

Bag_2 fibers, on the other hand, showed a high acid reactivity in juxtaequatorial and polar regions, whereas chain fibers consistently exhibited low acid reactivity throughout their lengths.

These histochemical staining properties were characteristic of both normal and dystrophic intrafusal fibers. In contrast to the histochemical changes seen in extrafusal fiber types in dystrophy, as noted in this study and in other murine dystrophies (Ovalle et al., 1983; Wirtz et al., 1983), the mATPase reactivity of intrafusal fibers appeared to be unaffected by the disease process. In mdx-dystrophic mice, bag_1, bag_2, and chain fibers could be clearly differentiated based on reactivity in an acid preincubation. Moreover, bag_1 fibers showed the same regional variability as that seen in its normal counterpart.

It has been postulated that the variations in mATPase staining of the intrafusal fibers are a consequence of their specific motor innervation (Yellin, 1969; Milburn, 1973). This fusimotor influence can be disputed since Zelena and Soukup (1974) have demonstrated that selectively de-efferented spindles at birth eventually differentiate and mature to obtain their normal histoenzymatic characteristics. Additionally, Kucera (1981) has shown no correlation between regional ATPase activity and the location, number, or type of motor endings determined by cholinesterase staining. The histochemical profiles of these fibers has, therefore, been suggested to be controlled by different neurotrophic factors than those of the extrafusal fibers. Intrafusal fiber integrity may instead be influenced by beta innervation in polar regions, or by sensory innervation in equatorial regions.

It has also been shown that the three types of intrafusal fibers exhibit different isoforms of myosin in capsular (Pierobon-Bormioli et al., 1980; te Kronnie et al., 1981; Rowlerson et al., 1985; Maier et al., 1988) and extracapsular polar (Kucera and Walro, 1989) regions. Furthermore, it has been reported that, like mATPase staining, the expression of myosin heavy chains varies along the lengths of intrafusal fibers, whereas in extrafusal fibers a uniform distribution of these isoforms is observed (Kucera and Walro,

1989). Their observations revealed that intracapsular regions of intrafusal fibers contained a relatively high concentration of slow-tonic and neonatal myosin isoforms while extracapsular regions of these fibers displayed a strong reactivity to antibodies specific for slow-twitch and fast-twitch myosin isoforms. A relationship between mATPase staining properties and the expression of myosin heavy chains has been described by Kucera and Walro (1989) in the extracapsular regions of intrafusal fibers, however, intracapsular regions were not shown to have such correlations. Similarly, extrafusal fibers have been shown to have a relationship between myosin composition and mATPase reactivity (Staron and Pette, 1986).

Immunolocalization of Dystrophin in Muscle Spindles

The topographic localization of dystrophin in the intrafusal fibers of muscle spindles from normal and dystrophic mice was determined in this study. The results from this work support the original findings of previous workers (Miike et al., 1989; Sammit and Bonilla, 1990; Tanaka et al., 1990; Zhao et al., 1992) that dystrophin is localized under the sarcolemma of the intrafusal fibers in normal muscle. However, in this study the consistency of dystrophin expression was not homogeneous along the lengths of these fibers. By serial inspection of transverse and longitudinal sections, all three intrafusal fiber types displayed a homogeneous sarcolemmal labelling for dystrophin along the regions of each fiber not in contact with sensory terminals. On the other hand, the sensory equatorial zone exhibited discontinuous immunolabelling marked by short dystrophin-negative segments of sarcolemma interposed with positive-staining regions. With the nerve-specific antibody, these dystrophin-deficient zones in the intrafusal fibers were clearly identified as areas of the sarcolemma immediately underneath sensory nerve-terminals.

Electron microscopic studies of mammalian spindles have revealed characteristics about the sensory nerve terminals which suggest that a neurosecretory process may be

involved at these sites (Boyd and Smith, 1984). Small membrane-bound vesicles with contents thought to be neurochemical in nature have been seen in both primary and secondary nerve endings. Additionally, this hypothesis is supported by deafferentation and developmental studies which have shown that sensory nerves to spindles are required for differentiation (Zelená, 1957; Milburn, 1973) and continued maintenance (Tower, 1932; Schröder, 1974a, b; Schröder et al., 1978) of the intrafusal fibers. It is possible that sensory nerve innervation plays a trophic role which promotes a modification in the structure of the subsynaptic membrane (i.e. one that inhibits dystrophin expression at this site). Alternatively, either a homologue of dystrophin (Pons et al., 1991) or a dystrophin-related protein (Ohlendieck et al., 1991b), both of which have been recently described at neuromuscular junctions of extrafusal fibers, may, in fact, assume the role of dystrophin at these locations.

Maier and Mayne (1993) have recently reported regional differences in the organization of some of the cytoskeletal components at the equator of chicken intrafusal fibers. In their study, the immunocytochemical expression of the cytoskeletal proteins, alpha-actinin and filamin, was primarily limited to the region subjacent to the myosensory junctions in the equatorial zones. Diametrically apposing nonsensory regions, on the other hand, did not exhibit significant labelling for these proteins. However, they did express a sharply delineated and narrow intrafiber crescent of vinculin colocalized with a crescent of talin. An asymmetric distribution of beta-1-integrin molecules in the membrane was also noted and coincided with the expression of vinculin and talin. They have proposed that this selective arrangement is related to the mooring of the individual components of the myosensory junction. Towards the end of the equator and in juxtaequatorial and polar regions, vinculin and the other proteins gradually became distributed equally around the intrafusal fibers, a change that paralleled the decreasing number of contacts made by sensory terminals.

In the present study, dystrophin exhibited a similar variation in distribution at the sarcolemma of normal murine intrafusal fibers. The absence of dystrophin at the myosensory junction suggests that similar sarcolemmal attachment modifications occur at these sites.

In several ultrastructural studies of muscle spindles, specialized junctions between the membranes of primary sensory terminals and adjacent intrafusal fibers have been described (Düring and Andres, 1969; Smith and Ovalle, 1972; Kennedy et al., 1975). Their structural resemblance to *fascia adherentes* in cardiac muscle (Kennedy et al., 1975) suggests that these membrane specializations in spindles may serve as mechanical anchoring sites, thereby inhibiting displacement of the apposed nerve terminal and muscle fiber membranes.

In a scanning electron microscopic study (Patten and Ovalle, 1991), the three-dimensional morphology of sensory endings has shown that these focal adhesion points appear as punctate bands that link the closely apposed membranes at irregularly spaced intervals. Kennedy and coworkers (1975, 1980) have proposed that these junctions transmit longitudinally applied shear forces directly to the nerve cell membrane. This distortion of the membrane results in an increase in Na^+ conductance which leads to generation of receptor potentials (Hunt et al., 1987). These junctions may also provide an alternate method of sarcolemmal adhesion to underlying myofibrillar structures since it is known that a peripheral rim of subsarcolemmal myofilaments characterizes the equatorial region of intrafusal fibers (Ovalle, 1972). Although dystrophin is thought to be a critical link between the sarcolemma and the underlying contractile elements of a muscle fiber (Hoffman et al., 1987), its absence under sensory nerve-terminals of spindles may be suggestive of a mechanical adaptation of the intrafusal fiber for its role as a sensory transducer.

Another ultrastructural feature of sensory nerve-terminals on intrafusal fibers is the absence of an intervening basal (external) lamina in the nerve-muscle cleft (Merrillees, 1960; Landon, 1966; Corvaja et al, 1967; Hennig, 1969). This is in contrast to motor nerve-

endings where an external lamina intervenes between the presynaptic terminal and the sarcolemma of both intrafusal (Landon, 1966) and extrafusal (Sanes and Chiu, 1983) fibers.

It has been shown that dystrophin maintains a tight association with integral membrane glycoproteins of 156 and 50 kD in skeletal muscle fibers (Ohlendieck et al., 1991a) and that dystrophin-associated glycoproteins link dystophin to the extracellular matrix (Ibraghimov-Beskrovnaya et al., 1992). The lack of an association of the subsynaptic sarcolemma at the sensory nerve-terminal with an external lamina might somehow be related to the absence of dystrophin at these sites. If incorporation of the glycoproteins is dependent on the presence of an external lamina, then the synaptic sarcolemma would be void of attachment points which may function as 'dystrophin receptors'. The functional role of the dystrophin-glycoprotein complex is not well established but it has been postulated to either stabilize the plasma membrane, maintain a non-uniform distribution of a membrane glycoprotein such as a cell receptor or ion channel, or it could be a link between dystrophin and the extracellular matrix (Campbell and Kahl, 1989).

Intrafusal fibers in Dystrophic Mice

The complete absence of dystrophin in spindles of the mdx mouse indicates that the intrafusal fibers exhibit the same primary defect in muscular dystrophy as seen in the surrounding extrafusal muscle fibers. From a physiological perspective, however, the absence of dystrophin in mdx intrafusal fibers may not be as detrimental as it is for the extrafusal fiber population. Muscle spindles in the dystrophic soleus observed in this study did not exhibit drastic morphological changes from their normal counterparts, and no abnormally-sized or necrotic intrafusal fibers were encountered. It is possible that their small diameters, substantially lower tension-outputs, and capsular investment may decrease the chance of

focal sarcolemmal tearing and subsequent fiber necrosis, the process that is thought to occur primarily in the larger diameter extrafusal fibers (Karpati and Carpenter, 1986).

Light and electron microscopic studies of muscle spindles in other murine dystrophies have revealed similar results. Failure to detect abnormalities in hindlimb spindles of either the dy (Meier, 1969; Yellin, 1974) or the dy^{2J} (James and Meek, 1979; Ovalle and Dow, 1986; Johnson and Ovalle, 1986) mouse, both autosomal recessive in nature, may be indicative of the lack of involvement of the receptors in the disease process. There has been, however, descriptions of atrophy in polar regions of intrafusal fibers and also capsular thickening in equatorial regions of spindles in the 1-year-old dy^{2J} mouse (Ovalle and Dow, 1986). The thickening of the capsule in equatorial regions has been postulated to be an adaptive response of the receptor to sequester the delicate sensory regions of the intrafusal fibers (Ovalle and Dow, 1986).

Sensory signal-transduction in spindles of the mdx mouse at the level of the sensory nerve/intrafusal fiber junctions is not likely to be affected in this animal model for dystrophy for two reasons. First, sensory-endings revealed immunofluorescently in double-labelled sections with the nerve-specific antibody appeared to be unaltered in dystrophy; they terminated in a normal annulospiral fashion similar to that observed in the age-matched control soleus. Secondly, even though dystrophin is absent in dystrophic intrafusal fibers, it is possible that this feature has little bearing on sensory transduction since, in normal muscle, dystrophin is also either significantly reduced or absent in the sarcolemma immediately underlying sensory terminals.

Denervation

The speculation that trophic factors emanating from the nervous system play a role in determining the expression of dystrophin was tested in the denervation experiments of normal mature mice. It was predicted that if trophic factors from sensory nerve terminals were absent, the expression of dystrophin at these sites would increase to a level equivalent to that seen in nonsynaptic regions of the sarcolemma. The results of this experiment revealed that at both 21-day and 42-day post-denervation time periods, absent or very low levels of dystrophin were still persistent in the equatorial region of the intrafusal fibers. From these observations, it was apparent that a neurotrophic influence was not the primary cause of the dystrophin deficiency and that alternate factors were involved.

There is the possibility that post-denervation times may not have been long enough for termination of this neurosecretory process. This, however, is unlikely since it has previously been shown by Barker et al. (1970) that all traces of motor and sensory nerve endings on cat intrafusal fibers disappear 96 hours after muscle nerve-section. In the present study, several morphological changes within the muscle had already occurred which were indicative of denervation. The atrophy of extrafusal fibers and, to a lesser degree, the intrafusal fibers, along with the absence of neurofilament protein expression in nerves and nerve terminals were obvious signs of a significant deprivation of nerve influences. Additionally, capsular thickening in equatorial regions and an overall increase in the cellular components of the endomysial and perimysial tissue were evident in both time periods with a significant increase seen in the 42-day denervated spindles. The changes observed in the overall morphology of muscle spindles emphasize the importance of innervation in the maintenance of spindle structure.

The unaltered immunoreactivity of the dystrophin antibody in both the extrafusal and intrafusal fibers of denervated normal muscle indicates that this protein is not affected by the absence of neural stimulation or neurotrophic factors. Moreover, the extrafusal fibers

continued to show elevated levels of dystrophin at distinct regions of their sarcolemma. These regions closely resembled the intensely-stained motor end-plate regions seen on the extrafusal fibers in the normal soleus and were presumed to be areas of degenerated motor neuromuscular junctions. These observations suggest that innervation does not play a crucial role in the adult in determining the expression of dystrophin in the subsarcolemmal lattice.

CONCLUSIONS AND FUTURE DIRECTIONS

The overall purpose of this study was to investigate more closely the localization of dystrophin in mouse muscle spindles in both normal and diseased states and, then to use this data to help elucidate the possible functions of dystrophin. The results can be summarized as follows:

1. In normal mouse skeletal muscle, dystrophin was found in all regions of the sarcolemma of intrafusal fibers except in those areas in contact with sensory nerve terminals.

2. In mdx-dystrophic muscle, the intrafusal fibers were deficient in dystrophin throughout their lengths, even though sensory innervation patterns appeared normal.

3. Chronic denervation of the normal mouse soleus did not significantly affect the expression or distribution of dystrophin in the intrafusal fibers of muscle spindles.

From these results several conclusions can be drawn regarding the function of dystrophin. First, the sensory innervation to an intrafusal fiber specifically affects the subsarcolemmal distribution of dystrophin. The exact reason why this occurs remains unclear, however, evidence from the chronically denervated spindles in this study indicates

that a neuromodulatory role in the form of a hormone or a chemical transmitter is unlikely. A more plausible explanation for this occurrence would be the unique ultrastructure of the sensory nerve/muscle junction itself. In contrast to motor endings where a basal lamina exists in the nerve-muscle cleft, sensory endings lack an intervening basal lamina. At these sites, the basal lamina of the intrafusal fiber is continuous with the external surface of the sensory nerve terminal. This feature of the sensory nerve/muscle junction suggests that dystrophin expression is dependent on the presence of a basal lamina. It is possible that components of the basal lamina are required for the mechanical linking of dystrophin via the integral membrane proteins to the extracellular matrix. The functional role for dystrophin would, therefore, predict one that is mechanical in nature.

Secondly, it can also be concluded that the sensory synaptic domains of the sarcolemma in equatorial regions of intrafusal fibers are structurally similar with respect to dystrophin expression in both normal and mdx muscle tissue, and, that the presence of dystrophin in these zones is not required for normal sensory nerve/muscle fiber interaction. This, however, does not eliminate the possibility of dystrophin-like proteins assuming the role of dystrophin at these sites. Further studies, involving high resolution microscopic techniques coupled with immunogold labelling may provide a better understanding of the exact localization of dystrophin at sensory junctions in muscle spindles. In addition, immunolocalization of other cytoskeletal components, such as cell adhesion proteins and dystrophin-associated and dystrophin-related proteins, would be beneficial for comparing and contrasting differences that exist between synaptic and nonsynaptic membranes in skeletal muscle.

LITERATURE CITED

Adal, M.N. 1969 The fine structure of the sensory region of cat muscle spindles. Ultrastruct. Res., *26*: 332-354.

Anderson, J.E., W.K. Ovalle, and B.H. Bressler 1987 Electron microscopic and autoradiographic characterization of hindlimb muscle regeneration in the mdx mouse. Anat. Rec., *219*: 243-257.

Anderson, J.E., B.H. Bressler, and W.K. Ovalle 1988 Functional regeneration in the hindlimb skeletal muscle of the mdx mouse. J. Mus. Res. Cell Mot., *9*: 499-515.

Bakker, G.J., and F.J.R. Richmond 1981 Two types of muscle spindles in cat neck muscles: a histochemical study of intrafusal fiber composition. J. Neurophysiol., *45*: 973-986.

Banks, R.W. 1986 Observations on the primary sensory ending of tenuissimus muscle spindles in the cat. Cell Tissue Res., *246*: 309-319.

Banks, R.W., D. Barker, and M.J. Stacey 1982 Form and distribution of sensory terminals in cat hindlimb muscle spindles. Philos. Trans. R. Soc. Lond. (Biol.), *299*: 329-364.

Banks, R.W., D.W. Harker, and M.J. Stacey 1977 A study of mammalian intrafusal muscle fibres using a combined histochemical and ultrastructural technique. J. Anat., *123*: 783-796.

Barker, D. 1948 The innervation of the muscle spindle. Q. J. Micr. Sc., *89*: 143-186.

Barker, D. 1974 The morphology of muscle receptors. In: Muscle Receptors. Handbook of Sensory Physiology. C.C. Hunt, ed. Springer-Verlag, Berlin, Vol. 3, Pt. 2, pp. 1-190.

Barker, D., and R.W. Banks 1986 The muscle spindle. In: Myology. A.G. Engel and B.Q. Banker, eds. McGraw-Hill, New York, Vol. 1, pp. 309-341.

Barker, D., and M.C. Ip 1963 A silver method for demonstrating the innervation of mammalian muscle in teased preparations. J. Physiol., *169*: 73-82.

Barker, D., M.J. Stacey, and M.N. Adal 1970 Fusimotor innervation in the cat. Philos. Trans. R. Soc. Lond. (Biol.), *258*: 315-346.

Baumbach, L.L., J.S. Chamberlain, P.A. Ward, N.J. Farwell, C.T. Caskey 1989 Molecular and clinical correlations of deletions leading to Duchenne and Becker muscular dystrophies. Neurology, *39*: 465-474.

Becker, P.E. 1964 Myopathien. In: Becker, P.E. (ed.) Humangenetik. Ein kurzes Handbuch, vol. 3. Georg Thieme, Stuttgart.

Becker, P.E. and F. Kiener 1955 Eine neue X-chromosomale Muskeldystrophie. Archiv fur Psychiatrie und Nervenkrankheiten, *193*: 427-48.

Bonilla, E., C.E. Samitt, A.F. Miranda, A.P. Hays, G. Salviati, S. DiMauro, L.M. Kunkel, E.P. Hoffman, and L.P. Rowland 1988 Duchenne muscular dystrophy: deficiency of dystrophin at the muscle cell surface. Cell, *54*: 447-452.

Boyd, I.A. 1962 The structure and innervation of the nuclear bag muscle fibre system and the nuclear chain muscle fibre system in mammalian muscle spindles. Phil. Trans. B., *245:* 81-136.

Boyd, I.A. 1981 The muscle spindle controversy. Sci. Prog. Oxf., *67*: 205-221.

Boyd, I.A., M.H. Gladden, P.N. McWilliam, and J. Ward 1975 "Static" and "dynamic" nuclear bag fibres in isolated cat muscle spindles. J. Physiol., *250*: 11-53.

Boyd, I.A., and R.S. Smith 1984 The muscle spindle. In: Peripheral Neuropathy. P.J. Dyck, P.K. Thomas, E.H. Lambert, and R. Bunge, eds. W.B. Saunders, Philadelphia, Vol. 1, pp. 171-202.

Bressler, B.H., L.G. Jasch, W.K. Ovalle, and C.E. Slonecker 1983 Changes in isometric contractile properties of fast-twitch and slow-twitch skeletal muscle of C57BL/6J dy^{2J}/dy^{2J} dystrophic mice during postnatal development. Exp. Neurol., *80*: 457-480.

Brooke, M.H., G.M. Fenichel, and R.C. Griggs 1989 Duchenne muscular dystrophy: patterns of clinical progression and effects of supportive therapy. Neurology, *39*: 475-481.

Brzezinski, D.K. von 1961a Untersuchungen zur Histochemie der Muskelspindeln. I. Mitteilung: Topochemie der Polysaccharide. Acta Histochem., *12*: 75-79.

Brzezinski, D.K. von 1961b Untersuchungen zur Histochemie der Muskelspindeln. II. Mitteilung: Zur Topochemie und Function des Spindelraulmes und der Spindelkapsel. Acta Histochem., *12*: 277-288.

Bulfield, B., B.G. Siller, P.A. Wight, and K.J. Moore 1984 X chromosome-linked muscular dystrophy (mdx) in the mouse. Proc. Natl. Acad. Sci. USA, *81*: 1189-1192.

Burghes, A.H., C. Logan, X. Hu, B. Belfall, R.G. Worton, and P.N. Ray 1987 A cDNA clone from the Duchenne/Becker muscular dystrophy gene. Nature, *328*: 434-437.

Byers, T.J., L.M. Kunkel, and S.D. Watkins 1991 The subcellular distribution of dystrophin in mouse skeletal, cardiac, and smooth muscle. J. Cell Biol., *115:* 411-421.

Cajal, S.R. 1888 Terminaciones nerviosas en los husos musculares de la rana. Riv. Trim. Histol. Norm. y Patol., 1.

Campbell, K.P., and S.D. Kahl 1989 Association of dystrophin and an integral membrane glycoprotein. Nature (Lond.), *338*: 259-262.

Carnwath, J.W., and D.M. Shotton 1987 Muscular dystrophy in the mdx mouse: histopathology of the soleus and extensor digitorum longus muscles. J. Neurol. Sci., *80*: 39-54.

Carpenter, S., G. Karpati, E. Zubrzycka-Gaarn, D.E. Bulman, P.N. ray, and R.G. Worton 1990 Dystrophin is localized to the plasma membrane of human skeletal muscle fibers by electron-microscopic cytochemical study. Muscle Nerve, *13*: 376-380.

Cazzato, G., and J.N. Walton 1968 The pathology of the muscle spindle: a study of biopsied material in various muscular and neuromuscular diseases. J. Neurol. Sci., *7*: 15-70.

Coons. A.H., and M.H. Kaplan 1950 Localization of antigens in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J. Exp. Med., *91*: 1-13.

Cooper, S., and P.M. Daniel 1963 Muscle spindles in man: their morphology in the lumbricals and deep muscles of the neck. Brain, *86*: 563-586.

Cooper, S., and M.H. Gladden 1974 Elastic fibres and reticulin of mammalian muscle spindles and their functional significance. Q. J. Exp. Physiol., *59*: 367-385.

Corvaja, N., V. Marinozzi, and O. Pompeiano 1969 Muscle spindles in the lumbrical muscles of the adult cat. Electron microscopic observation and functional considerations. Arch. Ital. Biol. *107*: 365-543.

Coulton, G.R., J.E. Morgan, T.A. Partridge, and J.C. Sloper 1988 The mdx mouse skeletal muscle myopathy: 1. A histological, morphometric and biochemical investigation. Neuropath. Appl. Neurobiol., *14*: 53-70.

Croft, P.B., R.A. Henson, H. Urich, and M. Wilkinson 1965 Sensory neuropathy with bronchial carcinoma: a study of four cases showing serological abnormalities. Brain, *88*: 501-522.

Cullen, M.J., J. Walsh, L.V.B. Nicholson, and J.B. Harris 1990 Ultrastructural localization of dystrophin in human muscle by using gold immunolabelling. Proc. R. Soc. Lond. B., *240:* 197-210.

DeReuck, J. 1974 The pathology of the human muscle spindle: a light microscopic, biometric and histochemical study. Acta Neuropath. (Berl.), *30*: 43-55.

Dow, P.R., S.L. Shinn, and W.K. Ovalle 1980 Ultrastructural study of a blood-muscle spindle barrier after sytemic administration of horseradish peroxidase. Am. J. Anat., *157*: 375-388.

Duchenne, G.B.A. 1868 Recherches sur la paralysie musculaire pseudohypertrophique ou paralysie myo-sclerosique. Arch. Gen. Med., *11*: 5-25; 179-209; 305-321; 421-443; 552-588.

Düring, M., and K.H. Andres 1969 Zur feinstruktur der muskelspindel von mammilia. Anat. Anz., *124*: 566-573.

Ervasti, L.M., K. Ohlendieck, S.D. Kahl, M.G. Gaver, and K.P. Campbell 1990 Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. Nature, *345*: 315-319.

Fukami, Y. 1982 Further morphological and electrophysiological studies on snake muscle spindles. J. Neurophysiol., *47*: 810-826.

Fukami, Y. 1986 Studies of capsule and capsular space of cat muscle spindles. J. Physiol. (Lond.), *376*: 281-297.

Geiger, B., and D. Ginsberg 1991 The cytoplasmic domain of adherens-type junctions. Cell Motil. Cytoskeleton, *20*: 1-6.

Gladden, M.H. 1970 A modified pyridine-silver stain for teased preparation of motor and sensory nerve endings in skeletal muscle. Stain Technol., *45*: 161-164.

Gladden, M.H. 1976 Structural features relative to the function of intrafusal muscle fibers in the cat. Progr. Brain Res., *44*: 51-67.

Hammond, R.G. 1987 Protein sequence of DMD gene is related to actin-binding domain of alpha-actinin. Cell, *51*: 1-9.

Harker, D.W., L. Jami, Y. Laporte, and J. Petit 1977 Fast conducting skeletofusimotor axons supplying intrafusal chain fibers in the cat peroneus-tertius muscle. J. Neurophysiol., *40*: 791-799.

Hassall, A.H. 1849 The Microscopic Anatomy of the Human Body. Vol. I. Samuel Highley, London.

Heilig, R., C. Lemaire, and J.L. Mandel 1987 A 230 kb cosmid walk in the Duchenne muscular dystrophy gene: detection of a conserved sequence and of a possible deletion prone region. Nucl. Acids Res., *15*: 9129-9142.

Hennig, G. 1969 Die Nervenendigungen der rattenspindel im elektronenmikroskopischen bild. Z. Zellforsch, *96*: 275-294.

Hoffman, E.P., R.H. Brown, Jr., and L.M. Kunkel 1987 Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell, *51*: 919-928.

Hoffman, E.P., S.C. Watkins, H.S. Slayter, and L.M. Kunkel 1989 Detection of a specific isoform of alpha-actinin with antisera directed against dystrophin. J. Cell Biol., *108*: 503-510.

Hsu, S.M., L. Raine, and H. Fanger 1981a A comparative study of peroxidase-antiperoxidase method and an avidin-biotin-complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am. J. Clin. Pathol., *74*: 32-40.

Hsu, S.M., L. Raine, and H. Fanger 1981b The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. A comparison between ABC and unlabelled antibody PAP procedures. J. Histochem. Cytochem., *29*: 577-580.

Huard, J., L.P. Fortier, C. Labrecque, G. Dansereau, and J.P. Tremblay 1991 Is dystrophin present in the nerve terminal at the neuromuscular junction? An immunohistochemical study of the heterozygote dystrophic (mdx) mouse. Synapse, *7*: 135-140.

Huard, J., L.P. Fortier, G. Dansereau, C. Labrecque, and J.P. Tremblay 1992 A light and electron microscopic study of dystrophin localization at the mouse neuromuscular junction. Synapse, *10*: 83-93.

Hunt, C.C. 1974 The physiology of muscle receptors. In: Muscle Receptors. Handbook of Sensory Physiology. C.C. Hunt, ed. Springer-Verlag, Berlin, Vol. 3, Pt. 2, pp. 191-234.

Hunt, C.C., R.S. Wilkinson, and Y. Fukami 1978 Ionic basis of the receptor potential in primary endings of mammalian muscle spindles. J. Gen. Physiol., *71*: 683-698.

Ibraghimov-Beskrovnaya, O., J.M. Ervasti, C.J. Leveille, C.A. Slaughter, S.W. Sernett, and K.P. Campbell 1992 Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. Nature (Lond.), *355*: 696-702.

Jasch, L.G., B.H. Bressler, W.K. Ovalle, and C.E. Slonecker 1982 Abnormal distribution of proteins in the soleus and extensor digitorum longus of dystrophic mice. Muscle Nerve, *5*: 462-470.

James, N.T. 1971 The histochemical demonstration of mucopolysaccharide in the lymph space of muscle spindles. J. Anat., *110*: 163.

James, N.T., and G.A. Meek 1979 Ultrastructure of muscle spindles in C57Bl/6J dy^{2J}/dy^{2J} dystrophic mice. Experentia, *35*: 108-109.

Johnson, M.I., and W.K. Ovalle 1986 A comparative study of muscle spindles in slow and fast neonatal muscles of normal and dystrophic mice. Am. J. Anat., *175*: 413-427.

Karpati, G., and S. Carpenter 1988 Small-caliber skeletal muscle fibers do not suffer deleterious consequences of dystrophic gene expression. Am. J. Med. Gen., *25*: 653-658.

Karpati, G., E. Zubrzycka, S. Carpenter, D.E. Bulman, P.N. Ray, and R.G. Worton 1990 Age related conversion of dystrophin negative to positive fiber segments of skeletal but not cardiac muscle fibers in heterozygote mdx mice. J. Neuropathol. Exp. Neurol., *49*: 96-105.

Kennedy, W.R., J. deF. Webster, and K.S. Yoon 1975 Human muscle spindles: fine structure of the primary sensory ending. J. Neurocytol., *4*: 675-695.

Kennedy, W.R., R.E. Poppele, and D.C. Quick 1980 Mammalian muscle spindles. In: The Physiology of Peripheral Nerve Diseases. A.J. Sumner, ed. Saunders, Philadelphia, pp. 74-133.

Khan, M.A., and T. Soukup 1979 Histoenzymatic study of rat intrafusal muscle fibers. Histochemistry, *62*: 179-189.

Koenig, M., E.P. Hoffman, C.J. Bertelson, A.P. Monaco, C. Feener, and L.M. Kunkel 1987 Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell, *50*: 509-517.

Koenig, M., A.P. Monaco, and L.M. Kunkel 1988 The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. Cell, *53*: 219-228.

Koenig, M., A.H. Beggs, and L. Moyer 1989 The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. Am. J. Hum. Genet., *45*: 498-506.

te Kronnie, G., Y. Donselaar, T. Soukup, and W. van Raamsdonk 1981 Immunohistochemical differences in myosin composition among intrafusal muscle fibers. Histochemistry, *73*: 65-74.

Kucera, J. 1980 Histochemical study of long nuclear chain fibers in the cat muscle spindle. Anat. Rec., *198*: 567-580.

Kucera, J. 1981 Histochemical profiles of cat intrafusal muscle fibers and their motor innervation. Histochemistry, *73*: 397-418.

Kucera, J., and K. Dorovini-Zis 1979 Types of human intrafusal muscle fibers. Muscle Nerve, *2*: 437-451.

Kucera, J., and J.M. Walro 1989 Nonuniform expression of myosin heavy chain isoforms along the length of cat intrafusal muscle fibers. Histochemistry, *92*: 291-299.

Kühne, W. 1864 Uber die Endigung der Nerven in den Nervenhügeln der Muskeln. Virchows Arch. Path. Anat., *30*: 187-220.

Kunkel, L.M., A.P. Monaco, W. Middlesworth, H.D. Ochs, and S.A. Latt 1985 Specific cloning of DNA fragments absent from the DNA of a male patient with an X chromosome deletion. Proc. Natl. Acad. Sci. (USA), *82*: 4778-82.

Landon, D.N. 1966 Electron microscopy of muscle spindles. In: Control and Innervation of Skeletal Muscle. B.L. Andrew, ed. Livingstone, Edinburgh, pp. 96-111.

Landon, D.N. 1972 The fine structure of developing muscle spindles in the rat. J. Anat., *111*: 512-513.

Lapresle, J., and M. Milhaud 1964 Pathologie du fuseau neuromusculaire. Rev. Neurol., *110*: 97-122.

Leksell, L. 1945 The action potential and excitatory effects of the small ventral root fibers to skeletal muscle. Acta Physiol. Scand. (Suppl.), *10*: 1-84.

Lidov, H.G.W., T.J. Byers, S.C. Watkins, and L.M. Kunkel 1990 Localization of dystrophin to postsynaptic regions of central nervous system cortical neurons. Nature, *348*: 725-727.

Maier, A., B. Gambke, and D. Pette 1988 Immunohistochemical demonstration of embryonic myosin heavy chains in adult mammalian intrafusal fibers. Histochemistry, *88*: 267-271.

Maier, A., and R. Zak 1990 Arrangement of cytoskeletal filaments at the equator of chicken intrafusal muscle fibres. Histochemistry, *93*: 423-428.

Maier, A, and R. Mayne 1993 Regional differences in organization of the extracellular matrix and cytoskeleton at the equator of chicken intrafusal muscle fibers. J. Mus. Res. Cell Mot., *14*: 35-46.

Matthews, P.B.C. 1962 The differentiation of two types of fusimotor fibre by their effects on the dynamic response of muscle spindle primary endings. Q. J. Exp. Physiol., *47*: 324-333.

Matsumara, K., J.M. Ervasti, K. Ohlendieck, S.D. Kahl, and K.P. Campbell 1992 Association of dystrophin-related protein with dystrophin-associated proteins in mdx mouse muscle. Nature, *360*: 588-591.

Merrillees, N.C.R. 1960 The fine structure of muscle spindles in the lumbrical muscles of the rat. J. Biophys. Biochem. Cytol., *7*: 725-742.

Meryon, E. 1852 On granular and fatty degeneration of the voluntary muscles. Med. Chir. Trans., *35*: 73-84.

Miike, T., M. Miyatake, J. Zhao, K. Yoshioka, and M. Uchino 1989 Immunohistochemical dystrophin reaction in synaptic regions. Brain Dev., *11*: 344-366.

Milburn, A. 1973 The early development of muscle spindles in the rat. J. Cell Sci., *12*: 175-195.

Monaco, A.P., R.L. Neve, C. Colletti-Feener, C.J. Bertelson, D.M. Kurnit, andL.M. Kunkel 1986 Isolation of candidate cDNAs for portions of the Duchenne muscular dystrophy gene. Nature, *323:* 646-650.

Nahirney, P.C., and W.K. Ovalle 1992a Immunocytochemical localization of dystrophin and neurofilament protein in muscle spindles of normal and dystrophic mice. Anat. Rec., *232*: 64A-65A.

Nahirney, P.C., and W.K. Ovalle 1992b The expression of dystrophin in intrafusal muscle fibers of normal and mdx mice. Proc. Can. Fed. Biol. Soc., *35*: 54A.

Nahirney, P.C., and W.K. Ovalle 1993 Distribution of dystrophin and neurofilament protein in muscle spindles of normal and mdx-dystrophic mice: an immunocytochemical study. Anat. Rec., *235*: 501-510.

Namba, T., T. Nakamura, and D. Grob 1967 Staining for nerve fiber and acetylcholinesterase activity in fresh frozen sections. Am. J. Clin. Path., *47*: 74-77.

North, A.J., B. Galazkiewicz, T.J. Byers, J.R. Glenney, Jr., and J.V. Small 1993 Complementary distributions of vinculin and dystrophin define two distinct sarcolemma domains in smooth muscle. J. Cell Biol., *120*: 1159-1167.

Ohlendieck, K., and K.P. Campbell 1991 Dystrophin-associated proteins are greatly reduced in skeletal muscles from mdx mice. J. Cell Biol., *115*: 1685-1694.

Ohlendieck, K., J.M. Ervasti, J.B. Snook, and K.P. Campbell 1991a Dystrophin-glycoprotein complex is highly enriched in isolated skeletal muscle sarcolemma. J. Cell Biol., *112*: 135-148.

Ohlendieck, K., J.M. Ervasti, K. Matsumura, S.D. Kahl, C.J. Leveille, and K.P. Campbell 1991b Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. Neuron, *7*: 499-506.

Onanoff, M.I. 1890 Sur la nature des faisceux neuromusculaires. C.R. Seanc. Soc. Biol., *42*: 432-433.

Ovalle, W.K. 1972 Fine structure of rat intrafusal muscle fibers. The equatorial region. J. Cell Biol., *52*: 382-396.

Ovalle, W.K., B.H. Bressler, L.G. Jasch, and C.E. Slonecker 1983 Abnormal distribution of fiber types in the slow-twitch soleus muscle of the C57Bl/dy^{2J}/dy^{2J} dystrophic mouse during postnatal development. Am. J. Anat., *168*: 291-304.

Ovalle, W.K., and P.R. Dow 1983 Comparative ultrastructure of the inner capsule of the muscle spindle and the tendon organ. Am. J. Anat., *166*:343-357.

Ovalle, W.K., and P.R. Dow 1986 Alterations in muscle spindle morphology in advanced stages of murine muscular dystrophy. Anat. Rec., *216*: 111-126.

Ovalle, W.K., and P.R. Dow 1988 The capsular sleeve of muscle spindles in mouse and man with special reference to the cytoskeleton. In: Mechanoreceptors. Development, Structure and Function. P. Hnik, T. Soukup, R. Vejsada, and J. Zelena, eds. Plenum, New York, pp. 255-261.

Ovalle, W.K., and R.S. Smith 1972 Histochemical identification of three types of intrafusal muscle fibers in the cat and monkey based on the myosin ATPase reaction. Can. J. Physiol. Pharm., *50*: 195-202.

Patel, A.N., V.S. Lalitha, and D.K. Dastur 1968 The spindle in normal and pathological muscle; an assessment of the histological changes. Brain, *91*: 737-750.

Patten, R.M., W.K. Ovalle 1991 Muscle spindle ultrastructure revealed by conventional and high-resolution scanning electron microscopy. Anat. Rec., *230*: 183-198.

Pierobon Bormioli, S., S. Sartore, M. Vitadello, and S. Schiaffino 1980 Slow myosins in vertebrate skeletal muscle. An immunofluorescence study. J. Cell Biol., *85*: 672-681.

Pons, F., N. Augier, J.O.C. Léger, A. Robert, F.M.S. Tomé, M. Fardeau, T Voit, L.V.B. Nicholson, D. Mornet, and J.J. Léger 1991 A homologue of dystrophin is expressed at the neuromuscular junctions of normal individuals and DMD patients, and of normal and mdx mice. FEBS (Fed. Eur. Biochem. Soc.) Lett., *282*: 161-165.

Roden, R.L., S.P. Donahue, G.A. Schwartz, J.G. Wood, and A.W. English 1991 200 kD neurofilament protein and synapse elimination in the rat soleus muscle. Synapse, *9*: 239-243.

Rowlerson, A., L. Gorza, and S. Schiaffino 1985 Immunohistochemical identification of spindle fibre types in mammalian muscle using type-specific antibodies to isoforms of myosin. In: The Muscle Spindle. I.A. Boyd and M.H. Gladden, eds. Macmillan, London, pp. 29-34.

Ruffini, A. 1898 On the minute anatomy of the neuromuscular spindles of the cat, and on their physiological significance. J. Physiol. (Lond.), *23*: 190-208.

Samitt, C.E., and E. Bonilla 1990 Immunocytochemical study of dystrophin at the myotendinous junction. Muscle Nerve, *13*: 493-500.

Sanes, J.R., and A.Y. Chiu 1983 The basal lamina of the neuromuscular junction. Cold Spring Harbor Symp. Quant. Biol., *48*: 667-678.

Sartore, S., L. Gorza, and S. Schiaffino 1982 Fetal myosin heavy chains in regenerating muscle. Nature, *298*: 294-296.

Schröder, J.M. 1974a The fine structure of de- and reinnervated muscle spindles. I. The increase, atrophy, and hypertrophy of intrafusal muscle fibers. Acta Neuropathol. (Berl.), *30*: 109-128.

Schröder, J.M. 1974b The fine structure of de- and reinnervated muscle spindles. II. Regenerated sensory and motor nerve terminals. Acta Neuropathol. (Berl.), *30*: 129-144.

Schröder, J.M., P.T. Kemme, and L. Scholz 1979 The fine structure of denervated and reinnervated muscle spindles: Morphometric study of intrafusal muscle fibers. Acta Neuropathol. (Berl.), *46*: 95-106.

Sherrington, C.S. 1894 On the anatomical constitution of nerves of skeletal muscles; with remarks on recurrent fibres in the ventral spinal nerve-root. J. Physiol. (Lond.), *17*: 211-258.

Shimuzu, T., K. Matsumura, Y. Sunada, and T. Mannen 1989 Dense immunostainings on both neuromuscular and myotendon junctions with an anti-dystrophin monoclonal antibody. Biomed. Res., *10*: 405-409.

Sicinski, P., Y. Geng, A.S. Ryder-Cook, E.A. Barnard, M.G. Darlison, and P.J. Barnard 1989 The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science, *244*: 1578-1580.

Smith, R.S., and W.K. Ovalle 1972 The structure and function of intrafusal muscle fibers. In: Progress in Muscle Biology. R.G. Cassens, ed. Marcel Dekker, New York, Vol. 1, pp. 147-227.

Soukup, T. 1976 Intrafusal fiber types in rat limb muscle spindles. Morphological and histochemical characteristics. Histochemistry, *47*: 43-57.

Squarzoni, S., P. Sabatelli, M.C. Maltarello, A. Cataldi, R. DiPrimio, and N.M. Maraldi 1992 Localization of dystrophin COOH-terminal domain by the fracture-label technique. J. Cell Biol., *118*: 1401-1410.

Staron, R.S., and D. Pette 1986 Correlation between myofibrillar ATPase activity and myosin heavy chain composition in rabbit muscle fibers. Histochemistry, *86*: 19-23.

Sugita, H., K. Arahata, T. Ishiguro, Y. Suhara, T. Tsukahara, S. Ishiura, C.H. Eguchi, I. Nonaka, and E. Ozawa 1988 Negative immunostaining of Duchenne muscle dystrophy (DMD) and mdx muscle surface membrane with antibody against synthetic peptide fragment predicted from DMD cDNA. Proc. Jpn. Acad., *69*: 37-39.

Swash, M., and K.P. Fox 1974 The pathology of the muscle spindle; effect of denervation. J. Neurol. Sci., *22*: 1-24.

Swash, M., and K.P. Fox 1976 The pathology of the muscle spindle in Duchenne muscular dystrophy. J. Neurol. Sci., *29*: 17-32.

Tanaka, H., K. Ikeya, and E. Ozawa 1990 Difference in the expression pattern of dystrophin on the surface membrane between the skeletal and cardiac muscles of mdx carrier mice. Histochemistry, *93*: 447-452.

Tinsley, J.M., D.J. Blake, A. Roche, U. Fairbrother, J. Riss, B.C. Byth, A.E. Knight, J. Kendrick-Jones, G.K. Suthers, D.R. Love, Y.H. Edwards, and K.E. Davies 1992 Primary structure of dystrophin-related protein. Nature, *360*: 591-593.

Torres, L.B.F. and L.W. Duchen 1987 The mutant mdx: inherited myopathy in the mouse. Morphological studies of nerves, muscles and endplates. Brain, *110*: 269-299.

Tower, S. 1932 Atrophy and degeneration in the muscle spindle. Brain, *55*: 77-90.

Tower, S. 1939 The reaction of muscle to denervation. Physiol. Rev., *19*: 1-48.

Vandesande, G. 1983 Immunohistochemical double staining techniques. In: Immunohistochemistry. A.C. Cuello, ed. Wiley & Sons, Chichester, pp. 257-272.

Watkins, S.C., E.P. Hoffman, H.S. Slayter, and L.M. Kunkel 1988 Immunoelectron microscopic localization of dystrophin in myofibres. Nature (Lond.), *333*: 863-866.

Wirtz, P., H.M. Loermans, P.G. Peer, and A.G. Reintjes 1983 Postnatal growth and differentiation of muscle fibers in the mouse: II. A histochemical and morphometrical investigation of dystrophic muscle. J. Anat., *137*: 127-142.

Worton, R.G. 1992 Duchenne muscular dystrophy: gene and gene product; mechanism of mutation in the gene. J. Inher. Metab. Dis., *15*: 539-550.

Yellin, H. 1969 A histochemical study of muscle spindles and their relationship to extrafusal fiber types in the rat. Am. J. Anat., *125*: 31-46.

Zelená, J. 1957 The morphogenetic influence of innervation on the ontogenetic development of muscle spindles. J. Embryol. Exp. Morphol., *5*: 183-292.

Zhao, J., K Yoshioka, M. Miyatake, and T. Miike 1992 Dystrophin and a dystrophin-related protein in intrafusal muscle fibers, and neuromuscular and myotendinous junctions. Acta Neuropathol., *84*: 141-146.

Zubrzycka-Gaarn, E.E., D.E. Bulman, G. Karpati, A.H.M. Burghes, B. Belfall, H.J. Klamut, J. Talbot, R.S. Hodges, P.N. Ray, and R.G. Worton 1988 The Duchenne muscular dystrophy gene product is localized in sarcolemma of human skeletal muscle. Nature (Lond.), *333*: 466-496.