

Expression of utrophin and its mRNA in denervated *mdx* mouse muscle

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Abstract Utrophin is a large cytoskeletal protein which shows high homology to dystrophin. In contrast to the sarcolemmal distribution of dystrophin, utrophin accumulates at the postsynaptic membrane of the neuromuscular junction. Because of its localization within this compartment of muscle fibers, expression of utrophin may be significantly influenced by the presence of the motor nerve. We tested this hypothesis by denervating muscles of *mdx* mouse and monitoring levels of utrophin and its mRNA by immunofluorescence, immunoblotting and RT-PCR. A significant increase in the number of utrophin positive fibers was observed by immunofluorescence 3 to 21 days after sectioning of the sciatic nerve. Quantitative analyses of utrophin and its transcripts in hindlimb muscles denervated for two weeks showed only a moderate increase in the levels of both utrophin (~2-fold) and its transcript (~60 to 90%). The present data suggest that although utrophin is a component of the postsynaptic membrane, its neural regulation is distinct from that of the acetylcholine receptor.

Key words: Synapse; Neuromuscular junction; Acetylcholine receptor; Regulation; Cytoskeleton

1. Introduction

The most severe and prevalent primary myopathy is the Duchenne form of muscular dystrophy (DMD) [1]. The genetic defect responsible for this disease is located on the short arm of the X chromosome and prevents the production of normal size dystrophin, a large cytoskeletal protein of 427 kDa (for review see refs. [2,3]). In 1989, Love et al. [4] showed the existence of an autosomal homologue to dystrophin subsequently named utrophin since it is expressed in a variety of tissues ([5,6], and see for review [7]). Further studies revealed that this gene encodes a large cytoskeletal protein highly homologous to dystrophin in its C-terminus [8] and that it presents a genomic organization similar to the structure of the dystrophin gene [9,10]. The utrophin gene is located on chromosome 6 in human and 10 in mouse [4,11].

In contrast to dystrophin which is found on the cytoplasmic face of the sarcolemma in normal muscles [12,13,14], utrophin accumulates specifically at the levels of the neuromuscular synapse and myotendinous junction in both normal and dystrophic adult muscles [15–24]. Additional studies have shown that utrophin is present in greater amounts in small caliber muscle fibers of *mdx* mice [18,25], small or regenerating muscle fibers of

DMD patients [19,26–29] as well as in embryonic and neo-natal muscles ([5,18,19,22,30,31], see also [27]). Recently, it was also reported that some muscles of *mdx* mice may contain significantly more utrophin in comparison to normal mouse muscles [32].

Although the distribution of utrophin in normal and dystrophic muscles appears well established, little is known concerning the cellular and molecular mechanisms involved in its regulation. Given the co-distribution of utrophin with the acetylcholine receptor (AChR) at the neuromuscular junction and the well-documented increase in AChR expression in denervated muscles [33–35], it may be hypothesized that expression of utrophin is significantly influenced by the presence of the motor nerve. A first indication that this may be the case was provided by Takemitsu et al. [18] who showed that denervation of *mdx* mouse muscle appears to increase expression of utrophin to a level similar to that observed in embryonic muscle fibers. However, in the latter study, the impact of muscle denervation was judged strictly by immunofluorescence experiments for which no quantitative estimates of the extent of reexpression was provided. Therefore, the purpose of the present study was to examine the expression of utrophin in denervated *mdx* mouse muscles by immunofluorescence and to quantify the impact of denervation by immunoblot analysis. In addition, we wished to gain insight into the mechanisms underlying the denervation-associated increase in utrophin expression by quantifying levels of mRNA encoding utrophin in denervated muscles.

2. Materials and methods

2.1. Antibodies

Rabbit anti-*Torpedo* dystrophin was produced in our laboratory (Cartaud et al., 1992). The rabbit anti-utrophin antibody was a kind gift of Dr. T. Khurana, Harvard University [5].

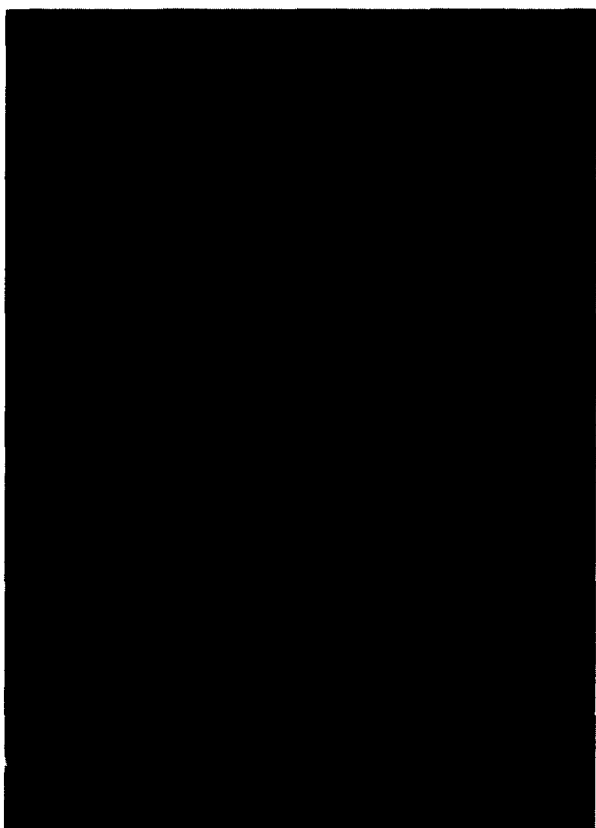
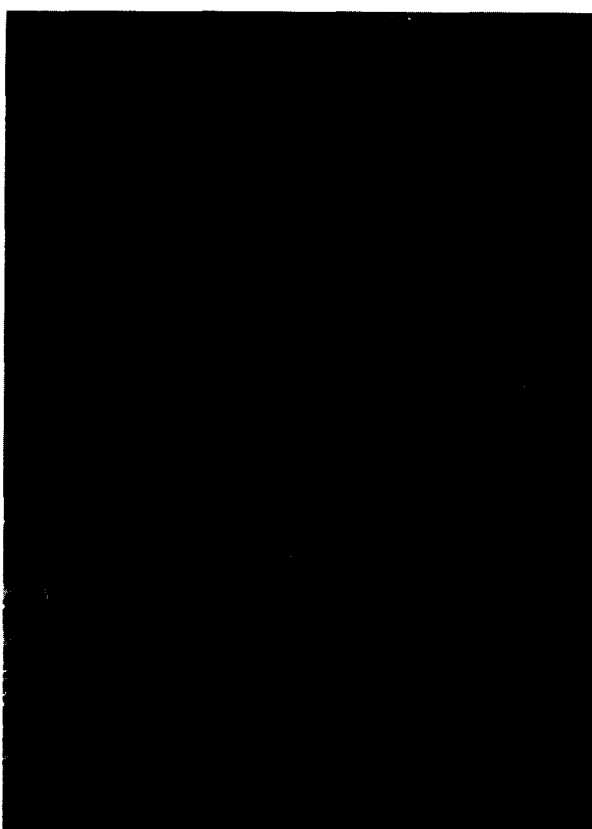
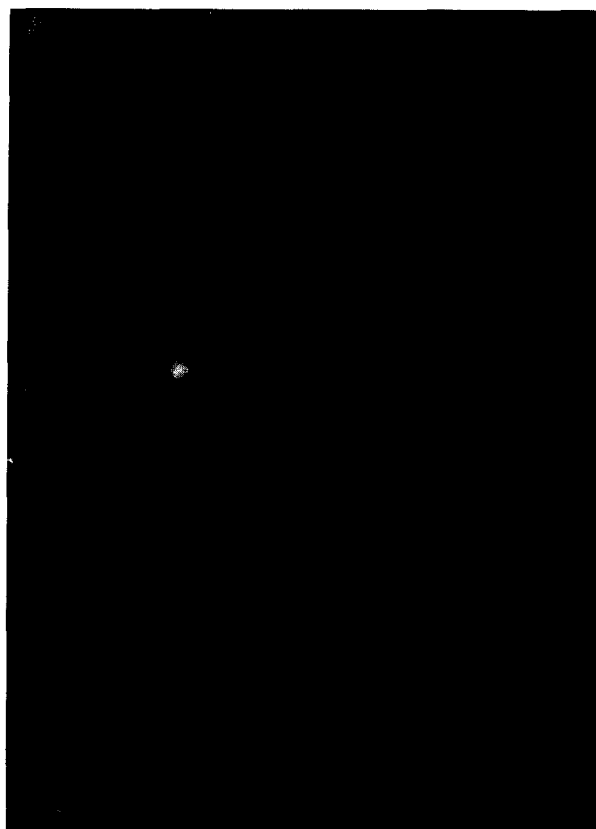
2.2. Denervation

Ten-week-old *mdx* mice were anesthetized with chloral hydrate (3.5%, 3 ml/kg of body weight, i.p.) or sodium pentobarbital (35 mg/kg of body weight, i.p.). An incision was made at the mid-thigh region and the sciatic nerve exposed by blunt dissection of the underlying musculature. A mid-point axotomy was made and a ~1 cm segment of the nerve was removed to prevent reinnervation. At varying time-points thereafter (1, 3, 5, 7, 14, 30, 45, 60 and 90 days), animals were euthanized, and both denervated and contralateral gastrocnemius muscles were removed. Muscles were snap frozen in melting isopentane pre-cooled with liquid nitrogen. Samples were kept at –80°C until used.

2.3. Immunocytochemistry

Antibodies to dystrophin (1/100) and utrophin (1/1000) were diluted in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA). These antibodies were applied onto separate serial cross-

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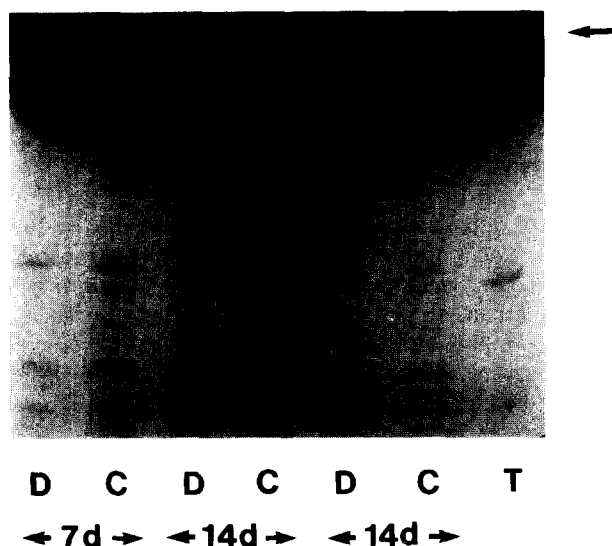


Fig. 2. Immunoblot analysis of utrophin levels in control and denervated hindlimb muscles of *mdx* mice. Utrophin was detected by chemiluminescence following incubation of the upper part of the immunoblot which corresponds to high molecular weight proteins (>200 kDa), with anti-utrophin antibodies. The bottom panel corresponds to Ponceau red staining of proteins in the lower part of the blot and shows that similar amounts of proteins were loaded in each pair of well. Each pair of lanes represents denervated (D) and contralateral control (C) limb muscles obtained 7 or 14 days after nerve sectioning. The arrow indicates the position of dystrophin (~ 400 kDa) from *Torpedo* electric tissue (lane T) which ran together with the muscle samples on the gel. As shown, denervated muscles displayed a noticeable increase in the levels of utrophin as compared to control muscles.

sections ($10\ \mu\text{m}$). After 1 h of incubation, sections were rinsed with PBS containing 0.1% Tween. This was then followed by an additional 1 h incubation with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG diluted (1/100) in PBS with 3% BSA. Sections were thoroughly rinsed with PBS, mounted in moviol and examined by epifluorescence using a Leitz photomicroscope.

2.4. Gel electrophoresis and immunoblotting

SDS/PAGE of proteins from denervated and contralateral control muscles was carried out as described [36] with the following modifications. Equivalent amount of control and 7 or 14 day-denervated muscles representing approximately 1.2 mg of total proteins, was homogenized in $300\ \mu\text{l}$ of Tris buffer (100 mM; pH 6.9) containing 25% glycerol, 13% SDS, 1 mM EDTA, 1 mM benzamide and 1 mM PMSF. Protein concentrations were determined according to the bicinchoninic acid (BCA) Protein Assay Reagent protocol (Pierce Laboratories). For gel electrophoresis, samples were heated to 60°C for 10 min. Following the addition of bromophenol blue and mercaptoethanol (5%), a total of $140\ \mu\text{g}$ of whole muscle proteins were loaded in each well. Samples were separated by one dimensional SDS/6% PAGE using the Mini Protean II slab gel system (BioRad).

Immunoblot analysis of utrophin was performed after transfer onto nitrocellulose [37]. Detection of utrophin was performed via enhanced chemiluminescence reaction (ECL detection kit, Amersham). The upper part of the nitrocellulose filter was incubated with the antibody against utrophin (1/2000), and a goat horseradish peroxidase-conju-

gated anti-rabbit IgG was used as a secondary antibody. The lower part of the same blot was stained with Ponceau red to show that equivalent amount of proteins was loaded for each sample. The luminescent signal on the XR film was scanned and quantified using the NIH image program.

2.5. RNA extraction and quantitative RT-PCR

For these studies, tibialis anterior and extensor digitorum longus muscles (TA/EDL) as well as triceps surae and plantaris muscles (TS/PL) were excised and rapidly frozen in liquid nitrogen. Total RNA was extracted using the acid guanidinium phenol chloroform procedure [38]. Both TA/EDL and TS/PL muscle groups were homogenized using a Polytron in 10 volumes of solution D. Following the last precipitation, the RNA was washed with 75% ethanol and stored at -20°C .

RNA pellets from control and denervated muscles were dissolved in $50\ \mu\text{l}$ of RNase-free water. From each stock RNA sample, $10\ \mu\text{l}$ was further diluted 100-fold and only $2\ \mu\text{l}$ of this dilution was used for reverse transcription and amplification with the polymerase chain reaction (RT-PCR) as described in detail elsewhere [39,40]. Briefly, a master reverse transcription mixture was prepared containing 5 mM MgCl_2 , $1\times$ PCR buffer II (50 mM KCl, 10 mM Tris-HCl (pH 8.3)), 1 mM dNTPs, 20 U RNase inhibitor, 50 U reverse transcriptase, and 2.5 mM of random hexamers (GeneAmp RNA PCR kit; Perkin Elmer Cetus). The master mix was aliquoted and the RNA subsequently added. Negative controls consisted of reverse transcription mixtures in which total RNA was replaced with RNase-free water. Reverse transcription was performed for 45 min at 42°C and heated to 99°C for 5 min to terminate the reaction.

A PCR master mix was then prepared with final concentrations of 2.5 U AmpliTaq DNA polymerase, 2 mM MgCl_2 and $1\times$ PCR buffer II. Utrophin cDNAs were specifically amplified using primers designed on the basis of the mouse utrophin sequence. The 5' (5', 3': GGGGAAGATGTGAGAGATT) and 3' (5', 3': GTGTGGTGAGAGATACGAT) primers amplify a 548 bp target sequence. PCR was performed in a DNA thermal cycler (Perkin Elmer) by adding $5\ \mu\text{l}$ of the reverse transcription mixture to $20\ \mu\text{l}$ of the PCR master mix. Each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 65°C for 1 min and extension at 72°C for 1 min. A final 10 min elongation step at 72°C was added after the last cycle. Typically, amplification was performed for 32 cycles for TA/EDL and TS/PL muscles since our experiments showed that under these reaction conditions, amplification was within the linear range (see also refs. [39,40]). PCR products were visualized on a 1.5% agarose gel containing ethidium bromide. The 100 bp molecular weight marker (Gibco BRL) was used to estimate the molecular weight of the PCR products. Quantitative PCR experiments under non-competitive conditions were performed as described [39,40] since in the present studies, we were primarily interested in comparing the relative abundance of utrophin mRNAs in control and denervated muscles. For these, 2×10^6 cpm of ^{32}P -end labelled primers were added to the PCR reaction mixture. After amplification, $10\ \mu\text{l}$ aliquots of the PCR reactions were Cerenkov counted and separated by gel electrophoresis on 1.5% agarose gels. The gels were then photographed, the appropriate gel bands excised and the amount of radioactivity determined by Cerenkov counting. The counts per minute were adjusted for the amount of sample loaded per gel lane and background activity. These final counts per minute are proportional to the relative abundance of utrophin transcripts per muscle groups.

3. Results

In a first series of experiments, we examined the distribution of utrophin in cryostat sections of control muscles obtained

Fig. 1. Immunofluorescence detection of dystrophin and utrophin in cryostat sections of control and denervated hindlimb muscles of 10-week-old *mdx* mouse. A and B represent immunolabelling of dystrophin and utrophin in control skeletal muscles, respectively. Note that in A, only a few revertant fibers were stained (arrow). In B, intensive immunostaining of nerves (N), blood vessels (BV) and neuromuscular junctions (arrows) was observed. Also, a heterogeneous staining pattern was seen at the periphery of myofibers. C and D show immunolabelling of utrophin in 3- and 14-day denervated muscles, respectively. As shown, denervation induces an increase in the number of utrophin-positive fibers at day 3 (C). A further increase was seen at day 14 (D).



Fig. 3. Effects of denervation on expression of utrophin transcripts in *mdx* mouse muscles. Shown are representative examples of ethidium bromide stained agarose gels of utrophin PCR products for denervated (D) and control (C) anterior (TA-EDL) and posterior (TS-PL) crural muscle groups of *mdx* mice. Negative control lane is marked with a minus.

from *mdx* mice. Transverse tissue sections were immunolabelled with either anti-dystrophin or anti-utrophin antibodies. Immunolabelling of *mdx* hindlimb muscles with anti-dystrophin antibodies showed that only a few revertant muscle fibers were decorated (arrow) thereby confirming the absence of this protein from the majority of muscle fibers (Fig. 1A). Anti-utrophin antibodies revealed in addition to labelling of neuromuscular (arrows) and myotendinous junctions (not shown), a heterogeneous pattern of immunostaining (Fig. 1B). Only a few fibers, presumably regenerating myofibers either isolated or grouped, showed utrophin immunoreactivity at the sarcolemma. A strong immunoreactivity was also observed in nerve trunks and blood vessels (arrow).

In denervated hindlimb muscles of *mdx* mice, anti-dystrophin antibodies showed the same pattern of labelling as that observed in control muscles (not shown). Using anti-utrophin antibodies, however, we observed a significant increase in the number of utrophin-positive fibers as early as 3 days after denervation (Fig. 1C). A steady increase of the number of utrophin-positive fibers was observed at later time-points and reached a plateau between 14 to 21 days following nerve transection (Fig. 1D). The number of utrophin-positive fibers remained elevated until the end of the third month after denervation.

In a series of complementary experiments, we quantitated the magnitude of this increase by comparing levels of utrophin expression in control and denervated muscles of *mdx* mice with immunoblotting assays. For these experiments, an equal amount of protein was loaded in each pair of well as evidenced by Ponceau red staining of the lower part of the blot (Fig. 2). Using the utrophin antibody, we detected the presence of a band of ~420 kDa in both control and denervated muscles. Densitometric analysis of the blots revealed a significant increase (~2- to 3-fold) in utrophin levels 14 days following sectioning of the sciatic nerve (Fig. 2).

To determine the influence of the motor nerve on expression of the utrophin gene, we examined in a separate set of experiments, levels of utrophin mRNAs in control and denervated muscles of *mdx* mice. For these studies, the relative abundance of utrophin transcripts was measured by quantitative RT-PCR in both anterior (TA-EDL) and posterior (TS-PL) crural muscle groups of *mdx* mice following 2 weeks of denervation (Fig. 3). The identity of the utrophin PCR product (548 bp) was confirmed by restriction enzyme mapping and predicted size

based upon the sequence of a partial cDNA clone (not shown). As illustrated in Fig. 4, denervation increased expression of utrophin transcripts by approximately 62% and 93% in anterior and posterior crural muscle groups, respectively.

4. Discussion

The aims of the present study were two fold. First, we quantitated the extent of utrophin re-expression in denervated *mdx* mouse hindlimb muscles. Second, we wished to gain insights into the mechanisms underlying the denervation-associated increase in utrophin expression. Our results show that, despite a noticeable increase in the number of utrophin positive fibers as observed by immunofluorescence, utrophin levels are moderately increased by two weeks of denervation as revealed by densitometric analysis of immunoblots. Furthermore, quantitative RTPCR experiments showed that this increase in protein levels is sustained by a parallel increase in the abundance of utrophin mRNAs thereby indicating the involvement of pre-translational regulatory mechanisms in utrophin re-expression.

During myo- and synaptogenesis, expression of utrophin appears modulated such that increased levels of expression have been reported in both embryonic and neo-natal muscles [5,18,19,22,27,30,31]. In *mdx* mouse muscles, levels of utrophin decrease progressively reaching minimal levels three to four weeks after birth [31]. Although the developmental regulation of utrophin appears well established, the influence that the nerve may exert on utrophin is unclear. To date, this issue has only been addressed using the denervation model. For instance, in muscles of normal mice, denervation leads to no [16] or modest [18] changes in utrophin expression and localization. By contrast, denervation of *mdx* mouse muscle has been reported to have a pronounced effect on utrophin expression. Indeed, Takemitsu and colleagues [18] have shown on the basis of immunofluorescence experiments, re-expression of utrophin in denervated muscles that appeared to reach levels as high as those observed in embryonic muscle fibers. Our quantitative estimates based on densitometric analysis of immunoblots do not support these findings. In fact, our results indicate that the denervation-associated increase in utrophin expression is considerably less than assumed from immunofluorescence experiments reaching only moderate levels. This is further supported

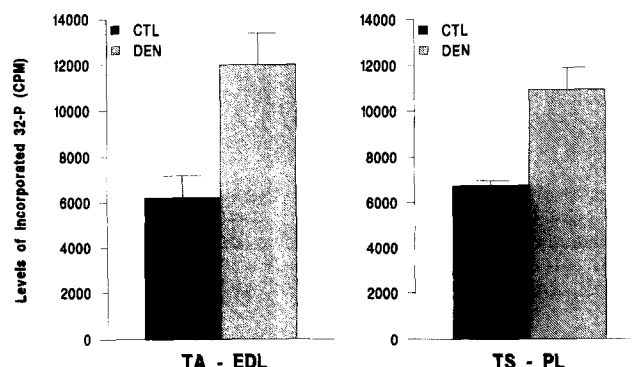


Fig. 4. Quantitation of utrophin mRNA levels in control and denervated hindlimb muscles of *mdx* mice. Utrophin mRNA expression was significantly increased in both the anterior (TA-EDL) and posterior (TS-PL) crural muscle groups of *mdx* mice following 2 weeks of denervation. Shown are the results of quantitative RT-PCR experiments performed on 3 control and 3 denervated mice. Values are mean \pm S.D.

by analysis of mRNA levels which in agreement with the immunoblot data shows a modest increase in utrophin transcripts. The apparent discrepancy may be reconciled if we consider the methods used to determine the impact of denervation on utrophin expression. In Takemitsu et al. [18], this was strictly done by immunofluorescence. Using this method, we similarly observed a rather strong re-expression of utrophin in numerous fibers of denervated muscles. However, the apparent increase of fluorescent fibers is difficult to quantify. Also, cross-reactivity of the anti-utrophin antibody with other membrane components is likely as seen in immunoblotting experiments. This fact should be taken into consideration when making a direct comparison between immunofluorescence observations and biochemical estimates of the protein or mRNA increases.

The impact of denervation on expression of AChR in muscles has been extensively studied and it is now well established that denervation leads to pronounced increases in the levels of AChR. The increases vary between five to 100 fold according to the species, muscle and time-point studied (see for review [33]). This increase in AChR expression in denervated muscles reflects de novo synthesis as exemplified by the large increases in levels of mRNAs encoding the various AChR subunits [34,35]. In this context, it has been suggested that utrophin expression in denervated muscles increases significantly [18] yet, our quantitative analyses clearly indicate that it is not the case. Thus, although utrophin is a synaptic component which may be involved in the organization of the postsynaptic membrane domain of the neuromuscular junction [41–43], its regulation is clearly distinct from that of AChR.

The 43 kDa protein is another important constituent of the postsynaptic membrane of the neuromuscular junction [44,45]. In both *Torpedo* electrocyte and differentiated mouse skeletal muscle, the AChR and 43 kDa protein are present in nearly equimolar amounts [46]. Because of its localization and intimate relationship with AChR, it has been also hypothesized that expression of this protein may be markedly influenced by the presence of the motor nerve. In separate experiments, however, Frail et al. [47] and Froehner [48] showed that denervation of rodent muscle resulted only in marginal increases in the expression of mRNA encoding the 43 kDa protein. These results obtained with a distinct cytoskeletal protein of the postsynaptic membrane are thus coherent with our observations. Taken together, these findings indicate that in striking contrast to the pronounced effect that innervation has on the expression of AChR, synapse-specific cytoskeletal proteins are only marginally influenced by denervation.

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