Expression of Neuronal Acetylcholine Receptor Genes in Vertebrate Skeletal Muscle during Development

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Of the 15 nicotinic ACh receptor genes identified in vertebrates, only four ($\alpha 1$, $\beta 1$, γ , and δ) have been shown to be expressed in embryonic skeletal muscle at early times. In mammalian muscle a fifth gene (ϵ) replaces the γ gene in expression at later times. The remaining 10 nicotinic receptor genes identified to date ($\alpha 2$ – $\alpha 8$, $\beta 2$ – $\beta 4$) are expressed in the nervous system and are considered neuronal genes. Using RNase protection assays, we show here that four of the neuronal-type genes ($\alpha 4$, $\alpha 5$, $\alpha 7$, and $\beta 4$) are expressed in developing chick skeletal muscle. Two of them ($\alpha 4$ and $\alpha 7$) decline substantially in transcript abundance between embryonic days 11 and 17, as does $\alpha 1$, while the other two ($\alpha 5$ and $\beta 4$) show only moderate decreases over the same time period. At embryonic day 8, $\alpha 7$ transcripts are nearly 20% as abundant as $\alpha 1$ transcripts. *In situ* hybridizations confirm the presence of $\alpha 7$ transcripts in muscle cells both in cell culture and in embryonic tissue. No evidence was found for expression of the $\alpha 2$, $\alpha 3$, $\alpha 8$, or $\beta 3$ genes in muscle. Immunoprecipitations and immunoblot analysis using subunit-specific monoclonal antibodies reveal $\alpha 7$ protein in muscle, and the amount of protein rises and declines with the amount of $\alpha 7$ mRNA during development. Sucrose gradient analysis demonstrates that the $\alpha 7$ protein is present in muscle as a species of 10S, the size expected for a nicotinic receptor. The $\alpha 7$ species in muscle binds α -bungarotoxin but does not contain $\alpha 1$ subunits, indicating that the two kinds of α -type gene products segregate during assembly. The results suggest that neuronal AChRs may play a role in early muscle development.

[Key words: nicotinic receptors, ACh receptors, $\alpha 7$ mRNA, $\alpha 7$ subunits, α -bungarotoxin, gene expression, transcription, neuronal, muscle]

Nicotinic acetylcholine receptors (AChRs) in vertebrate skeletal muscle have provided a model for studying how postsynaptic components are synthesized, assembled, and regulated (reviewed by Hall and Sanes, 1993). The receptors are pentameric membrane proteins containing four kinds of subunits and serve as cation-selective, ligand-gated ion channels that mediate fast, excitatory synaptic transmission to the muscle fibers. In em-

bryonic muscle the receptors are distributed throughout the plasma membrane at a relatively low density and are composed of two $\alpha 1$ subunits and one each of $\beta 1$, γ , and δ subunits. After the muscle becomes innervated and muscle activity ensues, several changes occur. Expression of the four AChR genes declines and becomes restricted to muscle nuclei in the vicinity of the neuromuscular junction. AChRs disappear from extrasynaptic regions on the muscle and become highly concentrated in the subsynaptic folds underlying the nerve terminal. Coincident with these changes, in rat and bovine muscle the γ gene ceases to be transcribed and in its place transcripts appear from the AChR ϵ gene. AChRs synthesized at subsequent times contain principally ϵ instead of γ subunits and have different single-channel properties from those of the γ -containing embryonic receptors.

Gene cloning efforts have identified 10 additional members of the AChR gene family in vertebrates (for review, see Sargent, 1993). Seven have been classified as α -type genes ($\alpha 2$ – $\alpha 8$) because they contain the two vicinal cysteines analogous to those at amino acid positions 191 and 192 in the $\alpha 1$ gene. Three have been designated β -type genes ($\beta 2$ – $\beta 4$) because they lack the two vicinal cysteines but are very similar in sequence to other members of the family. Expression studies in *Xenopus* oocytes together with subunit analysis of native receptors have confirmed that a number of the genes do indeed encode subunits of functional AChRs. Each of the 10 genes is expressed in the nervous system and therefore is thought to represent a neuronal AChR gene.

We report here that several neuronal AChR genes are expressed in embryonic skeletal muscle. One of these, $\alpha 7$, produces transcript levels nearly 20% as high as $\alpha 1$ during early stages of development. Moreover, the $\alpha 7$ transcripts are translated into protein, and the protein assembles into a putative receptor that binds α -bungarotoxin (α Bgt) and is distinct from conventional muscle AChRs containing $\alpha 1$ subunits. Both $\alpha 7$ transcripts and $\alpha 7$ protein decline in amount at late embryonic stages, as do $\alpha 1$ transcripts and protein. The finding of a putative neuronal AChR in embryonic muscle indicates that the tissue-specific division of AChR gene expression is less categorical than previously recognized and raises the possibility that neuronal AChRs participate in muscle development.

Materials and Methods

RNA probes. 32 P-labeled riboprobes used for RNase protection experiments were generated for the chicken $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 7$, $\alpha 8$, $\beta 2$, $\beta 3$, and $\beta 4$ genes by "runoff transcription" using methods and DNA constructs previously described (Corriveau and Berg, 1993). Similar methods were used to generate $\alpha 1$ riboprobe from linearized p $\alpha 7$ (Shieh et al., 1987), which contains exon VII of the chicken $\alpha 1$ gene. The sizes (number of bases) of the undigested probes and the corresponding species that would be protected by native transcript are as follows (probe,

Received Mar. 30, 1994; revised Aug. 1, 1994; accepted Aug. 10, 1994.

Grant support was provided by the National Institutes of Health (Grants R01 NS12601 and P01 NS25916) and the California Tobacco Related-Disease Research Program.

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undigested size/protected size): $\alpha 1$, 408/224; $\alpha 2$, ~350/300; $\alpha 3$, 511/384; $\alpha 4$, ~600/595; $\alpha 5$, 217/130; $\alpha 7$, 224/166; $\alpha 8$, 211/154; $\beta 2$, 235/216; $\beta 3$, 275/239; $\beta 4$, 414/341.

^{35}S -labeled $\alpha 1$ and $\alpha 7$ riboprobes were used for *in situ* hybridization analysis. Exon 7 of the chicken $\alpha 1$ gene was subcloned from p $\alpha 7$ (Shieh et al., 1987) into pSP72 (Promega) to generate $\alpha 1$ pSP72. Sense and antisense ^{35}S -labeled probes corresponding to exon 7 were generated using appropriately linearized $\alpha 1$ pSP72. $\alpha 7$ probes were generated using linearized pCH 34-1; sense probe consisted of 673 bases corresponding to amino acids 179–401, and antisense probe consisted of 684 bases of 3' untranslated sequence (Schoepfer et al., 1990). After synthesis probes were separated from unincorporated nucleotides by three consecutive ethanol precipitations, alkaline hydrolyzed, reprecipitated, quantified by scintillation counting, and used at about 5×10^6 cpm/ml.

RNA isolation. Muscle, brain, ciliary ganglia, and liver were rapidly dissected from chick embryos at the indicated times and immediately frozen in liquid nitrogen. For some experiments RNA was extracted immediately after dissection without freezing in liquid nitrogen. Total RNA was isolated from both dissected tissue and cell cultures by extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture as previously described (Chomczynski and Sacchi, 1987). RNA was quantified by measuring OD₂₆₀.

RNAse protection. RNAse protection experiments were carried out as previously described (Corriveau and Berg, 1993). Protected sequences were separated from digested products by ethanol precipitation and electrophoresis in 5% polyacrylamide gels containing 8 M urea. The gels were dried onto Whatman 3 mm paper and exposed at -70°C to Kodak XAR-5 x-ray film using a Du Pont Cronex intensifying screen. Signals were quantified directly from the gels using a Molecular Dynamics PhosphorImager. After taking the specific activities of the probes into account and normalizing for the amount of total RNA analyzed, the amounts of neuronal AChR transcripts were compared on a percent basis with the amount $\alpha 1$ transcript present. Control experiments demonstrated that the signals observed were proportional in a linear manner to the amount of transcript present (Fig. 1; Corriveau and Berg, 1993, and unpublished observations).

In situ hybridization. $\alpha 1$ and $\alpha 7$ RNA probes labeled with α - ^{35}S -UTP were used to detect hybridizing material in muscle using methods previously described (Simmons et al., 1989) with minor modifications. Briefly, muscle tissue was fixed in 4% paraformaldehyde/0.5% glutaraldehyde and embedded in paraffin. Ten-micrometer-thick sections were cut and mounted on Fisherbrand Superfrost/Plus microscope slides. The tissue sections were dewaxed, hydrated, permeabilized with proteinase K, and dehydrated with ethanol. Myotube cultures were prepared as described below (except they were plated on collagen-coated Fisherbrand Superfrost/Plus microscope slides), fixed in 4% paraformaldehyde, permeabilized with proteinase K, and dehydrated. Hybridization with labeled RNA probes was carried out for 16–18 hr at 50°C . After hybridization the samples were incubated with 20 $\mu\text{g}/\text{ml}$ RNase A and 10 U/ml RNase T1 for 30 minutes at 37°C to digest the residual probe and then washed in a series of SSC solutions of increasing stringency, ending with $0.1 \times \text{SSC}$ at 68°C . After exposure to Kodak XAR-5 x-ray film at room temperature, sections were coated with NTB-2 emulsion, exposed for 4 weeks, developed, and stained with hematoxylin and eosin using standard methods (Humason, 1979). Results were analyzed using a light microscope with bright-field and phase-contrast optics.

Solid phase immunoprecipitation. AChRs in embryonic day 11 (E11) chick muscle were solubilized using 2% Triton X-100 in PBS containing the following protease inhibitors: 1 mM iodoacetamide, 5 mM benzamide, 5 mM EDTA, 5 mM EGTA, 0.5 mM phenylmethylsulfonyl fluoride, and 10 $\mu\text{g}/\text{ml}$ soybean trypsin inhibitor. After a 2 hr incubation at 4°C , insoluble material was removed by centrifugation at $40,000 \times g$. Extracts were recovered and diluted to a concentration of 0.25 gm (wet weight) muscle per milliliter.

AChRs in the muscle extracts were quantified using a solid phase immunoprecipitation assay in which anti-AChR monoclonal antibodies (mAbs) tethered to Immulon 2 Removawells (Dynatech Laboratories, Inc.) were used to bind AChRs followed by incubation with ^{125}I - αBgt or ^{125}I -mAb 35 to quantify the number of receptors retained. For the assays, wells were first coated on a shaker overnight at 4°C with affinity-purified rabbit anti-rat or rabbit anti-mouse antibodies (Jackson Inc.) at a concentration of 20 $\mu\text{g}/\text{ml}$ in phosphate-buffered saline (PBS; 0.15 M sodium chloride plus 0.01 M sodium phosphate, pH 7.4) containing 0.02% azide (PBS-azide). The wells were then washed three times with

PBS containing 0.5% Triton X-100 (PBS-Triton) and incubated on a shaker overnight at 4°C with 50 μl of anti-AChR antibody diluted in PBS-azide. For $\alpha 7$ -containing receptors, mAbs 318 and 319 were used together for immunoprecipitation. The two mAbs recognize different epitopes and have previously been shown to be specific for the $\alpha 7$ gene product (Schoepfer et al., 1990; Vernallis et al., 1993). mAb 319 was used as a hybridoma culture supernatant while mAb 318 was used at a 1:400 dilution from a concentrated stock (Schoepfer et al., 1990). To immunoprecipitate $\alpha 1$ -containing AChRs, wells were incubated with mAb 35 at a concentration of 0.145 mM (544 $\mu\text{g}/\text{ml}$). To immunoprecipitate AChRs containing $\alpha 4$ or $\beta 4$ subunits, mAbs 289 and B4-1 were used, respectively, at 1:400 dilutions from concentrated stocks. The specificities of mAbs 35, 289, and B4-1 in immunoprecipitations have been documented previously (Schoepfer et al., 1990; Conroy et al., 1992; Vernallis et al., 1993).

After loading with the indicated mAbs, the wells were rinsed three times with PBS-Triton and incubated 30 min at 37°C with 3% (w/v) nonfat dry milk (Carnation Natural Nonfat Dry Milk) in PBS containing 0.1% Tween-20 as a blocking step. After an additional three rinses with PBS-Triton, muscle extracts (25–50 μl) were added to the wells and incubated overnight at 4°C on a shaker. The extracts were removed, and the wells were washed three times with PBS-Triton. Normal rat serum plus normal mouse serum at 2% (v/v) each in PBS-Triton were incubated in the wells for 30 min at 37°C followed by incubation with either 10 nM ^{125}I - αBgt in PBS-Triton plus 10 mg/ml BSA or 5 nM ^{125}I -mAb 35 in 2% (v/v) normal rat plus normal mouse serum solution for 2 hr at 37°C . Unbound probe was removed by four washes with PBS-Triton, and bound radioactivity was determined by gamma counting individual wells. Nonspecific binding was routinely determined by competing the radiolabeled αBgt or mAb 35 with an excess of unlabeled αBgt (1 μM) or mAb 35 (500 nM), respectively. Net specific binding was calculated by subtracting the nonspecific binding from the binding found in the absence of unlabeled probe. Wells in which the anti-AChR mAb was omitted did not bind ^{125}I - αBgt or ^{125}I -mAb 35 specifically. The concentration of ^{125}I - αBgt used in the assays was near-saturating for the binding sites examined (data not shown).

Immunopurification. Detergent extracts of E11 chick pectoral muscle were prepared as described above to solubilize AChRs, and aliquots of the extracts were incubated with 0.2 ml of Protein G-Sepharose (negative control) or 0.2 ml of mAb 319-Protein G-Sepharose to adsorb $\alpha 7$ -containing AChRs. The mAb 319-Protein G-Sepharose was prepared by cross-linking mAb 319 to Protein G-Sepharose 4B Fast Flow (Pharmacia) using dimethyl-pimelimidate as described by Harlow and Lane (1988). Incubations of extract with the resins were carried out in a 15 ml centrifuge tube for 16 hr at 4°C on a shaker. After incubation the resin was washed three times with PBS-Triton, three times with PBS-Triton containing 1 M NaCl, 5 mM EDTA, and 5 mM EGTA, and two more times with PBS-Triton. Bound material was eluted with two 1 ml aliquots of 0.1 M sodium citrate (pH 3) and then immediately neutralized by adding 1 M Tris (pH 8). Eluates were concentrated by ultrafiltration (Centricon-30, Amicon) to 50 μl , and aliquots were transferred to electrophoresis sample buffer for SDS-PAGE and immunoblotting as described below.

Immunoblot analysis. AChRs immunopurified from E11 pectoral muscle were analyzed on immunoblots by solubilizing the material in SDS sample buffer containing 5% β -mercaptoethanol, subjecting the material to SDS-PAGE on a 9% polyacrylamide gel, and electroblotting the separated components onto nitrocellulose as previously described (Towbin et al., 1979). The blots were blocked with 3% (w/v) nonfat dry milk in PBS containing 0.1% Tween-20, incubated with mAbs for 14 hr at 4°C diluted in the same solution, washed in PBS, 0.05% Tween-20, and incubated for 2 hr at room temperature with horseradish peroxidase coupled to goat anti-rat IgG (Jackson Laboratories, Inc.) to detect bound mAbs. Signals were visualized by enhanced chemiluminescence (Amersham). Prestained molecular weight markers (Bio-Rad, low range) and their estimated molecular weights (kDa) were, for phosphorylase B, 106; bovine serum albumin, 80; ovalbumin, 49.5; carbonic anhydrase, 32.5; and soybean trypsin inhibitor, 27.5.

Sucrose gradient analysis. Velocity sedimentation analysis of αBgt -binding components in muscle extracts was performed by layering 0.40 ml aliquots of E11 pectoral muscle extract onto 11 ml linear sucrose gradients (5–20% sucrose in PBS-Triton) and centrifuging for 17 hr in an SW41 Ti rotor (Beckman) at 41,000 rpm at 4°C . After collecting 17–26 fractions from the gradient, the αBgt -binding species present were quantified either with solid phase assays for $\alpha 1$ -containing species (see

Figure 1. Embryonic pectoral (*A*) and leg (*B*) muscles express the $\alpha 7$ gene. RNase protection experiments were carried out with 32 P-labeled $\alpha 3$ and $\alpha 7$ antisense probes (lanes 1 and 7) using 10 μ g of tRNA (lanes 2 and 8), 25 μ g of E17 liver RNA (lanes 3 and 9), 5 μ g of E15 ciliary ganglion RNA (lanes 4 and 10), and the indicated amounts of RNA isolated from E8 pectoral (lanes 5 and 6) and E11 leg muscle (lanes 11 and 12). Liver RNA and tRNA served as negative controls, and ciliary ganglion RNA as a positive control in each experiment. $\alpha 7$ transcripts are present in RNA extracted from both types of muscle, while $\alpha 3$ transcripts are present in neither. The expected shifts in size occur between the full-length probes (arrowheads) and the protected species (arrows). Similar results were obtained in eight additional experiments with pectoral muscle and one with leg muscle.

above) using 0.035–0.045 ml per fraction or with modified immunoprecipitation binding assays for $\alpha 7$ -containing species using 0.4–0.5 ml per fraction. For the modified assays, mAbs 318 and 319 were adsorbed to Protein G-Sepharose and washed with PBS-Triton to remove excess antibody. The adsorbed antibodies were incubated overnight at 4°C with the fraction aliquots, washed twice with 1 ml of PBS-Triton, and then incubated with 10 nM 125 I- α Bgt for 2 hr with shaking at room temperature, washed four times, and counted for gamma radiation. Nonspecific binding was determined either by omitting the gradient fraction or by including 1 mM nicotine in the incubation with 125 I- α Bgt; the values were subtracted from the total to obtain specific binding. Recoveries ranged from 25% to 90% among experiments for the two α Bgt-binding species.

Cell cultures. Myotube cultures were prepared from E11 chick pectoral muscle essentially as previously described (Fischbach, 1972). Briefly, the muscle tissue was minced with forceps, incubated at 37°C for 30 min in a divalent cation-free salt solution to promote cell dissociation, triturated to disperse cells, filtered twice through a Millipore Swinnex-13 filter to remove tissue fragments, and incubated 15 min at 37°C on plastic 60 mm culture dishes to remove fibroblast-like cells, which preferentially adhere to the plastic. The cell suspension (about 2×10^6 cells in 4 ml) was then plated on collagen-coated 60 mm culture dishes (Falcon) in culture medium containing 5% (v/v) embryo extract and 10% (v/v) heat-inactivated horse serum in Eagles Minimal Essential Medium supplemented with glutamine, glucose, and penicillin/streptomycin (GIBCO-Bethesda Research Labs). After 2 d the medium was replaced with new medium containing 2% embryo extract and 10^{-5} M cytosine β -D-arabino-furanoside (ara C) to inhibit proliferating cells. After 2 more days, the medium was again changed, substituting 3% (v/v) embryonic eye extract (Nishi and Berg, 1981) for the embryo extract and omitting ara C. The cultures were then maintained in the medium for up to 7 d with feeding (replacing 50% of the medium) at 2–3 d intervals.

Materials. White Leghorn embryonated chick eggs were obtained locally and maintained at 39°C in a humidified incubator as described

(Boyd et al., 1991). mAb 35 was purified and radioiodinated as previously described (Smith et al., 1985). α Bgt was purified from *Bungarus multicinctus* venom (Ravdin and Berg, 1979) and radioiodinated to a specific activity of 0.5 – 0.7×10^{18} cpm/mol using chloramine T. Tissue extracts and culture media were prepared as described previously (Nishi and Berg, 1981). $\alpha 7$, which contains exon VII of the $\alpha 1$ gene, was a generous gift of Dr. Jakob Schmidt (Shieh et al., 1987). Other compounds were obtained from Sigma unless otherwise indicated.

Results

Expression of AChR $\alpha 7$ gene transcripts in vertebrate muscle. RNase protection experiments demonstrated that the $\alpha 7$ gene is expressed in developing muscle. Total RNA extracted from both embryonic pectoral and leg muscle was hybridized with $\alpha 7$ and $\alpha 3$ antisense probes. The $\alpha 7$ probe was protected, indicating the presence of $\alpha 7$ transcripts in the muscle RNA samples (Fig. 1). The $\alpha 3$ probe was not protected. Total RNA extracted from ciliary ganglia and used as a positive control protected both probes. The likelihood of false positive results for $\alpha 7$ mRNA expression in muscle is remote because of the following considerations. (1) The $\alpha 7$ probe was protected as a single species of the predicted size. (2) No $\alpha 3$ transcripts were detected in muscle. (3) Neither liver RNA nor tRNA protected the $\alpha 7$ probe, indicating that the probe did not interact with itself or nonhomologous sequences in ways that protected a species of the size observed. (4) The chicken $\alpha 7$ probe used in these experiments was not protected by very similar rat $\alpha 7$ mRNA (Corriveau and Berg, 1993). Other AChR mRNA species present in muscle would be less similar and therefore should not protect the $\alpha 7$ probe.

Figure 2. *In situ* hybridizations demonstrating that the $\alpha 7$ gene is expressed in muscle cells. Cultures of skeletal muscle (*A–D*) and microtome sections (10 μ m) of E11 leg muscle (*E, F*) were hybridized with 35 S-labeled probe, coated with emulsion, exposed for 4 weeks, developed, and stained with hematoxylin and eosin. For muscle cultures, each horizontal pair of photomicrographs represents the same field of view: phase-contrast optics (*A, C*) emphasize cell morphology and bright-field optics (*B, D*) accentuate silver grains. Tissue sections are shown using bright-field optics (*E, F*), which reveal both cell morphology and silver grains. $\alpha 7$ antisense probe hybridized to myotubes (examples of labeled myotubes are delineated by arrows) both in cell culture (*A, B*) and *in vivo* (*E*). Myocytes present in cell culture were also labeled (*A, B*, arrowheads). Labeling of muscle cells by $\alpha 7$ antisense probe varied from little, if any, to relatively high levels such as is seen over the myocyte depicted in *A* and *B*. Heterogeneity of $\alpha 7$ expression among nuclei within the same myotube was also evident both in cell culture and *in vivo*. Hybridization to tissue sections of E11 pectoral muscle produced similar results. Background levels of silver grains were observed over extracellular spaces and cells hybridized with $\alpha 7$ sense probe (*C, D, F*). Scale bar: 40 μ m for *A–D*, 25 μ m for *E* and *F*.

Localization of $\alpha 7$ transcripts within muscle. While the RNase protections clearly demonstrated $\alpha 7$ mRNA in dissected embryonic muscle, this tissue typically includes nonmuscle cell types such as fibroblasts and chondroblasts along with myoblasts and myotubes. In addition, the unusual finding of a neuronal AChR mRNA in a non-neuronal tissue raised concerns about

neuronal contamination of the samples. Accordingly, it was important to determine whether muscle cells express the $\alpha 7$ gene. For localization, *in situ* hybridization experiments were initially carried out with muscle cell cultures where myotubes can be readily distinguished. Cultures were prepared with dissociated E11 pectoral muscle and treated with ara C to depress

Figure 3. RNase protection experiment comparing the amounts of $\alpha 7$ and $\alpha 1$ transcripts present in developing pectoral muscle at the indicated times. *A*, 32 P-labeled $\alpha 7$ and $\alpha 1$ antisense probes (lanes 2 and 9, arrowheads) were protected from RNase digestion with either 15 (upper) or 5 μ g (lower) of total RNA extracted from pectoral muscle at the indicated stages of development. Protections with equivalent amounts of tRNA were used as negative controls (lanes 1 and 8). The amount of radioactivity associated with each protected species (arrow) was measured directly from the gel using a Molecular Dynamics PhosphorImager. *B*, To compare the absolute amounts of $\alpha 7$ and $\alpha 1$ mRNAs as well as the patterns of expression during development, signals were corrected for the specific activities of the probes and normalized for the amount of RNA used. The expression patterns for $\alpha 7$ and $\alpha 1$ mRNAs were similar though $\alpha 7$ mRNA levels were 5–10-fold lower than $\alpha 1$ mRNA. Similar results were obtained in two additional experiments.

proliferation of dividing cells. Three separate RNase protection experiments demonstrated that $\alpha 7$ mRNA was present in the cultures (data not shown). *In situ* hybridizations were carried out with 35 S-labeled $\alpha 7$ riboprobes. The culture slides were dipped in emulsion, exposed, developed to reveal silver grains, and stained using a standard hematoxylin and eosin procedure. The $\alpha 7$ antisense probe specifically labeled multinucleated myotubes as well as occasional myocytes present in the cultures (Fig. 2*A,B*). Only background levels of silver grains were observed over cells hybridized with $\alpha 7$ sense probe as a negative control (Fig. 2*C,D*). Heterogeneity of labeling with antisense probe was evident; not all myotubes or myocytes expressed the $\alpha 7$ gene. Even among nuclei within a myotube, labeling varied from high levels down to background. Similar observations have been made for $\alpha 1$ expression in myotube cultures (Fontaine and Changeux, 1989; R. A. Corriveau, S. J. Romano, and D. K. Berg, unpublished observations).

In situ hybridizations on tissue sections of embryonic skeletal muscle demonstrated that *in vivo*, as in culture, a subset of myotubes express the $\alpha 7$ gene (Fig. 2*E*). In addition to myotubes, embryonic muscle tissue includes mononuclear interstitial cells, many of which contain mitotic bodies (Przybylski and Blumberg, 1966). $\alpha 7$ antisense probe specifically labeled a number of interstitial cells (data not shown). While the morphology and location of the cells were consistent with their being myoblasts, definitive identification was not possible with the resolution available. Antisense probe produced only background levels of silver grains over extracellular spaces and cartilage. No evidence was obtained for contaminating neurons, labeled or unlabeled, in the tissue. $\alpha 7$ sense probe produced background labeling over all portions of the sections including myotubes (Fig. 2*F*). $\alpha 1$ expression has previously been demonstrated both in myotubes and in myoblasts *in vivo* (Fontaine and Changeux, 1989); a similar pattern of $\alpha 1$ expression was found in the present experiments (data not shown).

Developmental time course of $\alpha 7$ gene expression. The finding that the $\alpha 7$ gene is expressed in embryonic muscle challenges the traditional division between neuronal and muscle AChR genes and raises questions regarding the abundance, purpose, and regulation of $\alpha 7$ transcripts in muscle. To compare $\alpha 1$ and $\alpha 7$ mRNA expression during muscle development, RNase protection experiments were performed using total RNA extracted from pectoral muscle at E7, E8, E11, E15, and E17 (Fig. 3*A*). The patterns of expression and the relative abundance of $\alpha 1$ and $\alpha 7$ transcripts were determined by quantifying the signals obtained using a Molecular Dynamics PhosphorImager, taking into account the specific activities of the probes.

$\alpha 7$ mRNA was detected at E7 and reached peak levels per unit total RNA at E8–E11 (Fig. 3). By E17, $\alpha 7$ levels had diminished almost to background. This pattern paralleled that observed for $\alpha 1$ mRNA, which, as reported previously (Moss et al., 1989), peaked at E11 and decreased about sixfold by E17 (Fig. 3). During the period of peak expression, the $\alpha 7$ transcript was about 15% as abundant as the $\alpha 1$ transcript.

Muscle expression of other neuronal AChR genes. Of the 10 known neuronal AChR genes, nine were tested for expression in muscle tissue. In addition to $\alpha 7$, these included $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$, $\alpha 8$, $\beta 2$, $\beta 3$, and $\beta 4$. One gene, $\alpha 6$ (Sargent, 1993), could not be tested because no sequence data were available to permit construction of probes.

RNase protection experiments indicated that both E11 and E17 pectoral muscle contained significant amounts of $\alpha 4$, $\alpha 5$,

Figure 4. RNase protection experiments demonstrating the presence of additional neuronal AChR transcripts in muscle and the absence of others. *A*, Protection experiments with $\alpha 4$, $\alpha 5$, and $\beta 4$ probes indicate the presence of the corresponding transcripts in E11 and E17 pectoral muscle. *B*, Protection experiments with $\alpha 2$, $\alpha 3$, $\alpha 8$, and $\beta 3$ probes failed to detect transcripts in either E11 or E17 pectoral RNA though positive controls with brain RNA demonstrated that the probes were competent. The experiments were carried out as described in preceding figures with negative control(s) in each case. All hybridizations used 15 μ g of RNA, and the lanes are labeled to indicate sample composition. Arrowheads indicate the positions of undigested probes and arrows indicate the protected species. Similar results were obtained in two or more additional experiments for each condition.

Figure 5. Relative amounts of neuronal AChR transcripts expressed in muscle at two developmental stages. *A*, Comparison of neuronal AChR transcript abundances with $\alpha 1$ mRNA levels in E11 and E17 pectoral muscle. *B*, Amount of each AChR transcript present at E17 as a percentage of E11 levels. Experiments were performed as described for Figures 1–4. Signals were quantified directly from dried gels using a Molecular Dynamics PhosphorImager, and corrected for the specific activities of the probes. Values represent the mean $\pm$ SEM of at least three separate experiments in each case.

and $\beta 4$ mRNA (Fig. 4*A*). The protections also suggested that $\beta 2$ transcripts were present ($n = 4$; data not shown), but the small amount of $\beta 2$ signal and the high backgrounds obtained (probably due to the high GC content of both the $\beta 2$ coding sequence and the probe) prevented a conclusive determination. No mRNA was detected from $\alpha 2$, $\alpha 3$, $\alpha 8$, and $\beta 3$ genes in either E11 or E17 pectoral muscle (Fig. 4*B*). The sensitivity of the assay was sufficient to detect transcripts present at levels as low as 1% of that observed for $\alpha 1$ at E11.

Of the four neuronal AChR genes clearly expressed in em-

brionic muscle ($\alpha 4$, $\alpha 5$, $\alpha 7$, and $\beta 4$), only $\alpha 5$ appeared to be expressed at any level in E17 liver (Figs. 2, 4). The small amount of $\alpha 5$ protection achieved by liver RNA was specific in that it could not be mimicked by tRNA. The significance of $\alpha 5$ expression at low levels in liver tissue is unclear.

The relative amounts of neuronal AChR gene transcripts in E11 and E17 muscle were quantified using a PhosphorImager as described above and compared to the amounts of $\alpha 1$ transcript. Control experiments demonstrated that the probes, conditions, and levels of RNA used generated signals in RNase

Table 1. Selective immunoprecipitation of putative muscle AChR subtypes with subunit-specific mAbs

Extract	Tethered mAb [Name (Spec)]	Probe	Bound sites (fmol/gm)		Efficiency [1st/(1st + 2nd)]
			1st pass	2nd pass	
Pectoral	318/319 ($\alpha 7$)	α Bgt	400 $\pm$ 50	20 $\pm$ 10	95%
Pectoral	35 ($\alpha 1$)	α Bgt	10,020 $\pm$ 390	1760 $\pm$ 410	85%
Leg	318/319 ($\alpha 7$)	α Bgt	270 $\pm$ 40	10 $\pm$ 10	96%
Leg	35 ($\alpha 1$)	α Bgt	8030 $\pm$ 440	1250 $\pm$ 280	87%
Pectoral	318/319 ($\alpha 7$)	mAb 35	10 $\pm$ 10		
35-depleted	318/319 ($\alpha 7$)	α Bgt	440 $\pm$ 30		
Pectoral	289 ($\alpha 4$)	α Bgt	20 $\pm$ 10		
Pectoral	289 ($\alpha 4$)	mAb 35	30 $\pm$ 10		
Pectoral	B4-1 ($\beta 4$)	α Bgt	10 $\pm$ 10		
Pectoral	B4-1 ($\beta 4$)	mAb 35	30 $\pm$ 10		

Receptors were retained in the solid phase immunoprecipitation assay by incubating the indicated muscle extract with the specified tethered mAb in a single incubation (1st pass) or, in some cases, after recovering the depleted extract from the first incubation and subjecting it to a second incubation in a new well (2nd pass). Adsorbed receptors were quantified using the indicated radiolabeled probes. The efficiency of the procedure was calculated by determining the proportion of the total receptor (1st + 2nd pass) that was retained in the 1st pass. Pectoral, E11 pectoral muscle; leg, E11 leg muscle; 35-depleted, E11 pectoral muscle extract subjected to one round with mAb 35 in a solid phase adsorption prior to the indicated solid phase assay. The subunit specificities of the mAbs for solubilized receptors are as indicated (Spec) except that mAb 35 also immunoprecipitates certain neuronal AChRs (see text). The data represent the mean $\pm$ SEM of at least eight determinations from three or more separate experiments, except for the 2nd pass results with leg muscle, which represent six determinations from two separate experiments. The results show that putative $\alpha 7$ -containing AChRs binding α Bgt are present in both pectoral and leg muscle and are distinct from AChRs containing $\alpha 1$ subunits. Neither class of receptors appears to contain either $\alpha 4$ or $\beta 4$ subunits. mAbs 289 and B4-1 were confirmed as competent by showing in the same experiments that they immunoprecipitated the expected receptor subtypes from chick brain and ciliary ganglia (data not shown).

protections that were related in a linear manner to the amount of mRNA present in the hybridization (Fig. 1; Corriveau and Berg, 1993, and unpublished observations). Several patterns emerged. The neuronal AChR transcripts were approximately an order of magnitude less abundant in muscle than was the $\alpha 1$ transcript, ranging from 3% to 18% depending on the gene and stage of development (Fig. 5A). Both the $\alpha 7$ and $\alpha 4$ transcripts mimicked the $\alpha 1$ transcript in undergoing a sharp decrease in abundance between E11 and E17 (Fig. 5B). The $\alpha 4$ and $\alpha 1$ transcripts decreased almost identical amounts, while $\alpha 7$ declined even further to levels barely detectable in the assay. In contrast, the levels of $\alpha 5$ and $\beta 4$ transcripts decreased only moderately and, as a result, increased in abundance relative to $\alpha 1$ during the same period (Fig. 5A,B).

Putative AChRs containing $\alpha 7$ protein in muscle. The existence of gene transcripts in a cell does not guarantee the presence of the encoded protein. Recently, the pheochromocytoma cell line PC12 has been shown to express high levels of NMDAR1 mRNA and yet to have only trace amounts of NMDAR1 protein (Sucher et al., 1993). Accordingly, it was important to determine whether $\alpha 7$ protein could be detected in embryonic muscle. This was done by relying on the fact that $\alpha 7$ -containing receptors should bind α Bgt and be immunoprecipitated by mAbs 318 and 319. Previous studies have shown the mAbs to be specific for the $\alpha 7$ gene product both on immunoblots and in immunoprecipitations (Schoepfer et al., 1990; Vernallis et al., 1993).

When detergent extracts of embryonic pectoral muscle were incubated with mAbs 318/319 in solid phase immunoprecipitation assays, significant numbers of α Bgt-binding sites were specifically adsorbed by the antibodies (Table 1). The numbers of such sites were far less than the numbers of sites immunoprecipitated by mAb 35, an mAb known to recognize the chicken AChR $\alpha 1$ gene product and to bind to receptors containing $\alpha 1$ subunits (Conroy et al., 1992). [mAb 35 also recognizes the $\alpha 5$ gene product on immunoblots and immunoprecipitates receptors containing $\alpha 3/\alpha 5/\beta 4$ subunits but such receptors do not bind α Bgt (Vernallis et al., 1993).] The α Bgt-binding components brought down by mAbs 318/319 did not represent a low-affinity cross-reaction of the mAbs with conventional muscle AChRs ($\alpha 1/\beta 1/\gamma/\delta$). If low-efficiency cross-reactions with conventional muscle AChRs had been responsible for the immunoprecipitation, significant amounts should also have been obtained in subsequent passes with mAbs 318/319. Instead, immunoprecipitations with mAbs 318/319 were highly efficient in a single pass ($\geq 95\%$; Table 1). As a result, it must be concluded that the population of α Bgt-binding sites in muscle extracts immunoprecipitated by mAbs 318/319 is distinct from the large class of $\alpha 1$ -containing receptors recognized by mAb 35. Similar results were obtained from an analysis of embryonic leg muscle extracts (Table 1).

Sucrose gradient analysis was used to determine whether the α Bgt-binding components containing $\alpha 7$ protein had the size expected for AChRs. Extracts prepared from E11 pectoral muscle were fractionated by sedimentation through sucrose gradients, and the fractions were examined for immunoprecipitable α Bgt-binding sites. A prominent species of about 10S was obtained for the toxin-binding component recognized by mAbs 318/319 (Fig. 6), a size similar to that reported for neuronal AChRs solubilized from brain and ciliary ganglia (Schneider et al., 1985; Smith et al., 1985; Anand et al., 1993). The major species migrated slightly ahead of the $\alpha 1$ -containing AChRs analyzed on the same gradients, confirming the separate identity

Figure 6. Sucrose gradients showing the size of the $\alpha 7$ -containing component that binds α Bgt in embryonic muscle. Extracts of E11 pectoral muscle were fractionated by sucrose gradient sedimentation, and the fractions were analyzed for $\alpha 7$ -containing (0.4 ml aliquots; solid squares) and $\alpha 1$ -containing (0.05 ml aliquots; open circles) components that bind α Bgt. Sedimentation was from right to left; specific binding is shown from a single gradient. Similar results were obtained in two experiments including a total of four gradients.

of the two classes of components distinguished by immunoprecipitation with mAbs 318/319 and 35.

Segregation of $\alpha 1$ and $\alpha 7$ gene products into separate muscle components. Expression of the $\alpha 7$ gene in *Xenopus* oocytes produces receptors that are thought to be homomeric (Couturier et al., 1990; Seguela et al., 1993). In chick brain a portion of the $\alpha 7$ protein is coassembled with $\alpha 8$ subunits to form a subpopulation of native AChRs that bind α Bgt (Schoepfer et al., 1990). The question of whether $\alpha 7$ subunits are present in muscle AChRs containing $\alpha 1$ subunits was addressed with both immunoprecipitations and immunoblots. In one set of experiments muscle extracts were incubated with mAbs 318/319 to adsorb receptor, and the bound receptor was tested for 125 I-mAb 35 binding to determine the amount of $\alpha 1$ protein present. No binding was detected over background (Table 1), suggesting that none of the adsorbed protein contained $\alpha 1$ subunits. A similar conclusion was reached in a second set of experiments in which muscle extracts were first incubated with mAb 35 to deplete $\alpha 1$ -containing AChRs. The depleted extracts were then incubated with mAbs 318/319 to determine whether the components recognized by the anti- $\alpha 7$ mAbs had been removed. This clearly was not the case (Table 1, "35-depleted"), indicating that the putative AChRs containing $\alpha 7$ subunits did not also contain $\alpha 1$ protein.

The conclusion that the $\alpha 7$ and $\alpha 1$ proteins are segregated into separate muscle components was confirmed by immunoblot analysis. $\alpha 7$ -Containing components were partially purified from muscle extracts by using mAb 319 coupled to Protein G-Sepharose. The bound protein was eluted, submitted to SDS-PAGE, and probed on immunoblots with mAbs 318, 319, and 210. Since mAbs 318 and 319 recognize distinct epitopes on the $\alpha 7$ subunit (McLane et al., 1992), their ability to detect a species of about 60 kDa in the immunopurified material indi-

Figure 7. Immunoblots demonstrating the presence of $\alpha 7$ protein in putative AChRs distinct from those containing $\alpha 1$ subunits in muscle. Extracts from E11 pectoral muscle were incubated either with mAb 319-Protein G-Sepharose to immunoprecipitate receptors containing $\alpha 7$ subunits (lanes 1–4) or with Protein G-Sepharose as a negative control (lanes 5–8). The precipitates were then eluted with 0.1 M sodium citrate, pH 3, containing 0.1% Triton X-100 and analyzed on immunoblots probed with mAbs 319 (lanes 1 and 5) and 318 (lanes 2 and 6), which recognize different epitopes on the $\alpha 7$ protein, mAb 210 (lanes 3 and 7), which recognizes the $\alpha 1$ subunit, and no primary mAb (lanes 4 and 8) as a negative control. Bound mAbs were detected using rabbit anti-rat antibodies coupled to horseradish peroxidase; signals were visualized with enhanced chemiluminescence (Amersham). The size of the immunopurified component identified as $\alpha 7$ (arrow, lanes 1 and 2) is about 60 kDa. The material at the top of lanes 1–4 is most likely cross-linked mAb leached from the resin and recognized by the secondary antibody; some high-molecular-weight material of unknown identity also binds mAb 318 (lane 3). Similar results were obtained in a second experiment. Molecular weight markers (from top to bottom, kDa): 106, 80, 49, 32.5, 27.5.

Figure 8. Putative AChRs containing $\alpha 7$ protein are expressed transiently in developing muscle. Solid phase assays with mAbs 318/319 and 35 were used to quantify the numbers of α Bgt-binding sites associated with $\alpha 7$ -containing components (open circles) and $\alpha 1$ -containing AChRs (solid squares), respectively, in muscle extracts of the indicated developmental stages as described in Table 1. Each point represents the mean $\pm$ SEM of at least eight determinations from three or more separate experiments. Though putative $\alpha 7$ -containing receptors are less abundant than conventional $\alpha 1$ -containing AChRs in muscle, the two types of receptors rise and fall together during development and parallel the levels of transcript present.

icates the presence of $\alpha 7$ protein (Fig. 7). mAb 210, which recognizes the chicken muscle $\alpha 1$ protein on immunoblots (Conroy et al., 1992), failed to detect a component in the purified material, indicating that little, if any, $\alpha 1$ protein copurified with $\alpha 7$ (Fig. 7). Taken together, the results of both the immunoprecipitation and immunopurification experiments indicate that $\alpha 1$ and $\alpha 7$ subunits are segregated into separate multimeric species in embryonic muscle.

Subunit-specific mAbs were also used to determine whether the other neuronal AChR genes expressed in muscle contributed subunits that coassembled with either $\alpha 1$ or $\alpha 7$ subunits. These included mAb 289, which recognizes the $\alpha 4$ gene product (Whiting et al., 1991); mAb B4-1, which recognizes the $\beta 4$ gene product (Vernallis et al., 1993); and mAb 268, which recognizes the $\alpha 5$ gene product (Conroy et al., 1992). Neither mAb 289 nor mAb B4-1 was able to immunoprecipitate muscle AChRs that bound 125 I- α Bgt or 125 I-mAb 35 (Table 1), though both antibodies recognize their respective antigens when present in receptors under such conditions. mAb 268, which recognizes its antigen only on immunoblots, failed to detect any $\alpha 5$ subunit in protein immunopurified with mAb 319-Protein G-Sepharose and subjected to immunoblot analysis (data not shown). Previous results showed that mAb 268 does not react with components of muscle AChRs affinity-purified with α Bgt (Conroy et al., 1992). The results indicate that the $\alpha 4$, $\beta 4$, and $\alpha 5$ proteins are absent from muscle AChRs that bind α Bgt.

Developmental changes in muscle levels of $\alpha 7$ protein. Quantification of $\alpha 7$ proteins that bind α Bgt in embryonic muscle

demonstrated that the pattern of accumulation during development follows that of conventional $\alpha 1$ -containing AChRs recognized by mAb 35. Both the $\alpha 1$ -containing AChRs and the putative AChRs containing $\alpha 7$ subunits are present at E7, peak at E11, and subsequently decrease in abundance (Fig. 8). The rate of decrease after E11 is greater, however, for putative $\alpha 7$ -containing AChRs. They are 8% as abundant as the $\alpha 1$ -containing receptors at E7 but less than 1% as abundant at E17. The results indicate that the putative $\alpha 7$ -containing AChRs are present at early times in muscle development and that the amounts are roughly proportional to the amounts of $\alpha 7$ transcript present at each stage (Figs. 3B, 8).

Discussion

The major findings reported here are that embryonic skeletal muscle expresses several neuronal AChR genes. In the case of the $\alpha 7$ gene, the resulting transcripts are translated into protein that assembles into a multimeric species that binds α Bgt and has the size expected for an AChR. The results reveal a pattern of expression for neuronal AChR genes more diverse than previously appreciated and raise questions regarding the function of neuronal AChRs in non-neuronal cells.

The stringency of the RNase protection assay leaves little doubt that the neuronal AChR gene transcripts were correctly identified. The amounts of neuronal AChR mRNAs are significant, in some cases approaching 20% of that observed for $\alpha 1$. The *in situ* hybridizations confirmed that the $\alpha 7$ gene is expressed in muscle cells both in cell culture where myotubes can be readily distinguished and in embryonic muscle where a variety of cell types and developmental stages coexist (Bourne, 1972).

The pattern of neuronal AChR gene expression in muscle appears unique. Of the four genes shown to be expressed in embryonic muscle, the transcript levels of two ($\alpha 4$ and $\alpha 7$) decline substantially between E11 and E17, as does the transcript level of $\alpha 1$, while the other two ($\alpha 5$ and $\beta 4$) decrease only moderately. In contrast, transcript levels of all five neuronal AChR genes expressed in chick ciliary ganglia increase significantly per neuron during the same time period (Corriveau and Berg, 1993). No transcripts were detected in muscle from four other neuronal AChR genes ($\alpha 2$, $\alpha 3$, $\alpha 8$, and $\beta 3$), and none of eight genes tested appeared to be expressed in E17 liver with the exception of $\alpha 5$ at very low levels. Of particular note is the absence of $\alpha 3$ transcripts in embryonic muscle though it appears in all classes of peripheral neurons tested to date including sympathetic, parasympathetic, and sensory neurons as well as adrenal chromaffin cells (Boulter et al., 1986; Boyd et al., 1991; Listerud et al., 1991; Corriveau and Berg, 1993). $\alpha 3$ transcripts are also absent from denervated muscle (Boulter et al., 1986). Several human non-neuronal carcinoma cell lines (Chini et al., 1992) as well as thymic tissues (Mihovilovic and Roses, 1993) have been shown to express certain neuronal AChR genes, although it is not clear which neuronal AChR proteins might be present in the cells. It is possible that neuronal AChRs play a role very early in development, since the $\alpha 7$ promoter is active in cultures of undifferentiated mesoderm stem cells (Matter-Sadzinski et al., 1992). The diversity in neuronal AChR gene expression among cell types suggests specific regulation to produce needed receptor subtypes rather than a lack of precision in transcriptional control at early times.

The immunoprecipitation and immunoblot experiments demonstrate that $\alpha 7$ protein is present in embryonic muscle

tissue. Furthermore, the developmental pattern of expression follows that of the $\alpha 7$ transcript with protein levels peaking at E11 and then falling substantially. Sucrose gradient analysis indicates that a large portion of the $\alpha 7$ -containing protein is present in a species having the size expected for a fully assembled AChR. Though it has yet to be shown that the protein is present in the plasma membrane and functions as a receptor, such an outcome seems likely. Expression of the $\alpha 7$ gene alone in *Xenopus* oocytes produces functional AChRs that are thought to be homomeric (Couturier et al., 1990; Seguela et al., 1993). The same may be true of $\alpha 7$ -containing AChRs in chick ciliary ganglion neurons, which are functional and contain only $\alpha 7$ of the known neuronal AChR gene products (Vernallis et al., 1993; Zhang et al., 1994). Alternatively, the $\alpha 7$ gene product may be coassembled with other AChR gene products normally expressed in muscle, including $\beta 1$, γ , and/or δ . Recent evidence indicates that such AChR hybrids, when expressed in *Xenopus* oocytes, are functional (Helekar et al., 1994). The sequential immunoprecipitation studies with mAbs 35 and 318/319 show that the $\alpha 7$ subunits are not coassembled with $\alpha 1$ in muscle.

It seems unlikely that presynaptic neuronal AChRs, if such receptors exist on motor nerve terminals, could account for the bulk of the $\alpha 7$ protein detected in muscle. *In situ* hybridizations detected $\alpha 7$ transcript in muscle cells both in culture and in embryonic tissue with no indication of neurons being present. The levels of $\alpha 7$ protein in the muscle are proportional to the amounts of $\alpha 7$ mRNA at all times examined. In addition, the level of $\alpha 7$ protein in the tissue declines with development rather than increasing as might be expected if the protein were largely contributed by the innervating nerve. Preliminary studies on myotube cultures indicate that $\alpha 7$ protein is produced in the absence of neurons (S. J. Romano and D. K. Berg, unpublished observations). Nonetheless, the present results do not exclude the possibility that a portion of the $\alpha 7$ protein detected in embryonic muscle derives from motoneuron terminals.

Functional receptors containing $\alpha 7$ protein in muscle would have gone undetected in electrophysiological studies because the receptors would have been blocked by many of the same antagonists that block conventional muscle AChRs, namely, α Bgt, α -cobrotoxin, and *d*-tubocurarine. The presence of $\alpha 4$, $\alpha 5$, and $\beta 4$ transcripts suggests that other types of neuronal AChRs may also be transiently expressed in developing muscle. The encoded subunits of the three genes would not be expected to produce receptors that bind α Bgt or to coassemble with $\alpha 7$ subunits (Vernallis et al., 1993). As a result, receptors containing $\alpha 4$, $\alpha 5$, and $\beta 4$ subunits would not have been detected in the present studies. The expression of AChRs displaying a considerable range of single-channel properties in embryonic muscle has been interpreted as meaning that incomplete muscle AChR species can be assembled in the cells and transported to the cell surface (Liu and Brehm, 1993). An alternative possibility that should be considered in light of the present results is that a portion of the single-channel classes observed may represent neuronal AChRs transiently expressed in the cells.

Changes in the combination of ligand-gated ion channel genes expressed in a cell may be a general feature of development. The reasons for such changes, however, are poorly understood. In the case of muscle AChRs, it has been suggested that the expression of an embryonic form of the receptor having a γ subunit may promote spontaneous receptor activity needed to establish neuromuscular junctions (Jaramillo et al., 1988). The early expression of ligand-gated ion channels has also led to

speculation that the receptors help mediate other aspects of neuronal development, including the shaping of neuronal connectivity patterns (Bettler et al., 1990; Matter et al., 1990; Killisch et al., 1991). Supporting evidence in the case of NMDA receptors has been provided by the finding that receptor antagonists disrupt eye-specific stripes in the optic tectum of three-eyed tadpoles and pattern formation in the mammalian lateral geniculate nucleus (Cline et al., 1987; Hahm et al., 1991).

Identifying a role for $\alpha 7$ -containing receptors in non-neuronal cells may suggest new functions for ligand-gated ion channels. An interesting possibility in developing muscle is that the receptors facilitate a specific step such as myoblast fusion or early events in synaptogenesis by virtue of their ability to elevate intracellular calcium levels. Expression of the $\alpha 7$ gene in *Xenopus* oocytes produces receptors with a calcium:sodium permeability much greater than that of conventional muscle AChRs (Bertrand et al., 1993; Seguela et al., 1993). As a result, even a few $\alpha 7$ -containing receptors might have profound effects on intracellular calcium levels at early times. Specific blockers of $\alpha 7$ -containing AChRs should help reveal the possible roles of such receptors in muscle development.

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