# Local Neurotrophic Repression of Gene Transcripts Encoding Fetal AChRs at Rat Neuromuscular Synapses

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***Abstract.*** The spatio–temporal expression patterns of mRNA transcripts coding for acetylcholine receptor (AChR) subunits and myogenic factors were measured in denervated rat soleus muscle and in soleus muscle chronically paralyzed for up to 12 d by conduction block of the sciatic nerve by tetrodotoxin (TTX). In denervated muscle the AChR α-, β-, γ-, and δ-subunit mRNAs were elevated with highest expression levels in the former synaptic and the perisynaptic region and with lower levels in the extrasynaptic fiber segments. In muscle paralyzed by nerve conduction block the α-, β-, γ-, and δ-subunit mRNA levels increased only in extrasynaptic fiber segments. Surprisingly, in the synaptic region the γ-subunit mRNA that specifies the fetal-type AChR, and α-, β-, δ-subunit mRNAs were not elevated. The expression of the gene encoding the ε-subunit, which specifies the adult-type AChR, was always restricted to synaptic nuclei. The mRNA for the regulatory factor myogenin showed after denervation similar changes as the subunit transcripts of the fetal AChR. When the muscle was paralyzed by nerve conduction block the increase of myogenin transcripts was also less pronounced in synaptic regions compared to extrasynaptic fiber segments. The results suggest that in normal soleus muscle a neurotrophic signal from the nerve locally down-regulates the expression of fetal-type AChR channel in the synaptic and perisynaptic muscle membrane by inhibiting the expression of the γ-subunit gene and that inhibition of the myogenin gene expression may contribute to this down-regulation.

DURING development, innervation of skeletal muscle fibers causes initially a restriction of acetylcholine receptor channels (AChRs)[1] in the muscle membrane to the site of the neuromuscular contact. This is followed by a change in AChR channel properties as the subunit composition of the AChR switches from $\alpha_2\beta\gamma\delta$ to $\alpha_2\beta\epsilon\delta$ (Mishina et al., 1986; for review see Hall and Sanes, 1993).

The expression of AChR subunits responsible for the developmental switch in channel properties, termed γ- and ε-subunits, is independently regulated by trophic factors from the nerve and by electrical muscle activity (Sakmann et al., 1992). The ε-subunit–specific mRNA is restricted throughout development to synaptic nuclei, suggesting that ε-subunit gene expression is primarily under neurotrophic control (Brenner et al., 1990, 1994; Martinou and Merlie, 1991; Sanes et al., 1991; Gundersen et al., 1993). The γ-subunit mRNA, on the other hand, is expressed transiently in developing muscle and, upon innervation, is thought to be down-regulated by nerve-induced electrical muscle activity (Goldman et al., 1988; Witzemann et al., 1991). However, when adult rat soleus muscles are chronically paralyzed by blocking impulse conduction in sciatic nerve by a nerve cuff containing tetrodotoxin (TTX), the increase in γ-subunit mRNA level is significantly smaller than when the nerve is transected (Witzemann et al., 1991). This suggests that in innervated adult muscle expression of the γ-subunit is controlled both by electrical activity of the muscle and by neurotrophic factor(s) from the nerve. Northern blot hybridization analysis (Eftimie et al., 1991; Witzemann and Sakmann, 1991) as well as promoter characterization of AChR subunit genes suggested that regulatory factors of the MyoD1 family are involved in the regulation of AChR gene expression in skeletal muscle (Piette et al., 1990; Numberger et al., 1991; Jia et al., 1992; Prody and Merlie, 1992; Berberich et al., 1993; Simon and Burden, 1993).

To address the question of whether the inhibitory neurotrophic influence acts locally restricted at the site of nerve–muscle contact or whether it acts globally along the entire fiber, we performed in situ hybridization experiments in adult muscles that were made electrically silent either by cutting the nerve and resulting in the degeneration of the nerve endings, or by block of nerve conduction

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1. *Abbrevations used in this paper*: AChE, acetylcholinesterase; AChR, acetylcholine receptor channels; ROI, region of interest; TTX, tetrodotoxin.

with TTX, but leaving the nerve endings morphologically intact. The two experimental procedures produced strikingly different spatial expression patterns of the γ-subunit mRNA in synaptic and perisynaptic nuclei as well as that of the α-, β-, and δ-subunit mRNAs suggesting that in normal adult muscle neurotrophic signals from the nerve inhibit locally and independently of nerve-induced muscle activity the expression of α-, β-, γ-, and δ-subunit genes. The concordant changes in the spatial distribution of myogenin mRNA and of γ-subunit mRNA in the two experimental conditions suggest also that repression of the myogenin gene mediates the neurotrophic down-regulation of fetal-type AChR subunit genes in synaptic nuclei of adult innervated muscle.

## Materials and Methods

Experiments were carried out on soleus muscles of adult Wistar and Sprague-Dawley rats. This muscle was chosen because it has been employed previously for studies on the neural regulation of AChR genes and their products, both during development and for synapse maintenance (Brenner et al., 1990; Witzemann et al., 1991; Kues et al., 1995). Furthermore, sections of total muscle can be easily processed for hybridization, and the synapses are located on a characteristic "band" that facilitates identification of synaptic regions.

### Hybridization Probes

The cDNAs of AChR subunits (Witzemann et al., 1990), MyoD1 (Davis et al., 1987), myogenin (Edmondson and Olson, 1989), MRF4 (Rhodes and Konieczny, 1989), and β-actin (Cleveland et al., 1981) were ligated in pSP72 plasmids (Promega), respectively. Radiolabeled sense or antisense cRNA probes were generated by run off transcription from the linearized plasmids, by the use of either SP6- or T7-RNA-polymerase and uridine 5′-(α-$^{35}$S)thiotriphosphate (800 Ci/mmol, Amersham).

### Preparation of Muscle Sections

Rats were killed by $CO_2$. The soleus muscles were excised, fixed on polystyrene support, and snap frozen in cooled isopentane (–80°C). Complete longitudinal sections of 10 μm thickness were cut on a cryostat and thaw mounted on poly-L-lysine coated microscope slides. The sections were fixed in 4% paraformaldehyde (in PBS) and stained for acetylcholinesterase activity according to Koelle and Friedenwald (1949). The synaptic sites were documented and sections were then proteinase K digested and acetylated (Fontaine et al., 1988). Sections were dehydrated through a series of graded ethanol solutions and allowed to air dry.

### In Situ Hybridization

40 μl hybridization solution, with 1.5 × 10 cpm of denatured probe in 50% formamide, 10% dextran sulfate, 300 mM NaCl, 25 mM dithiothreitol, 20 mM Tris (pH 7.5), 5 mM EDTA, 1× Denhardt's solution, and 1 mg/ml yeast tRNA were applied per slide and covered with cover slips. The hybridizations were performed overnight at 52°C. The washing steps were as described by Fontaine et al. (1988), except the RNase digestion, which was done for 60 min to reduce unspecific hybridization signals. Sections were then air dried and exposed to autoradiography film for 4–8 d. For cellular resolution the sections were dipped in Kodak NBT2 emulsion and exposed for 10–20 d.

### Denervation of Hind Leg Muscles and Exogenous Electrical Stimulation

The hind leg muscles of adult rats were denervated unilaterally under anesthesia, excising a 5-mm long piece of sciatic nerve. The denervated state of the leg was ascertained by the missing toe spreading reflex that was also controlled at the time of death. Before dissecting the denervated muscle the distal sciatic nerve stump was stimulated mechanically but in no case contractions of the lower leg muscles could be detected. Finally, muscles denervated for more than one week display obvious atrophy that further demonstrated complete inactivation due to denervation. The animals were killed after different postoperative survival times. The denervated, as well as the contralateral soleus muscles were removed and processed for in situ hybridization in the same experiment. Denervation data were obtained from five independent series of experiments in a total number of 23 animals.

Stimulation of denervated soleus muscles was performed as described (Goldman et al., 1988). In brief, electrodes were implanted and muscles were stimulated either directly for 7 d or left initially unstimulated for 5 d and then stimulated for 5 d, respectively. Electrical stimulation was in 100-Hz trains, 1-s duration, applied once every 100 s. The contralateral muscles served as controls. Electrical stimulation experiments were performed with two animals.

### Tetrodotoxin Block of Sciatic Nerve Conduction

Chronic blockade of impulse conduction in sciatic nerve was accomplished by application of TTX via a silastic cuff (inside diameter 1.8 mm, length 8 mm), which was placed around the nerve in the upper thigh (Witzemann et al., 1991). The cuff was connected to an osmotic minipump (Alzet 2002; Alza, Palo Alto, CA), implanted in peritoneum, filled with 370 μg/ml TTX, 200 IU penicillin, and 200 μg/ml streptomycin in Hank's solution. At a nominal pumping rate of 0.5 μl/h, this corresponds to a delivery of 4.5 μg TTX per day. Completeness of conduction block was tested as described previously (Witzemann et al., 1991). Data were obtained from three independent series of experiments with 12 animals.

### Densitometric Evaluation

To obtain semi-quantitative profiles of transcript distributions in muscle, adjacent longitudinal cryosections were analyzed after hybridization. The sections were first contact-exposed to x-ray film and then dipped in nuclear track photographic emulsion for cellular resolution. For densitometric evaluation the autoradiograms were captured with a slow scan CCD video camera (CD250; Photometrics Ltd., Tucson, AZ) installed on a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany), using a camera lucida equipment for scanning contact-autoradiograms or bright field optics for emulsion-dipped sections. The evaluation of the images were done on a Macintosh II (Apple) computer using the IPLAP-SU2 (Signal Analytics Corp., Vienna, VA) program to measure pixel intensity values in selected regions of interest (ROI). Rectangular ROIs corresponded to the length of the muscle and were 1 mm wide. Background values were measured on film areas outside the muscle section in the same ROI and subtracted from the data. Relative densitometric values of a rectangular ROI extending along the length of the autoradiograms are plotted as inverse intensity changes. Scale bar corresponds to maximum reduction in densitometric values of autoradiograms hybridized with ε-subunit–specific probe obtained in innervated muscle. To measure profiles through the synaptic regions of emulsion-dipped sections, several overlapping ROIs were combined to obtain a segment of 1.4 mm in length and 100 μm in width. Background intensity values were specified by measuring ROIs in those parts of the muscle that did not exhibit specific hybridization signals.

## Results

### Effects of Muscle Disuse Induced by Cutting the Nerve

Fig. 1 *A* illustrates the changes in muscular distribution of AChR subunit mRNAs 5 d after denervation. The α-, β-, γ-, and δ-subunit mRNAs were strongly elevated (Fig. 1 *A*, *right*) and showed an asymmetric distribution that was particularly prominent for the γ-subunit mRNA (Fig. 1 *A*, *third row*). The ε-subunit mRNA was also increased but expression remained sharply restricted to the former synaptic region (Fig. 1 *A*, *fifth row*). The ε-subunit transcript distribution was, therefore, used to identify the location of former synaptic nuclei in adjacent sections.

Fig. 1 *B* shows densitometric evaluations of autoradiograms of the same sections. They demonstrate that after 5 d denervation the highest levels of transcripts are found in the central part of the muscle where former synapses were located. The peak levels of all AChR mRNAs, however,

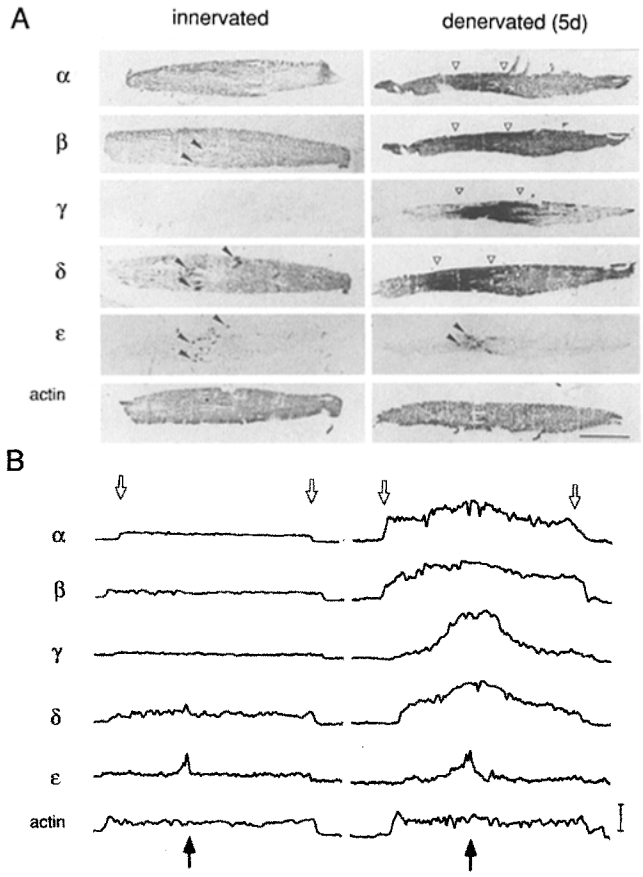


*Figure. 1.* Spatial expression patterns of AChR subunit genes 5 d after surgical denervation. (*A*) Longitudinal sections of innervated and 5 d denervated muscles were hybridized with radiolabeled antisense cRNA probes as indicated and were contact-exposed to autoradiographic film. Arrowheads indicate some of the synapse-specific accumulations of β-, δ-, and ϵ-subunit transcripts in the "synapse band" in innervated muscle. Open triangles mark the central muscle region with strongly increased levels of α-, β-, γ-, and δ-subunit transcripts after denervation. The ϵ-subunit mRNA is confined to synapses in innervated muscle and to sites of former synapses in denervated muscle. The uniform distribution of actin transcripts, hybridized with a β-actin probe, is not changed after denervation. (*B*) Densitometric evaluation of autoradiograms shown in *A*. Relative densitometric values of a rectangular ROI that extended along the length of the autoradiograms and was 1 mm in width. Densitometric values are plotted as inverse intensity changes (a decrease in light intensity on the x-ray film is plotted as an upward deflection of the trace). Scale bar corresponds to maximum reduction in densitometric values of autoradiograms hybridized with ϵ-subunit–specific probe measured in innervated muscle and including a synaptic spot of ϵ-subunit mRNA hybridization signal. Filled arrows indicate synaptic regions, open arrows mark the ends of sections. The profiles were obtained as detailed in Materials and Methods. Bar, 4 mm.

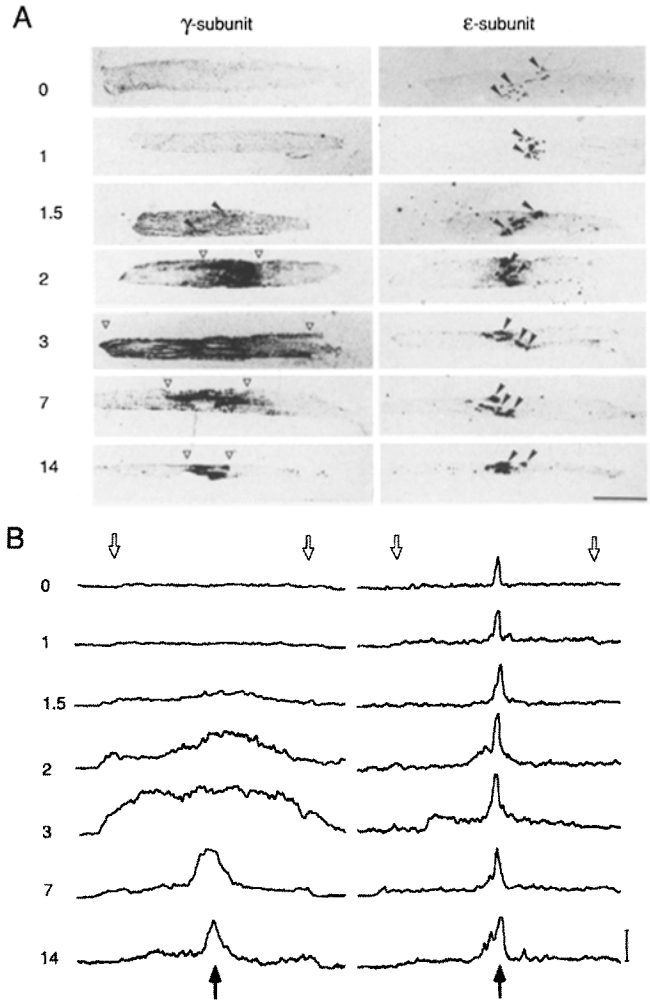


*Figure 2.* Time course of γ-subunit gene expression after surgical denervation. (*A*) Hind leg muscles of adult rats were denervated unilaterally, and soleus muscles were removed at various times after denervation as indicated (numbers on the left refer to days after denervation). Adjacent longitudinal sections were probed with γ- or ϵ-subunit–specific radiolabeled cRNAs. Arrowheads indicate γ- and ϵ-subunit transcripts in former synaptic regions. The γ-subunit transcripts spread as a "transcription wave" (*open triangles*) along the muscle. Accumulation of γ-subunit transcripts was first detected 1.5 d after denervation in former synaptic regions. Hybridized sections from day 0, 1, and 1.5 were exposed longer (8-d exposure) than sections from day 2–14 after denervation (4 d) to detect early γ-subunit mRNA expression. Maximal γ-subunit transcript spread was seen 3 d after denervation. At later times γ-subunit transcript levels are reduced again progressing from the fiber ends. 14 d after denervation γ-subunit transcripts are accumulated in the perisynaptic and synaptic region. (*B*) Densitometric evaluation of autoradiograms as in Fig. 1. Filled arrows indicate former synaptic regions, open arrows mark ends of sections. Scale bar was determined as described in Fig. 1. Bars, 4 mm.

clearly extended beyond the band of synapses, as marked by the ϵ-subunit mRNA distribution. The asymmetric distribution was specific for the AChR subunit transcripts, as actin mRNA expression in adjacent sections was uniform and did not depend on the state of innervation (Fig. 1 *B*, *sixth row*). Similar patterns of transcript distributions were seen in soleus muscles from five animals.

The spatial expression pattern of γ-subunit mRNA was examined at different times after denervation (Fig. 2 *A*, *left*). In innervated soleus muscle the γ-subunit mRNA could not be resolved (Goldman et al., 1988; Goldman and Staple, 1989). After 1.5 d of denervation expression was detected in the vicinity of the former synapses as marked

by the position of ε-subunit mRNA in a consecutive section (Fig. 2 *A*, *third row*). Between 1.5 to 3 d after denervation, γ-subunit transcript levels increased further and spread over the entire length of the muscle to the myotendinous insertions. At even longer denervation times transcript levels declined again progressively from the myotendinous ends but remained higher in the former synaptic and perisynaptic regions. At 14 d after denervation, γ-subunit mRNA could only be resolved in the immediate vicinity of the former synapses (Fig. 2 *A*, *seventh row*).

Fig. 2 *B* shows profiles of transcript distributions of the autoradiograms shown in Fig. 2 *A* that indicate that a spatio–temporal "transcription wave" of γ-subunit mRNA increase (Fig. 2 *B*, *left*) is triggered by denervation that begins in the former synaptic region and then spreads gradually towards the myotendinous insertions. The denervation-induced increase of γ-subunit mRNA thus occurs not uniformly. Comparable patterns were seen for δ-subunit mRNA and, less pronounced, for α- and β-subunit mRNAs (not shown). In contrast, the ε-subunit mRNA remained concentrated at the former synaptic nuclei at all times after denervation (Fig. 2, *A* and *B*; *right*).

### *Effects of Muscle Disuse Induced by Impulse Conduction Block of the Sciatic Nerve*

In muscle that was inactivated by impulse conduction block of the sciatic nerve, the spatial expression patterns of α-, β-, γ-, and δ-subunit mRNAs were different from those in denervated muscles. Transcript levels in the synaptic and perisynaptic region were barely elevated and similar as in innervated, electrically active muscle, while levels in extrasynaptic regions were strongly increased, comparable to the levels observed in denervated muscle (Fig. 3 *A*). The ε-subunit mRNA distribution remained unaffected (Fig. 3 *A*, *fifth row*). Densitometric evaluation of contact-autoradiograms and of emulsion-dipped sections showed that the γ-subunit mRNA remains low in synaptic and perisynaptic areas and is increased in extrasynaptic fiber segments (Fig. 3 *B*). For analysis at higher spatial resolution with emulsion-dipped sections the location of synapses was first marked by staining for acetylcholinesterase (AChE) activity. Then, after hybridization, the same regions were densitometrically scanned. As shown in Fig. 3 *C* (*left*) no or only a few hybridization-positive signals were detected in the segment around the synapse. This low grain density region extended in both directions from the synapse. In 18 fibers from 7 animals that were paralyzed by TTX-induced nerve conduction block for 3 or 5 d the γ-subunit transcript-free region around the synapse averaged 315 ± 25 μm (mean ± SEM) in length. In regions further away from the synapse the γ-subunit specific signals were strongly elevated.

In contrast, hybridization signals of ε-subunit mRNAs (Fig. 3 *C*, *right*) were strictly confined to the synaptic region. Accumulation of ε-subunit transcripts extended, on average, over a length of 55 ± 11 μm (mean ± SEM), as measured at 15 fibers from five different muscles.

Fig. 4 illustrates the time course of the increase in γ-subunit mRNA following nerve conduction block by TTX as measured by densitometric scanning of autoradiograms. In the extrasynaptic segments of the fibers the time course of

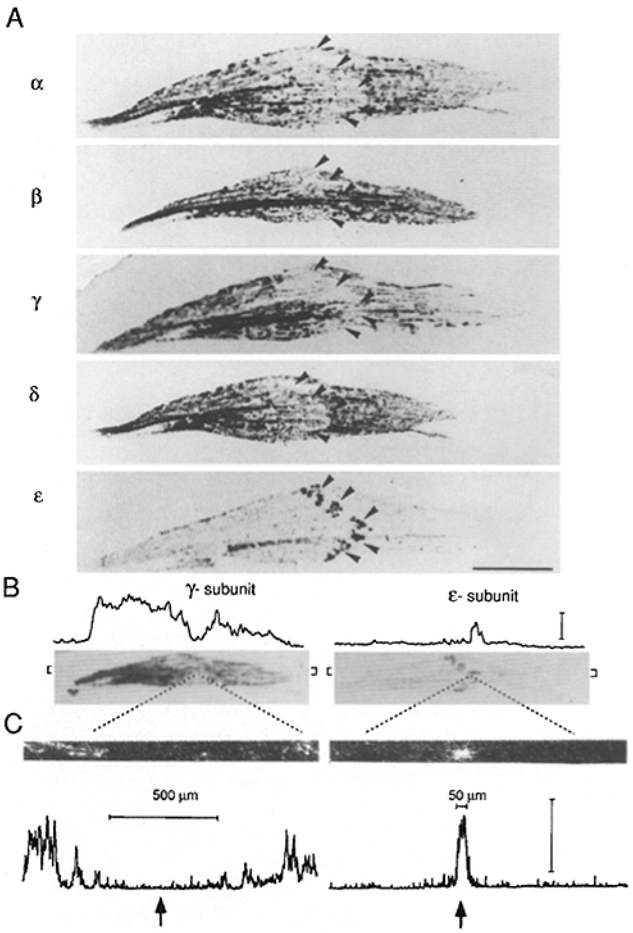


*Figure 3.* AChR-subunit gene expression 3 d after block of sciatic nerve impulse conduction. (*A*) Adjacent longitudinal sections of electrically silent muscles were hybridized with subunit-specific probes as indicated on the left. Arrowheads mark the position of synaptic regions according to the ε-subunit transcript locations. The α-, β-, γ- and δ-subunit transcript levels are increased in extrasynaptic regions, but are not elevated in synaptic regions (*arrowheads*). Sections of normal and denervated muscles were processed in parallel to ascertain specificity of hybridization patterns (not shown). Bar, 4 mm. (*B*) Densitometric evaluation of autoradiograms of sections hybridized with the γ- and ε-subunit–specific probes as described in Fig. 1. The location of ROIs is marked by square brackets in the video images. (*C*) Emulsion-dipped sections of synaptic regions as outlined in *B* and densitometric evaluation is shown below. Location of synapses (*arrows*) was identified by AChE activity (not shown). ROI was 1.4 mm in length and 100 μm in width. Scale bars in *B* and *C* correspond to maximal reduction in densitometric values of autoradiograms hybridized with ε-subunit-specific probe.

mRNA increase, as shown by the densitometric profiles of transcripts distributions, is comparable to that observed in denervated muscle. In the synaptic region transcript levels remained lower at all times after blocking nerve conduction. The mRNA changes were reflected in the differential perinuclear accumulation of γ-subunit gene transcripts in perisynaptic and extrasynaptic regions. Transcripts were not detectable around synaptic nuclei that were identified by colocalization with AChE (Fig. 8, *second row*). The level of expression of AChR subunit transcripts near the

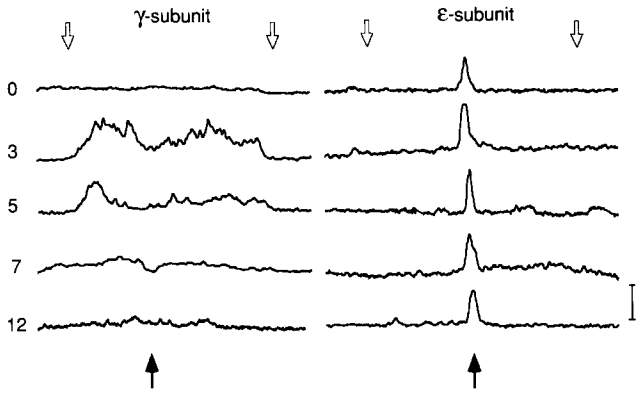


*Figure 4.* Transient extrasynaptic accumulation of γ-subunit mRNA after block of nerve impulse conduction. Adjacent longitudinal sections of muscles paralyzed by nerve impulse conduction block for 0, 3, 5, 7, and 12 d (as indicated on the left) were hybridized with γ- and ε-subunit–specific cRNA probes. The spatial mRNA distribution was evaluated densitometrically as described in Fig. 1. The ε-subunit mRNA accumulations identified synaptic regions (*filled arrows*). The level of γ-subunit mRNA in synaptic regions is low or mRNAs are not detectable. Increase of γ-subunit mRNA in extrasynaptic segments is transient and follows a time-course comparable to that observed after denervation (e.g., Fig. 2). Open arrows mark the end of sections.

fiber ends also decreased gradually at longer times after the conduction block (Fig. 4).

The observations illustrated in Fig. 3 and 4 were made in three independent series of experiments with a total of 12 animals and strongly suggest that neurotrophic factors from the nerve terminal, from Schwann cells or from fibroblasts inhibit the expression of α-, β-, γ-, and δ-subunit mRNAs in the synaptic and perisynaptic region even when the muscle is electrically inactive. In extrasynaptic muscle segments, the expression of these subunit mRNAs is elevated when the muscle was electrically inactive irrespective of whether paralysis is induced by denervation or by nerve conduction block. Fig. 5 (*left*) illustrates autoradiograms of denervated muscles that were, however, kept active by exogenous electrical stimulation. When stimulation was begun at the time of denervation and was then maintained for 7 d, γ-subunit mRNA was not detectable, neither in the former synaptic region nor in extrasynaptic portions (*upper left*). Similar results were obtained, when stimulation was begun 5 d after denervation and was then maintained for an additional 5 d (*lower left*).

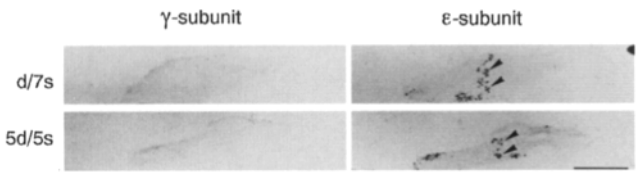


*Figure 5.* Exogenous electrical stimulation prevents increase of γ-subunit mRNA in denervated muscle. Soleus muscle was denervated and immediately stimulated electrically for 7 (d/7s) or 5 d denervated and then, in addition, stimulated for 5 d (5d/5s) as detailed in Material and Methods. Adjacent longitudinal thin sections were hybridized with γ- and ε-subunit–specific radiolabeled cRNAs. In these muscles γ-subunit mRNAs are not detected. Distribution of ε-subunit mRNA marks former synaptic regions and is almost unchanged after denervation (*arrowheads*). Bar, 4 mm.

### *Effects of Muscle Disuse Induced by Cutting the Nerve or by Nerve Conduction Block on mRNAs Encoding Myogenic Transcription Factors*

Myogenic transcription factors of the helix-loop-helix family participate in the expression of AChR subunit mRNAs (Piette et al., 1990; Numberger et al., 1991; Jia et al., 1992; Prody and Merlie, 1992; Berberich et al., 1993; Simon and Burden, 1993). If the differential changes of α-, β-, γ-, and δ-subunit mRNA distribution observed upon denervation and following nerve conduction block are mediated by myogenic factors one would expect to observe parallel changes in the spatio–temporal distribution patterns of transcripts encoding myogenic factors. Fig. 6 shows low resolution autoradiograms of sections of denervated muscle hybridized with probes for γ-subunit mRNA or for transcripts of different myogenic factors. They illustrate that 5 d after denervation (*right*), levels of transcripts encoding myogenin, MyoD1, and MRF4 were increased. As for the AChR subunit transcripts the highest levels are observed in former synaptic and perisynaptic regions and the denervation-induced increase was strongest for myogenin mRNA (Fig. 6, *second row*) and weakest for MRF4 mRNA (Fig. 6, *fourth row*). Similar observations were made in soleus muscles from five animals.

The densitometric profiles of myogenin mRNA and of synapse-specific ε-subunit mRNA in adjacent sections of TTX-paralyzed muscle are compared in Fig. 7. Myogenin mRNAs, as well as the transcripts of MyoD1 and MRF4 (not shown), were expressed significantly less in synaptic and perisynaptic region than in extrasynaptic regions. This compartmentalized expression of myogenin was comparable to that of γ-subunit (Fig. 7, *third row*) and α-, β-, and δ-subunit mRNA (not shown) and was observed in muscles from four animals treated with TTX for 5 d.

Fig. 8 compares, at higher spatial resolution, the differential distributions of, γ-subunit– and myogenin-specific

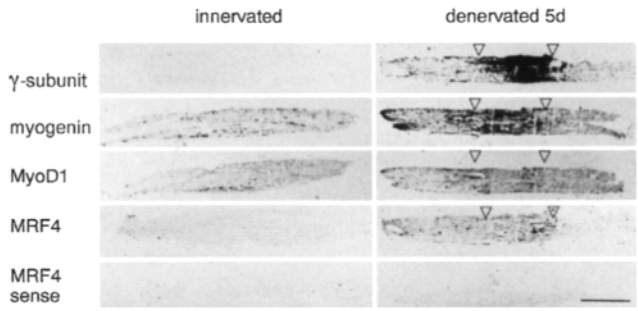


*Figure 6.* Spatial expression patterns of mRNAs encoding myogenic factors myogenin, MyoD1, and MRF4, in innervated and denervated soleus muscles. The distributions of mRNAs indicated on left were visualized in longitudinal muscle sections. The mRNAs of the myogenic factors are hardly detectable in innervated muscle. After 5 d of denervation the mRNAs levels are increased in the central region of muscle (*open triangles*). Control experiments with the MRF4 sense probe demonstrate specificity of hybridization signals. Bar, 4 mm.

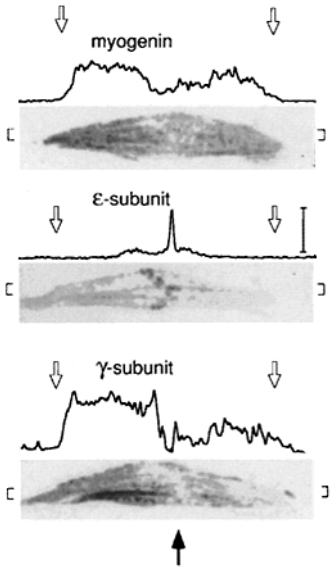


*Figure 7.* Myogenin transcripts are reduced in synaptic regions of muscle paralyzed by block of sciatic nerve impulse conduction. Densitometric evaluation of autoradiograms of sections hybridized with the myogenin, ε- and γ-subunit–specific probes as described in Fig. 1. Location of ROIs is indicated by square brackets in the video images. Scale bar as in Fig. 1. Filled arrow indicates synaptic region, open arrows mark the end of sections.

transcripts in denervated versus TTX-paralyzed muscle. The ε-subunit mRNA is used as a marker for synapses since it is concentrated at synaptic sites in both experimental conditions (Fig. 8, *first row*). In denervated muscle mRNA levels of the γ-subunit and of myogenin (Fig. 8, *second* and *third row*) were high in nuclei in the former synaptic region whereas in muscle paralyzed by nerve conduction block the levels of these transcripts were low or not detectable at synaptic, but elevated in extrasynaptic muscle portions.

## Discussion

Down-regulation of AChR γ-subunit gene transcription at the developing neuromuscular synapse contributes to the disappearance of fetal AChRs ($\alpha_2\beta\gamma\delta$) and their replacement by the adult AChR ($\alpha_2\beta\epsilon\delta$) subtype in the endplate. To resolve the role of nerve terminals in this switch in subunit composition, we have compared, by in situ hybridization techniques, the spatio–temporal changes in the levels of α-, β-, γ-, δ-, and ε-subunit mRNA in muscle fibers of rat soleus muscles that had been made electrically inactive either by denervation, which destroys nerve terminals, or by conduction block of the sciatic nerve, which leaves nerve terminals intact. In the present experiments, AChR subunit transcript distributions were analyzed semi-quantitatively on sections of whole muscle after hybridization with AChR subunit-specific probes and contact exposure to x-ray film. Unlike in sections from sternomastoid and diaphragm muscles, the synaptic localization of α- and β-subunit mRNAs was not resolved by this method, presumably due to significant extrasynaptic expression of these transcripts even in normally innervated muscle (Kues et al., 1995).

### *Compartmentalized mRNA Expression and Neurotrophic Factors*

The present experiments suggest that the transcription of the AChR γ-subunit gene in innervated skeletal muscle fibers is differently regulated in extrasynaptic and in synaptic fiber segments. In segments more distant than ~300 μm from the synapse, electrical activity is required for the down-regulation of γ-subunit mRNA. In contrast, at and near the synapse, muscle activity, although sufficient, is not necessary for the down-regulation of γ-subunit mRNA, if the nerve terminal is present. Rather, in the myonuclei under the synapse, the nerve down-regulates γ-subunit mRNA via a neurotrophic factor acting on the muscle. The repression of γ-subunit gene expression lasts at least for 12–14 d in the absence of electrical activity. Combined with previous electrophysiological results showing that the subsynaptic membrane of innervated but electrically inactive muscle does not contain functional fetal type AChRs (Witzemann et al., 1991), the present experiments indicate also that trophic factors are sufficient for the developmental switch from γ- to ε-subunit expression at and near the synapse, and thus, for the switch in endplate channel subunit composition from fetal to adult AChR (Mishina et al., 1986).

This conclusion assumes that denervation produced a strong increase in AChR subunit mRNAs in denervated muscle because of the removal of electrical muscle activity and negatively acting neurotrophic signals. However, other factors may contribute to denervation-induced mRNA increase. Products of nerve degeneration, which arise upon denervation and that in combination with electrical inactivity locally enhance the ACh sensitivity of the muscle fiber (Cangiano and Lutzemberger, 1977; Cangiano et al., 1984) may also contribute to the higher mRNA levels at former synapses. Denervation also causes a proliferation of fibroblasts in the vicinity of the former synapse (Murray and Robbins, 1982; Connor and McMahan, 1987), which in turn may contribute to the increased expression of γ-subunit mRNA in this region. Incorporation of newly synthesized AChRs after denervation occurs preferentially in perisynaptic membrane segments (Salpeter et al., 1988), reflecting, at least partially, the changes in the expression pattern of the γ-subunit gene. The subsequent decline in γ-subunit mRNA expression observed after 5 d of denervation may be related to changes in the metabolic state of the muscle. The differences in spatial expression patterns of subunit mRNAs may thus reflect not only the difference of muscle deprived of electrical activity and neurotrophic factors (in denervated muscle) and those deprived of electrical activity only (muscle with TTX block). However, the locally restricted inhibition of γ-subunit expression in TTX-paralyzed muscle, together with the dependence of the γ-subunit mRNA level on electrical activity, strongly indicates that the low mRNA levels in the endplate region of TTX-paralyzed muscle is due to the action of a neurotrophic factor.

Northern blot analysis of total muscle mRNA had shown previously that the increase in AChR subunit mRNA levels upon blockade of nerve activity alone, when nerve terminals were anatomically intact, was significantly less than that observed after denervation (Witzemann et al., 1991). This difference was most conspicuous for γ-subunit mRNA, suggesting that the γ-subunit gene is most susceptible to a repressing neurotrophic signal. The spatial expression pattern of γ-subunit mRNA in muscle blocked by a TTX cuff around the sciatic nerve show unequivocally that the difference in total RNA content between dener-

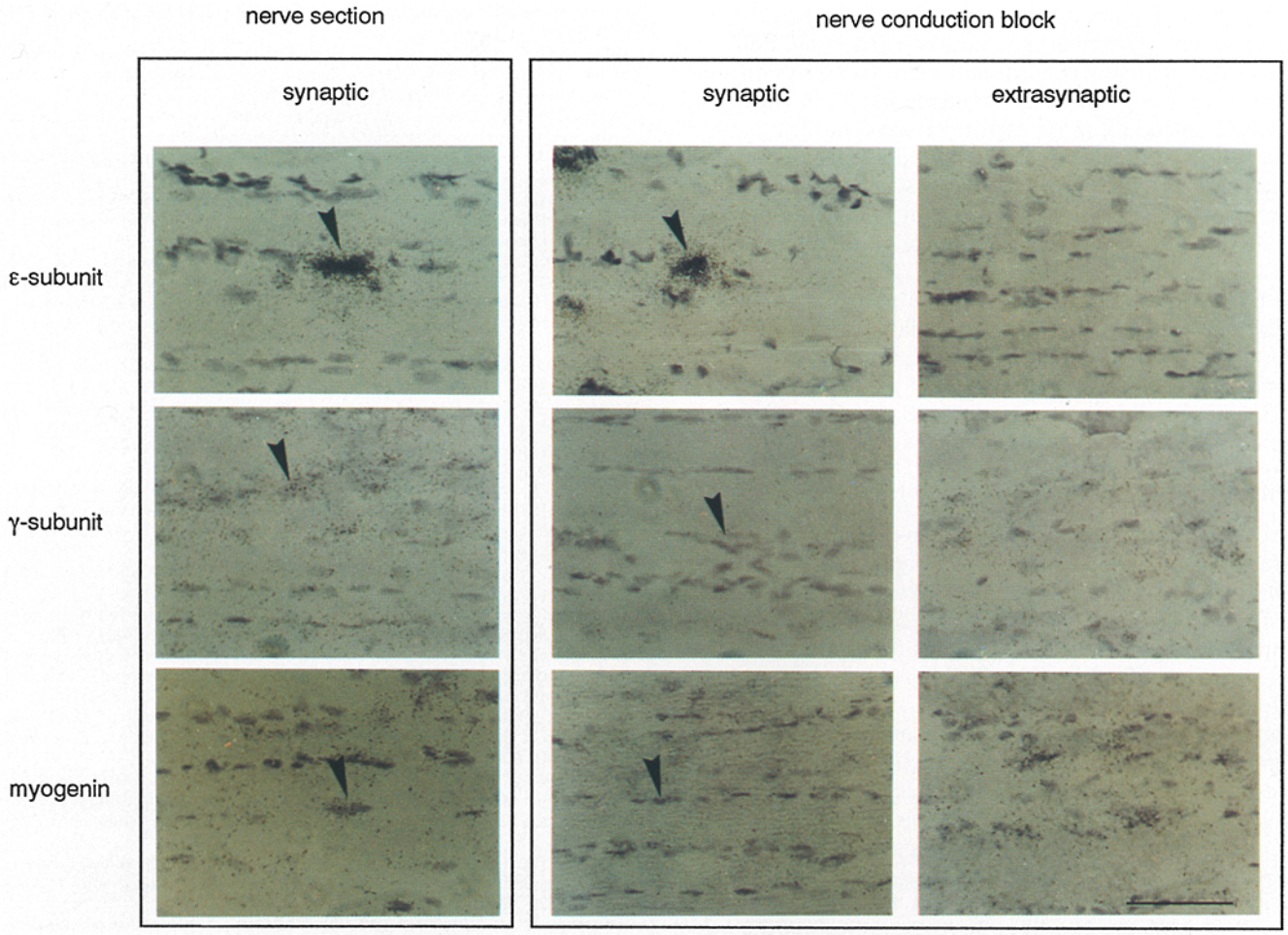


*Figure 8.* Distribution of ε-, γ-subunit, and myogenin transcripts in former synaptic regions in denervated muscle and synaptic regions of muscle paralyzed by nerve impulse conduction block in emulsion-dipped sections. (*Left*) ε-subunit, γ-subunit, and myogenin transcript distributions in muscle denervated for 5 d. (*Middle* and *right*) ε-subunit, γ-subunit, and myogenin transcript distributions in muscle paralyzed for 5 d by a TTX cuff around the sciatic nerve. Synaptic sites (*arrowheads*) were identified by AChE staining (not shown). The ε-subunit mRNA is strictly confined to synaptic nuclei in denervated and TTX-blocked muscle. In denervated muscle γ-subunit and myogenin mRNAs are expressed in former synaptic and perisynaptic nuclei at higher levels (*left*) than in extrasynaptic nuclei (not shown). In muscles paralyzed by impulse conduction block the γ-subunit and myogenin mRNA levels are low or mRNAs are absent in synaptic nuclei (*middle*), but are accumulated around extrasynaptic nuclei (*right*). Bar, 30 μm.

vated muscle and nerve-blocked muscle (Witzemann et al., 1991) could not have been produced by undetected electrical muscle activity due to incomplete nerve conduction block. Action potentials evoked at endplates by incompletely blocked nerves would have propagated and suppressed γ-subunit mRNA along the entire muscle fiber.

At the receptor level, this local nerve action is reflected by the absence of a slow component in the decay of miniature endplate currents recorded in muscle with nerve conduction block, clearly indicating the lack of functional fetal AChRs in the subsynaptic membrane (Witzemann et al., 1991). A comparable local repression in muscles paralyzed by a TTX cuff around the nerve was also seen for α-, β-, and δ-subunit mRNAs in perisynaptic nuclei, indicating a coordinated local negative control by the nerve of α-, β-, γ-, and δ-subunit genes that encode the fetal AChR subtype. As expected, an inhibitory effect on α-, β-, and δ-subunit genes in the subsynaptic nuclei, as observed for the γ-subunit gene, was not seen.

### *Inhibitory Signal Cascade*

Since in electrically silent muscle with complete block of neuromuscular transmission (i.e., evoked as well as spontaneous vesicular release) by botulinum toxin the AChR-subunit mRNAs increase strongly (Witzemann et al., 1991), the local repression depends on the presence of the nerve terminal with intact spontaneous vesicular release of ACh. One possibility is that the inhibitory effect is mediated by $Ca^{2+}$-influx across AChR channels activated by spontaneous ACh release (Miledi et al., 1980), as is the case for activity-induced down-regulation of fetal AChR subunit transcripts in extrasynaptic nuclei (Klarsfeld et al., 1989; Huang et al., 1992). However, previous Northern blot hybridization analyses of muscles in which AChRs were blocked by α-BuTX for 3-4 d showed, as in TTX-blocked muscle and in contrast to denervated muscle, only a small increase in γ-subunit mRNA, suggesting that ACh-induced $Ca^{2+}$-influx may not be sufficient to mediate the inhibitory effect (Witzemann et al., 1991).

Myogenic factors transactivate the γ-subunit gene promoter in vitro (Numberger et al., 1991; Jia et al., 1992) and the strong increase in γ-subunit gene transcription upon denervation is preceded by increases in the levels of mRNAs encoding these factors (Buonanno et al., 1992). Exogenous stimulation of denervated muscle that reduces γ-subunit mRNA levels also decreases the mRNA encoding myogenin (see also Eftimie et al., 1991; Buonanno et al, 1992), both being presumably mediated by protein kinase C (Huang et al., 1992; Neville et al., 1992). Together with the present observations these findings are consistent with the view that local down-regulation of myogenin by the nerve terminal either by inactivation of the transactivator and/or reduction of myogenin protein by the nerve terminal contributes to or even mediates the local repression of α-, β-, γ-, and δ-subunit genes.

### *Functional Significance of a Neurotrophic Factor*

The functional implication of down-regulation of α-, β-, and δ-subunit genes in the perisynaptic region of innervated muscle is not obvious at present, whereas γ-subunit gene repression in synaptic nuclei could be functionally important. Synaptic nuclei respond to two kinds of neurotrophic signals. An activating signal, exemplified by its effects on ε-subunit mRNA (Brenner et al., 1990) "imprints" only synaptic nuclei of fetal muscle for permanent expression of α-, β-, δ-, and ε-subunit genes early during muscle development (Brenner et al., 1990; Kues et al., 1995) or in ectopic synapses of adult muscle (Brenner et al., 1994). Alternatively, the nerve leaves a "trace" on the muscle (Lømo and Slater, 1980), e.g., in its basal lamina (Goldman et al., 1991; Brenner et al., 1992; Jo and Burden, 1992). The early activating signal requires only a brief (about one day) neuromuscular contact (Brenner et al., 1990, 1994). The additional repressing signal acts later in synapse development and this signal requires permanent nerve–muscle contact to be effective. After neuromuscular transmission has been established, it prevents γ-subunit expression locally and might accelerate the elimination of fetal AChR channels from the subsynaptic membrane, thus, accelerating the time-course of the AChR subtype switch. In support of this view, it has been observed that early during postnatal development, the ACh sensitivity declines more rapidly in perisynaptic than in extrasynaptic fiber segments (Yoshioka and Miyata, 1983). In adult muscle, the repressing neurotrophic signal would ensure permanent expression of adult type AChRs in the endplate of innervated muscle, irrespective of changes in electrical muscle activity.

We thank Drs. W. Betz (Univ. of Colorado School of Medicine, Denver, CO) and T. Lømo (Univ. of Oslo, Oslo, Norway) for critically reading the manuscript. We also thank Drs. E. Olson (Univ. of Texas, Houston, TX), A. Lassar (Harvard Medical School, Boston, MA) and S. Konieczny (Purdue Univ., West Lafayette, IN) for providing the plasmid DNAs for myogenin, MyoD1 and MRF4 plasmid DNA.

This work was supported in part by the Deutsche Forschungsgemeinschaft and by the Swiss National Science Foundation.

Received for publication 14 April 1995 and in revised form 7 May 1995.

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