Assembly of an adult type acetylcholine receptor in a mouse cell line transfected with rat muscle ε-subunit DNA

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The mouse muscle cell line BC3H-1 expresses an acetylcholine receptor (AChR) composed of α-, β-, γ- and δ-subunits [1]. The functional characteristics of this AChR are comparable to the non-synaptic AChR subtype in mouse muscle [2,3]. To investigate the role of the ε-subunit, which is believed to replace the γ-subunit in forming the adult AChR subtype [4], BC3H-1 cells were stably transfected with cDNA encoding the rat muscle AChR ε-subunit. Expression of this cDNA was under the control of a heat shock promoter, and the plasmid carried the neomycin resistance gene for selection. Several clones were isolated that had integrated the plasmid DNA in a stable form and produced ε-subunit specific RNA after heat induction. Single-channel current recording from cells which contained abundant ε-subunit mRNA identified a novel AChR channel having a larger conductance than the native AChR in these cells. These results suggest that the rat muscle ε-subunit may assemble with mouse muscle α-, β- and δ-subunits to form a mouse-rat hybrid AChR with properties similar to that of end-plate channels in the mature mammalian neuromuscular synapse. The novel AChR channel appears in the surface membrane within a few hours following the rise in ε-subunit mRNA. Thus, the notion that replacement of the γ-subunit by the ε-subunit during development is the result of the postnatal rise in the level of ε-subunit specific mRNA is further supported.

Acetylcholine receptor; Muscle development; Transfection

1. INTRODUCTION

The nicotinic acetylcholine receptor (AChR) of the neuromuscular junction is a ligand-gated ion channel mediating synaptic communication between nerve and muscle. Whereas in adult innervated muscle the AChR is predominantly localized at the neuromuscular junction, in fetal muscle and in denervated adult muscle the AChR is also present at the non-junctional membrane. AChR channels of the junctional and extrajunctional fiber segments have different electrophysiological properties (for review [5]). Two classes of AChR channels have also been observed at the same muscle fiber during muscle development [5]. Moreover, expression in *Xenopus laevis* oocytes of the cloned subunit specific cDNAs of the bovine muscle AChR has provided direct evidence for the different molecular composition of the observed subtypes of AChR channels ([4,6] for review). These studies, together with the developmental changes observed in the muscular content of the subunit specific mRNAs [4,7,8], suggest that the substitution of the γ-subunit in the fetal AChR, presumably with a $\alpha_2\beta\gamma\delta$ structure, by the ε-subunit to form a channel with $\alpha_2\beta\delta\epsilon$ structure is, at least partially, responsible for the functional differences between fetal and adult AChRs. The non-fusing mouse muscle cell line BC3H-1 [9,10] expresses an AChR with functional properties similar to the fetal channel type [2,11]. This cell line has been extensively used in studies of the developmental regulation of AChR gene transcription [12], RNA processing and protein assembly [13]. Its AChR is composed of α-, β-, γ-and δ-subunits. By stable transfection of BC3H-1 cells with the cDNA coding for the ε-subunit of the rat muscle AChR, we have constructed several cell lines that produce ε-subunit specific transcripts after heat shock activation. These cells also express a novel AChR channel with larger conductance which is characteristic for the end-plate channel in adult mammalian muscle. This result confirms, in a different expression system, the role played by the ε-subunit in determining the functional properties of the AChR in mature neuromuscular synapses.

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2. MATERIALS AND METHODS

The plasmid pεHS was constructed by isolating the coding region of the rat muscle AChR ε-subunit as well as the flanking 5′ and 3′ untranslated sequences [14] by *SalI-XbaI* digestion and inserted in the right orientation at the corresponding *SalI* and *XbaI* sites of the heat shock expression vector p17SPneo (a kind gift of Dr. M. Dreano, Batelle Res. Institute, Geneva) [15,16]. The most important features of this vector are (Fig. 1): (1) The neomycin resistance gene driven by SV 40 regulation (2) the heat shock promoter hsp 70 [17], and (3) a SV 40 polyadenylation site cloned downstream from the polylinker sequence.

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BC3H-1 cells were maintained in Dulbecco modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum. Twenty μg of pϵHS DNA was used to transfect 5×10^5 cells in a 100 mm culture dish by the calcium phosphate procedure [18]. Two days after transfection, the medium was changed and supplemented with 400 μg/ml geneticin (Sigma Co., St. Louis, USA). Foci were isolated, and cell clones expanded for further characterization. Heat shock activation was performed by incubating cells at 41°C while maintaining controls at the usual culture temperature of 36°C. All other culture parameters were maintained unchanged. After 90 min at 41°C, activated cells were incubated at 36°C for 2 additional hours and their mRNA extracted. Genomic DNA of selected cell lines was isolated as described by Hogan et al. [19] and used for Southern analysis. Poly(A)$^+$ RNA of the different cell lines was prepared either from heat shock treated or untreated control cells. Further details are found at the corresponding figure legends.

ACh-activated single-channel currents were studied at 20–22°C in cells of young cultures [2] of BC3H-1 cells and cells from transfected cell lines Bϵ15 and Bϵ16. Briefly, cells were plated on small plastic Petri dishes and maintained in DMEM medium supplemented with 0.5% fetal calf serum for 8–10 days. Single-channel currents were recorded with standard patch-clamp techniques in the cell-attached configuration [20]. The bath and the pipette solutions had the following ion composition (in mM): KCl 140; $CaCl_2$ 1.8, $MgCl_2$ 1.8, Hepes 10. The high K^+ bath solution ensured a cell resting potential close to 0 mV [2]. Either 0.5 or 1 μM ACh was added to the pipette solution. Channel conductance was measured by linear regression of current–voltage plots in the range of 30–120 mV pipette potential. The measurements on heat shocked Bϵ16 cells were made 2–10 h after the end of the heat-shock interval.

3. RESULTS

BC3H-1 cells express an AChR composed of α-, β-, γ- and δ-subunits with electrophysiological properties similar to those of AChRs in fetal muscle [2,11]. To be able to study the functional properties of channels formed when ϵ-subunit specific RNA is also present the ϵ-subunit DNA was cloned under control of a heat shock promoter and BC3H-1 cells were stably transfected. Fig. 1 shows the recombinant plasmid used for transfection. Addition of geneticin allowed the selection of foci which appeared to have stably integrated the transfected DNA. Eight of the isolated clones were chosen for further characterization.

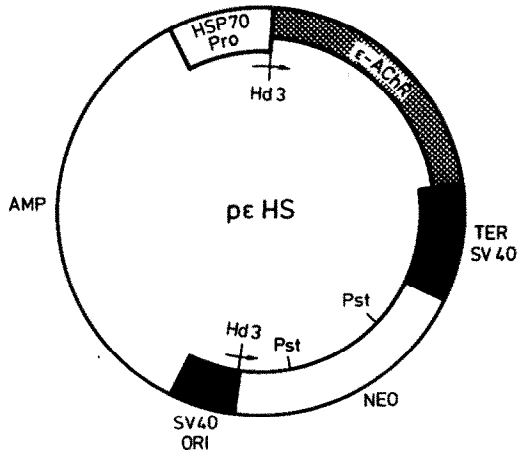

Fig. 1. Heat shock expression vector for the rat muscle AChR ϵ-subunit used for BC3H-1 cell transfection. Plasmid pϵHS contains two transcription units: (1) the ϵ-subunit AChR gene (ϵ-AChR) under the control of the human hsp70 promoter (HSP 70 Pro) and ended by the SV40 terminator (TER SV 40); and (2) the neomycin resistance gene (NEO) driven by a SV40 promoter (SV40 ORI). Other elements: AMP, β lactamase gene; Hd3 and Pst, sites cleaved by restriction endonucleases *Hind*III and *Pst*I, respectively.

3.1. *Southern blot analysis*

Southern analysis of genomic DNA isolated from the 8 clones is shown in Fig. 2. Incorporation of plasmid DNA into the genome was tested by hybridization using a *Pst*I fragment of the neo gene as probe (see Fig. 1). Given that there are two *Hind*III sites at the p17SP neo vector (Fig. 1) and none at the ϵ-subunit insert, *Hind*III digestion should produce a fragment of about 3800 base pairs (ϵ-subunit DNA included). Indeed, DNAs of each tested cell line showed a hybridizing fragment of the mentioned size after *Hind*III digestion. As expected, the amount of hybridizing DNA varied extensively from clone to clone, indicating differences in copy number of plasmid integrated into the genome (Fig. 2). Further hybridization of another blot containing the same DNAs with an ϵ-subunit cDNA probe confirmed that the *Hind*III genomic fragment mentioned above

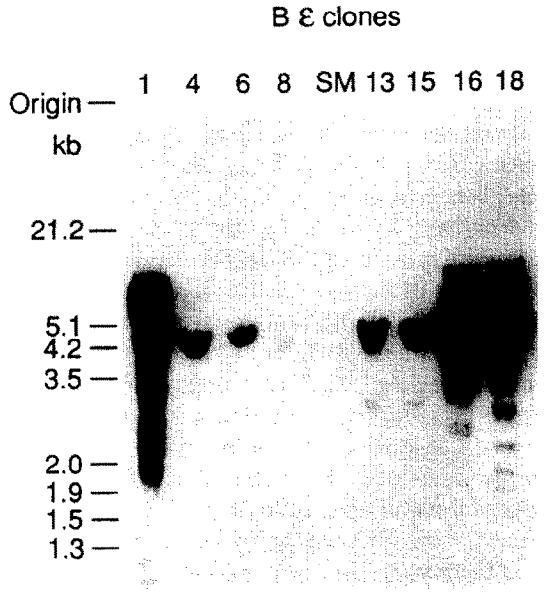

Fig. 2. Autoradiograph of Southern blot hybridization using genomic DNA isolated from transfected BC3H-1 cells (as indicated above each lane). Cells growing at confluence in four 100-mm plastic dishes were trypsinized and collected. DNA was extracted and dissolved in 0.3 ml of 10 mM Tris-Cl (pH 8), 1 mM EDTA. Twenty μl of each DNA solution were digested overnight with 40 U of *Hind*III. Samples were applied to a 0.8% agarose gel and run at 75 V (SM, size marker). After transfer to nitrocellulose the blot was hybridized in 10% dextran sulfate, 50% formamide at 42°C to the radiolabelled *Pst*I fragment of the neo gene (see Fig. 1). After hybridization, the filter was washed with 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate pH 7.0), 0.1% SDS at 24 and 42°C and with 0.1 × SSC, 0.1% SDS at 42, 50 and 65°C. Autoradiography was carried out on Kodak XAR5 X-ray film with intensifying screen at −70°C for 4 h. Overexposure was necessary to show the weak hybridization of the Bϵ8 cell line.

contained the ε-DNA sequence (data not shown), as expected from the cloning strategy used. Hybridization intensity was parallel to the one observed with the neo fragment. Thus, all the analyzed clones had stably integrated the plasmid DNA, although in different copy numbers. For example, clones Bε1, Bε16 and Bε18 showed strong hybridization, whereas in Bε8 hybridization was barely perceptible.

3.2. *Northern blot analysis*

Poly $(A)^+$ RNA from 5 transfected clones was isolated and subjected to Northern blotting. Cells were maintained at 36°C (controls) or heated at 41°C for 90 min. Two hours after heatshock poly$(A)^+$ RNA was extracted, blotted and hybridized either with an ε- or γ-subunit specific cDNA probe [21]. Fig. 3 shows that a transcript of about 2.5 kb is detected in cells from the clone Bε16 with the ε-subunit probe, only after they were incubated at 41°C. Similar results were obtained with cells from other clones (data not shown). The clone Bε15 is an exception, since the same transcript is already observed without heat shock, suggesting that this clone expresses the ε-subunit mRNA independently of the heat shock promoter. A possible explanation for this result could be the reading from an endogenous promoter through the heat shock promoter into the ε-subunit gene. Despite this effect ε-subunit gene transcription is still heat inducible. Another band of about 4.5 kb hybridized with the ε-subunit probe in all cases, with or without heat shock. In general this band is less prominent than the 2.5 kb transcript and seems to be the result of a polymerase start site inside the plasmid sequence 2000 bp upstream. Hybridization with a γ-subunit specific fragment (Fig. 3) shows, as expected, the presence of γ-subunit specific transcripts in all conditions, with and without heat shock activation. Similarly, no differences were observed on the expression of the α-, β- and δ-subunit-specific mRNA before or after heat shock (data not shown). In most cases hybridization signals are weaker than the ones obtained with the ε-subunit specific probe after activation. The differences observed in hybridization intensity are not due to a different amount of mRNA applied into each slot, since an actin-specific probe showed the same hybridization pattern in all cases (data not shown). Therefore, it may be concluded that, at least at the time of collecting cells for RNA isolation, the different clones were producing different amounts of ε- and γ-subunit specific RNAs.

The cell line Bε16 carries the ε-subunit cDNA under the control of heat-shock promoter, offering the possibility of examining the change in the level of ε-subunit transcripts following induction by the heat shock. Fig. 4 shows Northern blots of RNA isolated

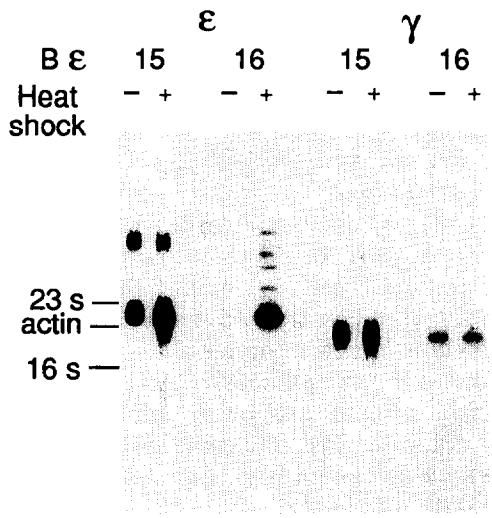

Fig. 3. Autoradiograph of Northern blot hybridization using poly$(A)^+$ RNA isolated from transfected cell lines Bε15 and Bε16. Cell material from four 100-mm dishes submitted or not to heat shock (+ or −) was used to isolate poly$(A)^+$ RNA. Three μg of mRNA were applied into each slot of a formaldehyde gel. After RNA transfer to a Nytran membrane (Schleicher and Schüll, FRG) the blot was hybridized in the same conditions as the ones described for Fig. 2 with a rat muscle AChR ε- or a γ-subunit specific cDNA probe [21] as indicated. Wash and autoradiography conditions were also the same as in Fig. 2, but a 16-h exposure was used. In all cases an additional band of about 4.5 kb, presumably a result of transcription start sites in plasmid sequences upstream of the heat shock promoter, hybridized with the ε-specific probe.

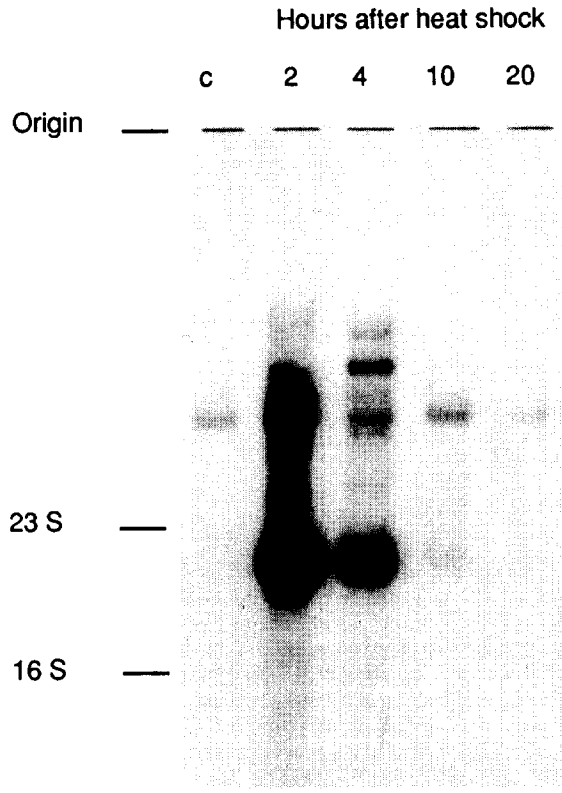

Fig. 4. Rise and time-dependent decrease of ε-subunit specific mRNA level following heat shock. Autoradiograph of Northern blot analysis of poly$(A)^+$ RNA isolated from transfected cell line Bε16 without heat shock (C) and heat-shocked cells harvested at different times after heat shock as indicated by the number above each lane. Hybridizing conditions as described for Fig. 2.

from Bε16 cells at various times after heat shock. The signal is highest immediately after the heat shock and, surprisingly, the mRNA levels decline rapidly to undetectable levels within about 10 h following the heat shock.

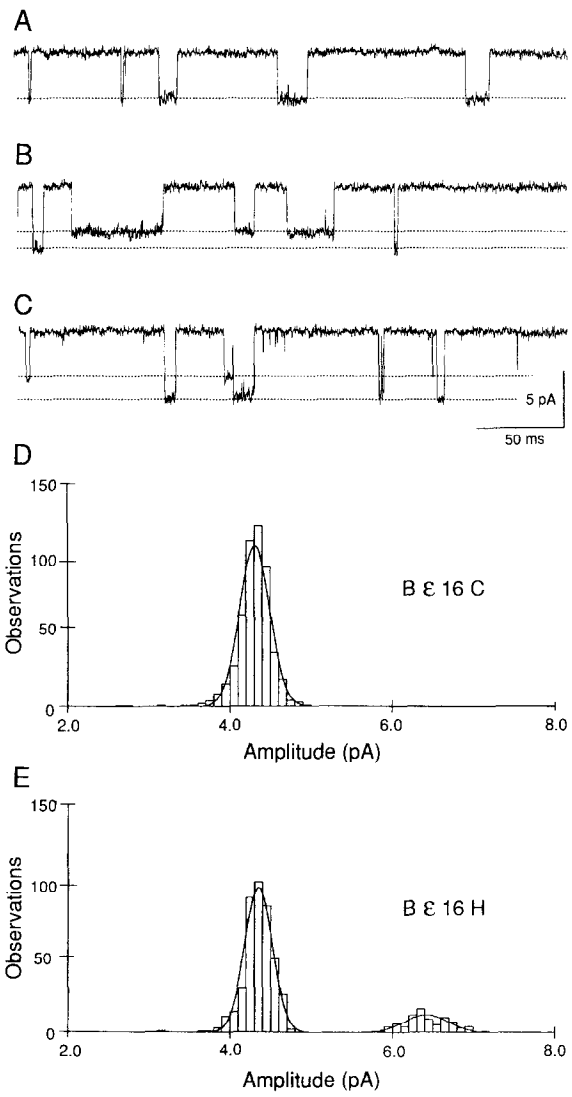

Fig. 5. Single-channel currents activated by ACh in Bε16 cells. (A): Records of ACh-activated currents in cell line Bε16 cells without heat shock (Bε16 C). A single class of currents of 4.23 ± 0.21 pA ($n = 582$) mean amplitude is observed at 80 mV pipette potential. Reversal potential was at −12 mV pipette potential. (B): Current recording from a Bε16 cell about 6 h after the heat shock (Bε16 H). Note occurrence of two classes of currents, characterized by mean amplitudes of 4.35 ± 0.17 pA ($n = 412$) and 6.42 ± 0.27 pA ($n = 81$), respectively. Pipette potential was 80 mV, reversal potential −6 mV. The broken lines indicate the mean current amplitudes of the two classes of currents. (C): Record from another patch of the same cell. In this patch mostly (>95%) large amplitude currents were observed, mean amplitude was 6.25 ± 0.31 pA ($n = 221$). (D,E): Histograms of amplitude distributions of single-channel currents in Bε16 cells from a cell without heat shock (Bε16C) and from a cell 6 h after heat shock (Bε16H). Pipette potential was 80 mV in both experiments.

3.3. *Functional properties of AChR channels in transfected cells*

AChR channels comprising the ε-subunit are characterized in other expression systems by a larger channel conductance and shorter mean open times than the γ-subunit containing AChR channel [4]. The lower conductance AChR is the endogenous channel of BC3H-1 cells [2]. ACh-activated single-channel currents were measured to test whether the increase in the level of the rat muscle ε-subunit specific RNA in the transfected mouse cell line was sufficient to produce a rat–mouse hybrid AChR with novel properties.

Fig. 5 shows a comparison of ACh activated currents recorded from Bε16 cells, either without heat shock (Bε16C) or after heat shock (Bε16H). In Bε16C cells a single class of currents is observed with amplitudes indicating the opening of endogenous AChR channels (Fig. 5A,D). The slope conductance of the endogenous AChR channel of Bε16C cells was 50 ± 3 pS ($n = 7$) which is close to the value measured for BC3H-1 cells (51 ± 4 pS, $n = 3$) confirming a previous report [2]. In contrast in Bε16H cells two classes of currents, characterized by different amplitudes, are seen (Fig. 5B,C). The currents with the larger amplitude indicate the opening of a novel type AChR channel which has a conductance which is nearly 50% larger than that of the endogenous channel (Fig. 5E). In 4 of 9 patches studied in Bε16H cells within the first 10 h following the heat shock the two classes of channels were activated whereas in 3 patches only the large conductance class and in 2 patches only the low conductance class was observed. The two classes of channels corresponded in their conductance to a novel class of AChR channel, characterized by a larger conductance of 69 ± 7 pS ($n = 7$), and to that of the endogenous AChR channel (49 ± 4 pS, $n = 6$).

ACh-activated currents recorded in two patches of the clonal cell line Bε15, which transcribes ε-subunit cDNA without heat activation, showed that the amplitudes of ACh-activated single-channel currents also fell into two well separated classes. They also corresponded in their amplitudes to those mediated by the endogenous channel and by an additional, less frequently occurring class of channels which have a conductance which is about 50% larger than that of the endogenous AChR channel.

4. DISCUSSION

We used the mouse BC3H-1 cell line that expresses endogenously mRNAs coding for the mouse muscle α-, β-, γ- and δ-subunits and where only the low conductance, fetal type of AChR is found. After transfection of BC3H-1 cells with the cDNA coding for the ε-subunit of the rat muscle AChR, several clones were isolated which had stably integrated the ε-subunit cDNA and in which upon heat activation the respective ε-subunit

coding mRNA was transcribed. In these cells two classes of AChR channels are present, a lower conductance class representing the endogenous channel and a novel channel with higher conductance. This suggests that the ε-subunit specific mRNA directs the synthesis of the ε-subunit polypeptide which assembles with the endogenously expressed AChR subunits into a mouse–rat hybrid channel composed of α-, β-, and δ-subunits of mouse and the ε-subunit of rat muscle. This hybrid channel has conductance properties resembling those of AChR channels in the end-plate of adult bovine, rat and mouse skeletal muscle [3,4,21].

Previously it was reported that two subclasses of AChR channels, defined by different conductance and gating properties, are formed in *Xenopus* oocytes injected with α-, β-, δ- and either γ- or ε-subunit specific mRNAs from bovine muscle AChR [4]. The two subtypes, comprising either the γ- or the ε-subunit, had functional properties similar to those observed in fetal and adult muscle, respectively [4]. Given the opposite changes in the contents of the γ- and ε-subunit mRNAs during development in calf and rat muscle [4,21] it was suggested that a transient low conductance AChR channel in fetal muscle is assembled from the α-, β-, γ- and δ-subunits, whereas the end-plate channel in innervated adult muscle is composed of α-, β-, δ- and ε-subunits ([6] for review).

By using a different host system for AChR expression (*Xenopus laevis* oocyte vs mouse cell line) and AChRs from different species (calf vs mouse–rat hybrid), the notion that the presence of the ε-subunit in the AChR complex constitutes the molecular distinction between fetal and adult forms of mammalian muscle AChR channels has been further supported. The experiments demonstrate that the ε-subunit competes with the γ-subunit in forming a channel and show that the adult form of the AChR assembles and is inserted into the plasma membrane within a few hours following the rise in ε-subunit mRNA level. Despite the similar levels of ε- and γ-subunit specific mRNA, the rat mouse hybrid channels appear with lower frequency than the endogenous mouse channels suggesting inefficient assembly of the rat mouse hybrid. The presence of two AChR subtypes may mimic the situation at the developing neuromuscular synapse: initially, a low conductance fetal type of receptor is expressed. Activation of ε-subunit specific RNA transcription at subsynaptic nuclei [8] gives rise to a new high conductance type of receptor, with functional properties similar to those in the receptor in the adult end-plate. Both receptor subtypes are co-expressed during a short postnatal period [22] during which γ-subunit mRNA levels decrease and ε-subunit mRNAs increase [7]. As a consequence the fetal subtype disappears and finally only the adult subtype is present in the mature end-plate.

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