Development 124, 5075-5086 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV9549

Deficient development and maintenance of postsynaptic specializations in mutant mice lacking an 'adult' acetylcholine receptor subunit

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SUMMARY

At many synapses, 'fetal' neurotransmitter receptor subunits are replaced by 'adult' subunits as development proceeds. To assess the significance of such transitions, we deleted the gene encoding the adult acetylcholine receptor (AChR) ε subunit, which replaces its fetal counterpart, the γ subunit, at the skeletal neuromuscular junction during early postnatal life. Several aspects of postnatal maturation, including synapse elimination, proceeded normally in the absence of the adult AChR, but structural development of the endplate was compromised. Later, inadequate compensation by the γ subunit led to severely reduced AChR density in mutant endplates relative to controls. This decreased density led to a profound reorganization of AChR-associated components of the postsynaptic membrane and cytoskeleton. Together, these results suggest novel roles for AChRs in assembly of the postsynaptic apparatus.

Key words: acetylcholine receptor, neuromuscular junction, synapse formation, mouse

INTRODUCTION

At synapses throughout the nervous system, neurotransmitter receptors are concentrated in the postsynaptic membrane. Many of these receptors are hetero-oligomers drawn from large multigene families (Ortells and Lunt, 1995). In several cases, the subunit composition of such receptors has been shown to change as development proceeds, with 'fetal' subunits being replaced by their 'adult' counterparts. At glycinergic synapses in the rat spinal cord, for example, replacement of α2 homomultimers by α1 homomultimers appears to account for a shortening of inhibitory postsynaptic current decays during development (Takahashi et al., 1992; Rajendra et al., 1997). Similar transitions occur in gamma-aminobutyric acid type A ($GABA_A$) receptors (Brooks-Kayal and Pritchett, 1993), in the N-methyl-D-aspartate (NMDA) subclass of glutamate receptors (Sheng et al., 1994), and in neuronal nicotinic acetylcholine receptors (AChRs; McGehee and Role, 1995). Thus it seems likely that changes in subunit composition are important for the structural or functional maturation of synapses.

Like many aspects of synaptic development, receptor subunit transitions have been best characterized at the skeletal neuromuscular junction. Initially, electrophysiological studies revealed developmental changes in the biophysical properties of the acetylcholine-gated channels at motor endplates: the channel mean open times decrease from approx. 5 mseconds to approx. 1.5 mseconds during the first two postnatal weeks while the mean channel conductance increases by approx. 50% (Fischbach and Schuetze; 1980 Siegelbaum et al., 1984; reviewed in Schuetze and Role, 1987). These changes were subsequently shown to arise from the substitution of an adult ε subunit for the fetal γ subunit in the AChR, leading to the replacement of $\alpha_2\beta\delta\gamma$ receptors by $\alpha_2\beta\delta\varepsilon$ receptors (Gu and Hall, 1988; Mishina et al., 1986). The basis of this transition appears to be transcriptional, as expression of the γ subunit gene is down-regulated, and the ε subunit gene is activated postnatally (Martinou and Merlie, 1991; Missias et al., 1996; Sanes et al., 1991; Witzemann et al., 1989). In addition to their different temporal regulation, the γ and ε subunit genes show differences in their spatial regulation and response to activity: the γ subunit gene is expressed by extrasynaptic nuclei in developing, paralyzed, and denervated muscles, whereas expression of the ε subunit gene is restricted to subsynaptic nuclei under all of these circumstances (Brenner et al., 1990; Goldman and Staple, 1989; Gundersen et al., 1993; Missias et al., 1996; Witzemann et al., 1987).

Because so much is known about the neuromuscular junction, it has been possible to formulate specific hypotheses about the functional significance of the AChR subunit switch. For example, the longer endplate currents produced by fetal receptors might permit newly formed, small synaptic contacts to evoke muscle contraction (Jarramillo et al., 1988). The transition to a shorter current might then be important in protecting the synaptic region from the harmful effects of excessive calcium influx through the AChR channel (Schuetze and Role, 1987; but see Villarroel and Sakmann, 1996). Additionally, the coincidence in timing of the AChR γ to ε subunit switch with numerous other postnatal steps in the maturation of the neuromuscular junction raises the possibility that these processes might be interrelated at the mechanistic level. For example,

molecular differences between the two subunits might allow them to recruit different proteins to the synaptic membrane; differences in calcium influx through the two channel types might affect structural maturation of the sole plate; or changes in activity might affect the competitive processes that lead to synapse elimination (Hall and Sanes, 1993).

To test these hypotheses, we generated AChR ε subunit-deficient mutant mice. During the early postnatal period, the mice had normal AChR numbers, but maintained γ subunit-containing receptors at their endplates beyond the usual time of transition. Examination of mice in this interval allowed us to determine which aspects of synaptic maturation were dependent on the appearance of adult AChRs. Later in life, AChR density fell dramatically at mutant endplates, which continued to grow without net addition of receptors. Thus, adult animals displayed a delayed-onset, progressive congenital myasthenia. Analysis of these adult mutants allowed us to assess the effects of decreased receptor density on other features of synaptic organization.

Recently, Witzmann et al. (1996) also generated ε subunit deficient mice, and reported that mutants retained γ subunit-containing receptors and exhibited severe muscle weakness. Our results are consistent with theirs, and extend them by documenting several of the molecular, developmental, and ultrastructural abnormalities that result from epsilon subunit deficiency and contribute to neuromuscular pathology.

MATERIALS AND METHODS

Generation of mutant mice

The targeting vector was constructed using an AChR ε subunit genomic clone, isolated from a mouse strain 129 genomic library (PCC4, Stratagene). The structure of this gene fragment was the same as that reported previously for another mouse strain (Buonanno et al., 1989). The long arm of homology consisted of a 7 kb *Bam*HI fragment that included exon 1. The short arm was an 829 bp *Hin*dIII to *Bam*HI fragment that contained exons 6-8. The two genomic arms were inserted into cloning sites situated on either side of the PGKneo cassette of vector pPNT (Tybulewicz et al., 1991); the completed targeting vector thus lacked the approx. 1.4 kb *Bam*HI to *Hin*dIII region spanning exons 2-5 (Fig. 1a). The placement of the neo cassette was such that splicing together of the remaining exons 1 and 6 around the insertion would produce a message out of frame for translation. Moreover, the N-terminal region of AChR subunits is required for proper subunit 'recognition' and assembly (Kreienkamp et al., 1995; Verrall and Hall, 1992; see also Kuhse et al., 1993). Thus, even if a C-terminal fragment of the ε subunit were generated, it should not appear at the muscle surface. The vector was transfected into R1 embryonic stem cells (Nagy et al., 1993), and homologous recombinants were selected with G418 and FIAU. Characterized recombinant ES cells were injected into mouse blastocysts to generate chimeras. Germline chimeric mice were then crossed with C57BL/6J mice to give rise to heterozygous and finally homozygous mutants.

Molecular analysis

Southern blot analysis was performed using liver genomic DNA made from homozygous mutant mice, heterozygous littermates and unrelated wild-type mice. The genomic DNA was digested with a combination of *Sac*I and *Cla*I, and probed with a fragment containing exon 8-10 sequences, which were external to the targeting vector.

RNAse protection assays were performed as described by Donoghue et al. (1991) using RNA isolated by the acid-phenol method of Chomczynski and Sacchi (1987; also see Martinou and Merlie, 1991); 70-100 μg of total RNA from pooled limb and trunk muscles was used per reaction. Antisense probes for the γ and ε subunits were derived from sequences lying between M3 and M4.

Immunocytochemistry

The molecular architecture of the synapse was assessed by an immunofluorescent method. For cross sections, muscles were frozen unfixed in liquid nitrogen-cooled isopentane and sectioned at 5-6 μm in a cryostat. For longitudinal sections, muscles were fixed for 20 minutes in phosphate-buffered saline (PBS) containing 1% paraformaldehyde, rinsed briefly, sunk in 25% sucrose/PBS, frozen, and cut at 8-40 μm. Sections were incubated with primary antibody at 4°C overnight, washed extensively, and then reincubated for 2-4 hours in a mixture of fluorescein-conjugated species-specific secondary antibody plus rhodamine-α-bungarotoxin (rBtx). For staining with anti-AChRγ, sections were fixed for 10 minutes with methanol at –20°C, and then treated with 50 mM ethylamine-HCl (pH 11) for 5 minutes at room temperature as recommended by Gu and Hall (1988), before incubation with primary antibody.

Sources of antibodies are as follows: AChR ε-subunit-specific antiserum was a gift from Dr Zach Hall (UCSF) and has been described previously (Gu and Hall, 1988). Anti-acetylcholinesterase and anti-synaptophysin were gifts from Drs T. Rosenberry (Case Western Reserve) and A. Czernik (Rockefeller University), respectively. Antibodies to the AChR γ subunit, rapsyn, agrin, and collagen α3 (IV) were generated in our laboratory (Noakes et al., 1993; Miner and Sanes, 1994; Gautam et al., 1996; Missias et al., 1996). Rabbit-anti-MuSK (muscle-specific kinase) was a generous gift of Dr G. Yancopoulus (Regeneron). Anti-erbB4 616 was a gift from Dr S. Burden (Zhu et al., 1995). Monoclonal antibodies to the AChR α (mab35) and β (mab 148) subunits were a kind gift from Dr John Lindstrom (Tzartos et al., 1981, 1986), and a monoclonal antibody to the AChR δ subunit (mab 88b) was provided by Dr Stanley Froehner (U. North Carolina; Froehner et al., 1983). Monoclonal antibodies to utrophin and β-dystroglycan were purchased from Novacastra (Newcastle upon Tyne, UK). Antiserum to neurofilaments and fluorescein-labeled VVA-B4 lectin were purchased from Sigma (St.Louis, MO). Antiserum to erbB3 was purchased from Santa Cruz (Santa Cruz, CA).

Electrophysiology

P17-18 mice were killed by cervical dislocation, and intact diaphragm muscles (with ribs) were dissected in HEPES-buffered saline solution (HBSS) containing 145 mM NaCl, 3.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes pH 7.3. Miniature endplate currents (mepcs) were recorded at room temperature with a focal extracellular pipette filled with HBSS (Fischbach and Schuetze, 1980; Katz and Miledi, 1973). Pipette resistances ranged from 1-3 MΩ. Signals were amplified (Axoclamp 2A, Axon Instruments), low pass filtered at 2-4 kHz, and digitized at 10 kHz. Up to 85 mepcs were analyzed from each recording site (average 29), and at least 4 sites were recorded from each animal. Mepcs were analyzed using the program 'MINI' (written in the Steinbach lab). The extracted mepcs were sorted into two size classes for each record. For each class the averaged mepc was calculated, by aligning the mepcs at the half-amplitude time for the rising phase. The falling phase of the mepcs was analyzed using the non-linear curve fitting program DELP (provided by D. J. Maconochie, University of Leicester). No differences were found between fits for the averages from small or large classes; because the larger events had a better signal to noise ratio, they were analyzed further. The falling phase of each mepc was fit with a single exponential, to estimate the time constant for decay. The averaged mepc for each record was also fit with the sum of two exponentials, to see if there was any indication of multiple components in the falling phase; mutant mepcs appeared by eye to be better fit by two exponentials, but there was no consistent pattern for the components.

Quantification of endplate AChR

Quantification of receptors was based on the method of Fumigalli et al. (1990). Mutant mice and age-matched controls were killed by an overdose of anesthetic (Nembutol), then sternomastoid muscles were dissected out and pinned in small dishes in HBSS. Muscles were rinsed with oxygenated HBSS, and then incubated in 50 nM ^{125}I-Btx (Amersham; obtained at >2000 Ci/mmol and diluted with cold Btx to 200-600 Ci/mmol) in oxygenated HBSS. Labeling was done for 2 hours, followed by extensive washing, and fixation with 4% paraformaldehyde plus 5% sucrose in PBS (pH 7) at 4°C, for 1-16 hours. Muscles were then washed and stained for AChE (Karnovsky, 1964). Subsequently, the endplate portion of the muscle was cut out, teased into bundles, and re-stained for better visualization of all endplates. Bundles of fibers containing 2-15 endplates were teased free from the larger muscle pieces, cut into endplate-containing and endplate-negative segments, and counted with a Beckman γ-counter. Values for endplate-free portions were subtracted from the corresponding values for endplate-positive portions, and the resulting specific counts were divided by the number of endplates in the sample. In most cases, counts from end-plate free portions were not significantly above machine background, and there was no significant difference between end-plate free values from mutants and controls.

Electron microscopy

For ultrastructural study, muscles were fixed in 4% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer, washed, fixed in 1% osmium tetroxide (OsO_4), dehydrated, and embedded in resin. Thin sections were stained with lead citrate and uranyl acetate. To quantify fold density, the total length of terminal contact was measured on photomicrographs, and the number of junctional folds that could be directly seen to open into the cleft was recorded. The height of the sole plate was measured perpendicular to the postsynaptic plasma membrane, as the shortest distance in each electron micrograph from the most superficial myofilament bundle or myonucleus to the primary synaptic cleft.

To label AChRs, animals were perfused with Ringers, followed by 2% paraformaldehyde plus 0.1% glutaraldehyde in PBS. Sternomastoid muscles were then dissected out and fixed for another 15-30 minutes. After washing, muscles were incubated in Biotin-Btx (Molecular Probes, Eugene, OR) for 4 hours at room temperature, followed by incubation with Avidin-HRP (Sigma) overnight at room temp. Following labelling, muscles were fixed in 4% glutaraldehyde for 1 hour, and then teased into small bundles. The bundles were incubated for 20 minutes in 2 mg/ml diaminobenzidine in 0.05 M Tris-HCl, 0.1 M imidazole, pH 7.4 (Straus, 1982). 0.01% hydrogen peroxide was then added and the incubation continued for 40 min. Fiber bundles were fixed with 1% OsO_4 and embedded in resin. Sections were examined without further staining.

RESULTS

An AChR ε subunit-deficient mutant

A targeting vector was constructed in which exons 2 to 5 of the AChR ε subunit gene, encoding the N-terminal 150 amino acids of the mature protein, were replaced by a neomycin resistance gene (Fig. 1a). The mutated allele was introduced into embryonic stem (ES) cells by homologous recombination, and germline chimeras were generated. Heterozygotes were outbred to C57 mice for 3 generations to ensure segregation of the mutant allele from other potential deficiencies in the ES cells. The resulting heterozygotes, which appeared normal in all respects, were bred to obtain homozygotes. Southern analysis of DNA from homozygous mutant offspring confirmed disruption of the gene (Fig. 1b). RNAse protection,

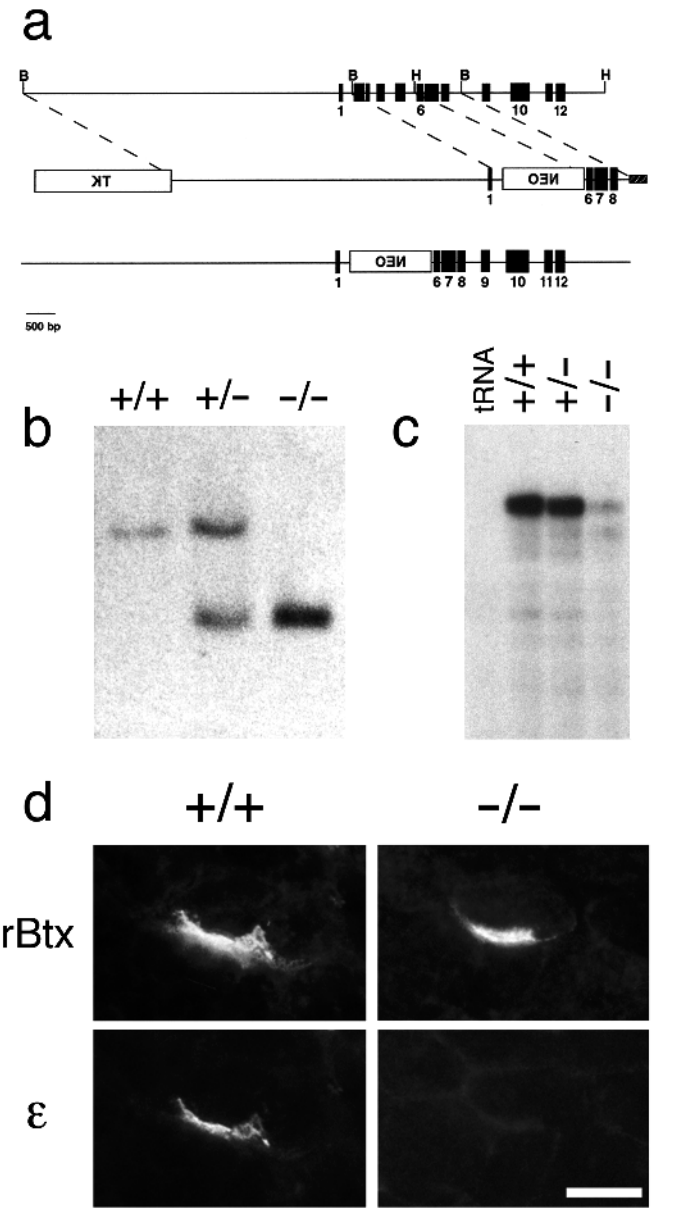

Fig. 1. Generation of ε subunit-deficient mice. (a) Structure of the AChR ε subunit gene (top), the targeting vector (middle), and the predicted product of homologous recombination (bottom). Black boxes indicate exons, and lettering reflects the direction of transcription of inserted genes. B and H indicate *Bam*HI and *Hin*dIII sites used in vector construction. (b) Southern blot of DNA from wild type (+/+), heterozygote (+/–), and homozygous mutant (–/–), showing a 4.7 kb wild-type band, and the 3.7 kb recombinant band. (c) RNAse protection of RNA from P21 mutant and control muscles, using an AChR ε subunit probe spanning exons 8-10. tRNA is included as a negative control. ε subunit mRNA levels are markedly reduced in homozygous mutants. (d) Sections of tibialis anterior muscle from P16 wild-type and mutant littermates, co-stained with rBtx to label all receptors (top panels), and an antibody to the AChR ε subunit (bottom panels). The ε subunit is absent in the mutant. Bar in d, 20 μm.

using an antisense probe located downstream of the disruption, indicated greatly reduced levels of AChR ε subunit message in mutant muscle when compared to wild-type or heterozygote

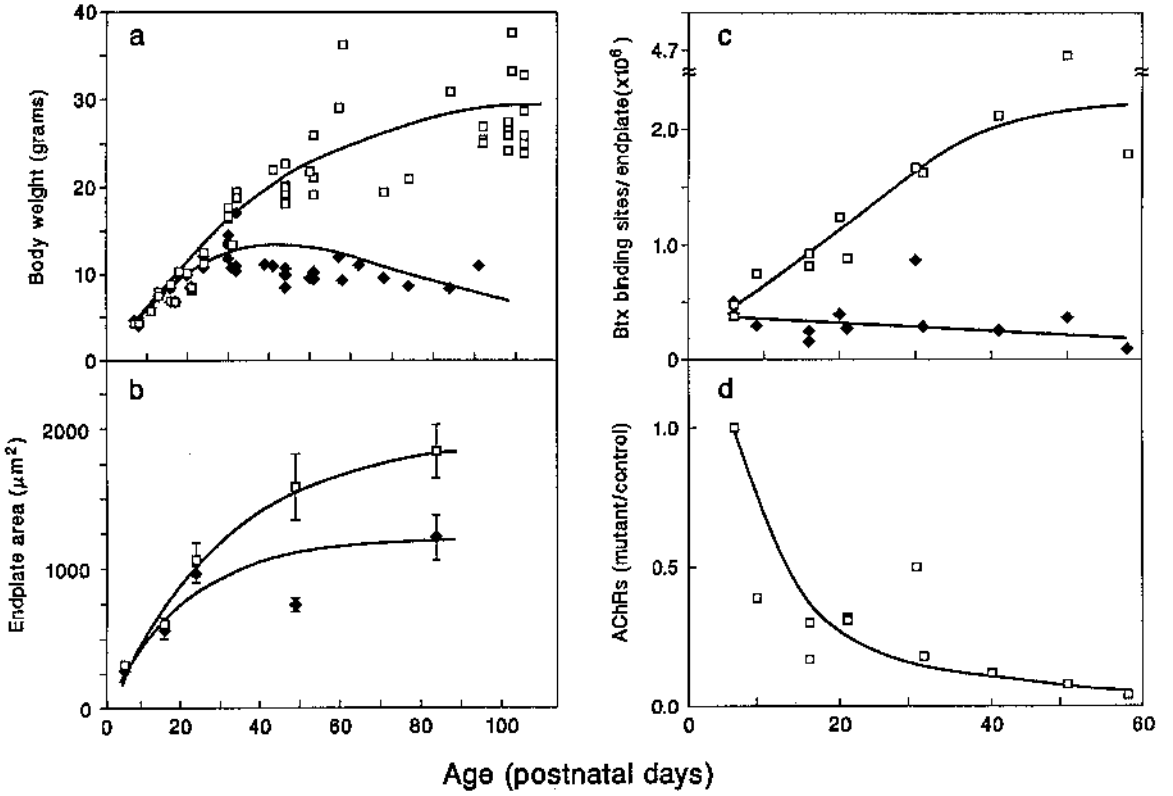

Fig. 2. Growth and AChR levels in normal and ε subunit-deficient mice. (a) Weights of ε mutant mice (filled symbols) and their littermates (+/– and +/+; open symbols). Control and mutant weights are indistinguishable for approx. 1 month postnatally, but mutant weights then plateau as controls continue to grow. (b) Endplate area in sternomastoid muscles of mutants and littermate controls. Major and minor diameters were measured on micrographs of longitudinal sections; the product of these two measurements served as an estimate of area. Each point represents measurements from a single muscle; error bars give s.e.m. Mutant and control endplates grow at similar rates for approx. 3 weeks. (c) Numbers of ^{125}I-Btx binding sites per endplate in control and mutant muscle. AChR numbers increase until approx. P40 in controls, but mutants show no increase after P7. (d) Ratio of AChR number at mutant and age-matched control endplates. By P50, mutant mice have <5% of wild-type receptor numbers.

muscle (Fig. 1c). To test for presence of the ε subunit protein, muscle cross-sections were double-labeled with rhodamine-α-bungarotoxin (rBtx), to mark the location of all AChRs, and with an ε subunit-specific antibody directed against epitopes C-terminal to the deleted sequences (Gu and Hall, 1988). As expected, the ε subunit was absent from neuromuscular junctions of homozygous mutants (Fig. 1d).

Phenotype of ε subunit-deficient mice

Homozygous mutant mice appeared externally normal for the first few weeks after birth, but at around 1 month of age they become noticeably weaker and less active than their littermates. The mutants also ceased to gain weight at this time, and eventually weighed less than half as much as age-matched controls (Fig. 2a). Starting at about 5 weeks of age, mutant muscle became thin and atrophic. However, analysis with a panel of histological stains used in the diagnosis of human muscle biopsies revealed no obvious deficits other than muscle atrophy (data not shown), indicating that ε-deficient muscles were free from gross pathology. Likewise, no signs of muscle necrosis or apoptosis were evident by light or electron microscopy. Some mutant mice died at 8 weeks of age, most died at 10-12 weeks, and none lived past 14 weeks.

Muscle weakness seemed likely to result from a decreased number and/or altered composition of AChRs. To measure the total number of AChRs at mutant endplates, we incubated live muscles with ^{125}I-Btx and quantified specific binding. In normal mice, the number of Btx binding sites per endplate increased approx. 5-fold between postnatal day P6 and P50 (Fig. 2c), roughly in proportion to the growth of the endplate itself (Fig. 2b). In the mutant, in contrast, end-plate area increased several-fold over the same period but the number of Btx binding sites did not increase at all (Fig. 2b,c). As a result, mutant endplate had significantly fewer AChRs than wild-type endplates by approx. P10 (Fig. 2d). The difference between mutants and controls increased steadily with age. By 8 weeks of age, when mutant mice were beginning to die, their endplates had only 5% of wild-type AChR numbers, distributed over 50-60% of the wild-type area. Thus, the density of receptors at mutant endplates must fall precipitously with increasing age.

Lack of the ε subunit causes retention of γ-containing AChR

In normal fast-twitch muscles, replacement of the AChR γ subunit by the ε subunit occurs largely during the first postnatal week, and the γ subunit is undetectable by 2 weeks of age (Gu and Hall, 1988; Missias et al., 1996). Because muscles of mutant mice showed no staining for the ε subunit, but did show localized reactivity for Btx (Fig. 1d), we asked whether AChRs

at mutant endplates contained the γ subunit. Staining with a γ subunit-specific antibody showed that levels of the γ subunit were similar in controls and mutants at P5 (Fig. 3a,b). By P13, no γ subunit was detectable in control muscles (Fig. 3c), but mutant muscles remained strongly γ-positive (Fig. 3d). Thereafter, the intensity of endplate staining by rBtx declined progressively, consistent with the ^{125}I-Btx binding data, and the intensity of staining for the γ subunit declined in parallel (Fig. 3f). Distinct AChR γ subunit reactivity was, however, still present at P74, the oldest age examined (data not shown). At all ages, mutant endplates were stained by monoclonal antibodies specific for the α, β, and δ subunits (data not shown), suggesting that AChRs were of the fetal ($\alpha_2\beta\delta\gamma$) composition.

To determine whether AChRs in ε subunit-deficient muscle were functionally of the fetal type, we examined their electrophysiological properties. Focal extracellular recordings were made from endplate sites in diaphragms of P17-18 mutants and littermates controls. As expected, mepcs were smaller in mutants than in controls; they also appeared to be less frequent, although this difference was not quantitated. Importantly, as shown in Fig. 3g,h, miniature endplate currents (mepcs) decayed significantly more slowly in mutant than control muscles. The falling phases of the mepcs could be described by single exponentials (Fig. 3g); currents in wild-type muscle decayed with a $t_{1/2}$ of 1-1.5 mseconds (τ approx. 1.8 mseconds), while mutant currents decayed with a $t_{1/2}$ of 4-5 mseconds (τ approx. 6.5 mseconds). The difference between wild-type and mutant distributions was highly significant ($P<0.001$, by a one-way nested classification ANOVA; Snedecor and Cochran, 1967), but differences within each group were not ($P>0.1$). These values are similar to those obtained previously from developing mice, in which mepcs recorded from muscles of early postnatal animals have time constants of approx. 6 mseconds while those recorded from animals older than P12 have time constants of approx. 1.5 mseconds (Fischbach and Schuetze, 1980), a difference attributed to the switch in subunit composition from γ-containing to ε-containing AChR (Kopta and Steinbach, 1994; Mishina et al., 1986). Thus, electrophysiological evidence supported and extended the conclusion that ε subunit-deficient mice maintain functional γ subunit-containing AChRs at their neuromuscular junctions.

The basis for the maintenance of γ-containing AChRs is unclear. RNAse protection analysis failed to detect γ subunit message in mutants (or controls) between P16 and P74, even though this assay readily detected γ message in both mutants and controls at P5, when levels have already declined several-fold from their peak (Martinou and Merlie, 1991). By comparison to standards, levels of γ-mRNA in P16 mutants were <5% those in 1-week denervated wild-type muscle. It may be, therefore, that the γ subunit staining seen in mutant muscle primarily reflected the retention of AChRs that had been in the membrane prior to down-regulation of the γ

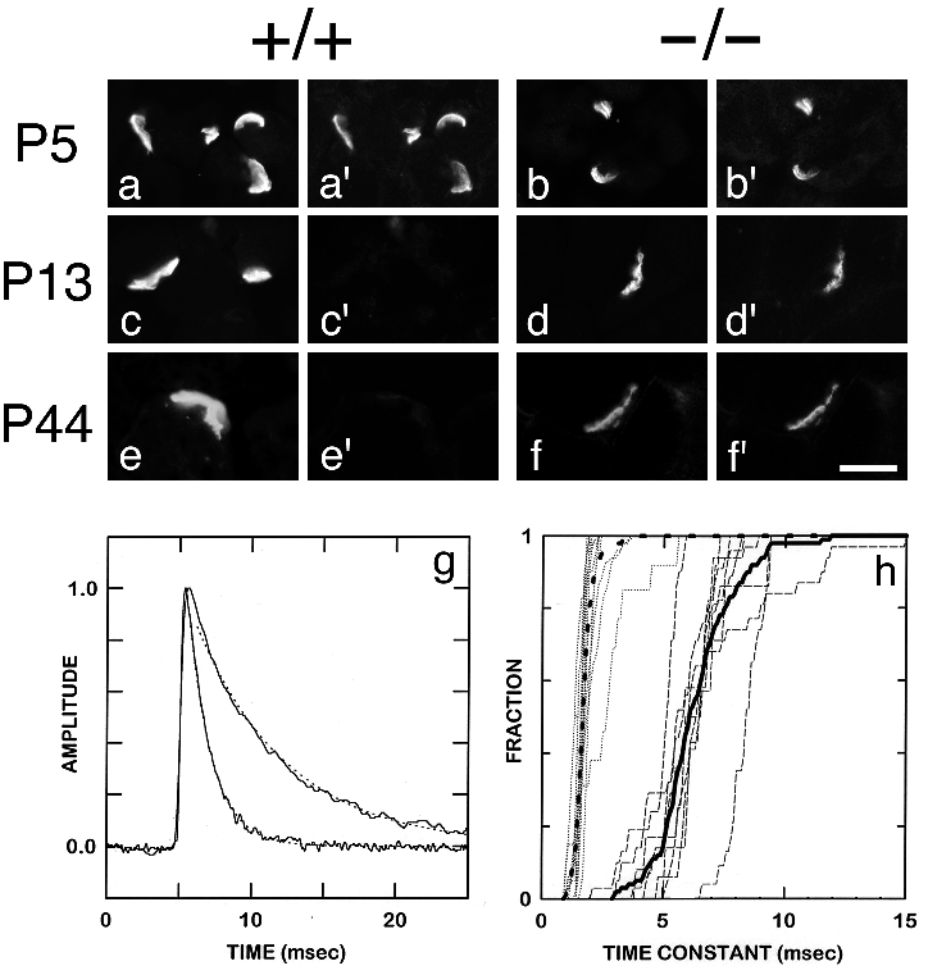

Fig. 3. Maintenance of γ subunit-containing AChRs at mutant junctions. (a-f) Cross sections of tibialis anterior muscle stained for receptors. Pairs of micrographs show the same fields stained with rBtx, which labels all AChRs (on left), and fluorescein-tagged antiserum specific for the γ subunit (on right). Both wild-type and mutant mice stain for the γ subunit at P5 (a,b). By P13, wild-type muscle no longer stains with γ subunit antiserum (c), while mutant muscle continues to show strong γ staining (d). This difference is maintained in adulthood (e,f). (g) Representative miniature endplate currents (mepcs) from P18 control and mutant diaphragm, normalized to the same amplitude. Each trace shows average of all mepcs recorded at a single site. The mutant currents (top trace) decay at the slow rate characteristic of γ-containing AChRs, while wild-type currents (bottom trace) decay at the fast rate characteristic of ε-containing AChRs. Dotted lines represent curve fits for each trace, using a single exponential component. (h) The cumulative distribution of time constants for mepcs at all control (dotted lines) and mutant (dashed lines) sites. Each line summarizes the distribution of time constants obtained from a single site: the ordinate shows the fraction of the total events at that site having a time constant less than or equal to the abscissal value. Median time constants from all control sites are faster than those from any mutant sites. Overall averages for the control and mutant data sets are represented by the heavy line in each cluster. Scale bar in f′ = 20 μm.

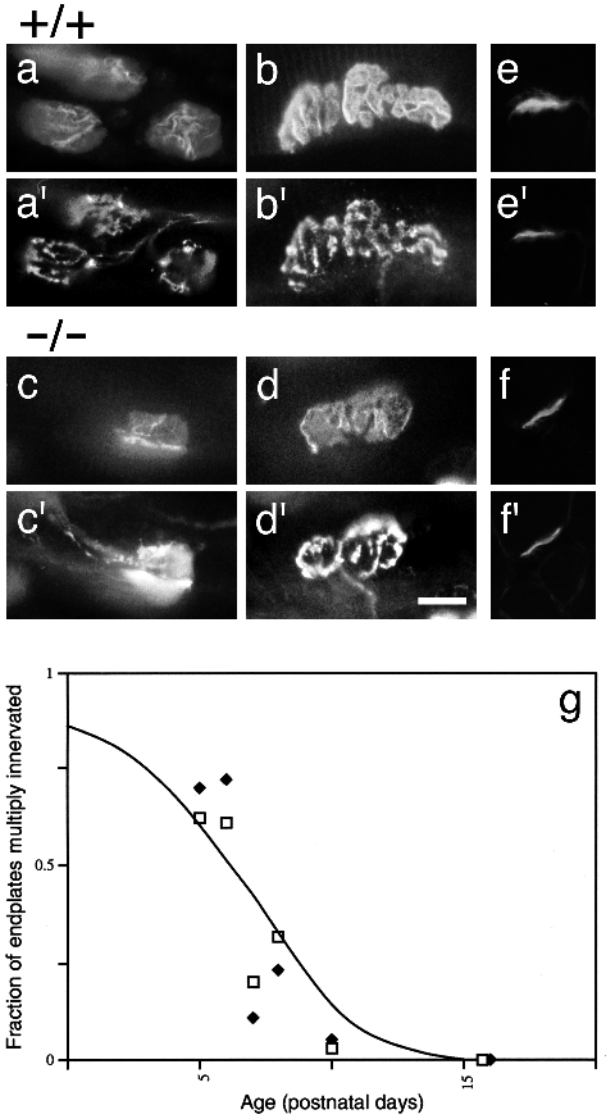

Fig. 4. Several aspects of postnatal synaptic maturation occur normally in ε mutants. (a-d) Thick longitudinal sections of sternomastoid taken between P5 and P16, stained with rBtx (upper panel of each pair), and fluorescein-tagged antibodies to neural antigens (lower panels). A similar distribution of simple (a,c) and more complex (b,d) morphologies are found in mutants and controls. (e,f) Cross sections from P13 tibialis anterior, stained with rBtx (upper panels) and the lectin VVA-B4 (lower panels), which binds to the sugar GalNAc. GalNAc-terminated glycoconjugates become localized to the synapse in both control (e) and mutant (f) muscles. (g) The degree of multiple innervation at control (open symbols) and mutant (filled symbols) endplates stained as in a-d. Each point represents data from a single sternomastoid muscle. The rate of synapse elimination was similar in mutants and controls. The line shows the average rate of synapse elimination in a larger sample of normal mice (redrawn from Balice-Gordon and Lichtman, 1993). Bar in d′, 10 μm for a-d; 02 μm for e,f.

subunit gene. Alternatively, the γ subunit gene may be expressed at low levels undetectable by RNAse protection assay. In this case, some ongoing AChR insertion might occur at mutant endplates. In this regard, it is noteworthy that Witzemann et al. (1996) did detect low levels of γ-mRNA in an independently generated ε-deficient mutant mouse. In any event, our results show that down regulation of the AChR γ subunit gene does not require expression of the ε subunit.

Consequences of altered AChR composition at developing neuromuscular junctions

Numerous aspects of synaptic maturation occur coincident with the AChR γ to ε subunit transition during the early postnatal period. These include elaboration of a branched geometry, elimination of multiple innervation, molecular maturation of the postsynaptic apparatus, and formation of an infolded, raised sole plate. During much of this period (until P7), the number of AChRs at mutant endplates was normal, even though their composition was not. We were therefore able to ask whether any of these postnatal developmental steps were affected by the absence of an AChR ε subunit.

The geometry of the endplate undergoes a striking change during postnatal development: simple plaque-like shapes evolve into more branched and elaborate morphologies (Steinbach, 1981; Slater, 1982; Balice-Gordon and Lichtman, 1993). We monitored this transition by co-staining longitudinal sections with rBtx to mark AChRs, plus fluorescein-tagged antibodies to antigens concentrated in axons (neurofilaments) and nerve terminals (synaptophysin; Gautam et al., 1995). The transition from plaques to more complex shapes occurred during the same period in both mutants and controls, and mature endplates of both genotypes appeared equally intricate (Fig. 4a-d). Thus, the elaboration of a complex, branched endplate does not require the usual AChR γ to ε subunit switch.

Synapse elimination begins about the time of birth and is completed by the end of the second postnatal week, at which time all muscle fibers are singly innervated (Thompson, 1985; Balice-Gordon and Lichtman, 1993). AChR-mediated activity and redistribution of AChRs have been hypothesized to trigger removal of overlying nerve terminals (Balice-Gordon and Lichtman, 1994). To ask whether AChR subunit composition affects the rate of synapse elimination, endplates were identified in sections doubly stained for receptors and neural antigens (Fig. 4 a-d), and scored for multiple innervation. At each age tested, levels of multiple innervation were similar in mutant and control muscles, falling from approx. 70% at P5 to <10% at P10 (Fig. 4g). Thus the timing of synapse elimination is not affected by the AChR subunit composition.

The AChR γ to ε switch is only one of several changes in the molecular architecture of the postsynaptic apparatus that take place in the early postnatal period. For example, collagen α3 (IV) appears in the basal lamina during the first postnatal week (J.H. Miner and J.R.S., unpublished), the receptor tyrosine kinase erbB3 becomes detectable in the synaptic membrane soon after birth (Moscoso et al., 1995), and N-acetylgalactosamine- (GalNAc-) terminated sugars recognized by the lectin VVA-B4 become restricted to the synapse during the second postnatal week (Scott et al., 1988). It seemed possible that the appearance of the ε subunit triggered some of these other developmental events. However, all three molecules were synaptically localized by P13 in mutants, as they were in controls (Fig. 4e,f and data not shown). Thus, the molecular maturation of the postsynaptic apparatus occurs in the absence of the AChR ε subunit.

Finally, we examined the ultrastructure of mutant endplates. At mature control synapses, nerve terminals are capped by

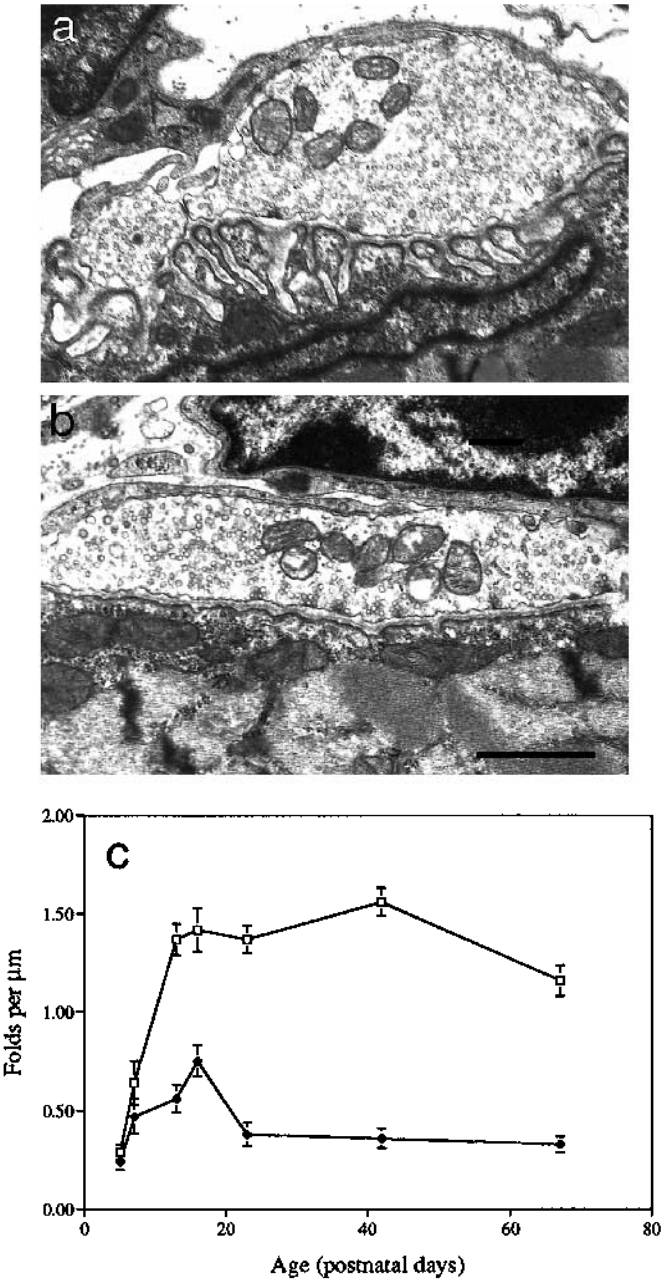

Fig. 5. Defective ultrastructure of the mutant postsynaptic apparatus. (a) A control (+/–) endplate at P13. The postsynaptic membrane is extensively infolded, and sarcomeres are separated from the folds by cytoplasmic organelles and a myonucleus. (b) A mutant endplate at P13. The postsynaptic membrane has few folds and sarcomeres extend nearly to the synaptic cleft. In both mutants and controls, nerve terminals are fully differentiated, bear active zones, and are capped by nerve terminals. (c) Numbers of junctional folds per µm of primary synaptic cleft. The density of folds in mutants (filled symbols) increases little after P7, and is lower than control values (open symbols) at all ages examined. Bar is 1 µm.

Schwann cell processes, and overlie a specialized postsynaptic apparatus marked by infolding of the plasma membrane, a raised, myofilament-poor sole plate, and aggregates of transcriptionally specialized myonuclei (Fig. 5a; Hall and Sanes, 1993). In mutants, the nerve terminal and Schwann cells appeared normal, and synaptic nuclei were normal in appearance and number (as assessed also by use of a nuclear dye; see Moscoso et al., 1995b). However, the postsynaptic apparatus was altered in two ways. First, the density of junctional folds underlying nerve terminals was markedly decreased (Fig. 5b). At P5, the earliest age examined, the density of folds seen at mutant endplates was slightly less than that in controls (Fig. 5c). During the subsequent week, the number of folds increased more than two-fold in heterozygotes, but changed little in mutants. This difference in fold accumulation did not merely represent a delay in postsynaptic maturation, as the lower density persisted into adulthood (Fig. 5c). Second, a distinct subsynaptic cytoplasmic compartment failed to form: there appeared to be little distance and few organelles intervening between the postsynaptic plasma membrane and the myofibrillar network in mutants (Fig. 5b). To quantify this difference, we measured the shortest distance from the primary synaptic cleft to the most superficial myofilament bundle (sarcomere) or myonucleus in the electron micrographs used to generate Fig. 5c. This distance was <0.3 µm in half (131/263) of the mutant endplates but in only 5% (11/240) of control endplates. Thus, different steps in the postnatal development of the postsynaptic apparatus display distinct requirements for the AChR ε subunit: several aspects of molecular and geometric maturation proceed normally in the absence of ε, whereas at least some aspects of structural maturation do not.

Consequences of decreased AChR number at mature neuromuscular junctions

As noted above, the number of AChR per endplate was lower in mutant than control muscle by P10, and the difference increased thereafter (Fig. 2c,d). To determine the distribution of the residual receptors, we stained longitudinal sections with rBtx. Mutant and control endplates were similar in morphology, although the former were smaller, probably as a result of muscle atrophy. As expected, mutant endplates were considerably dimmer than controls when viewed at low magnification (Fig. 6a,b). At higher magnification, however, it became apparent that the dim staining did not reflect a uniform decrease in AChR density. Instead, AChRs were concentrated in patches or islands, separated by relatively receptor-poor regions of membrane (Fig. 6c-f). This pattern first became evident around P24 (younger mutant endplates appearing qualitatively similar to controls; see, for example, Fig. 4d), and remained pronounced at all subsequent ages. Thus, as the endplate grew without net addition of AChRs, the residual receptors were redistributed into smaller regions of the postsynaptic membrane.

Despite the abnormal distribution of AChRs within mutant endplates, the geometry of nerve endings was remarkably normal. In mutants, as in controls, virtually all of the primary gutters were directly apposed by nerve terminal branches (Fig. 6c′ and d′). Nerve terminals in mutant animals did, however, differ from those in controls in two ways. First, there was some tendency toward more discrete boutons in mutant terminals, although this was not a consistent feature. Second, although all AChR-containing regions of the postsynaptic membrane were covered by nerve terminals, some thin axonal processes extended beyond the endplate region (not shown), forming 'terminal sprouts' as previously described (Brown et al., 1981). Such sprouts often form in paralyzed muscle, or following

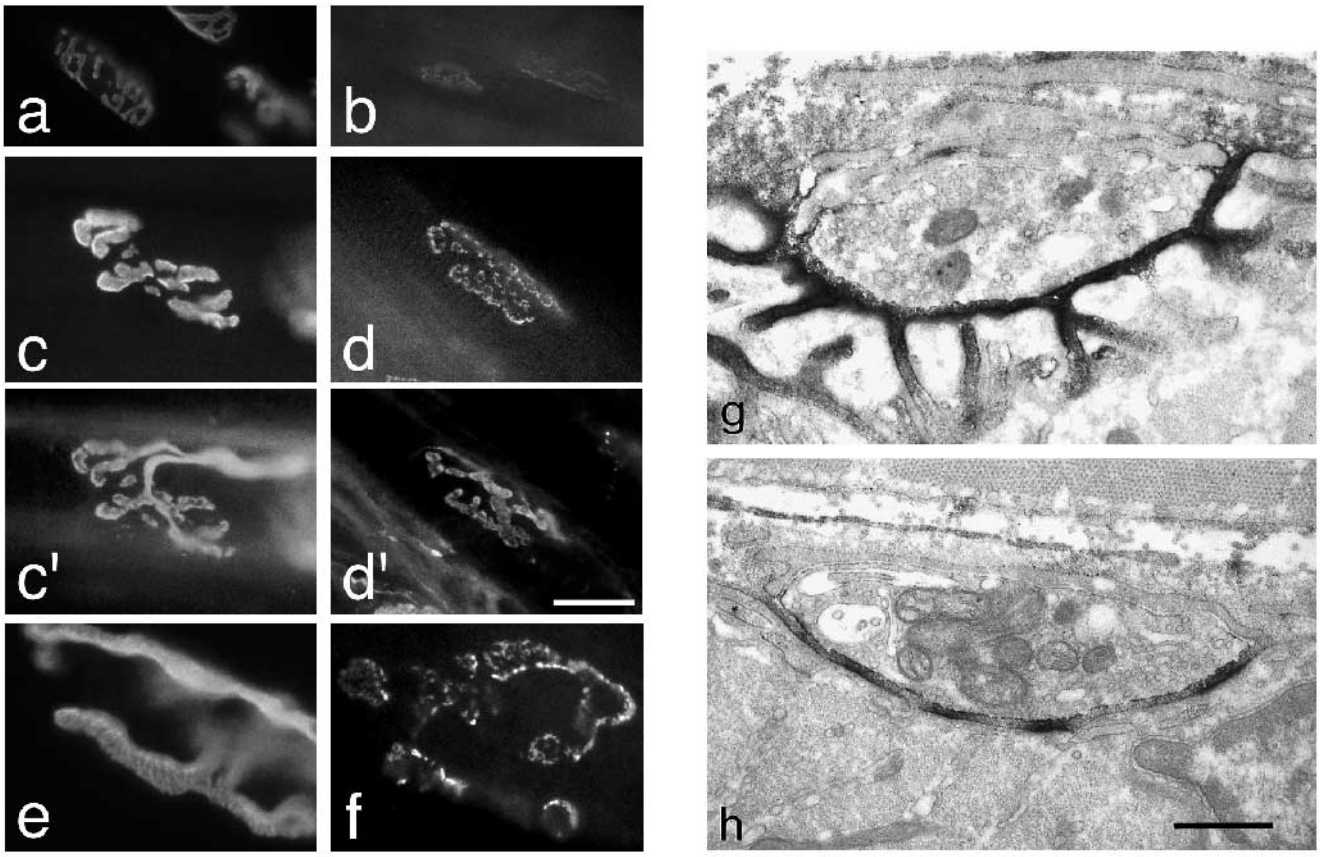

Fig. 6. AChR-rich endplates of ε mutants. (a-f) Longitudinal sections from control (left) and mutant (right) adult sternomastoid muscle, stained with rBtx (a-f) and fluorescein-tagged antibodies to neural antigens (c′,d′). At low magnification, mutant endplates (b) appear much dimmer than controls (a). At higher power, the decreased intensity can be seen to reflect fragmentation of the postsynaptic membrane into small AChR-rich and -poor patches (d,f). In contrast, staining of control endplates is relatively homogeneous (c,e). Faint striations in wild-type endplates represent junctional folds (e). Nerve terminals precisely overlie the endplate in mutants (d′) as well as controls (c′). (g,h) Electron micrographs of neuromuscular junctions stained with HRP-tagged Btx. At control endplates (g), AChRs are present throughout the primary cleft, and partway down the sides of junctional folds. At mutant endplates (h), alternating AChR-rich and AChR-poor regions of the postsynaptic membrane lie beneath a single terminal bouton. Bar in d′ 40 μm for a,b; 20 μm for c,d; 8 μm for e,f. Bar in h is 0.5 μm for g,h.

partial denervation, where innervated endplates extend processes to contact nearby unoccupied endplate sites. Thus, sprouting from nerve terminals of ε subunit mutants might represent either a direct response to an abnormal target surface, or an indirect effect of decreased muscle activity.

To assess the relationship of AChR-rich islands to other features of synaptic ultrastructure, we labeled muscles with horseradish peroxidase-(HRP-) tagged Btx for electron microscopy. This method confirmed that in control muscle AChR are present throughout the primary synaptic cleft and partway down the sides of the junctional folds (Fig. 6g). In contrast, mutant endplates showed discontinuous patches of AChRs in the postsynaptic membrane (Fig. 6h). No consistent correspondence was seen between these patches and either junctional folds or presynaptic active zones. Importantly, however, AChR-rich and AChR-poor areas were both present beneath single terminal boutons, and the synaptic cleft was no wider in AChR-poor than in AChR-rich areas. Thus the redistribution of AChRs neither reflects nor results in a decrease in the total synaptic contact area or a fragmentation of the synapse as a whole.

Localization of other synaptic molecules in fragmented endplates

Numerous studies have examined the involvement of various synaptic components, such as rapsyn, agrin and utrophin, in AChR aggregation (reviewed by Sanes, 1997). The decreased density of AChRs in the ε mutant allowed us to ask how alteration in AChR distribution affected other elements of the synaptic complex. Two staining patterns were observed. First, several synaptic molecules showed a patchy distribution within mutant endplates, closely matching that of AChRs. Proteins in this category were two cytoskeletal elements closely associated with AChRs, rapsyn and utrophin, and two receptor tyrosine kinases concentrated in the postsynaptic membrane, erbB4 and MuSK (Fig. 7b and data not shown). Antibodies to phosphotyrosine also stained fragments corresponding to receptor islands (data not shown). Second, some molecules were homogeneously distributed within mutant endplates, extending over both AChR-rich and AChR-poor patches. In this category were three components of the synaptic basal lamina: AChE, agrin and GalNAc-terminated glycoconjugates recognized by the lectin VVA-B4 (Fig. 7d and data not shown). These two

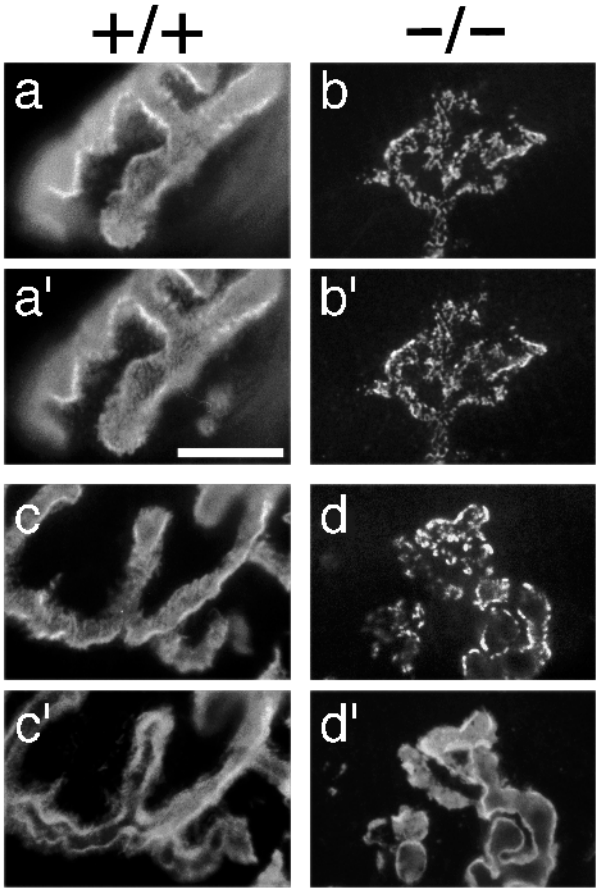

Fig. 7. Membrane and cytoskeletal antigens redistribute with AChR fragments, while basal lamina components do not. Longitudinal sections of control and mutant sternomastoid muscle, co-stained with rBtx (top in each pair) and fluorescein-tagged antibodies to a cytoskeletal protein, rapsyn (a′b′), or a basal lamina component, AChE (c′, d′). Both antigens are colocalized with AChRs in controls (a,c), although AChE often shows a 'railroad track' pattern around the nerve terminal. In mutants, rapsyn and AChRs are colocalized in the small AChR-rich regions (b), whereas AChE remains distributed throughout the synaptic cleft (d). Bar in a′, 10 μm.

patterns of staining seem to correspond with the subcellular localization of the molecules: transmembrane and cytoskeletal elements were redistributed with AChR, while components of the basal lamina remained synaptically localized but were not redistributed. Thus, the process of synaptic fragmentation appears to involve reorganization of many but not all synaptic molecules.

DISCUSSION

The mammalian neuromuscular junction forms in the embryo and is functional at birth. During early postnatal life, however, the composition of AChRs is altered, as the 'adult' ε subunit replaces the 'fetal' γ subunit (Gu and Hall, 1988; Mishina et al., 1986). These two subunit genes are homologous (52% sequence identity in the mouse), but the substitution of ε for γ transforms the channel properties of the AChR, decreasing mean open time and increasing channel conductance (Fischbach and Schuetze, 1980; Siegelbaum et al., 1984; Schuetze and Role, 1987). Similar transitions in receptor subunit composition and channel properties occur at developing synapses in many parts of the central nervous system (see Introduction). To explore the functional significance of such subunit switches, we disrupted the AChR γ-to-ε transition at the neuromuscular junction by deleting the gene for the ε subunit. Retention of γ subunit-containing AChRs transiently compensated for the lack of insertion of new adult-type receptors. The formation of junctional folds and the raised sole plate, which normally begins during this period, was impaired at mutant endplates, suggesting that structural maturation of the synapse is promoted by the γ- to ε-subunit switch. In contrast, several other molecular and morphological steps in the development of the neuromuscular junction occurred on schedule in mutants. Later, the density of AChRs in the postsynaptic membrane fell as mutant endplates continued to grow without net increase in receptors number. This decreased density led to a profound rearrangement of the endplate itself, with receptors and other synaptic components forming discontinuous patches within the synaptic gutter. Thus, normal AChR density is required for maintenance of postsynaptic integrity. Together, these results suggest that in addition to their best studied function in signaling, AChRs play structural roles in synaptic assembly and maintenance.

The transition from γ to ε subunit-containing AChRs occurs between P1 and P10 in most mouse muscles (Missias et al., 1996). In the ε mutant, γ-containing AChRs were retained in the junctional membrane long after their usual time of disappearance: AChRs levels were 100% of control values at P6, when only half of AChRs in normal mice contain the γ subunit, and 30% of control at P16, when no γ-containing AChRs are detectable in wild types. However, expression of the γ subunit gene was down-regulated on schedule in the mutant, resulting in little or no net increase in AChR number per endplate after P7. Thus, insertion of ε-containing AChRs may normally promote removal of γ-containing AChRs, but down-regulation of AChR γ subunit expression does not require accumulation of the ε subunit. Given the lack of substantial γ-AChR synthesis, the retention of γ-AChRs in the membrane is likely to reflect a process of metabolic stabilization. Normal fetal (γ-containing) AChRs turn over with a half-life of approx. 24 hours, while the half-life of ε-containing receptors in normal adult muscle is approx. 10 days (reviewed in Salpeter, 1987). The transition in receptor stability occurs around the time of birth (Reiness and Weinberg, 1981), and could correspond to the appearance of the ε subunit. In the absence of new AChR insertion, the residual γ-AChRs would have to turn over more slowly than normal adult receptors to account for the very slow decline in numbers seen at mutant endplates. In that these animals probably have low levels of γ gene expression, undetectable by RNAse protection (Witzemann et al., 1996), some γ-AChRs may be inserted locally at the synapse, or migrate from extrasynaptic to synaptic sites. Even in this case, however, it seems unlikely that γ subunit production is sufficient to offset AChR turnover at the fetal rate. Unfortunately, poor health and low receptor numbers have so far precluded direct measurement of receptor half-life in the ε mutants by established methods (Salpeter et al., 1988).

The normal AChR γ to ε subunit transition coincides with

several other postnatal events in neuromuscular maturation. These include the elaboration of a branched geometry, synapse elimination, localization of synaptic antigens, formation of junctional folds, and generation of a raised 'sole plate' with an expanded cytoplasmic compartment (Hall and Sanes, 1993; Zacks, 1973). It seemed possible that ε subunit-containing AChR might be important for some of these processes. For example, differences in protein sequences between the γ and ε subunits might cause them to interact with components of the cytoskeleton or extracellular matrix in different ways, altering recruitment of other synaptic antigens. Alternatively, differences in the postsynaptic currents generated by fetal and adult channels could affect the activity-dependent competition that leads to synapse elimination (reviewed by Colman and Lichtman, 1993). The ε subunit knock-out mice allowed us to test these hypotheses. In fact, the absence of an AChR subunit switch elicited no change in the acquisition of mature endplate geometry, the localization of synaptic antigens, or the rate of synapse elimination. However, mutant endplates did show a marked decrease in the density of junctional folds, and in the depth of the sole plate.

Several mechanisms might link absence of the ε and/or retention of the γ subunit to these ultrastructural changes. First, specific interactions between ε-subunit-containing AChRs and components of the cytoskeleton might promote infolding of the membrane or elaboration of a sole plate. Second, the greater calcium ion influx through the retained fetal-type receptors (Decker and Dani, 1990) might alter the distribution of structural elements. Precedent for this possibility comes from humans with mutant AChRs that generate abnormally long synaptic currents; these longer currents lead to excessive calcium influx and calcium-mediated necrotic alterations in the postsynaptic apparatus (Engel, 1994a; Engel et al., 1982; Ohno et al., 1995; see also Gomez et al., 1996). No such necrosis is apparent in the ε mutant, presumably because the γ-containing channels do not carry enough calcium, or because the decrease in AChR density offsets the increased single channel calcium flux. However, slightly increased calcium influx though γ-AChRs might compromise formation of the sole plate. Third, previous work has demonstrated that formation of the sole plate is impaired by paralysis (Brown et al., 1982; Duxson, 1982). It is therefore possible that decreased muscle activity plays a role. Because structural defects were profound before effects of inactivity (e.g., atrophy) were detectable, however, we believe that other factors predominate. Finally, even though minor structural defects are visible in mutants at P5, when AChR number is nearly normal, it is possible that the more substantial effects seen in the following week result from altered AChR number rather than from altered subunit composition.

Starting in the second postnatal week, the density of AChRs at mutant endplates fell relative to that of control animals. This decrease reflects a slowly declining number of receptors combined with near-normal growth in synaptic area. Surprisingly, however, AChR density did not fall uniformly. Instead, endplates underwent a profound reorganization into interspersed AChR-rich and AChR-poor areas. Other components of the synaptic cytoskeleton and membrane were redistributed along with receptors. Of special interest is rapsyn, which shows a close physical association with AChRs and is required for receptor clustering in vivo (Gautam et al., 1995). When expressed in non-mammalian cells, rapsyn forms clusters in the absence of AChRs; when rapsyn and AChR are co-expressed, they co-cluster (Froehner et al., 1990; Phillips et al., 1991). Based on these studies, one might expect the distribution of rapsyn to be maintained as AChR density falls. The fact that this does not happen, but that rapsyn is reorganized along with AChRs, implies that the AChR is not merely a ligand of other molecules that form the postsynaptic apparatus, but plays a structural role in synapse assembly. Likewise, the coordinated redistribution of other cytoskeletal (utrophin) and membrane (erbB4 and MuSK) proteins indicates that the multimolecular complex comprising the postsynaptic apparatus may assemble with a defined stoichiometry. Interestingly, Westerfield et al. (1990) showed that neuromuscular junctions can form in mutant zebrafish that apparently lack all AChRs; although these mutants are larvally lethal, it should be feasible to assess the distribution of other synaptic components at early stages of their development.

Finally, it is important to note that ε subunit-deficient mice developed a severe, delayed-onset congenital myasthenic syndrome (see Engel, 1994a for review). Mutants first began to show weakness at about 4 weeks of age, at which point they had approx. 20-30% of the receptor levels of wild-type mice (Fig. 2d). This finding corresponds well with previous estimates of the safety margin for neuromuscular transmission, which indicate that no effects on muscle contraction are detectable until 70% of AChR are inactivated (Lingle and Steinbach, 1988; Paton and Waud, 1967). Examination of human myasthenic patients has also yielded values of <30% of normal receptor numbers for symptomatic patients (Fambrough et al., 1973). It is perhaps surprising that the mutant mice remained viable as AChR numbers fell well below this level, to approx. 5% of control by two months of age. Further experiments will be required to determine whether increased transmitter release, and/or the reorganization of AChRs in the endplate, act to compensate for decreased levels of synaptic transmission.

Although, as noted above, a few humans have been found with congenital myasthenia due to AChR mutation, the more prevalent form, myasthenia gravis, results from autoimmune attack on AChRs, leading to a decline in their number and/or activity (Engel, 1994b). Congenital and autoimmune myasthenias differ fundamentally in pathogenesis, in that only the latter show immune-mediated disruption of the postsynaptic membrane. However, the clinical manifestations of the two forms are similar. Therefore, the ε subunit mutant mice described here may provide a good model for study of interventions designed to relieve myasthenic symptoms.

We thank Mia Nichol for skilled management of the animal facility; Michelle Elam and Barb Klocke for technical assistance; Andres Nagy for ES cells; J. Planar and A. Pestronk for diagnostic pathological analysis of muscle samples; and S. Burden, A. Czernik, S. Froehner, J. Lindstrom, T. Rosenberry, and G. Yancopoulos for gifts of antibodies. This work was supported by grants from M. D. A. and NIH to J. P. M. and J. R. S. J. P. M. died on May 27, 1995.

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(Accepted 28 September 1997)