

RAPID DISPERSAL OF CLUSTERED POSTSYNAPTIC NUCLEI FOLLOWING DISSOCIATION OF SKELETAL MUSCLE FIBERS

CHRISTIAN BRÖSAMLE* AND DAMIEN P. KUFFLER†

Institute of Neurobiology and Department of Physiology, Medical Sciences Campus, University of Puerto Rico, San Juan, PR 00901, USA

Accepted 25 June 1996

Summary

The vertebrate neuromuscular junction is a highly specialized structure containing many unique proteins and an underlying cluster of nuclei. Part of this specialization results from the expression of the genes for these proteins in nuclei clustered in the postsynaptic region. Contractile activity, as well as molecules located in the synaptic extracellular matrix (ECM), have been implicated in the induction of gene expression in these clustered nuclei. The present experiments were aimed at examining whether the presence of the synaptic ECM and presynaptic cells play a role in maintaining the clustering of the nuclei. We describe the normal distribution of nuclei clustered in the synaptic region of intact adult frog, *Rana pipiens*, skeletal muscle fibers and show that innervation is not required to maintain the nuclear clusters. Even after long-term (4 week) denervation, the clusters remain unchanged. **Dissociation of the muscle fibers with proteases that remove**

ECM, Schwann cells and other satellite cells from the synaptic sites is followed by a rapid (within approximately 1.5 h) and almost complete dispersal of the clustered nuclei. Attempts to recluster the postsynaptic nuclei by the application of ECM components to muscle fibers *in vitro* were not successful. We propose that a factor or factors, localized in the synaptic ECM as a result of synapse formation and acting *via* the transmembrane or cytoplasmic domains of their respective receptors, induces the formation of a specialized cytoskeleton in the postsynaptic region that is capable of pulling in or 'trapping' nuclei. The removal of these factors from the ECM by proteases brings about the disorganization of the cytoskeleton and the freeing of the 'trapped' nuclei.

Key words: muscle fiber isolation, skeletal muscle fibers, *Rana pipiens*, cell culture, frog, extracellular matrix.

Introduction

The vertebrate neuromuscular junction is a highly specialized structure in which more than 40 proteins are concentrated (see Hall and Sanes, 1993, for a review). While there are many nuclei within a single muscle fiber, the synthesis of acetylcholine receptor (AChR) mRNA may be controlled independently by individual nuclei (Bursztajn *et al.* 1989; Fontaine and Changeux, 1989). The localization of some of these molecules, for example the α -subunit of the acetylcholine receptor (AChR) (Klarsfeld *et al.* 1991), the AChR ϵ -subunit (Sanes *et al.* 1991), the AChR δ -subunit (Simon *et al.* 1992) and acetylcholine esterase (Rossi and Rotundo, 1992; Jasmin *et al.* 1993), results from the expression of the genes for these proteins in the muscle fiber nuclei located only in the synaptic region. Owing to the localized gene expression and protein synthesis, these proteins are found predominantly in the synaptic region. Nuclei in the extrasynaptic regions of the same muscle fibers show no, or only very low, expression of these genes, and thus their associated proteins are absent from the extrasynaptic regions

of the muscle fibers. Several factors have been implicated in the regulation of muscle fiber gene expression. The regulation is influenced by the contractile activity of the muscle fibers (Goldmann *et al.* 1988) and the induction of gene expression by molecules located in the synaptic basal lamina (Jo and Burden, 1992).

In addition to differences in gene expression patterns in the synaptic and extrasynaptic regions of muscle fibers, the distribution of nuclei throughout the muscle fiber is not uniform. For several vertebrate species, the nuclei of the synaptic region have been shown to be clustered at a higher density than those of the extrasynaptic region (e.g. in mammals, Cardasis, 1979; snakes, Roberts, 1987; and quail, Jasmin *et al.* 1993).

In developing chick muscle cells, moving nuclei become 'trapped' under AChR clusters (Englander and Rubin, 1987), and such AChR clusters are induced by factors released into the synaptic ECM by nerve terminals (Harris *et al.* 1989; Wallace, 1989). These findings suggest that innervation of a

*Present address: Brain Research Institute, University of Zurich, August-Forel-Strasse 1, 8029 Zurich, Switzerland.

†Author for correspondence (e-mail: d_kuffler@rcmaca.upr.clu.edu).

muscle fiber leads to the introduction of molecules into the ECM that bring about a specialization in the postsynaptic cytoplasm causing it to 'trap' migrating nuclei. Agrin, which becomes localized to the synaptic ECM with innervation, can trigger the formation of specializations containing at least seven components of the postsynaptic apparatus within 24 h of application (Burden *et al.* 1979; Wallace, 1989; Nitkin and Rothschild, 1990). Other molecules in the ECM are connected to the actin-based skeletal muscle fiber cytoskeleton *via* transmembrane linkers. One such linker is the dystrophin–glycoprotein complex, which is capable of binding both actin to the cytoskeleton and laminin in the ECM (Ervasti and Campbell, 1993). The introduction of such molecules into the synaptic ECM at the time of innervation could induce a modification of the postsynaptic cytoskeleton, giving it the ability to trap and retain the nuclei. Therefore, removal of the synaptic ECM could result in the loss of the cytoskeleton organizing factor(s), bringing about disruption of the specialized nuclear trapping properties of the cytoskeleton and allowing release of 'trapped' nuclei.

In this paper, we describe the distribution of nuclei in normal intact adult frog skeletal muscle fibers and their rapid declustering upon protease treatment and muscle fiber dissociation, as well as attempts to recluster the dissociated nuclei. Although innervation may be involved in the initial triggering of nuclear clustering, we demonstrate that it is not required for the maintenance of the nuclear clusters. However, factors in the ECM appear to play critical roles in the maintenance of the nuclear clusters, since removal of the ECM results in the rapid and almost complete dispersal of the clustered nuclei. Attempts to recluster the postsynaptic nuclei were unsuccessful.

Materials and methods

Animals

Muscle fibers were isolated from adult North American frog, *Rana pipiens*, kept in running water at 10 °C and fed live crickets twice a week. For the denervation experiments, the frogs were kept at room temperature (20–21 °C) following nerve section.

Muscle fiber isolation

Frogs were bathed in water containing 0.4 mg ml⁻¹ tetracycline hydrochloride for 24 h before isolating the muscle fibers. The frogs were cold-anesthetized, decapitated and pithed. The lumbricalis muscles (extensor) of the second and third toes were dissected free and rinsed five times in Leibowitz 15 (L-15) culture medium (Gibco, Grand Island, NY) with an osmolarity of 240 mosmol l⁻¹ (77% L-15), containing 0.25% garamycin (Gibco), and pinned at resting length to a sterile dish coated in Sylgard (Dow Corning, Seneffe, Belgium). The muscles were bathed for 1 h in 3 ml of a sterile filtered solution of 8 mg ml⁻¹ neutral dispase and 1.3 mg ml⁻¹ collagenase P (Boehringer Mannheim, Mannheim, Germany) in a nominally Ca²⁺-free Ringer's solution

(119 mmol l⁻¹ NaCl, 2 mmol l⁻¹ KCl, 1 mmol l⁻¹ NaH₂PO₄). 2 ml of sterile filtered 30 mmol l⁻¹ K⁺ Ringer's solution (90 mmol l⁻¹ NaCl, 30 mmol l⁻¹ KCl, 2 mmol l⁻¹ CaCl₂, 1 mmol l⁻¹ NaH₂PO₄) was then added to the protease solution, and after 15 min this solution was completely replaced by 30 mmol l⁻¹ K⁺ Ringer's solution. After 1 h, the pins were removed from the dish and the muscles were dissociated into single fibers by gentle trituration using a 1 ml polyethylene pipette with a fire-polished tip. The dissociated muscle fibers were plated in 1 ml of L-15 medium in ultraviolet-sterilized, modified 35 mm tissue culture dishes with a 20 mm diameter glass coverslip for the bottom. The dishes were sealed with Parafilm and kept in an incubator at 27 °C in an ambient air atmosphere.

Labeling nuclei and original synaptic sites

Two nuclear stains were used, one on fixed muscle fibers (in the time course and denervation experiments) and the other on living cultured muscle fibers. Nuclei were labeled in muscle fibers that had either been teased apart from fixed muscles (denervation experiments) or isolated by protease treatment and then fixed after various times in culture (time course experiments) using Hoechst 33258 nuclear stain. Fibers were incubated overnight in 5 µg ml⁻¹ Hoechst 33258 in a 0.1% Triton solution in normal frog Ringer's solution (NFR). Dissociated cultured muscle fibers were treated with labeled SYTO 13 (Molecular Probes, Eugene, OR). The SYTO 13 was added to their L-15 medium to achieve a final concentration of 100 mmol l⁻¹. Good labeling of the nuclei was obtained after approximately 1 h and persisted for several hours. By the next day, the labeling was too faint to work with, or had disappeared, but the nuclei could be relabeled by adding fresh SYTO 13. The advantage of SYTO 13 over Hoechst 33258 is that it readily permeates living muscle fibers and does not appear to cause any deleterious effects. Even after the labeling of nuclei, the muscle fibers could be maintained in culture and relabeled at later times, rather than having to be fixed before labeling, as is required for Hoechst 33258. Hoechst 33258 was used in some experiments, however, because of its greater stability and resistance to photo-bleaching.

The acetylcholine receptors (AChRs) at the original synaptic sites were labeled with rhodamine-tagged α -bungarotoxin (rh-BTX) (Molecular Probes, Eugene, OR) at a concentration of 100 nmol l⁻¹ in normal frog Ringer's solution for times varying from 1 h to overnight.

Time course and analysis of the dispersal of nuclei

In analyzing the time course of the dispersal of synaptic nuclei, time zero was set as the time at which the frogs were decapitated. To determine the distribution of nuclei in normal undissociated muscle fibers (time zero), the skin of the leg was stripped off and the foot immediately fixed in 3% paraformaldehyde. Single muscle fibers were teased from the fixed muscles using micro hooks and the nuclei were labeled as above. The isolated muscle fibers were examined at 40× magnification under phase optics and fluorescence illumination

to determine whether single fibers had been obtained and whether there were any Schwann or satellite cells adhering to them. If there were any doubts about the teased muscle fibers being single and free of adhering cells, they were not included in the analysis. Consequently, most teased fibers had to be discarded, since we were not convinced that they were single or clean. For other time points, muscle fibers were isolated as described above and rinsed for 2 min in 2 mol l^{-1} formamide in NFR to prevent the muscle fibers from contracting upon exposure to fixative. The fibers were fixed and stained as described above. The formamide treatment had no influence on the distribution of the nuclei.

In vivo denervation of lumbricalis muscle

For experiments in which the lumbricalis muscle fibers were denervated for only 7.5 h, the frogs were pithed but their spinal cords were left intact. For experiments requiring longer denervation times (up to 4 weeks), the frogs were anesthetized for 20 min in 0.1% tricaine methanesulfonate (MS 222) (Sigma, St Louis, MO) and the lumbricalis motor nerve was then sectioned and the frogs allowed to recover.

To denervate the lumbricalis muscles, a small incision was made in the skin above the lumbricalis muscle of the second toe, the innervating nerve was exposed and sectioned where it entered the muscle and the incision sutured shut. The control muscles of these frogs received a sham operation.

Movement of nuclei

The experiments to monitor the movement of nuclei were carried out on a computer-controlled, motorized x,y,z stage with a precision of positioning of $0.1 \mu\text{m}$. The nuclei were observed with a $40\times$ objective, with which we could reliably detect movements at rates above $1 \mu\text{m h}^{-1}$. Movements faster than this would have been readily detected, especially at the rates of movement required for the declustering of nuclei.

For these experiments, the coordinates of 22 nuclei labeled with SYTO 13 in seven fields from four different muscles were established. Images of each field were taken at 10 min intervals for 3 h. The image from each site was stored on an optical disc in separate time-lapse directories, one for each site. To determine whether the nuclei had moved, images from the time-lapse series were overlaid to compare the relative positions of the nuclei over time.

Exposure of cultured muscle fibers to extracellular matrix preparations

Muscle fibers were dissociated and cultured overnight in L-15 to allow for the dispersal of the synaptic nuclei. Various extracellular matrix (ECM) preparations were then added to the culture medium of the muscle fibers. At intervals, nuclei were labeled with SYTO 13 and the AChRs with rh-BTX, and the nuclear distribution was then analyzed.

The ECM preparations were tested at two different concentrations. The highest concentration was as concentrated as possible without giving rise to any apparent adverse effects on the morphological appearance of the muscle fibers and their

survival times in culture. The second concentration was an order of magnitude less concentrated than this.

ECM preparations

Matrigel

Matrigel (Beckton Dickinson, Bedford, MA) is a preparation of basement membrane matrix extracted from the Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. It contains laminin, fibronectin, collagen IV, heparan sulfate proteoglycans and a variety of other components. Matrigel was used at a concentration of $200 \mu\text{l ml}^{-1}$ ($2.8 \text{ mg protein ml}^{-1}$).

Frog skeletal muscle ECM extract

An extracellular matrix extract was prepared from the leg muscles of adult *Rana pipiens*, as described previously (Kuffler and Luethi, 1992). The extract was then fractionated on a molecular sizing column. Fractions 19 and 25, which have been shown to be enriched for frog ECM proteins (laminin, collagen IV and fibrillin) and to promote outgrowth from adult frog spinal motoneurons, were used at concentrations of 4 and $40 \mu\text{l ml}^{-1}$.

Laminin

EHS laminin (Sigma, St Louis, MO) was tested at 12 and $120 \mu\text{g ml}^{-1}$.

Agrin

Conditioned medium (kindly provided by the laboratory of Dr Z. Hall, UCSF) from a cos-cell line that expresses and secretes a truncated form of rat agrin (4-8 form) was added to muscle fiber cultures at dilutions of 1:100 and 1:10. This conditioned medium has been shown to cause the clustering of AChRs on C-2 myoblasts at concentrations as low as 1:1000.

Electron microscopy

Isolated muscle fibers were fixed in 0.8% glutaraldehyde in phosphate buffer, followed by fixation in 1% OsO_4 , dehydration and then embedding in Epon 812 (Fullam, Schenectady, NY). Ultrathin sections were counter-stained in uranyl acetate and lead citrate.

Analysis of densities of nuclei in muscle fibers

Nuclei and AChRs in labeled muscle fibers were viewed on a Leitz Fluovert FS inverted microscope with a $40\times$ Fluotar objective under appropriate fluorescence illumination. Images were taken through a silicon-intensified target (SIT) camera (Hamamatsu), displayed on a monitor and stored on an optical disc drive (Panasonic) using Image-1/AT (Universal Imaging, West Chester, PA) imaging software run on a 486-PC.

Since the synaptic arborizations vary from fiber to fiber, the density of synaptic nuclei was defined as the number of nuclei in a $100 \mu\text{m}$ length of muscle fiber lying in the middle of the synaptic arborization (as defined by the α -bungarotoxin labeling). Extrasynaptic nuclei were counted in 4–6 regions $100 \mu\text{m}$ long of the same muscle fiber on each side of the

synaptic site. The values for these regions were averaged to determine the density of nuclei in the extrasynaptic region of the muscle fiber. The nuclei were counted from the live video image displayed on a monitor while focusing through the fiber.

The absolute numbers of nuclei in both the synaptic and extrasynaptic regions were found to vary with the diameter of the muscle fiber (20–80 μm). Therefore, the density of nuclei in the synaptic region was normalized to the density in the synaptic segment.

Results

Isolated muscle fibers in vitro

Following protease treatment, the muscle fibers appeared to be completely dissociated from one another. This was confirmed by examination at the light microscope level at 40 \times magnification under both phase optics and fluorescence illumination. Only completely dissociated single muscle fibers, free of Schwann or satellite cells, were seen. Preparations stained with rh-BTX had only one labeled synaptic site per muscle fiber, further indicating that they were only single

muscle fibers. This was confirmed by examining the muscle fibers in the electron microscope (see next section).

Protease-treated and dissociated lumbricalis muscle fibers survived *in vitro* and retained their ability to contract when electrically stimulated for more than 4 weeks. The original synaptic sites on the isolated muscle fibers were clearly identifiable for up to 33 days by the binding of rh-BTX to the synaptic AChRs, and they were always located in the middle third of the fibers (Fig. 1A). Although the length and pattern of the postsynaptic arborizations remained normal, the intensity of the fluorescence from the freshly rh-BTX-labeled AChRs decreased the longer the muscle fibers were held *in vitro*. In addition, with increasing time *in vitro*, the rows of labeled AChRs at the crests of the postsynaptic folds of the muscle fibers appeared to become slightly more widely spaced.

Electron microscopic examination of dissociated and cultured muscle fibers

Dissociated muscle fibers were prepared for electron microscopy, both immediately after dissociation and after they

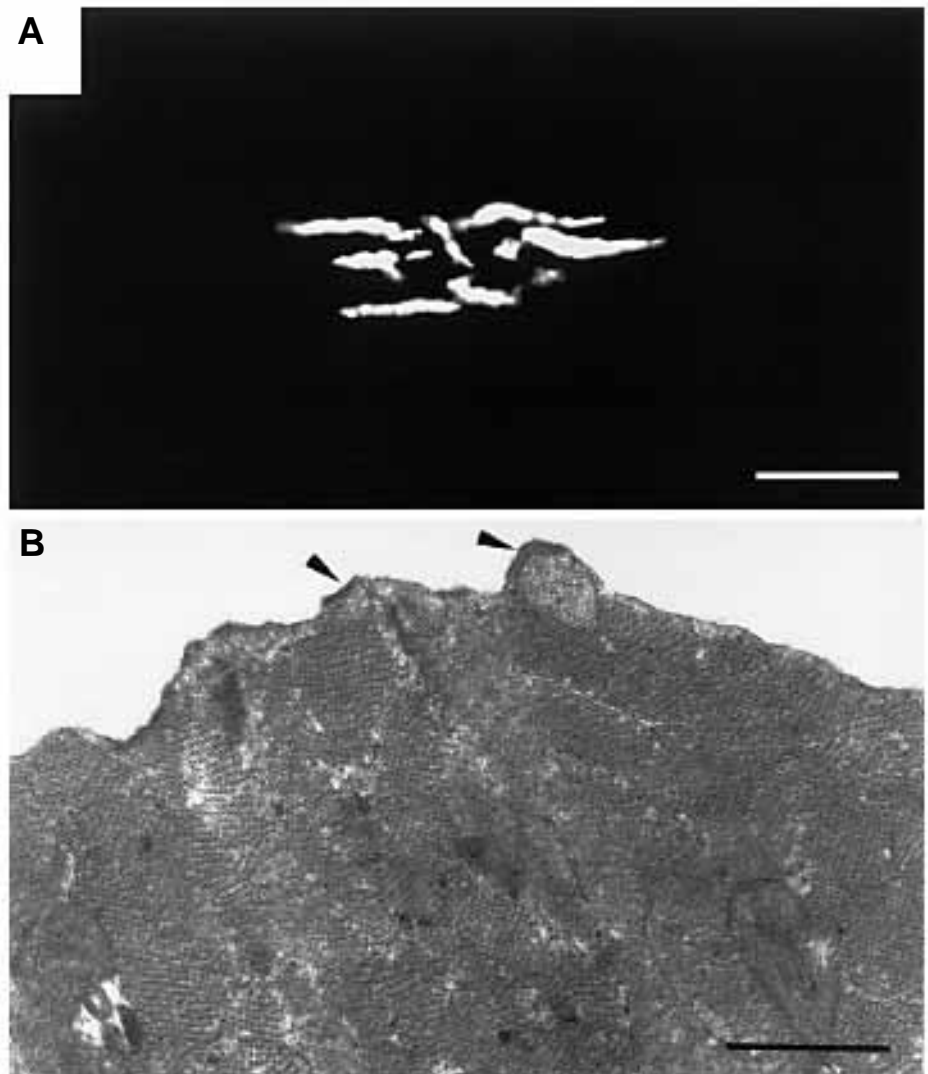


Fig. 1. Light and electron micrographs of long-term cultured lumbricalis muscle fibers. (A) The original synaptic site on an isolated skeletal muscle fiber after 26 days in culture, visualized by labeling of the acetylcholine receptors (AChRs) with α -bungarotoxin. Scale bar, 50 μm . (B) Electron micrograph of the synaptic region of a muscle fiber in culture for 4 weeks. The muscle fiber morphology was well preserved and no extracellular matrix was visible. Shallow synaptic folds were still present, with slight thickenings at the crest of the folds that may represent the locations of the remaining synaptic AChRs (arrowheads). Scale bar, 1 μm .

had been *in vitro* for up to 5 weeks. Each muscle fiber believed to be a single fiber at the time of embedding (12 of 12) was indeed found to be only a single fiber when examined in the electron microscope. These findings increased our confidence that the protease treatment truly dissociated the muscle fibers from one another and that the dissociated muscle fibers examined at the light microscope level for the distribution of their nuclei were single fibers.

The dissociated muscle fibers examined in the electron microscope were completely free of satellite cells and had no

detectable ECM along their entire length, even at the old synaptic sites. Even after up to 5 weeks *in vitro*, the muscle fibers still had no ECM. These results indicate that the protease treatment completely dissociated the muscle fibers from one another, and from satellite cells and ECM, and that the muscle fibers did not synthesize a new ECM *in vitro*.

The original synaptic sites on the dissociated muscle fibers, found in the middle of the muscle fibers, could be localized in the electron microscope for up to 4 weeks, the oldest cultured muscle fibers examined (Fig. 1B). After 4 weeks *in vitro*, the

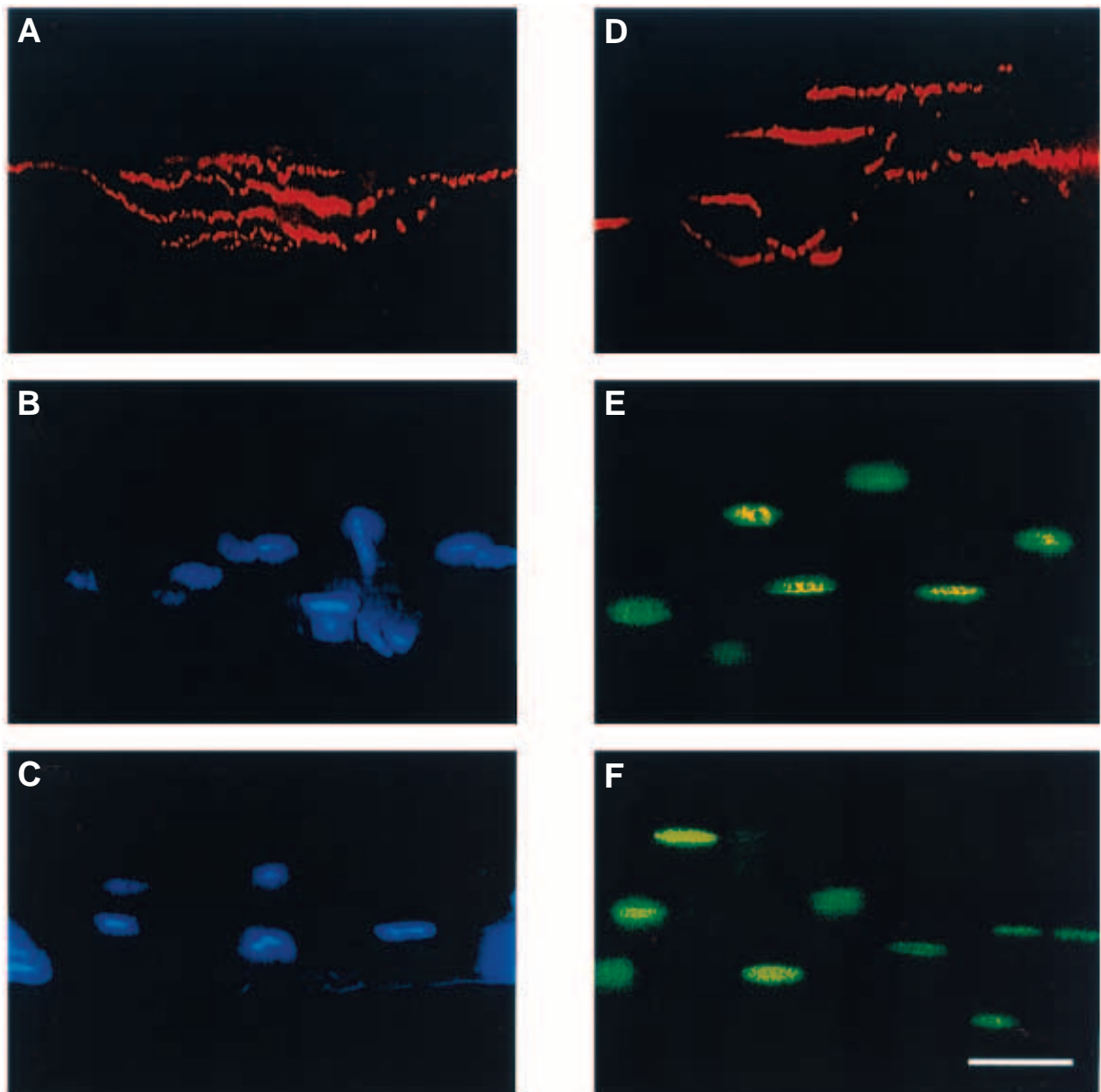


Fig. 2. Comparison of the distribution of nuclei of non-dissociated and dissociated muscle fibers. (A) Synaptic site of a muscle fiber fixed *in vivo*, labeled with rhodamine-tagged α -bungarotoxin (rh-BTX) and isolated by dissection when viewed under rhodamine illumination. (B) Identical region of the muscle fiber in A under fluorescence illumination to show the distribution of postsynaptic nuclei. The nuclei were clustered under the labeled acetylcholine receptors (AChRs) of the synaptic site. (C) The extrasynaptic region of the muscle fiber seen in B showed no clustering of nuclei. (D) rh-BTX-stained original synaptic site of a muscle fiber 6 h after isolation. (E) Labeled nuclei underlying the synaptic site in D. No clustered nuclei were seen. (F) The distribution of nuclei in the extrasynaptic region of the same fiber was virtually identical to that seen in both C and E. Scale bar, 50 μ m.

postsynaptic folds were shallow but still had thickenings at their top (Fig. 1B, arrowheads). The thickenings were similar to those seen on the crests of the postsynaptic folds of normal innervated muscle fibers. Such membrane thickenings were only seen in the central region of the muscle fibers and only in association with the crest of the membrane folds. These results are consistent with the observation that the spacing of the bands of rh-BTX-labeled AChRs at the synaptic site appeared to become wider as the muscle fibers were maintained *in vitro* for increasing periods. They also suggested that these dense membrane areas were remnants of the original synaptic sites and were not the result of the spontaneously formed AChR clusters.

Normal distribution of postsynaptic nuclei and their redistribution after muscle fiber dissociation

To establish the normal distribution of muscle fiber nuclei, it was critical to count only the nuclei belonging to the muscle fibers and not those of the perijunctional myosatellite cells and fibroblasts that are associated with intact muscle fibers. Lumbricalis muscles were, therefore, fixed *in situ*, the muscle dissected free, and individual muscle fibers teased from it. Only muscle fibers that were completely free of adhering cells when viewed under phase optics were used for these experiments.

Although nuclei in normal intact lumbricalis muscle fibers were distributed throughout their entire length, there was always a nuclear cluster in the central postsynaptic region (Fig. 2A–C). The density of clustering of the nuclei was 2.46 ± 0.03 (S.E.M., $N=45$, four different experiments) times greater than that of the nuclei in the extrasynaptic regions of the same muscle fibers (Fig. 3).

Following the initiation of the protease treatment and muscle fiber dissociation, there was a rapid dispersal of the clustered nuclei (Fig. 2E). By 1.5 h, the earliest time at which single fibers could be examined, the density of synaptic nuclei had decreased to 1.31 ± 0.07 (S.E.M., $N=10$, one experiment) times that of the extrasynaptic region. By 6 h, the nuclear density had further decreased to 1.17 ± 0.01 (S.E.M., $N=30$, three experiments). No further changes were seen in nuclear density with increasing time *in vitro*.

Influence of in vivo denervation on nuclear distribution

To determine whether innervation, or the absence of muscle contractile activity, played a role in maintaining the clusters of postsynaptic nuclei, the nuclear distribution was analyzed after *in vivo* denervation for 7.5 h and 3, 7 and 15 days. The contralateral innervated lumbricalis muscles served as controls. For each time point, 10 muscle fibers, from each of three frogs, from both the innervated and denervated muscles, were analyzed. In all, 240 muscle fibers from 12 frogs were analyzed. None of the muscle fibers showed a statistical change in the ratio of synaptic to extrasynaptic nuclei ($P < 0.05$, Student's *t*-test). These results indicate that denervation does not play a role in the rearrangement of synaptic nuclei.

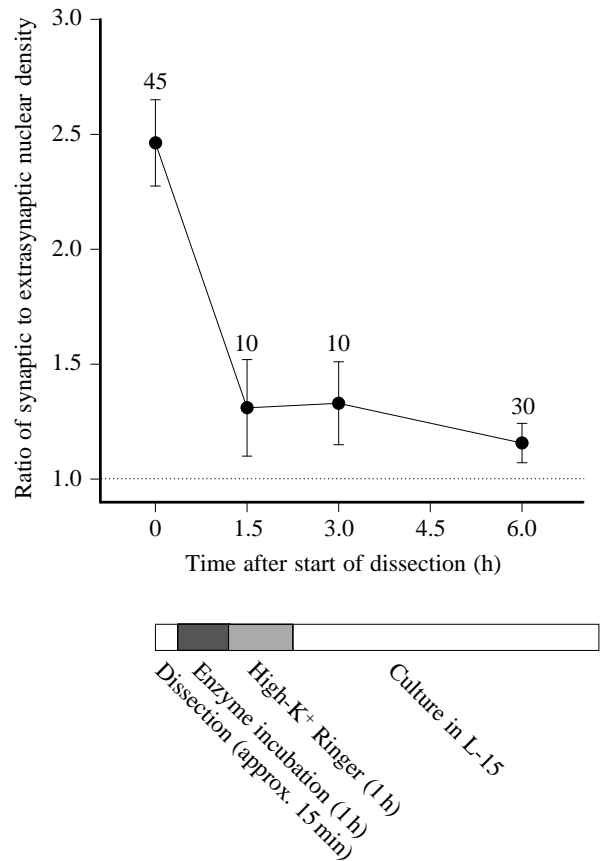


Fig. 3. Time course of redistribution of synaptic nuclei. Plot of the ratio of the nuclear density in the synaptic region to the density in the extrasynaptic region of the muscle fibers against time after beginning to prepare the muscle fibers for isolation. At time zero (for a muscle fiber fixed *in vivo*), the synaptic nuclear density was 2.46 times greater than that of the extrasynaptic region. Within 1.5 h of beginning to isolate the muscle fibers, the density of synaptic nuclei fell to only 1.31 times that of the extrasynaptic regions. This redistribution did not change significantly over the following 4.5 h. Values are given as means \pm S.E.M. The numbers of muscle fibers examined at each time point are given. The bar under the plot gives a reference of the times required to prepare the isolated muscle fibers.

Nuclear movement

The nuclei in cultured chick myoblasts were found to move (Englander and Rubin, 1987). Individual labeled nuclei of dissociated muscle fibers, which had already become dispersed, were observed under time-lapse microscopy to determine whether the nuclei continued to move following their dispersal. Images of the nuclei were captured at 10 min intervals over a 3 h period. The images were then overlaid and the positions of the nuclei compared. None of the nuclei observed moved at a rate of more than $1 \mu\text{m h}^{-1}$ ($N=22$, four fibers). It should be noted that many of the muscle fibers that were labeled with SYTO 13 contracted slowly under fluorescence illumination. Only muscle fibers that showed no contraction during the time-lapse examination were used for the analysis of nuclear movement.

Influence of ECM preparations on nuclear distribution in cultured muscle fibers

A number of molecules in the synaptic ECM play important roles in organizing both the pre- and postsynaptic components of the neuromuscular junction. This, combined with the observation that the nuclei declustered in parallel with the removal of the muscle fiber ECM, suggested that components of the ECM might be involved in inducing and maintaining the clustering of the postsynaptic nuclei. Therefore, isolated muscle fibers were exposed to a number of different ECM preparations to see whether they could bring about the reclustering of nuclei at the original synaptic sites.

After 1, 6 and 12 days of exposure to ECM preparations, the nuclei were labeled with SYTO 13 and the AChRs with rh-BTX. The distribution of nuclei in 10 muscle fibers per time point was analyzed. No reclustering of nuclei was observed ($P < 0.05$, Student's *t*-test).

Discussion

The present study examined the declustering of postsynaptic skeletal muscle fiber nuclei that was found to be related to dissociating the muscle fibers from one another with proteases. Although it would be of interest to follow the declustering of the nuclei in a single muscle fiber in real time, this was not possible. This was due to our inability to determine which nuclei belonged to a single muscle fiber of interest. Such observations were made difficult owing to the simultaneous labeling of the nuclei of the Schwann and other cells associated with the neuromuscular junction of the muscle of interest as well as those of muscle fibers lateral to, above and below the fiber of interest. Therefore, the study consisted of an examination of single muscle fibers after their complete dissociation or of single muscle fibers teased from fixed muscles.

For the present study, it was critical that we were certain that the muscle fibers were completely dissociated to ensure that we were examining nuclei of only single muscle fibers. This was confirmed by light microscopic examination of both muscle fibers that had been protease-dissociated and those that had been teased apart following fixation of the intact muscle. For the protease-treated muscles, electron microscopic examination confirmed that the treatment yielded completely dissociated fibers. In the case of the fixed muscle fibers, it was much more difficult to achieve completely dissociated muscle fibers by teasing, and only the few single fibers that could be clearly be seen to be free of satellite cells were used in the analysis.

Although skeletal muscle fibers have nuclei distributed along their length, it is well established that there is a cluster of nuclei in the postsynaptic region (Cardasis, 1979; Roberts, 1987; Jasmin *et al.* 1993). The mechanisms that give rise to and maintain these nuclear clusters are unknown. The present experiments were designed to examine whether muscle activity or the ECM influences the maintenance of the nuclear clusters or affects reclustering.

Within 1.5 h of initiating the protease treatment to dissociate lumbricalis muscle fibers, the density of postsynaptic nuclei decreased from 2.5 to 1.3 times that in the extrasynaptic regions. By 6 h, the density had fallen further, to 1.2 times that of the extrasynaptic regions. Once the nuclei had declustered, they were never observed to recluster even after the muscle fibers had been *in vitro* for up to 4 weeks.

Muscle activity has been shown to be involved in the differential activation and inhibition of gene expression in muscle fiber nuclei. This activity-dependent mechanism plays a role in the repression of AChR mRNA expression in extrasynaptic regions of muscle fibers (Goldmann *et al.* 1988). Once nuclear clusters have formed, it is possible that muscle activity is required to maintain the clusters. Therefore, nuclear distribution was examined in muscle fibers after denervating them to block all evoked contractions. Muscle fibers denervated *in vivo* for up to 4 weeks showed no redistribution of the clustered nuclei. Thus, the maintenance of nuclear clusters is independent of synaptic activity, evoked contraction and factors released from the innervating nerve terminal.

The Schwann cells at the neuromuscular junction could play a role in maintaining the nuclear clusters by the continuous release of clustering factors. However, after long-term denervation, the Schwann cells retract from the synaptic site (Kuffler, 1986). In the present experiments, in spite of long-term denervation and the expected withdrawal of the Schwann cells from the synaptic sites, the nuclear clusters remained. These results suggest that the Schwann cells are not responsible for maintaining the nuclear clusters.

The ECM plays a number of important roles in the synaptic regions of muscle fibers, influencing the differentiation of the muscle fiber postsynaptic structures as well as differentially regulating a variety of genes in muscle fiber synaptic and extrasynaptic nuclei. AChR-inducing activity causes an increase in the expression of the α -subunit of the AChR mRNA, but only in a subset of nuclei in cultured chick embryonic myotubes (Harris *et al.* 1989), while it has been proposed that another molecule in the synaptic basal lamina induces the expression of AChR mRNA exclusively in synaptic nuclei (Brenner *et al.* 1992; Jo and Burden, 1992). Agrin, and possibly other molecules released into the synaptic ECM by nerve terminals (Harris *et al.* 1989; Wallace, 1989), brings about the clustering of AChRs in the muscle fiber membrane and the development of synaptic folds.

In developing chick muscle cells, moving nuclei become 'trapped' under AChR clusters (Englander and Rubin, 1987). This observation suggests that the cytoskeleton underlying the ACh clusters plays a role in trapping the nuclei. Transmembrane linkers, such β -1-integrin (Gehlsen *et al.* 1989) or the dystrophin-glycoprotein complex that links the cytoskeleton and the laminin in the ECM (Ervasti and Campbell, 1993), could be involved in influencing the organization of the cytoskeleton to 'trap' the postsynaptic nuclei. Activation of the β -1-integrin receptor which accumulates at the neuromuscular junction (Gehlsen *et al.*

1989), or induction into the ECM of the same or different factors from those that induce AChR clustering and postsynaptic specializations, may induce the formation of the specialized postsynaptic cytoskeleton, giving it the ability to 'trap' migrating nuclei.

Following enzyme dissociation of adult rat and mice skeletal muscle fibers, which removes their ECM, their AChR clusters begin to disaggregate (Lupa and Caldwell, 1991). Our similar findings in frog muscle, where protease-treated muscle fibers examined in the electron microscope also appear to be completely free of ECM, suggest that specialized cytoskeletal proteins (such as ankyrin and the 43 kDa protein) depend on an extracellular cofactor to anchor the AChR and Na⁺ channels in the membrane (Lupa and Caldwell, 1991) and the nuclei in the postsynaptic cytoplasm. Removal of the cytoskeleton-organizing factors from the ECM could bring about the disruption of the specialized cytoskeleton and the release of the trapped nuclei.

If the removal of an ECM component resulted in the declustering of the nuclei, it is possible that the application of these factors to the muscle fibers might induce the reclustering of the dispersed nuclei. Although we failed to induce nuclear reclustering with a variety of ECM preparations, this could have resulted from our failure to apply the correct factor in an appropriate manner or from the receptor for the ECM factor having been modified or removed by the protease treatment. Although agrin induces the clustering of AChRs when added diffusely to the medium of cultured muscle fibers (Nitkin *et al.* 1983), it has been shown that basic fibroblast growth factor must be applied focally to bring about receptor clustering (Dai and Peng, 1992). Thus, initiation of nuclear clustering may require the appropriate ECM factor to be applied to a small area of the muscle membrane rather than diffusely to the entire muscle fiber. This was not possible in the present experiments in which the muscle fibers were free-floating since they would not adhere to the substratum. Therefore, although no nuclear clustering was observed, the factors used in the present study must be retested using different application techniques on substratum-attached muscle fibers.

The nuclei of chick embryonic muscle fibers have an average rate of movement of 8–9 $\mu\text{m h}^{-1}$, with rates reaching 16 $\mu\text{m h}^{-1}$ (Englander and Rubin, 1987). However, in the present experiments, although nuclei showed a period of rapid movement during the period of cluster dispersal, they did not subsequently move significantly ($<1 \mu\text{m h}^{-1}$, $N=22$ in four fibers). In the absence of any movement of nuclei through the synaptic region, no nuclei could have become 'trapped' and reclustered in these muscle fibers, even if the specialized postsynaptic cytoskeleton had been established by the ECM components. It is possible that nuclear movement only occurs when the muscle fibers are under resting tension. It will be important to improve the adhesion of the muscle fibers to the culture dish to see whether this gives rise to nuclear movement. It will also be interesting to observe whether nuclear clustering occurs in the postsynaptic region when these isolated muscle

fibers become innervated *in vitro*, suggesting that ECM components other than those tested are able to trigger the clustering of the postsynaptic nuclei.

We thank Dr Zach Hall for generously providing the agrin and Mr Sixto Quiles for assistance with the graphics. Supported by ARO DAAL03-90-G-0189, ONR NB-91-12-101, NIH 5 PO1 NS07464-25 and NSF-EPSCoR EHR9108775.

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