Identification of Acetylcholine Receptor Subunits Differentially Expressed in Singly and Multiply Innervated Fibers of Extraocular Muscles

Sven Fraterman,[1,2] *Tejvir S. Khurana,*[2,3] *and Neal A. Rubinstein*[1,2]

Purpose. To identify the acetylcholine receptor (AChR) isoforms among the neuromuscular junctions (NMJs) of singly and multiply innervated fibers (SIFs and MIFs) of rat extraocular muscles (EOMs).

Methods. EOMs were dissected from adult rats and serially sectioned. Sections were simultaneously stained for acetylcholinesterase and with an antibody to the slow myosin heavy chain to identify NMJ topography and fiber types in the same section. Synapses and subsynaptic regions of SIFs and MIFs were isolated by laser capture microdissection and the AChR subunits identified by RT-PCR.

Results. The en plaque endings of SIFs expressed only the adult ε subunit, not the fetal γ subunit, of the AChR, whereas the en grappe endings of the MIFs expressed only the γ subunit, and not the ε subunit. Although the expression of the ε subunit was confined to the NMJ region of the SIFs, the γ subunit was expressed both synaptically and extrasynaptically within the MIFs. The γ subunit in MIFs correlated with the expression of the myogenic regulatory factor myogenin. Moreover, an unusual neuronal AChR subunit, $\alpha 9$, was found in the EOMs, but not in the limb muscles.

Conclusions. The adult ε and fetal γ subunits of the AChRs are segregated into distinct synapses on distinct fiber types. The maintenance of the fetal subunit in a population of fibers is probably linked to the expression of myogenin and is a unique attribute of the EOM allotype. (*Invest Ophthalmol Vis Sci.* 2006;47:3828–3834) DOI:10.1167/iovs.06-0073

With few exceptions, adult mammalian skeletal muscle fibers contain a single neuromuscular junction (NMJ), usually situated at the center of the fiber, and these singly-innervated fibers (SIFs) have been the paradigms for investigating the assembly, structure, and function of the NMJ.[1] Although several proteins are involved in the structure of the mature NMJ, the most important is the nicotinic acetylcholine receptor (AChR), which allows the transmission of nerve impulses into the fiber. The concentration of the AChRs at the subsynaptic membrane is the result of a complex process involving receptor migration, restricted nuclear capabilities, and changes in receptor gene expression.[1–3] In immature fibers, nuclei throughout the length of the fiber are capable of synthesizing the AChR subunits. Assembled receptors are found throughout the fiber; and the fibers are polyneuronally innervated.[4] With maturation, the polyneuronal innervation is lost, the preformed receptors migrate to the synaptic region and synthesis of receptor subunits is largely restricted to subsynaptic nuclei. Subsynaptic nuclei have long been considered specialized in their synthetic capabilities; and, in fact, in innervated adult skeletal muscle, AChR subunits are synthesized only by these subsynaptic nuclei.[5–9] Finally, replacement of the fetal γ receptor subunit with the adult ε subunit results in a pentameric receptor, $\alpha_2\beta\delta\varepsilon$, with properties that are are distinct from those in the more immature fiber.[10,11]

The extraocular muscles (EOMs) are a group of specialized skeletal muscles used by the visual system to locate and accurately track objects.[12] Because these muscles are adapted to their role in the control of eye movement, they exhibit fundamental differences from other skeletal muscles, including differences in the AChRs and NMJs.[11,13–16] Adult EOMs express both the γ and ε subunits[17]; moreover, only 80% of their fibers are singly innervated fibers (SIFs), whereas the remainder are multiply innervated (MIFs).[18] The MIFs are not usually found in healthy adult mammalian muscles, but are found in muscles of birds, reptiles, and amphibians,[19,20] as well as a consequence of denervation and age dependent atrophy of mammalian muscle.[20–24]

In addition to the AChR subunits, several other molecules are selectively transcribed by a small number of spatially restricted and specialized subsynaptic nuclei, which are tethered to the subsynaptic region by an anchoring protein, syne-1.[25] Other synapse-specific molecules include utrophin, sodium channels, acetylcholinesterase, and even TGFβ.[26,27] It is likely that many, if not most, of the molecules with synapse-specific regulation are yet to be identified.[8,9] In prototypical (i.e., limb) skeletal muscles, the regulation of the synapse specific transcription of the AChRs is the most studied. The regulatory mechanisms described for other skeletal muscles, however, may not be relevant to the EOMs with their multiple types of NMJs and their maintenance of both the fetal γ and the adult ε subunit expression into adulthood.

Studies of MIFs and the formation of their NMJs are severely limited. In fact, expression and localization of the γ and ε subunits among adult EOM fiber types are uncertain, since immunohistochemical studies using subunit specific antibodies have yielded conflicting results.[11,14] To begin to address the control of synapse-specific synthesis in EOMs, we used laser capture microdissection (LCM) to isolate the subsynaptic regions from the NMJs of multiply and singly innervated fibers of adult rat EOMs. We then used RT-PCR to determine the AChR subunit mRNAs expressed by these nuclei.

From the Departments of [1]Cell and Developmental Biology and [3]Physiology, and the [2]Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania.

Supported by National Eye Institute Grants EY11779 and EY01386, Vision Research Center Core Grant EY01583, and National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR051696.

Submitted for publication January 24, 2006; revised April 20, 2006; accepted July 13, 2006.

Disclosure: **S. Fraterman**, None; **T.S. Khurana**, None; **N.A. Rubinstein**, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Neal A. Rubinstein, Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104; nrubinst@mail.med.upenn.edu.

TABLE 1. Primer Sequences

	Product Size (bp)	Forward Primer Reverse Primer	Annealing Temperature (°C)
α1	288	5′-TGGAAGCACTGGGTGTTTTA	53
		5′-AACATGTACTTCCCGATCAGG	
α2	157	5′-CCTCTGAAGCCATGACCCTTTC	56
		5′-CGATTGTAGCCTCCAAACAGGTG	
α3	526	5′-CGTACTGTACAACAACGCTGATGGGG	59
		5′-GGAAGGAATGGTCTCGGTGATCACCAGG	
α4	483	5′-GTTCTATGACGGAAGGGTGCAGTGGACA	59
		5′-GGGATGACCAGCGAGGTGGACGGGATGAT	
α5	684	5′-GTTCCTTCGGACTCCCTGTGGATCCC	59
		5′-GGGCGCCATAGCGTTGTGTGTGGAGG	
α6	609	5′-TCTTAAGTACGATGGGGTGATAACC	59
		5′-AAGATGGTCTTCACCCACTTG	
α7	510	5′-GTGGAACATGTCTGAGTACCCCGGAGTGAA	59
		5′-GAGTCTGCAGGCAGCAAGAATACCAGCA	
α9	288	5′-TTATCTGGGAGAGCGTGACCTG	56
		5′-GTGGCAGTGAGGGTGTTTTGAG	
α10	209	5′-TAGCCAGTCTCTCCCCAAA	53
		5′-GCTGGAATTACCGTGCTCA	
β1	355	5′-ATAGCTCAGTAAGGCCGGCG	57
		5′-TAGGTGACCTGGATGCTGCA	
β2	498	5′-CTTCTATTCCAATGCTGTGGTCTCCTATG	59
		5′-AGCGGTACGTCGAGGGAGGTGGGAGG	
β3	466	5′-CCATCAGAATCGGCTCTGGCTGCCGGAC	59
		5′-CGTCCGAGGGTAGGTAGAACACCAGG	
β4	495	5′-TGTCTACACCAACGTGATTGTGCGTTCCA	59
		5′-AGCGGTACGTCGAGGGAGGTGGGAGG	
γ	114	5′-GGGTCCGCAAGGTGTTTTTG	57
		5′-AGGAAGAGCCATTCTGGAGTCG	
δ	223	5′-CAATGAGGAGAAGGACAACTGGAAC	56
		5′-GCCGTCTGCCTGACTGTTTTACTC	
ε	215	5′-TGTTTGAGGGACAGAGGCATCG	57
		5′-CACCAACGCTGCCCAAAAAC	
Myogenin	438	5′-TGGGCGTGTAAGGTGTGTAAGAGG	59
		5′-CATATCCTCCACCGTGATGCTG	
GAPDH	195	5′-CCATGGAGAAGGCTGGGG	60
		5′-CAAAGTTGTCATGGATGACC	
Slow myosin	190	5′-GAGNAGGTCGAGNACGACCTAGGGC	54
		5′-CGTNAAAAGNAACTTNNCTTAAGCG	

METHODS

Tissue Preparation and LCM

Adult Sprague-Dawley rats were killed by CO_2 inhalation. Animals were maintained in approved accommodations at the School of Medicine, University of Pennsylvania, and used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Using sterile conditions, the extensor digitorum longus (EDL) from the distal hindlimb, the brain, and the globes and their associated muscles were isolated and frozen in 2-methylbutane chilled in liquid nitrogen. Twelve- to 14-μm sections were mounted on electrostatically charged slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). Sections were then washed in water and dehydrated sequentially in 75%, 95%, and 100% ethanol followed by 100% xylene. Sections were allowed to air dry and were stored in a desiccator at room temperature until they were used for LCM. For some experiments, distal hindlimbs were denervated by removal of a 2-mm segment of the sciatic nerve in the proximal thigh after halothane anesthesia.[28] Animals were killed 30 days after surgery. EDL muscles were excised and treated as described for the orbit.

Staining of Sections

We developed a method that allowed visualization and discrimination of the different types of endings in EOMs and also allowed later analyses of laser capture microdissected tissue at the nucleic acid and protein level. Previous results from our laboratory and the laboratories of others have shown that the MIFs of EOMs express a slow twitch or tonic MyHC.[16,29] Hence, we used AChE staining[30] as well as a mouse monoclonal anti-slow MyHC antibody[31] conjugated with Alexa Fluor 488 for unequivocal detection of synaptic regions of SIFs and MIFs as well as extrasynaptic regions of both fiber types. Both labels were visualized simultaneously using the blue filter cube (excitation, 455–495 nm/emission, >510 nm) of the fluorescence package and dimmed visual light. Antibody was conjugated to Alexa Fluor 488 by using Zenon technology (Invitrogen, Carlsbad, CA). To preserve the tissue for LCM, the entire staining procedure was kept as brief as possible.

To avoid any confusion with staining emanating from AChE enriched at the myotendinous junctions—found at the ends of muscle fibers—only the central third of each EOM was examined on LCM. Even within the central third, we limited NMJ capture to an area within 2 mm of the endplate band. EOMs have a well-defined endplate band in which most of the motoneurons terminate with single synapses on individual muscle fibers.

For double staining with antibodies to myogenin and to slow MyHC, frozen sections were fixed in 50% methanol/50% acetone at −20 °C for 10 minutes, then air dried. Sections were blocked at room temperature for 1 hour in prestain solution (90% PBS/10% fetal calf serum/0.1% Triton X-100). Monoclonal anti-myogenin antibody F5D (Developmental Studies Hybridoma Bank, Iowa City, IA) was coupled to Alexa Fluor 488, while the anti-slow MyHC antibody was directly

coupled to Alexa Fluor 546. Antibodies were diluted in prestain solution, and sections were stained for 2 hours at room temperature and washed in PBS before visualization.

Identification of En Grappe and En Plaque Endings

The en grappe and en plaque classification of EOM fibers was originally an electron microscopic observation.[15,32] In EOMs, en plaque endings are those found in SIFs, whereas en grappe endings are found on MIFs. Because this fact is so accepted among the workers in this field, endings visualized by light microscopy can be categorized as en plaque or en grappe by their location on SIFs or MIFs, respectively.[12,18] We have suggested[29,33] and Khanna et al.[16] and Khanna and Porter[34]have observed that the MIFs of mammalian EOMs are the ones that stain with an anti-slow myosin antibody and that this observation alone (staining with anti-slow MyHC) can identify MIFs and, by definition, their en grappe endings.

Microdissection

For LCM, we used a commercial system (PixCell II; Acturus Engineering, Mountain View, CA) and followed methods described previously.[9] For each type of NMJ (from EDL, EOM SIF, and EOM MIF) we captured 20 to 25 NMJs on each plastic cap and combined 8 to 10 caps for each experiment. All 8 to 10 caps contained material from one animal. In any experiment in which we compared the various NMJ subunits, all samples came from the same animal. Each experiment was repeated three times. The laser beam was adjusted to a 7-μm spot diameter, power was 75 mW, and pulse duration was 2.5 ms. To avoid cross contamination of MIF NMJs by SIF NMJs, MIF NMJs were captured outside, but within 2 mm, of the endplate band. Caps were examined under the microscope to check for contamination by other than the desired tissue. The orbital and global layers of the EOM were dissected as previously described.[35]

Reverse Transcription–Polymerase Chain Reaction

RNA was isolated from laser captured material using the PicoPure RNA Isolation Kit (Arcturus Engineering), as recommended by the manufacturer. Total RNA was isolated from whole EDL and brain tissue (Trizol reagent and protocol; Invitrogen). The amount of total RNA was determined by UV spectroscopy.

Fifty nanograms of amplified cDNA was subjected to a singleplex PCR reaction for the different AChR subunits, GAPDH and myogenin. The exact annealing temperature is provided for each primer pair in Table 1. Primers were designed by computer (MacVector; Accelrys, Marietta, GA). All primers lie in different exons or on an exon–exon boundary. To retrieve the position of an exon within a gene sequence, the mRNA and gene sequences were aligned (PromotorWise software at the EBI server; www.ebi.ac.uk/Wise2/promoterwise.html/ provided in the public domain by the European Bioinformatics Institute, Hinxton, UK). The correctness of the alignment of the gene and mRNA was checked by the graphic output view of the Gene database (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=gene) at the National Center for Biotechnology Information (NCBI) server.

Twenty microliters of the PCR products were run on a 50-mL 2.5% agarose gel cast with 2.5 μL ethidium bromide (10 mg/mL).

Results

Figure 1 shows low-power views of a normal and a denervated EDL as well as the endplate region and the peripheral region of an adult rat EOM. In the innervated EDL (Fig. 1A), large, slightly c-shaped endings stained strongly for AChE, while in denervated EDL (Fig. 1B) the size of the endings was decreased and small, grapelike endings were present. In the endplate band of the EOM (Fig. 1C), two types of endplates were seen: large chevron- or c-shaped endings (also referred to as en plaque endings) which resemble the NMJs of the intact EDL and the smaller grape-like (en grappe) endings, which resemble the multiply innervated endings of the denervated EDL. Peripherally (Fig. 1D), only the en grappe endings were seen.

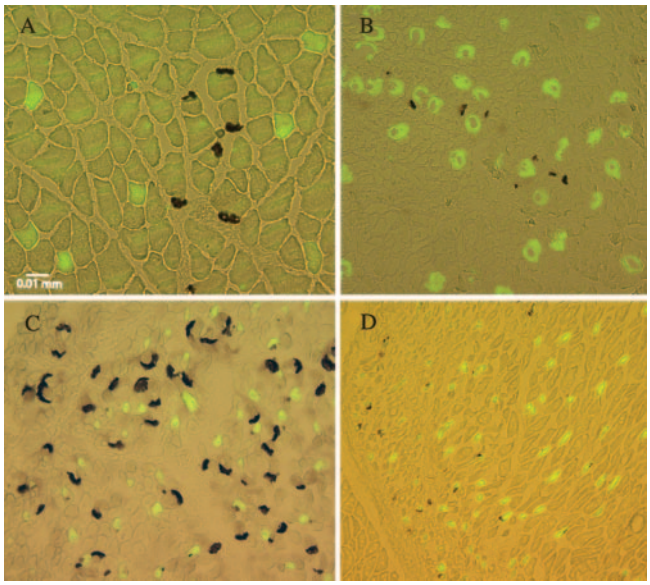

Figure 1. Sections of adult rat muscles stained for AChE and for slow MyHC. In the innervated EDL (**A**), large chevron-shaped (or c-shaped) endings stain strongly for AChE, whereas in denervated EDL (**B**) the size of the endings is decreased and small, grapelike endings are present. In the endplate band of the EOM (**C**), two types of endplates were visible: large chevron-shaped endings (also referred to as en plaque endings) which resemble the NMJs of the intact EDL; and the smaller grapelike, or en grappe, endings which resemble the multiple NMJs of the denervated EDL. Peripheral to the EOM endplate band only the en grappe endings were seen (**D**).

At higher power, we observed that neither the NMJs of intact EDLs (Fig. 2A) nor the NMJs of denervated EDLs (Fig. 2B) showed any correlation with specific fiber types, as expected. The c-shaped endings in the endplate regions of both global (Fig. 2C) and orbital (Fig. 2D) layers of EOMs, however, are not associated with fibers that stain with the anti-slow MyHC antibody. In contrast, the smaller en grappe endings are invariably associated with slow-MyHC positive fibers, whether they are located in the endplate region (Fig. 2D) or in the peripheral region of the global (Fig. 2E) or orbital (Fig. 2F) layer. In Figure 3A, an area in the middle of the endplate region, one large c-shaped ending, not associated with a slow MyHC positive fiber, can be seen. This ending and the entire cross section of the fiber beneath it were removed for analysis by LCM (Fig. 3B).

We used RT PCR to identify the AChR subunits of whole innervated and denervated adult EDL muscles, as well as those of the LCM-isolated orbital and global layers of EOMs[35] (Fig. 4). All muscles or muscle parts contained the ε subunit, as well as the α, β, and δ subunits. As expected, the innervated EDL did not express the γ subunit, which had been replaced by the ε subunit during postnatal development.[11] The denervated EDL, however, reactivates synthesis of the γ subunit. In the adult innervated EOM global and orbital layers, however, both ε and γ subunits were present.

Because previous results have been conflicting,[11,14] we analyzed the NMJs of the EDL as well as of EOM MIFs and SIFs to determine the expression of the ε versus the γ subunits of the AChR (Fig. 5). As described earlier, the subsynaptic regions were isolated by LCM, RNA was isolated, and RT-PCR was

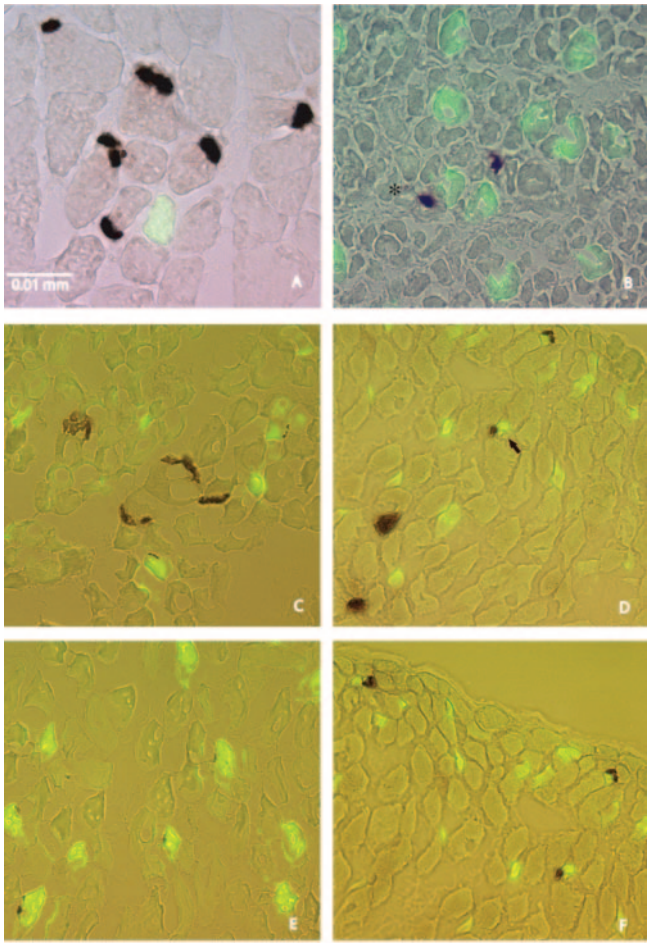

FIGURE 2. Higher-power magnifications of adult rat muscle sections stained for AChE and for slow MyHC. In the EDL (**A**) and in the denervated EDL (**B**), there was no consistent association of the endings with slow MyHC containing fibers. In the EOM, en plaque endings in the endplate region of the global (**C**) or orbital (**D**) layers were never associated with slow-MyHC–containing fibers. In contrast, the smaller en grappe endings were invariably associated with slow-MyHC positive fibers, whether they were located in the endplate region (**D**) or in the peripheral region of the global (**E**) or orbital (**F**) layer.

performed using appropriate primers. In the adult EOM (Fig. 5, right panel), in the NMJ of SIFs (the c-shaped endings in the endplate band), only the ε, not the γ, mRNA was detected.

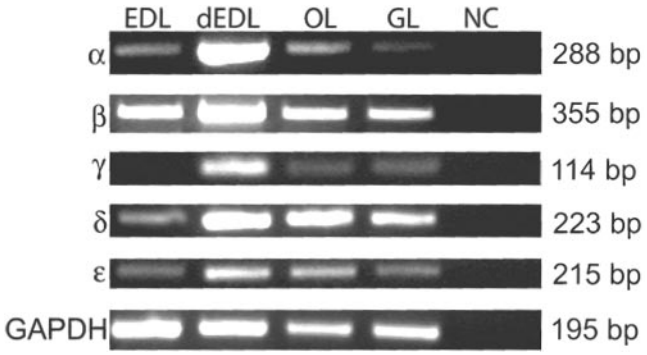

FIGURE 4. Demonstration of AChR subunits by RT-PCR. Total RNA isolated from entire muscles (EDL, dEDL) or laser captured material (OL, GL) was amplified and reverse transcribed into cDNA. Primers (Table 1) were used to amplify the entire set of AChR subunits from (EDL) adult rat EDL, (dEDL) denervated adult rat EDL, (OL) orbital layer and (GL) global layer of adult rat EOMs. A negative control (NC, no template) was also used. Whereas all muscles contained the normal adult α, β, δ, and ε subunits, only the denervated EDL and the various EOM layers also contained the γ subunit. The relative quantity of each subunit's mRNA was comparable to quantities demonstrated earlier.[36,37]

Away from the endplate band (the extrajunctional region of fibers lacking slow MyHC), SIFs did not synthesize any AChR subunit mRNAs. In the en grappe endings of the MIFs, however, only γ, not ε, mRNAs were detected. In the extrajunctional regions of the MIFs (slow+), γ subunits were detected. In the EDL (Fig. 5, left), only the ε subunit mRNA was detected; moreover, no receptor mRNAs were detected in the extrajunctional region.

Because the synthesis of the γ subunit in limb muscles has been shown to be dependent on myogenic regulatory factors,[22,38] we examined the NMJ samples for the expression of myogenin mRNA (Fig. 6). Significant PCR product was seen after 40 cycles in the orbital and global MIFs, although barely detectable amounts were seen in the SIFs of either layer. Moreover, in the areas of MIFs between NMJs, no myogenin could be detected. To confirm these findings, cross sections of adult EOMs were double stained with antibodies to slow MyHC and to myogenin (Fig. 7). Fibers positive for slow MyHC were also strongly positive for myogenin, whereas non–slow-MyHC fibers, the SIFs, were generally negative for myogenin.

We investigated the possibility that neuronal nicotinic AChRs may be expressed in EOM fibers, because several neuronal subunits have been found at rodent and avian NMJs.[39] As previously reported,[40] the $\alpha 4$ and $\beta 2$ subunits can be found in

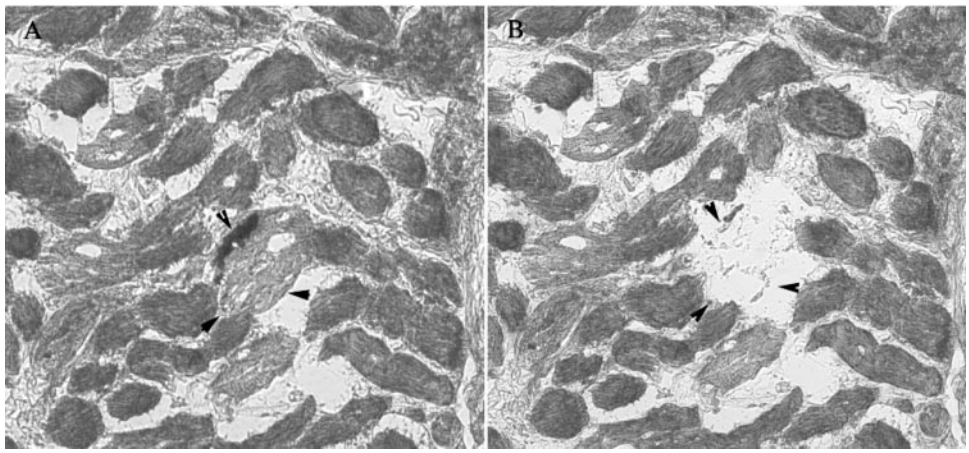

FIGURE 3. Removal of an individual EOM fiber in the NMJ region. An adult EOM was stained for AChE and an individual fiber with its associated NMJ removed by laser capture microdissection. (**A**) The fiber (*arrowheads*) with AChE staining clearly marking the NMJ; (**B**) the same field after removal of the fiber.

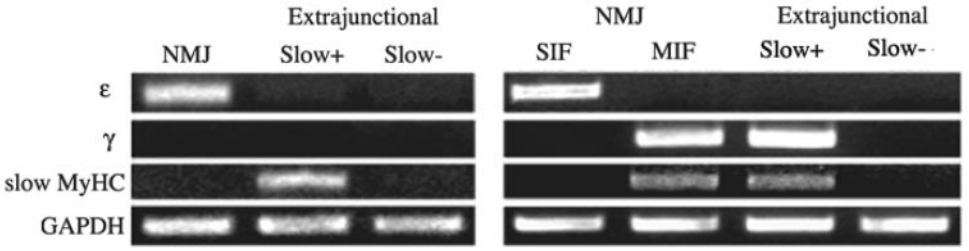

FIGURE 5. The γ and ε subunits in SIFs and MIFs of adult rat EOMs. NMJs were identified by staining with AChE, and fibers were identified with an antibody to slow MyHC. The fibers underlying NMJs were isolated by LCM, as were the extrajunctional region of slow positive (slow+) fibers and slow negative (slow−) fibers. In the EDL (*left*), only the ε subunit was identified by RT-PCR, and no subunits were detected in the extrajunctional region of any fiber type. In the EOMs (*right*) SIFs contained only the ε, not the γ, subunit, and SIFs did not contain either subunit mRNA in the extrajunctional region (extrajunctional slow−). MIFs, however, contained only the γ, not the ε, subunit; they also contained γ subunit mRNA in the areas between junctions (extrajunctional, slow+).

the innervated and denervated EDLs (Fig. 8). These were not found, however, in the adult rat EOM. In contrast, the α9 subunit was found in both the global and orbital layers of EOMs, but not in the EDL. This was confirmed by Western blot analysis (not shown).

DISCUSSION

In embryonic mammalian skeletal muscles, the fetal AChR contains five subunits: $\alpha_2\beta\gamma\delta$. Postnatally, the γ subunit is replaced by the ε subunit to produce the pentameric adult receptor $\alpha_2\beta\varepsilon\delta$.[8] In EOMs, however, whereas the ε subunit is synthesized postnatally, the muscle also retains synthesis of the γ subunit,[14,17] perhaps because of the presence of the unusual multiply innervated fibers in these muscles; but previous work has not resolved the question of distribution of the γ and ε subunits among the NMJs of SIFs and MIFs. Using polyclonal antibodies produced against peptide fragments of the γ and ε AChR subunits, Kaminski et al.[14] found binding of both antibodies to most en plaque and en grappe endings of adult rat EOMs. A subset of en plaque endings in the global region of the muscle stained with only one antibody, the anti-ε antibody. Their conclusion was that all en grappe endings and most en plaque endings in adult EOMs contained both the ε and the γ subunits. Using the same anti-ε antibody plus a distinct anti-γ antibody to study mouse EOMs, Missias et al.[11] presented an alternative conclusion: all AChRs of SIFs contain only the ε subunit, while nearly all MIFs contain only the γ subunit. A few en grappe endings stained strongly for the γ subunit and weakly for the ε subunit. Missias et al.[11] also produced transgenic mice bearing the promoter elements of the ε or γ subunits coupled to nuclear-localized β-galactosidase. In mice with the ε transgene, nuclear staining occurred only in the endplate band. With the γ transgene, nuclear staining occurred throughout the entire length of the muscle. Surprisingly, weakly positive staining with the γ transgene also occurred frequently in nuclei underlying the en plaque endings of SIFs.

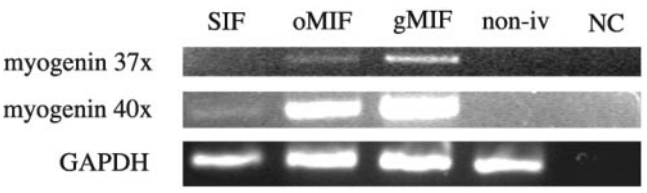

FIGURE 6. Identification of myogenin mRNA in MIFs. Because the synthesis of the γ subunit has been shown to be dependent on myogenic regulatory factors, we used primers to test the NMJ samples for the presence of myogenin mRNA. Significant PCR product was seen after 40 cycles in the orbital (oMIF) and global MIFs (gMIF), whereas barely detectable amounts were seen in the SIFs (SIF) of either layer. Moreover, in the noninnervated (non-iv) areas of MIFs, no myogenin was detected. This result is distinct from the finding of the γ subunit in these same areas (i.e., non-NMJ areas). NC, no template.

Our results for the localization of the γ and ε mRNAs are similar, but not identical, with the transgenic results of Missias et al.[11] Comparison of our results with those of Kaminski et al.[14] is not possible, since those investigators localized the protein products of the γ and ε genes, whereas we were localizing their mRNAs. Because we used LCM to identify unequivocally and remove subsynaptic regions of en grappe and en plaque endings of MIFs and SIFs, we were able to show that the subsynaptic areas underlying the c-shaped NMJs of SIFs expressed a great preponderance of the ε subunit, whereas the areas underlying the grapelike en grappe endings of MIFs expressed the γ subunit. We did not look at individual NMJs, as the previous investigators did. Although we cannot rule out that a small subset of NMJs in our samples expressed a different subunit, we would have expected that RT-PCR of approximately 200 subsynaptic regions would have revealed any minor components. Our conclusion, then, is that γ and ε subunits are predominantly synthesized by the nuclei beneath the NMJs of MIFs and SIFs, respectively.

It has been shown in other adult muscles that the ε subunit is synthesized in a very small subsynaptic domain and cannot be found in extrajunctional regions; of note, in EOM MIFs the γ subunit was found not only subsynaptically, but also in extrajunctional regions (Fig. 5). This finding is not due to the lack of resolution by LCM, because we found myogenin expressed exclusively in subsynaptic regions of MIFs, not between the synapses (Fig. 6). Furthermore, in Missias et al. (Ref. 11, Fig. 6a) it appears that the γ transgene is expressed in most, if not all, nuclei of MIFs, not just by subsynaptic nuclei. It is possible, then, that the SIF and MIF AChRs have distinct regulatory mechanisms, with synthesis of the ε subunit in SIFs restricted to subsynaptic nuclei, whereas synthesis of the γ subunit in MIFs shows no such restricted expression.

There is some evidence that myogenin, one of the myogenic regulatory factors, plays a role in the control of γ subunit synthesis. After denervation and during age dependent atrophy, levels of myogenin and the γ subunit transcript increase simultaneously[22]; and this increase in expression of the subunit and myogenin is colocalized along the fiber.[38] Furthermore, in the denervated muscle, the half-life of myogenin is increased. MyoD, in contrast, shows only minor upregulation after denervation.[41]

Considering the repetitive forces EOMs are subject to and reports that MIFs in frogs and atrophic muscle are dynamic structures,[19,24] it appears that myogenin may be an endogenous factor expressed in response to muscle fiber remodeling, stress, and damage. Myogenin itself binds with other regulators of transcription to the E-box in the promotor region of the γ subunit and increases its expression levels.[42] Nerve-induced electrical activity causes a large influx of Ca^{2+} which abolishes myogenin activity within minutes through the PKC-mediated

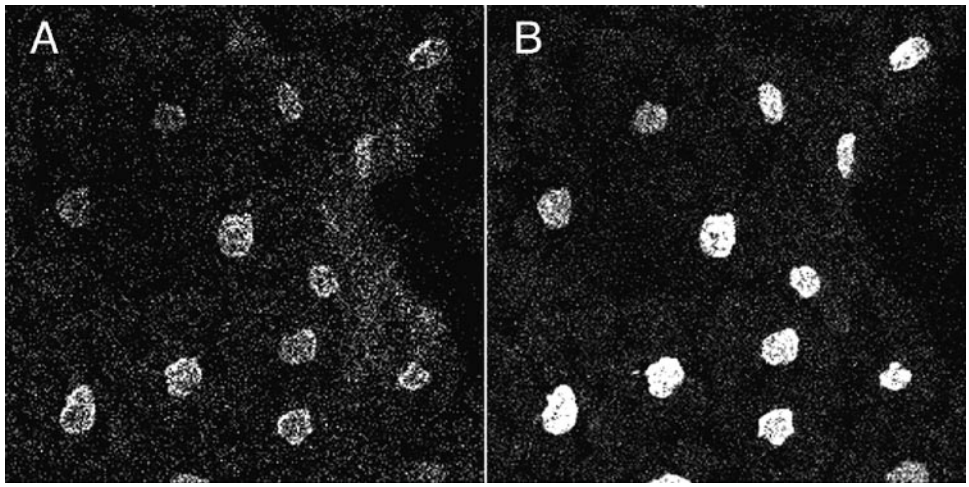

FIGURE 7. Immunohistochemical localization of myogenin. To confirm the PCR findings above, cross sections of adult EOMs were double stained with antibodies to myogenin (**A**) and to slow MyHC (**B**). Fibers positive for slow MyHC (MIFs) were also positive for myogenin, whereas non–slow MyHC fibers (SIFs) were generally negative for myogenin. Identical staining was seen with a rabbit polyclonal antimyogenin antibody, M-225 (Santa Cruz Biotechnology, Santa Cruz, CA).

phosphorylation of the myogenin.[43] It is possible that the different channel properties of the γ-containing AChR with the much lower influx of Ca^{2+} with each stimulus[10] allows the MIFs of EOMs to sustain myogenin activity and, hence, γ subunit synthesis in the adult.

The nicotinic AChRs can be categorized as neuronal or muscular by their anatomic localization and their specificity for inhibitors.[44] The typical mammalian NMJ contains the muscular subunits, whereas the neuronal subunits are widely distributed in the central nervous system and in parts of the peripheral nervous system, such as the ciliary ganglia[45] and cardiac ganglia.[46] Homology between the muscular and neuronal subunits in the same species can range from 48% to 70%, although the homology can approach 100% in the conserved transmembrane regions.[47] It should be noted that the nomenclatures for the muscular and the neuronal subunits are independent, and similar names do not define structural or functional similarities. A unique member of the neuronal subunit family is the α9 subunit. First described in cochlear hair cells, embryonic tongue, and the nasal epithelium, it shows only 39% identity to other subunits. It normally can form homopentamers or heteropentamers with α10. It shows unique pharmacological properties: It is blocked by antagonists of $GABA_A$, 5-HT3, and glycine, but is insensitive to nicotine and muscarine.[48] The α9 receptor plays a key role in innervation of the cochlear hair cells.[49] The cochlear hair cell is innervated by multiple nerve terminals; however, when α9 is knocked out in a transgenic animal, only a single nerve terminal remains.[50] In addition to this role in establishment or maintenance of multiple innervation of the cochlear cells, α9 is also responsible for modulating auditory nerve responses to acoustic stimulation and protection from overstimulation by calcium dependent potassium conductance and hyperpolarization.[51] Roles for the α9 subunit in EOMs are only hypothetical, especially since the cell localization of the subunit is not yet known and the channel built by α9 is most effective in the presence of the atypical α10 subunit, a subunit expressed in cochlear cells but not in EOMs.

The EOM allotype, then, shows an atypical content of AChR isoforms, both the fetal γ and the adult ε subunits are present in the adult, unlike the situation in other muscles. Moreover, these subunits are segregated into distinct fiber types, with the fetal (γ) subunit being expressed synaptically and extrasynaptically in MIFs. The presence of the neuronal subunits, however, suggests that the composition of AChRs in EOMs is more complex than previously imagined.

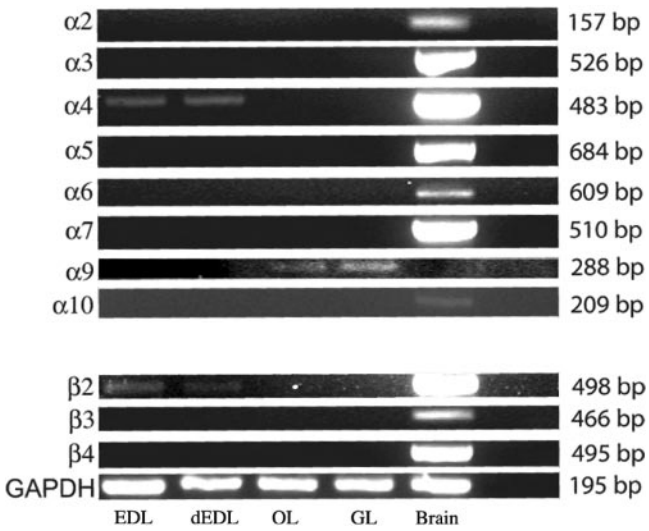

FIGURE 8. Neuronal nicotinic AChR subunits in EOMs. The nicotininc AChRs can be divided into muscular and neuronal types of subunits. The muscular subunits are those described earlier and found predominantly at the NMJ. As previously reported by others, the α4 and β2 subunits can be found in the innervated (EDL) and denervated (dEDL) EDLs. These were not found, however, in the orbital layer (OL) or global layer (GL) of adult rat EOM. In contrast, the α9 subunit was found in both the global and orbital layers of EOMs, but not in the EDL.

References

1. Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat Rev Neurosci*. 2001;2:791–805.
2. Sanes JR, Lichtman JW. Development of the vertebrate neuromuscular junction. *Annu Rev Neurosci*. 1999;22:389–422.
3. Burden SJ. The formation of neuromuscular synapses. *Genes Dev*. 1998;12:133–148.
4. Brown MC, Jensen JKS, Van Essen DC. Poly-neuronal innervation of skeletal muscle in new-born rats and its elimination during maturation. *J Physiol*. 1976;261:387.
5. Goldman D, Staple J. Spatial and temporal expression of acetylcholine receptor RNAs in innervated and denervated rat soleus muscle. *Neuron*. 1989;3:219–228.
6. Fontaine B, Sassoon D, Buckingham M, Changeux JP. Detection of the nicotinic acetylcholine receptor alpha-subunit mRNA by in situ hybridization at neuromuscular junctions of 15-day-old chick striated muscles. *EMBO J*. 1988;7:603–609.
7. Merlie JP, Sanes JR. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibres. *Nature*. 1985;317:66–68.

8. McGeachie AB, Koishi K, Andrews ZB, McLennan IS. Analysis of mRNAs that are enriched in the post-synaptic domain of the neuromuscular junction. *Mol Cell Neurosci.* 2005;30:173–195.
9. Nazarian J, Bouri K, Hoffman EP. Intracellular expression profiling by laser capture microdissection: three novel components of the neuromuscular junction. *Physiol Genom*. 2005;21:70–80.
10. Mishina M, Takai T, Imoto K, et al. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature*. 1986;321:406–411.
11. Missias AC, Chu GC, Klocke BJ, Sanes JR, Merlie JP. Maturation of the acetylcholine receptor in skeletal muscle: regulation of the AChR gamma-to-epsilon switch. *Dev Biol*. 1996;179:223–238.
12. Porter JD, Baker RS, Ragusa RJ, Brueckner JK. Extraocular muscles: basic and clinical aspects of structure and function. *Surv Ophthalmol*. 1995;39:451–484.
13. Oda K. Motor innervation and acetylcholine receptor distribution of human extraocular muscle fibres. *J Neurol Sci*. 1986;74:125–133.
14. Kaminski H, Kusner L, Block C. Expression of acetylcholine receptor isoforms at extraocular muscle endplates. *Invest Ophthalmol Vis Sci*. 1996;37:345–351.
15. Spencer RF, Porter JD. *Neuroanatomy of the Oculomotor System.* New York: Elsevier; 1989.
16. Khanna S, Richmonds CR, Kaminski HJ, Porter JD. Molecular organization of the extraocular muscle neuromuscular junction: partial conservation of and divergence from the skeletal muscle prototype. *Invest Ophthalmol Vis Sci*. 2003;44:1918–1926.
17. Horton RM, Manfredi AA, Conti-Tronconi BM. The 'embryonic' gamma subunit of the nicotinic acetylcholine receptor is expressed in adult extraocular muscle. *Neurology*. 1993;43:983–986.
18. Spencer RF, Porter JD. Structural organization of the extraocular muscles. *Rev Oculomot Res*. 1988;2:33–79.
19. Letinsky MS, Morrison-Graham K. Structure of developing frog neuromuscular junctions. *J Neurocytol*. 1980;9:321–342.
20. Miledi R, Uchitel OD. Induction of action potentials in frog slow muscle fibres paralysed by alpha-bungarotoxin. *Proc R Soc Lond B Biol Sci*. 1981;213:243–248.
21. Fagg GE, Scheff SW, Cotman CW. Axonal sprouting at the neuromuscular junction of adult and aged rats. *Exp Neurol*. 1981;74:847–854.
22. Gomes RR Jr, Booth FW. Expression of acetylcholine receptor mRNAs in atrophying and nonatrophying skeletal muscles of old rats. *J Appl Physiol*. 1998;85:1903–1908.
23. Pestronk A, Drachman DB. The effect of aging on nerve sprouting. *Trans Am Neurol Assoc*. 1978;103:14–18.
24. Pestronk A, Drachman DB, Griffin JW. Effects of aging on nerve sprouting and regeneration. *Exp Neurol*. 1980;70:65–82.
25. Grady RM, Starr DA, Ackerman GL, Sanes JR, Han M. Syne proteins anchor muscle nuclei at the neuromuscular junction. *Proc Natl Acad Sci USA*. 2005;102:4359–4364.
26. Duclert A, Changeux JP. Acetylcholine receptor gene expression at the developing neuromuscular junction. *Physiol Rev*. 1995;75:339–367.
27. Gramolini AO, Dennis CL, Tinsley JM, et al. Local transcriptional control of utrophin expression at the neuromuscular synapse. *J Biol Chem*. 1997;272:8117–8120.
28. Rubinstein NA, Kelly AM. Myogenic and neurogenic contributions to the development of fast and slow twitch muscles in rat. *Dev Biol*. 1978;62:473–485.
29. Rubinstein NA, Hoh JFY. The distribution of myosin heavy chain isoforms among rat extraocular muscle fiber types. *Invest Ophthalmol Vis Sci*. 2000;41:3391–3398.
30. Karnowsky MJ, Roots LA. A "direct coloring" thiocholine method for cholinesterase. *J Histochem Cytochem*. 1964:219–221.
31. Narusawa M, Fitzsimons RB, Izumo S, Nadal-Ginard B, Rubinstein NA, Kelly AM. Slow myosin in developing rat skeletal muscle. *J Cell Biol*. 1987;104:447–459.
32. Spencer RF, Porter JD. Innervation and structure of extraocular muscles in the monkey in comparison to those of the cat. *J Comp Neurol*. 1981;198:649–665.
33. Felder E, Bogdanovich S, Rubinstein NA, Khurana TS. Structural details of rat extraocular muscles and three-dimensional reconstruction of the rat inferior rectus muscle and muscle-pulley interface. *Vision Res*. 2005;45:1945–1955.
34. Khanna S, Porter JD. conservation of synapse-signaling pathways at the extraocular muscle neuromuscular junction. *Ann NY Acad Sci*. 2002;956:394–396.
35. Budak MT, Bogdanovich S, Wiesen MH, Lozynska O, Khurana TS, Rubinstein NA. Layer-specific differences of gene expression in extraocular muscles identified by laser-capture microscopy. *Physiol Genom*. 2004;20:55–65.
36. Numberger M, Durr I, Kues W, Koenen M, Witzemann V. Different mechanisms regulate muscle-specific AChR gamma- and epsilon-subunit gene expression. *EMBO J*. 1991;10:2957–2964.
37. Fischer MD, Gorospe JR, Felder E, et al. Expression profiling reveals metabolic and structural components of extraocular muscles. *Physiol Genom*. 2002;9:71–84.
38. Adams L, Carlson BM, Henderson L, Goldman D. Adaptation of nicotinic acetylcholine receptor, myogenin, and MRF4 gene expression to long-term muscle denervation. *J Cell Biol*. 1995;131:1341–1349.
39. Campos-Caro A, Carrasco-Serrano C, Valor LM, Ballesta JJ, Criado M. Activity of the nicotinic acetylcholine receptor alpha5 and alpha7 subunit promoters in muscle cells. *DNA Cell Biol*. 2001;20:657–666.
40. Sala C, Kimura I, Santoro G, Kimura M, Fumagalli G. Expression of two neuronal nicotinic receptor subunits in innervated and denervated adult rat muscle. *Neurosci Lett*. 1996;215:71–74.
41. Witzemann V, Brenner HR, Sakmann B. Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. *J Cell Biol*. 1991;114:125–141.
42. Lu PY, Taylor M, Jia HT, Ni JH. Muscle LIM protein promotes expression of the acetylcholine receptor gamma-subunit gene cooperatively with the myogenin-E12 complex. *Cell Mol Life Sci*. 2004;61:2386–2392.
43. Huang CF, Tong J, Schmidt J. Protein kinase C couples membrane excitation to acetylcholine receptor gene inactivation in chick skeletal muscle. *Neuron*. 1992;9:671–678.
44. Lukas RJ. Heterogeneity of high-affinity nicotinic [3H]acetylcholine binding sites. *J Pharmacol Exp Ther*. 1990;253:51–57.
45. Jacob MH, Berg DK, Lindstrom JM. Shared antigenic determinant between the electrophorus acetylcholine receptor and a synaptic component on chicken ciliary ganglion neurons. *Proc Natl Acad Sci USA*. 1984;81:3223–3227.
46. Sargent PB, Pang DZ. Acetylcholine receptor-like molecules are found in both synaptic and extrasynaptic clusters on the surface of neurons in the frog cardiac ganglion. *J Neurosci*. 1989;9:1062–1072.
47. Sargent PB. The diversity of neuronal nicotinic acetylcholine receptors. *Annu Rev Neurosci*. 1993;16:403–443.
48. Rothlin CV, Katz E, Verbitsky M, Elgoyhen AB. The alpha9 nicotinic acetylcholine receptor shares pharmacological properties with type A gamma-aminobutyric acid, glycine, and type 3 serotonin receptors. *Mol Pharmacol*. 1999;55:248–254.
49. Elgoyhen AB, Johnson DS, Boulter J, Vetter DE, Heinemann S. Alpha 9: an acetylcholine receptor with novel pharmacological properties expressed in rat cochlear hair cells. *Cell*. 1994;79:705–715.
50. Vetter DE, Liberman MC, Mann J, et al. Role of alpha 9 nicotinic ACh receptor subunits in the development and function of cochlear efferent innervation. *Neuron*. 1999;23:93–103.
51. Sridhar TS, Brown MC, Sewell WF. Unique postsynaptic signaling at the hair cell efferent synapse permits calcium to evoke changes on two time scales. *J Neurosci*. 1997;17:428–437.