Spontaneous muscle action potentials fail to develop without fetal-type acetylcholine receptors

Masazumi Takahashi⁺, Tai Kubo[‡], Akira Mizoguchi¹, C. George Carlson², Katsuaki Endo³ & Katsunori Ohnishi³

International Institute for Advanced Studies, Numa Project, 1 Nishinokyo-Kuwaharacho, Shimadzu N80, Nakagyo, Kyoto 604-8442, ¹Department of Anatomy and ³Department of Physiology, Kyoto University Faculty of Medicine, Yoshida-Konoe, Sakyo, Kyoto 606-8501, Japan and ²Department of Physiology, Kirksville College of Osteopathic Medicine, Kirksville, MO 63501, USA

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In mammals, two combinations of muscle nicotinic acetylcholine receptors (AChRs) are used: $\alpha_2\beta\gamma\delta$ (γ -AChR) or $\alpha_2\beta\epsilon\delta$ (ϵ -AChR). After birth, γ -AChRs are replaced by ϵ -AChRs (γ/ϵ -switch). The two receptors have different conductances and open times. During perinatal period, the long open time γ -AChRs generate random myofiber action potentials from unquantal miniature end-plate potentials (mEPPs). ϵ -AChRs are suitable for strong adult muscle activities. Since the effect of the γ/ϵ -switch on neuromuscular development was unclear, despite the many differences in channel characteristics, we carried out this study to generate γ -subunit-deficient mice. Homozygotes born alive survived for 2 days in a stable condition, and were able to move their forelimbs. Endplate AChRs included ϵ -subunits, and muscle fibers had multiple neuromuscular junctions. Both pre- and postsynapses were abnormal and spontaneous action potentials generated from mEPPs were totally absent. Results suggest a requirement for γ -AChRs in mediating synaptically-induced action potential activity critical for neuromuscular development.

INTRODUCTION

The muscle-type nicotinic acetylcholine receptor (AChR), a ligand gated ion channel, triggers muscle action potentials in response to the release of quantal packets of acetylcholine (ACh) from motor nerve endings (Sanes and Lichtman, 1999). In mammals, the receptor takes two different forms: $\alpha_2\beta\gamma\delta$ (γ -AChR) or $\alpha_2\beta\epsilon\delta$ (ϵ -AChR). The mouse γ -AChR is replaced by the ϵ -AChR (γ/ϵ -switch) within the first 2 weeks after birth (Mishina *et al.*, 1986; Martinou and Merlie, 1991; Bouzat *et al.*, 1994). Primary structures of γ - and ϵ -subunits are more highly conserved

between species than between the two subunits (Numa, 1986). The γ - and ϵ -AChRs have 39 and 59 pS single-channel conductances and 10.4 and 5.3 ms open times, respectively (Mishina *et al.*, 1986). The difference in conductance is ascribed mainly to the transmembrane domains M1 and M2, the channel open time to the M4 domain and the amphipathic helix (HA) between M3 and M4. Although ϵ -AChRs are concentrated at the adult neuromuscular junction (NMJ), γ -AChRs are also expressed outside the junctional membrane prior to innervation. In adult, γ -subunits are re-expressed after skeletal muscle denervation.

The accumulation of γ -AChRs at the NMJ is nerve-induced. The neuronal isoform of agrin induces AChR clustering through muscle-specific kinase (MuSK) and myotube-associated specificity component (MASC) complex in the muscle cell membrane (Gautam *et al.*, 1996; Glass *et al.*, 1996). Rapsyn forms a subsynaptic cytoskeletal complex with AChRs and agrin-induced accumulation of AChRs requires rapsyn (Apel *et al.*, 1995; Gautam *et al.*, 1995). As the original NMJ is a preferred re-innervation site, it is thought that the synaptic complex remains at the same site after denervation (Sanes *et al.*, 1978).

Neuromuscular activity represses extrasynaptic γ -AChR expression and induces the regression of polyneuronal innervation, leading ultimately to a single contact per muscle cell in mature NMJs (Witzemann *et al.*, 1991; Gan and Lichtman, 1998). Complete blockage of the NMJ in adult muscle induces sprouting of the motor nerve endings (Brown and Ironton, 1977) and transgenic mice that overexpress neuronal GAP43 show nerve sprouting that is independent of muscle activity (Aigner *et al.*, 1995). These data suggest that paralyzed muscle produces the nerve sprouting factor that promotes the sprouting mechanism.

⁺Corresponding author at present address: Centre National de Genotypage, 2 rue Gaston Cremieux-CP5721, 91057 Evry Cedex, France. Tel: +33 1 60 87 83 33; Fax: +33 1 60 87 83 83; E-mail: masazumi@cng.fr

[‡]Present address: National Institute of Bioscience and Human-Technology, AIST, MITI, 1-1 Higashi, Tsukuba 305-8566, Japan

Fig. 1. *AChR* γ targeting. (A) *AChR* γ and the targeting vector structures. Closed boxes indicate exons. pCR γ and *acrg5* probes are shown. Arrows indicate transcriptional orientations. Restriction enzymes are: B, *Bam*HI; RI, *Eco*RI; Sca, *Sca*I; Sm, *Sma*I; others are as described. (B) The first screening of pooled DNAs. Lanes 40 and 43 have a recombination band. (C) Southern blot of the subclone G10-5, G38-2, G40-7 and ES (control). (D and E) Southern blot of tail DNAs (lane 1, *AChR* γ $^{+/+}$; lane 2, $+/+$; lane 3, $-/-$) probed with *acrg5* (D) and pCR γ (E). (F) Northern analysis of the muscle RNA with pCR γ probe. Samples loaded: lanes 1 and 2, *AChR* γ $^{+/+}$; lanes 3 and 4, $+/+$; lanes 5 and 6, $-/-$; lane 7, $+/+$ (P16); lane 8, $+/+$ (P16, denervated). Each pair in lanes 1–6 is from different individuals. β -actin (2.6 kb) is a control. The size of mature γ -subunit mRNA is 2.3 kb. There are 1.4 kb faint and broad bands related to the targeted allele, which are thought to be alternatively spliced transcripts.

Table I. Heterozygous intercrosses and their offspring

ES line	G10-5	G38-2	G40-7	Total
Offspring	432 ^a	176	69	677
<i>AChR</i> γ $^{-/-}$	63 (14.6%) ^b	22 (12.5%)	13 (18.8%)	98 (14.5%)
Survived	16 (3.7%) ^c	12 (6.8%)	8 (11.6%)	36 (5.3%)

^aNumber of offspring from each line.

^bNumber and percentage (in parentheses) of homozygotes in relation to the total number of offspring.

^cNumber and percentage (in parentheses) of homozygotes who survived in relation to the total number of offspring.

The situation in embryonic synaptogenesis differs slightly because descending motor impulses are rarely available in developing muscle. Instead, unquantal miniature end-plate potentials (mEPPs) produced by the spontaneous release of ACh dominate in generating spontaneous muscle action potentials primarily because of the long open time of the γ -AChR (Jaramillo *et al.*, 1988). Although the significance of mEPPs is still unknown, it is possible that spontaneous mEPPs, in conjunction with the γ/ϵ -subunit switch and receptor-specific differences in single channel function, may contribute directly to NMJ development.

Although the γ/ϵ -switch is observed commonly in different mammalian species, there is no agreement as to the functions and consequences of the switch. To elucidate the systemic role

Fig. 2. *AChR* γ $^{-/-}$ mouse and its littermate at P0. (A) *AChR* γ $^{-/-}$. (B) Control. *AChR* γ $^{-/-}$ mice cannot ingest milk as controls (arrow).

of the γ/ϵ -switch in neuromuscular development, we generated γ -subunit gene (*AChR* γ) disrupted mice.

RESULTS AND DISCUSSION

The gene targeting strategy is shown in Figure 1A. Three independent clones were identified through Southern blot analysis of a pool of transfected embryonic stem (ES) cells (Murray *et al.*,

Table II. AChR subunit expression in the limb muscles

	γ	ϵ	β	δ
Forelimb muscle				
P0 ^a +/+	0.29 ^b	0.09	1.4	0.6
P0+/-	nd ^c	0.09	1.3	0.5
P0-/-	nd	0.06	1.4	0.5
P6+/+	0.35	0.47	4.6	1.3
P17+/+	0.03	1.73	5.0	0.4
Hindlimb muscle				
P0+/+	0.43	0.04	1.0	0.6
P0+/-	nd	0.04	0.7	0.4
P0-/-	nd	0.03	0.9	0.3
P6+/+	0.51	0.32	3.7	1.4
P17+/+	0.09	2.73	8.7	0.8

^aP0, P6 and P17 are post-natal days 0, 6 and 17, respectively. Remarks are: +/+, wild type; +/-, heterozygote; -/-, homozygote.

^bRadioactivity was measured at each band using a BAS-1500 (Fuji Film) and background count was subtracted. Each value was then divided by the count of the β -actin band.

^cnd, Not done.

1992). Selected ES clones gave rise to germline chimera. Heterozygotes (*AChR γ /-*) were inter-crossed and homozygotes (*AChR γ /-*) were obtained at about half the frequency expected from Mendelian segregation. About half of these were born alive (Table I). Northern hybridization analysis revealed no mature γ -subunit messenger RNA (mRNA) in homozygotes (Figure 1F) (Evans et al., 1987).

Heterozygotes were obviously normal. *AChR γ /-* mice had a characteristic lunar-shaped body profile (Figure 2) and reduced body weight $18 \pm 8\%$ (mean $\pm$ SD, $n = 12$). They showed a shallow, irregular and paradoxical respiration (the thorax was depressed corresponding to abdominal distension) and a problem in suckling. Strong cyanosis was observed in the mutants that were dead at birth, suggesting severe respiratory failure. Mutants born alive were stable for up to 2 days, were able to move their forelimbs and reacted to stimulation. The phenotype was similar in the three lines. Because respiration was irregular but continuous, dehydration by impaired milk intake seemed to limit their survival. This idea is supported by observations of an extended survival following intraperitoneal saline infusion (data not shown).

As the mutant mice were able to move their forelimbs, but not hindlimbs, RNA was extracted separately from each muscle (Table II). In both control and mutant mice, γ -subunit expression was lower in the forelimb muscle than in the hindlimb muscle, whereas the opposite was true for ϵ -subunit expression, as was suggested by the chronological order of the γ/ϵ -switch from rostral to caudal muscle (Sakmann and Brenner, 1978). However, no compensatory increase of the ϵ -subunit was observed in *AChR γ /-* mice, in contrast to the γ -subunit compensation seen in the ϵ -subunit gene defect (Witzemann et al., 1996; Missias et al., 1997). In mutant mice, the expression of β and δ subunits was at the same level as that of control mice.

Fig. 3. Staining of the AChR with rhodamine-labeled BTX. (A and B) Intercostal muscle. (C–F) Diaphragm. (G and H) Forelimb muscle. (A, C, E and G) Littermates. (B, D, F and H) *AChR γ /-* mice. (A) In the control, AChRs are clearly stained along the intercostal nerve trunk (n) between ribs (r). AChR clusters are distributed along the central endplate band in (C) and (D). (E–H) The sliced view of 10 μ m depth using laser scanning confocal microscopy. Round synaptic patches were seen, one on each muscle fiber (E). In *AChR γ /-* mice, the unequal size and shape of patches are seen (F) and multiple patches are seen on one muscle fiber (arrow heads). Scale bar is 500 μ m in (A)–(D) and 50 μ m in (E)–(H). Actin is shown in green (E and F), BTX in red and PGP9.5 in blue in (E) and (F) and in green in (G) and (H).

AChR labeling using rhodamine-labeled α -bungarotoxin (BTX) revealed clearly stained foci in intercostal muscle fibers in controls, but foci were barely visible in *AChR γ /-* mice (Figure 3A and B). In the diaphragm muscle, the patches of labeled AChRs were distributed in a broader range along the central end-plate band and were unequal in size and shape compared with those of controls (Figure 3C and D). These

Fig. 4. The phrenic nerve and AChR clusters of the diaphragm at P0. (A and C) Control. (B and D) *AChR γ ^{-/-}*. Tissue staining with rhodamine-labeled BTX and FITC-labeled PGP9.5 were represented by red and green, respectively. The runs of the phrenic nerve were indicated by arrow heads. Whole tissue was used in (A) and (B) and sections in (C) and (D). Scale bar is 400 μ m in (A) and (B), and 25 μ m in (C) and (D).

staining data show that only the diaphragm muscle was able to support respiration, and hence explain the paradoxical respiration.

To reveal the NMJ on each muscle fiber, muscle actin was simultaneously stained and the section was observed by a laser scanning confocal microscopy (MRC-1024, Bio-Rad). Muscle fibers of *AChR γ ^{-/-}* mice had unequal sizes and lower density cross-striation than those of control mice. In addition, multiple AChR clusters were seen on a single muscle fiber, which normally has only one cluster per synaptic region (Figure 3E and F). Anti-neuronal antibody PGP9.5 staining showed the motor neuron wandering among muscle fibers and sprouting many branches that ended at AChR clusters (Figures 3E–H and 4). Therefore, the γ -AChR is not necessary to initiate synaptic formation (Hashemolhosseini *et al.*, 2000).

AChRs were also stained with subunit-specific antibodies. Since staining with anti- δ -subunit antibody (88B) was localized at the synaptic region, extrasynaptic AChRs were suppressed in both wild type and mutant (Figure 5A and F). Staining with anti- ϵ -subunit antiserum and BTX were colocalized in the mutants, thus suggesting that the expressed AChRs included the ϵ -subunit (Figure 5G–I). Although functional channels exist with only α , β and δ subunits experimentally (Kurosaki *et al.*, 1987; Liu and Brehm, 1993), the single channel conductance is reduced 20- to 30-fold, the binding affinity of BTX is reduced to one-fifth or less than that of the γ -AChR (Bouzat *et al.*, 1994) and channel activity

disappears after exposure to ACh (drop-out phenomenon). Therefore, the viability of the mutant may depend on the level of ϵ -subunit expression.

Communication between pre- and postsynapses leads to synaptic specialization. Since AChRs were clustered and the clusters were innervated in *AChR γ ^{-/-}* mice, the cytoskeletal complex is formed and targeted by the motor neuron in the absence of AChR γ -subunit expression. Because the γ -subunit can be expressed elsewhere than the NMJ, which is not the case for the ϵ -subunit, there could be a molecule that interacts specifically with either the γ - or the ϵ -subunit. However, since such molecules have not been identified, one cannot explain the synaptic abnormalities seen in *AChR γ ^{-/-}* mice simply by γ -subunit interacting molecules.

Examination of fine structure revealed a rather well-developed presynapse with active zones, mitochondria and accumulations of synaptic vesicles, although the polarity of the vesicles was not clear (Figure 6B). Despite movements of the forelimb and diaphragm, indicating good conduction of motor nerve impulses to these muscles, the presence of well-developed but sprouted nerve terminals indicates a history of inactivity. This could be explained by the loss of a fraction of action potentials generated by other than descending neural impulses.

To examine this possibility, intracellular recordings were obtained from proximal forelimb muscle (triceps) and the

Fig. 5. Antibody staining of AChR subunits. (A, B, F and G) Diaphragm. (C and H) Forelimb muscle. (D and I) Hindlimb muscle. (E) Adult control forelimb muscle. Control mice (A–D) and *AChRγ*^{−/−} mice (F–I) at P0. AChRs were doubly stained with rhodamine-labeled BTX and FITC-labeled anti-δ-subunit antibody (A and F) or anti-ε-subunit antiserum (B–E and G–I). BTX staining is represented in red and antibody stainings are in green. In *AChRγ*^{−/−} mice, it is difficult to get NMJs in a slice view because they are not aligned. Scale bar is 15 μm in (A) and (F), and 10 μm in the others.

diaphragm, which indicated the presence of functional neuromuscular contacts. There was no significant difference ($p > 0.05$) in the resting potentials (RP) between the normal littermate, which had an average of -52 ± 4.6 mV (mean $\pm$ SD, $n = 5$ fibers), and the mutant mouse, which had an average of -45 ± 4.2 mV (mean $\pm$ SD, $n = 7$ fibers). Recordings from normal P0 littermates indicated that the large amplitude unquantal mEPPs characteristic of this stage of development (Muniak *et al.*, 1982) often elicited spontaneous action potentials (Figure 7A and C). These recordings also showed clusters of irregularly spaced action potentials (Figure 7A, traces 5–7) with inflections indicative of synaptic activity (Figure 7A, trace 5). This suggests that the spontaneous clusters of large synaptic unquantal activity seen in tetrodotoxin-treated fibers at this stage elicit irregular bursts of action potentials in untreated fibers (Carlson, 1992). In other instances, isolated action potentials were produced by single large-amplitude mEPPs, which occurred at an overall frequency of 1–2 events/min (Figure 7C). Approximately 45% (5/11 using five fibers) of the mEPPs recorded from normal endplates produced action potentials in these P0 fibers (Jaramillo *et al.*, 1988). In *AChRγ*^{−/−} fibers, mEPPs were recorded at a frequency similar to those of normal littermates, but the mEPPs were subthreshold and never (0/12 using seven fibers) resulted in action potentials (Figure 7B). The absence of action potentials elicited by mEPPs in *AChRγ*^{−/−} fibers was not secondary to a reduction in membrane excitability, since *AChRγ*^{−/−} fibers were actually hyperexcitable and occasionally exhibited fibrillatory action potentials immediately upon impalement (data not shown).

Although it is impossible to measure the average mEPP amplitude in the control muscle without blocking muscle action potentials, mEPPs in *AChRγ*^{−/−} fibers had an average value of

0.87 ± 0.47 mV (mean $\pm$ SD, $n = 12$ mEPPs). The average time constant of decay for the subthreshold mEPPs was 9.1 ± 1.6 ms (mean $\pm$ SD, $n = 4$ mEPPs) and 6.2 ± 0.9 ms (mean $\pm$ SD, $n = 7$ mEPPs) for control and mutant muscle fibers, respectively. This is consistent with shorter open times of the mutant AChR.

Since the first description of spontaneous quantal release (Fatt and Katz, 1952), the physiological role of the mEPP itself has remained unclear (Jaramillo *et al.*, 1988; Kidokoro and Saito, 1988). Synapses are formed and maintained without action potentials (Cohen, 1972), but action potentials suppress γ -subunit expression in non-endplate regions. There is also evidence that mEPP-triggered spontaneous muscle action potentials are related to cross-striation formation of the myocytes (Kidokoro and Saito, 1988). The γ -subunit is highly conserved in mammalian species, even though it is normally expressed only during the fetal to neonatal period. We have shown here that spontaneous mEPPs in conjunction with endplate γ -AChRs may play an important role in synapse elimination and muscle and synaptic maturation. Expression of γ -AChR can therefore be critical for early neuromuscular development, while high conductance, short open time ϵ -AChRs are optimized for adult skeletal muscle fibers.

METHODS

Gene targeting. The mouse AChR γ -subunit gene was isolated from a 129 genome library (a gift from T. Doetschman) using the human γ -subunit cDNA as a probe (Shibahara *et al.*, 1985). The 11 kb *EcoRI*–*SmaI* fragment was subcloned (Figure 1A). PGKneo (Stratagene) was inserted at the *AatI* site of exon 4. HSV-*tk* was inserted at the 3′-end of the insert. Feeder-dependent RW4 ES cells (6.6×10^7 ; Genome Systems) were transfected with 25 μg

Fig. 6. The fine structure of NMJs at P0. **(A)** Control. **(B)** *AChR γ ^{-/-}*. An asterisk indicates the nerve terminal. The doubly magnified views of the nerve terminals are shown on the right. Arrowheads indicate active zones. Scale bar is 1.7 μ m in (A) and 2 μ m in (B).

of the linearized (*NotI*) targeting vector by electroporation (625 V/cm, 25 μ F; Easylect, EquiBio), divided into 64 wells and cultured against G418 (200 μ g/ml) for 1 week. Twenty micrograms of genomic DNA extracted from each pool (Blin and Stafford, 1976) were digested with *Bam*HI, electrophoresed and transferred to nitrocellulose filters. The filters were hybridized with the riboprobe, which was prepared as described previously (Melton, 1985) using the 1 kb *Eco*RI fragment subcloned from the 5' flanking region as a template. The pools that showed a faint recombinant band (Figure 1B) were subcloned. Southern blot analysis was carried out for each subclone with an *acr*g5 probe, which is the 0.4 kb *Eco*RI–*Pst*I fragment 5 kb upstream of the γ -subunit gene (Figure 1C), as described previously (Honjo *et al.*, 1979). Recombination was confirmed with *acr*g5 and pCR γ probes (Figure 1D and E).

Generation of null mutant mice. Each clone was injected into C57BL/6 blastocysts. Chimeric male mice were crossed with C57BL/6 females to obtain agouti offspring. Targeted alleles were determined by Southern blot hybridization or PCR of tail DNAs (data not shown).

Northern blot hybridization. Total RNA was extracted from the fore- and hindlimbs using guanidine HCl and organic solvents (Chomczynski and Sacchi, 1987). Ten micrograms each was electrophoresed after glyoxal treatment and transferred to nylon membranes (McMaster and Carmichael, 1977). Membranes were hybridized with ³²P-labeled DNA probes containing the 3'-part of β , γ , δ or ϵ -subunit cDNA, which were obtained by

cloning PCR amplification products (Table III), and were confirmed by sequencing (ABI373S, Perkin Elmer). Stripping off the probe, each filter was re-hybridized with β -actin probe. For denervation, the sciatic nerve of *AChR γ ^{+/-}* mice was cut on P12. After 4 days (P16), RNA was extracted from the hindlimb muscle.

Immunohistochemical and ultrastructural analysis. Neonates were killed following the institutional guidelines and fixed with 1% formaldehyde for 10 min. Muscles were stained with 1 μ g/ml of rhodamine-labeled BTX (Molecular Probes) overnight. 88B (Affinity BioReagents), PGP9.5 (Ultraclone) and AChR ϵ -subunit-specific antiserum (a gift from J.R. Sanes) were used for staining as described by Mizoguchi *et al.* (1994). Actin was stained with phalloidin. For ultrastructural studies, neonates were fixed by transcardial perfusion with 2% paraformaldehyde and 2.5% glutaraldehyde in 100 mM phosphate buffer pH 7.4 for 10 min. Diaphragms were dissected out, soaked with the same fixative for 4 h, washed, post-fixed with 1% OsO₄ for 1 h, dehydrated and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed under an electron microscope (JEM-1200EX, JEOL).

Electrophysiological recordings. Intracellular recordings were obtained from P0 muscle fibers by using fiber-filled glass micro-pipettes (Warner Instruments, 3 M KCl; resistances, 30–45 M Ω), and the recordings were amplified using a Warner Model IE201 Intracellular Electrometer and a Grass P15 amplifier. Both the diaphragm and triceps muscles were placed in a normal Ringer

Fig. 7. Intracellular recordings from muscle fibers. **(A)** Large mEPPs and associated spontaneous action potentials recorded from normal littermate triceps muscle fibers at P0. Traces 1, 2 and 5–7 were recorded from the same fiber and traces 3 and 4 show mEPPs recorded from two additional fibers. The action potentials shown in traces 5–7 occurred within a spontaneous cluster of irregularly spaced action potentials that lasted ~1.75 s. The first and second arrows in trace 5 indicate inflections, which suggest that this cluster of activity was initiated by at least two large mEPPs recorded from a distant endplate. The third downward arrow in this trace indicates continuous membrane depolarization consistent with spontaneous and prolonged synaptic activity. The RP was -53 ± 4.6 mV (mean $\pm$ SD, $n = 5$ fibers). **(B)** Recordings from *AChRγ*^{-/-} fibers at P0 revealed mEPPs that were subthreshold for inducing spontaneous action potential activity in individual triceps and diaphragm muscle fibers. The second to fifth traces were recorded from a fiber in the diaphragm, while the mEPP in the first trace was recorded from another diaphragm fiber. The mEPPs in the sixth and seventh traces were recorded from two different fibers in the triceps muscle. The RP was -45 ± 4.2 mV (mean $\pm$ SD, $n = 7$ fibers). **(C)** An isolated action potential that was induced by a large (6–8 mV) spontaneous mEPP in another normal triceps muscle fiber at P0. Calibrations: (A) and (B) 2 mV, 100 ms; (C) 2 mV, 5 ms.

saline solution (125 mM NaCl, 5 mM KCl, 24 mM NaHCO₃, 2 mM CaCl₂, 1 mM NaH₂PO₄, 11 mM glucose pH 7.3; $T = 23$ – 25°C), continually bubbled with 95% O₂ and 5% CO₂. To obtain recordings from the triceps muscle, the entire forelimb and scapula were removed and pinned to a Sylgard base. Intracellular recordings were obtained in the usual way from the synaptic region of visually identified fibers (Carlson, 1992) and were identified by resting potential measurements while the electrode was advanced in order to sample spontaneous quantal activity. Because of fibrillatory activity after the muscle preparation, it was necessary to incubate 1–2 h until mEPP recordings started, a procedure that which would not affect the mEPP

Table III. Primer combinations used for PCR to make probes

Subunit	Forward	Reverse
β	5'-AAC TCC CTG AAC CAC ATC AC	5'-GCA AAT GGT ACG TGG CAT CT
γ (pCRγ)	5'-GAA TGG CTC TTC CTC AGG GT	5'-ATT GGT TGG CCT CAG TCT GG
δ	5'-CGC AGT GAC CTC ATG TTT GA	5'-CTT GTC CTG TTC GCT ATA GG
ε	5'-CTG CTG GGC AGG TAT CTT AT	5'-GGT AAG GGA GAT CAG GAA CT

activity (Purves and Sakmann, 1974). Subsequently, fibrillatory activity was seen in some fibers within the first few minutes after impalement. Only fibers in which impalements could be maintained beyond this period were used for measuring mEPPs. The data were stored on a PCM recorder (Vetter Model 200T modified Toshiba recorder) and examined using PCLAMP software (v 4.0) and a Labmaster A-D convertor. Records were presented using Sigmaplot v 5. The decay time constant (τ) was determined by negative exponential curve fits ($V = A e^{-t/\tau}$; V , voltage; t , time; A , constant) to individual mEPP decay phases.

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REFERENCES

- Aigner, L., Arber, S., Kapfhammer, J.P., Laux, T., Schneider, C., Botteri, F., Brenner, H.R. and Caroni, P. (1995) Overexpression of the neural growth-associated protein GAP-43 induces nerve sprouting in the adult nervous system of transgenic mice. *Cell*, **83**, 269–278.
- Apel, E.D., Roberds, S.L., Campbell, K.P. and Merlie, J.P. (1995) Rapsyn may function as link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex. *Neuron*, **15**, 115–126.
- Blin, N. and Stafford, D.W. (1976) A general method for isolation of high molecular weight DNA from eukaryotes. *Nucleic Acids Res.*, **3**, 2303–2308.
- Bouzat, C., Bren, N. and Sine, S.M. (1994) Structural basis of the different gating kinetics of fetal and adult acetylcholine receptors. *Neuron*, **13**, 1395–1402.
- Brown, M.C. and Ironton, R. (1977) Motor neurone sprouting induced by prolonged tetrodotoxin block of nerve action potentials. *Nature*, **265**, 459–461.
- Carlson, C.G. (1992) Early postnatal changes in presynaptic potassium sensitivity. *Dev. Brain Res.*, **68**, 183–191.
- Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, **162**, 156–159.
- Cohen, M.W. (1972) The development of neuromuscular connexions in the presence of D-tubocurarine. *Brain Res.*, **41**, 457–463.
- Evans, S., Goldman, D., Heinemann, S. and Patrick, J. (1987) Muscle acetylcholine receptor biosynthesis. *J. Biol. Chem.*, **262**, 4911–4916.
- Fatt, P. and Katz, B. (1952) Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.*, **117**, 109–128.

- Gan, W.-B. and Lichtman, J.W. (1998) Synaptic segregation at the developing neuromuscular junction. *Science*, **282**, 1508–1511.
- Gautam, M., Noakes, P.G., Mudd, J., Nichol, M., Chu, G.C., Sanes, J.R. and Merlie, J.P. (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. *Nature*, **377**, 232–236.
- Gautam, M., Noakes, P.G., Moscoso, L., Rupp, F., Scheller, R.H., Merlie, J.P. and Sanes, J.R. (1996) Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. *Cell*, **85**, 525–535.
- Glass, D.J. *et al.* (1996) Agrin acts via a MuSK receptor complex. *Cell*, **85**, 513–523.
- Hashemolhosseini, S., Moore, C., Landmann, L., Sander, A., Schwarz, H., Witzemann, V., Sakmann, B. and Brenner, H.R. (2000) Electrical activity and postsynapse formation in adult muscle: γ -AChRs are not required. *Mol. Cell. Neurosci.*, **16**, 697–707.
- Honjo, T., Obata, M., Yamawaki-Kataoka, Y., Kataoka, T., Kawakami, T., Takahashi, N. and Mano, Y. (1979) Cloning and complete nucleotide sequence of mouse immunoglobulin γ 1 chain gene. *Cell*, **18**, 559–568.
- Jaramillo, F., Vicini, S. and Schuetze, S.M. (1988) Embryonic acetylcholine receptors guarantee spontaneous contractions in rat developing muscle. *Nature*, **335**, 66–68.
- Kidokoro, Y. and Saito, M. (1988) Early cross-striation formation in twitching *Xenopus* myocytes in culture. *Proc. Natl Acad. Sci. USA*, **85**, 1978–1982.
- Kurosaki, T., Fukuda, K., Konno, T., Mori, Y., Tanaka, K., Mishina, M. and Numa, S. (1987) Functional properties of nicotinic acetylcholine receptor subunits expressed in various combinations. *FEBS Lett.*, **214**, 253–258.
- Liu, Y. and Brehm, P. (1993) Expression of subunit-omitted mouse nicotinic acetylcholine receptors in *Xenopus Laevis* oocytes. *J. Physiol.*, **470**, 349–363.
- Martinou, J.C. and Merlie, J.P. (1991) Nerve-dependent modulation of acetylcholine receptor ϵ -subunit gene expression. *J. Neurosci.*, **11**, 1291–1299.
- McMaster, G.K. and Carmichael, G.G. (1977) Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl Acad. Sci. USA*, **74**, 4835–4838.
- Melton, D.A. (1985) Injected anti-sense RNAs specifically block messenger RNA translation *in vivo*. *Proc. Natl Acad. Sci. USA*, **82**, 144–148.
- Mishina, M., Takai, T., Imoto, K., Noda, M., Takahashi, T., Numa, S., Methfessel, C. and Sakmann, B. (1986) Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature*, **321**, 406–411.
- Missias, A.C., Mudd, J., Cunningham, J.M., Steinbach, J.H., Merlie, J.P. and Sanes, J.R. (1997) Deficient development and maintenance of postsynaptic specializations in mutant mice lacking an ‘adult’ acetylcholine receptor subunit. *Development*, **124**, 5075–5086.
- Mizoguchi, A., Yano, Y., Hamaguchi, H., Yanagida, H., Ide, C., Zahraoui, A., Shirataki, H., Sasaki, T. and Takai, Y. (1994) Localization of Rabphilin-3A on the synaptic vesicle. *Biochem. Biophys. Res. Commun.*, **202**, 1235–1243.
- Muniak, C.G., Kriebel, M.E. and Carlson, C.G. (1982) Changes in MEPP and EPP amplitude distributions in the mouse diaphragm during synapse formation and degeneration. *Dev. Brain Res.*, **5**, 123–138.
- Murray, M.G., Pitas, J.W., DeMars, S.J. and Cramer, J.H. (1992) Long-lived Southern blots using RNA probes. *Plant Mol. Biol. Reporter*, **10**, 173–177.
- Numa, S. (1986) Evolution of ionic channels. *Chemica Scripta*, **26B**, 173–178.
- Purves, D. and Sakmann, B. (1974) Membrane properties underlying spontaneous activity of denervated muscle fibres. *J. Physiol.*, **239**, 125–153.
- Sakmann, B. and Brenner, H.R. (1978) Change in synaptic channel gating during neuromuscular development. *Nature*, **276**, 401–402.
- Sanes, J.R. and Lichtman, J.W. (1999) Development of the vertebrate neuromuscular junction. *Annu. Rev. Neurosci.*, **22**, 389–442.
- Sanes, J.R., Marshall, L.M. and McMahan, U.J. (1978) Reinnervation of muscle fiber basal lamina after removal of myofibers. Differentiation of regenerating axons at original synaptic sites. *J. Cell Biol.*, **78**, 176–198.
- Shibahara, S., Kubo, T., Perski, H.J., Takahashi, H., Noda, M. and Numa, S. (1985) Cloning and sequence analysis of human genomic DNA encoding γ subunit precursor of muscle acetylcholine receptor. *Eur. J. Biochem.*, **146**, 15–22.
- Witzemann, V., Brenner, H.R. and Sakmann, B. (1991) Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. *J. Cell Biol.*, **114**, 125–141.
- Witzemann, V., Schwarz, H., Koenen, M., Berberich, C., Villarroel, A., Wernig, A., Brenner, H.R. and Sakmann, B. (1996) Acetylcholine receptor ϵ -subunit deletion causes muscle weakness and atrophy in juvenile and adult mice. *Proc. Natl Acad. Sci. USA*, **93**, 13286–13291.

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