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Acetylcholine receptor-inducing activity stimulates expression of the ε-subunit gene of the muscle acetylcholine receptor

(neuron/neurotrophic/synapse/transcription/calcitonin gene-related peptide)

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ABSTRACT **Motor neurons regulate the transcription of acetylcholine receptor subunit genes in postsynaptic muscle fibers both through muscle electrical activity produced by motor neuron acetylcholine release and by mechanisms independent of such transmitter release. Factors secreted by the motor neuron may mediate activity-independent regulation, including the postnatal switch from $\alpha_2\beta\gamma\delta$ (embryonic type) to $\alpha_2\beta\varepsilon\delta$ (adult type) receptors. We have investigated the effect of putative trophic factors, agents affecting second-messenger systems, and muscle activity on the levels of acetylcholine receptor subunit mRNAs in primary mouse muscle cultures. We found that ARIA (acetylcholine receptor-inducing activity), a 42-kDa glycoprotein purified on the basis of its ability to increase the synthesis of acetylcholine receptors in chick myotubes, increases ε-subunit mRNA levels up to 10-fold. In addition, ARIA stimulated α-, γ-, and δ-subunit mRNA levels 2-fold but had no effect on the expression of the β-subunit gene. These effects of ARIA were independent of muscle activity, and they were not mimicked by calcitonin gene-related peptide nor by thyroxine, forskolin, phorbol 12-myristate 13-acetate, the calcium ionophore A23187, basic fibroblast growth factor, or transforming growth factor β. Based on these data, we suggest that ARIA may act at the mammalian neuromuscular junction to induce adult-type acetylcholine receptors.**

Acetylcholine receptors (AcChoRs) at mature mammalian neuromuscular junctions are pentameric protein complexes composed of four subunits in the ratio of $\alpha_2\beta\varepsilon\delta$ (1, 2). Most, if not all, embryonic AcChoRs contain a different subunit named "γ" in place of the ε subunit (1, 2). It is likely that this change in subunit composition, which occurs during the first 2 weeks after birth (2), accounts for the switch in properties of acetylcholine-activated channels from low-conductance, long open time to high-conductance, brief open time that occurs over approximately the same time course (for reviews, see refs. 3 and 4).

ε-Subunit mRNA is present at low levels in neonatal mouse and rat myotubes, whereas γ-subunit mRNA is present at relatively high levels (5, 6). During the first 2 weeks after birth, the amount of ε-subunit mRNA rises 10-fold and γ-subunit mRNA falls to undetectable levels (5, 6). The increase in ε-subunit mRNA appears to be confined to the developing motor endplate (7). At mature endplates, the expression of α-, β-, δ-, and ε-subunit mRNAs is maintained in the face of increasing muscle activity (7–9). This is quite remarkable considering that activity profoundly decreases the expression of AcChoR subunit genes in extrasynaptic regions of the muscle fiber (10–14). The γ subunit is apparently the exception, as its expression decreases at the endplate as well as at extrasynaptic regions (9). It is reasonable to suppose that the expression of AcChoR subunit genes at the endplate is regulated by one or more factors released from the motor nerve terminals.

We have characterized a glycoprotein purified from chick brain that can stimulate the synthesis of AcChoRs in cultured chick myotubes (15, 16) and that might mediate, therefore, the observed increased rate of AcChoR appearance at developing neuromuscular junctions (17, 18). This protein, called "ARIA" (for acetylcholine receptor inducing activity), increases the level of the α-subunit mRNA in chick myotubes (19, 20). The effect is selective in that no change in δ- or γ-subunit mRNA was detected. In chick muscle, although γ-subunit mRNA is not detectable in mature innervated muscle (11), no ε-subunit gene has been reported, and no change in channel conductance or open time occurs during development *in vitro* or *in vivo* (21). Therefore, we have studied the effect of ARIA on the level of ε-subunit mRNA in mouse myotubes. Cultured mouse myotubes express ε-subunit mRNA at levels similar to those found in neonatal mouse muscle (6).

No factors have been identified previously that alter the maximum level of ε-subunit mRNA. Thyroxine speeds the postnatal appearance of ε-subunit mRNA by a few days, but the peak level is unchanged (6). In addition to ARIA, we tested other agents that affect AcChoRs in chick myotubes, including calcitonin gene-related peptide (CGRP), which increases the levels of chick α-subunit mRNA (22) and, to a lesser extent, γ- and δ-subunit mRNAs (23); tetrodotoxin (TTX), which blocks impulse activity; forskolin, which stimulates protein kinase A; phorbol esters, which stimulate protein kinase C; and the calcium ionophore A23187.

METHODS

Mouse Myotube Cultures. Mononucleated myogenic cells were dissociated from limb muscle of mouse embryos (embryonic days 17–19) as described (6). After dissociation and filtration, the cells were plated at a density of 7×10^6 per 6-cm collagen-coated plate in Dulbecco's modified Eagle's medium (DMEM) containing 5% (vol/vol) newborn calf serum and 10% (vol/vol) horse serum. Cytosine arabinonucleoside (araC) (5 μM) was added for 48 hr starting on day 4 after plating. On day 6, the medium was changed to DMEM containing 5% horse serum and ARIA, or other test substances were added. Cultures were harvested 2 days later. In some experiments, 5 μM TTX (Sigma) was added. Synthetic rat CGRP (Sigma) was tested at a final concentration of 0.1 μM.

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Abbreviations: ARIA, acetylcholine receptor-inducing activity; AcChoR, acetylcholine receptor; α-BgTX, α-bungarotoxin; CGRP, calcitonin gene-related peptide; TTX, tetrodotoxin; HFBA, heptafluorobutyric acid.

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ARIA Purification. The ARIA used in these experiments was prepared as described (16) and was the fraction eluted from a C_4 column equilibrated with heptafluorobutyric acid (HFBA), which we have called "C_4/HFBA" fraction material. Briefly, an acid extract of delipidated adult chicken brains was desalted on a Vydac C_{18} column and then purified by the following chromatographic steps: a CM-Sepharose column was equilibrated in 25 mM Mes (pH 6) and eluted with a gradient of NaCl, a Vydac C_4 reverse-phase column was equilibrated in 0.1% trifluoroacetic acid and eluted with a gradient of isopropyl alcohol, and finally, the same C_4 column was equilibrated in 0.13% HFBA and eluted with a gradient of isopropyl alcohol ("C_4/HFBA"). For treatment of cultures, the C_4/HFBA fraction material was dried by vacuum centrifugation and then redissolved in culture medium. The concentration of C_4/HFBA protein in treated cultures was $\approx$10 μg/ml.

RNase Protection Assay. Cells were scraped from the 6-cm tissue culture plates with a rubber policeman, and total RNA was isolated by the method of Chomczynski and Sacchi (24) as described (6). The quality of RNA preparations was assessed by the size and relative ratio of 18S and 28S ribosomal RNAs fractionated on formaldehyde-containing agarose gels and detected by ethidium bromide staining. The RNase protection protocol, modified from that of Melton *et al.* (25) and a technical bulletin provided by Promega, has been described in detail (6, 26). An 800-base-pair (bp) fragment of the ε-subunit gene (27) containing exons 2, 3, and 4 was subcloned into the transcription vector Bluescribe (Stratagene). A 970-bp fragment of the δ-subunit gene (28) containing exon 1 and part of exon 2 was subcloned into pGEM-2 (Promega). An 1100-bp fragment of the γ-subunit gene (28) containing exons 1 and 2 was subcloned into the plasmid pGEM-1 (Promega). A 2000-bp fragment of the β-subunit gene (27) containing exons 1, 2, and 3 was subcloned into Bluescribe (+). A 1750-bp fragment of the α-subunit gene (C. Prody & J.P.M., unpublished data) containing exons 2 and 3 was subcloned into Bluescribe (+).

Radiolabeled antisense probes were synthesized from plasmids linearized with appropriate restriction enzymes. T7, T3, and SP6 phage polymerases were used for transcription, and 4×10^5 cpm of each probe was added to hybridize with 5–60 μg of total RNA in 30 μl of hybridization buffer (80% deionized formamide/40 mM Pipes, pH 6.7/0.4 M NaCl/1 mM EDTA) for 15–17 hr at 45°C. After hybridization, 300 μl of RNase digestion buffer containing 12 μg of RNase A (Sigma) and 0.6 μg of RNase T_1 (Sigma) was added, and the digestion proceeded for 60 min at room temperature. The digestion was stopped with the addition of 50 μg of proteinase K and 20 μl of 10% SDS for 15 min at 37°C. Samples were then extracted with 1:1 (vol/vol) phenol/chloroform and precipitated in the presence of 20 μg of tRNA. The precipitated RNA was washed twice in 70% ethanol, dried, and analyzed on 7.5% polyacrylamide/7 M urea gels. Gels were dried, exposed to Kodak XAR film with an intensifying screen, and the autoradiograms were quantitated by densitometry. The figures show the regions of the gel corresponding to exon 2 for all subunits; it is this band which was quantitated. All of the probes used were at least twice the size of the quantitated band, and there was never any significant quantity of residual probe. In some cases, bands that had high radioactivity were cut out and assayed for radioactivity. Moles of subunit mRNA per mg of total RNA were calculated from a standard curve of the type shown in ref. 6.

AcChoR Assay. The rate of appearance of new α-bungarotoxin (α-BgTX) binding sites on the surface membrane of cultured mouse myotubes was assayed as described (15, 16). All exposed receptors present at the start of the assay were blocked with native α-BgTX. After the unbound native α-BgTX was washed off, 5 nM ^{125}I-labeled α-BgTX (in culture medium) was applied, and the cultures were returned to the incubator for 5 hr. At the end of this period, the cultures were washed, solubilized, and assayed for radioactivity.

For these experiments, a suspension of mononucleated mouse myoblasts was prepared as described above. One set of assays was performed with each of the following four sets of parameters: (*i*) plating density of 5×10^4 cells per well, 96-well plate, 24 hr of treatment with the C_4/HFBA preparation; (*ii*) plating density of 5×10^4 cells per well, 96-well plate, 48 hr of treatment; (*iii*) plating density of 1×10^6 cells per well, 48-well plate, 24 hr of treatment; (*iv*) plating density of 2×10^6 cells per well, 48-well plate, 24 hr of treatment.

RESULTS

The species specificity of purified fractions of chick ARIA has not been determined previously to our knowledge. Therefore, we first examined the effect of ARIA on the appearance of new ^{125}I-labeled α-BgTX binding sites in mouse myotubes 5 hr after all exposed receptors were blocked with nonradioactive α-BgTX. In each of four experiments, an increase in the rate of AcChoR insertion into the surface membrane was observed that ranged between 120% and 150% of control values. The effect of ARIA was comparable to that of TTX assayed in sister cultures. The maximal effects of ARIA and TTX on mouse myotubes were less than the 3- to 5-fold effects usually observed in chick myotube cultures. This may reflect the fact that the basal rate of receptor incorporation is higher in mammalian myotubes than in chick myotubes (unpublished data). In any case, it is clear that the effect of ARIA is not species specific.

We next tested if ARIA altered AcChoR subunit mRNA levels. The effect on ε-subunit mRNA was most dramatic (Fig. 1). Exposure to ARIA for 48 hr resulted in 4- to 10-fold increases of ε-subunit mRNA levels in three experiments [(7.2 ± 1.7)-fold; mean ± SEM]. In three other experiments, a prominent signal was seen in cultures treated with ARIA, but a determination of the fold increase was not possible as no signal was detected in the parallel control cultures. The

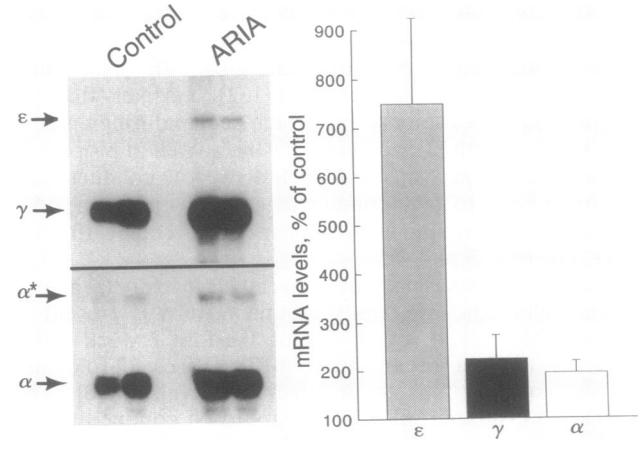

FIG. 1. Effect of ARIA on the expression of ε-, γ-, and α-subunit mRNA levels in mouse myotubes. The levels of ε- and γ-subunit mRNAs were quantitated in the same hybridization reaction by RNase protection with 60 μg of total RNA. α-Subunit mRNA levels were assayed separately with 30 μg of total RNA. (*Left*) Autoradiogram showing the protected exon 2 bands in replicate plates from the same experiment. In addition, for the α subunit, we show an aberrantly spliced fragment (labeled α*) that is regulated like the mature RNA by ARIA. (*Right*) Increase in mRNA level due to ARIA, expressed as the percent of control. Data are means ± SEM of $n = 3$ for ε and $n = 4$ for α and γ subunits with duplicate plates in each experiment. Autoradiographic exposures of the gels were 16 hr.

levels of α- and γ-subunit mRNAs were also increased by ARIA (Fig. 1), but only (1.9 ± 0.3)- and (2.2 ± 0.4)-fold, respectively (mean ± SEM; $n = 4$).

To determine if the effect of ARIA on ε-subunit mRNA accumulation could be explained indirectly by an inhibition of spontaneous electrical/contractile activity, we also assayed mRNA levels in myotubes treated with TTX. As expected from studies of chick myotubes (12, 19, 30), inhibition of voltage-gated sodium channels increased α-subunit mRNA relative to control cultures (Fig. 2). A significant increase was also observed in γ-subunit mRNA. In contrast, TTX produced only modest changes in ε- and δ-subunit mRNAs. Likewise, no change in β-subunit mRNA was detected. Thus, it is unlikely that the effect of ARIA on mouse ε-subunit mRNA is mediated by the chain of events that follows inhibition of electrical activity. Of the five mouse genes, the γ-subunit gene is most efficiently down-regulated by spontaneous activity *in vitro*, a characteristic that correlates well with the observation that γ-subunit mRNA is most dramatically affected by activity *in vivo* (11, 13, 31).

Fig. 3 and Table 1 show the amount of mRNA (in amol/mg of total RNA in Table 1) for each of the five subunits in innervated and in contralateral denervated leg muscle samples prepared from an adult mouse. Denervation produced large increases in the amount of α-, β-, γ-, and δ-subunit mRNAs with little change in the level of ε-subunit mRNA. The amounts of all five subunit mRNAs in innervated and denervated mouse muscle have not been reported previously to our knowledge. The changes upon denervation in the relative levels of mouse muscle AcChoR-subunit mRNAs are similar to changes reported for the rat (refs. 13 and 32; however, see

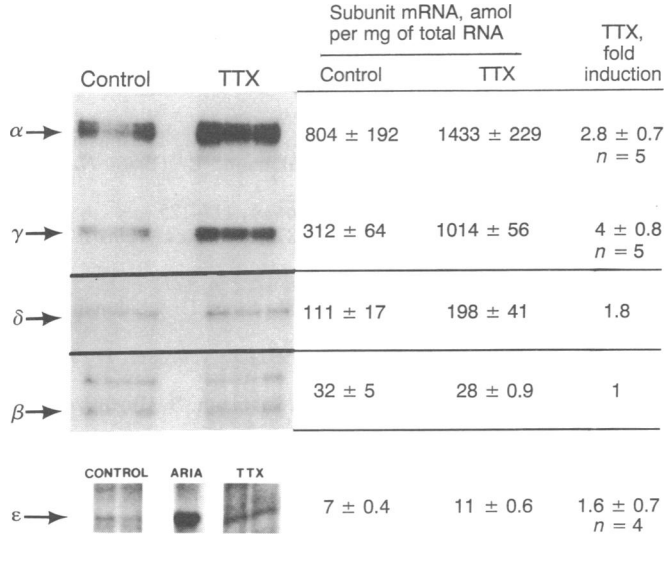

FIG. 2. (*Left*) Effect of TTX on the expression of the different AcChoR subunit mRNAs. Mouse myotubes were treated with 5 μM TTX for a period of 48 hr before the cells were harvested. α-, β-, γ-, and δ-subunit mRNAs were quantitated in sister cultures with 20 μg (α, γ, and δ) or 30 μg (β) of the same RNA preparation; α and γ subunits were assayed in the same hybridization reaction. ε-Subunit mRNA results, obtained with 40 μg of RNA, are from a different experiment (plating); and for this latter experiment, the ε-subunit mRNA level in an ARIA-treated plate shows the difference in the effects of TTX and ARIA in parallel cultures. Autoradiographic exposures are 1.5 days for α, β, γ, and δ subunits and for 3.5 days for the ε subunit. (*Right*) Attomoles of subunit transcript per mg of total RNA for control and TTX-treated plates are presented as well as their ratios. Although similar from plate to plate in a single experiment, the abundance of subunit mRNAs relative to total RNA varied up to 5-fold between experiments. We believe this to be due to the variation in relative abundance of myoblasts and fibroblasts in different plating cell suspensions.

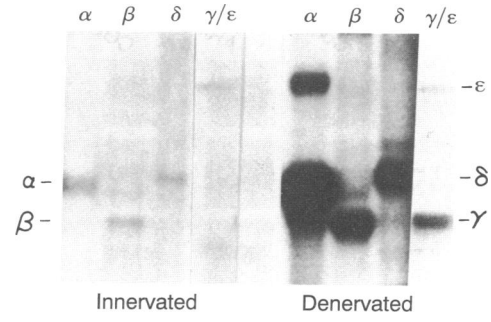

FIG. 3. The expression of AcChoR subunit mRNAs in innervated and denervated mouse muscle. Lower leg muscles were denervated unilaterally after transecting the sciatic nerve (6). Four days after denervation, the different subunit mRNA levels were measured in 30 μg of total RNA prepared from denervated and from contralateral innervated muscles. Autoradiographic analysis is shown of RNase protection assays for the level of subunit RNA in innervated and denervated muscle of a single mouse. The levels of ε- and γ-subunit mRNAs were quantitated in the same hybridization reaction. Autoradiographic exposures were 48 hr for α-, β-, and δ- and 16 hr for γ- and ε-subunit mRNAs.

the discussion in ref. 6 concerning the change in ε-subunit mRNA in mouse versus that in rat) and the chicken (11, 12). Based on these data and the results presented in Figs. 1 and 2, we estimate that the concentration of ε-subunit mRNA in ARIA-treated cultures approached the concentration (in amol/mg of total RNA) attained *in vivo*. However, treatment with ARIA did not result in ε-subunit mRNA concentrations that exceeded a small fraction of the γ-subunit mRNA concentrations. We attribute this to a lack of sufficient spontaneous electrical/contractile activity in cell culture to maximally inhibit expression of the γ-subunit gene. Indeed, the basal levels of γ- and also of α- and δ-subunit mRNAs are higher in cultures of contracting myotubes than in mature innervated myofibers *in vivo* (compare Figs. 2 and 3).

Finally, we also tested whether ARIA's efficacy in increasing ε-subunit mRNA was mimicked by other mediators thought to play a role in neuromuscular junction formation and/or muscle differentiation. Fig. 4 shows a comparison of the effects of CGRP and ARIA on the expression of all five subunit mRNAs. CGRP, at a concentration that maximally stimulates AcChoR synthesis and subunit mRNA levels in chick myotubes (22, 23), had no effect on any of the mouse subunit mRNAs. The lack of effect on ε-subunit mRNA is in marked contrast to the 7-fold effect of ARIA in the same experiment. To exclude effects due to changes in electrical activity, these cultures were treated with TTX. Thus, this experiment confirms that the ARIA effect on expression of the ε-subunit gene is independent of activity. ARIA produced ≈2-fold increases of α-, γ-, and δ-subunit mRNAs in these cultures, similar to the increase of α- and γ-subunit mRNAs seen in nonparalyzed cultures (compare Figs. 1 and 4). The level of β-subunit mRNA in the TTX-treated cultures was unchanged by ARIA. There also was no effect of CGRP on subunit mRNA levels in cultures not treated with TTX (data not shown).

No significant effect on ε-subunit mRNA levels was observed after a 48-hr exposure to optimal concentrations of thyroxine, forskolin, phorbol 12-myristate 13-acetate,

Table 1. Quantitation of data in Fig. 3 by densitometric scanning

Muscle	Subunit mRNA, amol per mg of total RNA				
	α	γ	δ	β	ε
Innervated	57	ND	27	35	57
Denervated	3606	1000	864	699	23

ND, not detectable.

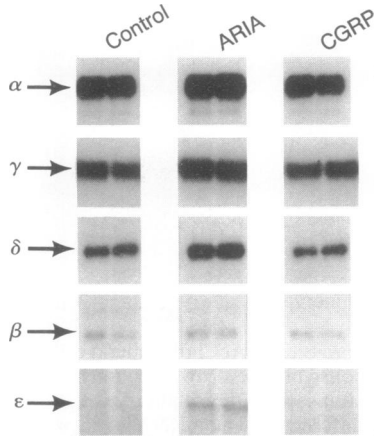

FIG. 4. Comparisons of the effects of ARIA and CGRP on the expression of the different AcChoR subunit mRNAs. Mouse myotubes were treated with ARIA or CGRP (0.1 μM) for 48 hr before the cells were harvested. In addition, TTX was added to the cultures for the same period of time to avoid increases in mRNA expression mediated by changes in muscle activity. The amount of RNA used for quantitation was: 40 μg for ε- and γ-subunit mRNAs, 5 μg for α-subunit mRNA, and 15 μg for β- and δ-subunit mRNAs. Results from duplicate plates in a single experiment are shown. Autoradiographic exposures are 16 hr.

A23187, basic fibroblast growth factor, or transforming growth factor β.

DISCUSSION

We have found that highly purified chick ARIA can increase the level of ε-subunit mRNA in cultured mouse myotubes. No other substance tested—including CGRP, a peptide found in a subpopulation of motor neurons that can stimulate the synthesis of AcChoRs in chick myotubes (33, 34), and TTX—mimicked ARIA's effect in this regard.

We do not know if the ARIA-induced increase in ε-subunit mRNA is due to an increase in the rate of gene transcription, but this seems likely as there is indirect evidence that ARIA's effect on chick muscle α-subunit mRNA is due to increased transcription (19), and nuclear run-on experiments have shown that the increase in subunit mRNA levels that occurs during the differentiation of mouse muscle cell lines is due to an increased rate of transcription (35).

The magnitude of the ARIA-stimulated increase in ε-subunit mRNA that we observed in cultured mouse myotubes (7-fold) is comparable to that measured in intact rat (5) and mouse (6) muscles between birth and postnatal day 15. Moreover, the amounts of ε-subunit mRNA (normalized to total RNA) at the two time points are similar in intact and cultured muscle. However, it should be noted that the increase in ε-subunit mRNA *in vivo* is apparently restricted to the motor endplate (7), whereas the increase observed in ARIA-treated cultures presumably occurs throughout the entire myotube.

ARIA also induced a small but reproducible increase in the rate of insertion of AcChoRs, a measure of the rate of AcChoR synthesis, into mouse myotube surface membranes. It is unlikely that ARIA's effect on AcChoR synthesis can be explained by its effect on ε-subunit mRNA, the control and stimulated levels of which are low compared with those of the mRNAs that encode the other subunits. Moreover, the level of γ-subunit mRNA, which is relatively abundant in control myotubes, was further increased by ARIA. Indeed, we do not know if any of the receptors synthesized in the presence of ARIA are of the $\alpha_2\beta\varepsilon\delta$ type. Single-channel recordings (32) and subunit-specific antibodies (2) should resolve this issue.

Three mechanisms may largely govern the spatial and temporal expression of AcChoR subunit genes (see ref. 6): (*i*) all five genes are expressed as part of the program of muscle development—α, β, γ, and δ at relatively high levels and ε at a low level; (*ii*) muscle activity down regulates the α-, β-, γ-, and δ-subunit genes in nuclei throughout the muscle fiber; and (*iii*) trophic factors released by the nerve and perhaps bound in the synaptic cleft produce a local up-regulation of all five genes. The data presented here raise the possibility that ARIA or an ARIA-like factor might be an important "trophic" regulator of mammalian AcChoR subunit genes during the process of nerve–muscle synapse formation, from the time that motor axons first contact muscle cells on embryonic days 15–17 through the postnatal period of junctional maturation.

It is thought that ε-subunit mRNA (and polypeptide) do not appear at developing motor endplates until after birth (2). This presents a puzzle for a single-factor hypothesis, as the first nerve-induced clusters appear shortly after innervation (29). However, the delay between the initial accumulation of AcChoRs and the subunit switch might be accounted for in several ways. First, the expression of the ε-subunit gene may be limited by muscle transcription factors that appear or increase after birth. Second, an early (prenatal) accumulation of ε-subunit mRNA and its protein at developing synapses may simply have not been detected by the Northern blots, *in situ* hybridization, and immunochemical methods used. The temporal relation between ε-subunit appearance and AcChoR channel function must be further explored.

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