Development 135, 1957-1967 (2008) doi:10.1242/dev.018119

Essential roles of the acetylcholine receptor γ-subunit in neuromuscular synaptic patterning

Yun Liu[1], Daniel Padgett[1], Masazumi Takahashi[2], Hongqiao Li[1], Ayaz Sayeed[3], Russell W. Teichert[4], Baldomero M. Olivera[4], Joseph J. McArdle[5], William N. Green[3] and Weichun Lin[1,*]

Formation of the vertebrate neuromuscular junction (NMJ) takes place in a stereotypic pattern in which nerves terminate at select sarcolemmal sites often localized to the central region of the muscle fibers. Several lines of evidence indicate that the muscle fibers may initiate postsynaptic differentiation independent of the ingrowing nerves. For example, nascent acetylcholine receptors (AChRs) are pre-patterned at select regions of the muscle during the initial stage of neuromuscular synaptogenesis. It is not clear how these pre-patterned AChR clusters are assembled, and to what extent they contribute to pre-and post-synaptic differentiation during development. Here, we show that genetic deletion of the AChR γ-subunit gene in mice leads to an absence of pre-patterned AChR clusters during initial stages of neuromuscular synaptogenesis. The absence of pre-patterned AChR clusters was associated with excessive nerve branching, increased motoneuron survival, as well as aberrant distribution of acetylcholinesterase (AChE) and rapsyn. However, clustering of muscle specific kinase (MuSK) proceeded normally in the γ-null muscles. AChR clusters emerged at later stages owing to the expression of the AChR epsilon-subunit, but these delayed AChR clusters were broadly distributed and appeared at lower level compared with the wild-type muscles. Interestingly, despite the abnormal pattern, synaptic vesicle proteins were progressively accumulated at individual nerve terminals, and neuromuscular synapses were ultimately established in γ-null muscles. These results demonstrate that the γ-subunit is required for the formation of pre-patterned AChR clusters, which in turn play an essential role in determining the subsequent pattern of neuromuscular synaptogenesis.

KEY WORDS: Neuromuscular junction, Nicotinic acetylcholine receptor, Synaptic patterning, Synaptogenesis

INTRODUCTION

During development, focal sites of pre- and postsynaptic cells undergo reciprocal interactions that ultimately lead to the establishment of functional synaptic connections. In developing mammalian muscles, neuromuscular synapses are generated within a narrow end-plate band that is often localized to the central region of the muscle fibers (Burden, 2002; Sanes and Lichtman, 2001). How is such a synaptic pattern established during development? Why does neuromuscular synaptogenesis take place at select sites of the muscle? It has long been thought that ingrowing nerves determine the site of synaptogenesis. For example, in vitro studies of nerve-muscle co-culture have demonstrated that innervation leads to accumulation of postsynaptic AChRs at sites of nerve-muscle contact, independent of muscle activity (Anderson et al., 1977), or the presence of pre-existing AChR clusters (Anderson and Cohen, 1977; Frank and Fischbach, 1979). Further studies have led to the discovery of agrin, a heparan sulfate proteoglycan, a potent nerve-derived factor that induces AChR clustering (McMahan, 1990; Nitkin et al., 1987; Rupp et al., 1991). Agrin activates the MuSK complex (Glass et al., 1996) and promotes neuromuscular synaptogenesis (reviewed by Bezakova and Ruegg, 2003; Bowe and Fallon, 1995). Genetic deletion of agrin (Gautam et al., 1996) or MuSK (DeChiara et al., 1996) leads to abnormal pre- and post-synaptic development at the NMJ. Agrin may also play a role in stabilizing AChRs by counteracting the dispersion effect of neurotransmitter ACh (Lin et al., 2005; Misgeld et al., 2005).

Several lines of evidence, however, challenge this long-held 'neurocentric view' and suggest that post-synaptic muscle cells independently prepare pre-patterned sites at the central region of the muscle (reviewed by Arber et al., 2002; Ferns and Carbonetto, 2001; Goda and Davis, 2003). Indeed, AChR clusters are detected in aneural muscles in vitro (Bekoff and Betz, 1976), or in vivo when motor innervation was prevented either by neurotoxin injection (Braithwaite and Harris, 1979) or neuroectomy (Creazzo and Sohal, 1983). However, these manipulations did not exclude the possibility that some muscle fibers were transiently contacted by motor axons, which could provide neural signals to induce postsynaptic differentiation.

Burden and his colleagues have provided the first genetic evidence for the presence of pre-patterned AChR clusters in developing muscles (Yang et al., 2000). Subsequent studies have confirmed this observation, and further demonstrated that the formation of pre-patterned AChR clusters is independent of agrin, but requires MuSK (Lin et al., 2001; Yang et al., 2001). Furthermore, ectopic expression of MuSK in muscles induces formation of ectopic synapses independently of agrin (Kim and Burden, 2008). In addition, studies of developing zebrafish NMJs in vivo elegantly demonstrate that pre-patterned AChR clusters are assembled prior to the arrival of the nerves; these AChRs are not only incorporated into the developing neuromuscular synapses but also map to the track of motor axonal growth (Flanagan-Steet et al., 2005; Panzer et al., 2005; Panzer et al., 2006). Together, these studies suggest that pre-patterned postsynaptic sites marked by nascent AChR clusters may serve as a primary determinant for subsequent innervation pattern. It is therefore essential to determine

[1]Department of Neuroscience, UT Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75235-9111, USA. [2]Centre National de Genotypage, 91057 Evry Cedex, France. [3]Department of Neurobiology, The University of Chicago, Chicago, IL, USA. [4]Department of Biology, University of Utah, Salt Lake City, UT, USA. [5]Department of Pharmacology and Physiology, UMDNJ-New Jersey Medical School, Newark, NJ, USA.

*Author for correspondence (e-mail: weichun.lin@utsouthwestern.edu)

Accepted 11 April 2008

how pre-patterned AChR clusters are initiated and to what extent these clusters contribute to pre- and postsynaptic differentiation of the NMJ.

AChRs of the embryonic vertebrate muscles are pentamers composed of five membrane-spanning subunits in a stoichiometry of two α-, one β-, one δ- and one γ-subunit (γ-AChR, $\alpha_2\beta\delta\gamma$). The γ-subunit distinguishes the embryonic from the adult AChR (ε-AChR, $\alpha_2\beta\delta\varepsilon$). Normally, the γ-subunit is expressed during embryonic and neonatal stages, and is replaced by the ε-subunit during the first 2 weeks after birth (γ/ε switch) (Changeux et al., 1992; Mishina et al., 1986). The physiological role of this switch is not well understood, but evidence suggests that the γ-subunit and the ε-subunit each play specific roles in embryonic and adult NMJs, respectively. Deletion of the ε-subunit gene in mice ($\varepsilon^{-/-}$ mice) leads to progressive neuromuscular weakness and death 2-3 months postnatally (Missias et al., 1997; Witzemann et al., 1996). Expressing human AChR γ-subunit in $\varepsilon^{-/-}$ mice rescues the lethality of the ε-subunit gene deletion, but the rescued mice continue to display AChR deficiency and develop phenotypes similar to human congenital myasthenic syndromes (Cossins et al., 2004). Furthermore, mutations in human AChR γ-subunit gene (*CHRNG* – Human Gene Nomenclature Database) cause severe prenatal myasthenia (Escobar or multiple pterygium syndrome) (Hoffmann et al., 2006; Morgan et al., 2006).

Deletion of the mouse γ-subunit ($\gamma^{-/-}$ mice) leads to perinatal lethality (Takahashi et al., 2002). Genetically replacing the γ-AChRs with ε-AChRs in mice (γ^{ε} chimeric mice) preserves normal end-plate formation but alters the patterning of the motor nerves at postnatal stages (Koenen et al., 2005). Although these results suggest that the γ-subunit is required for normal development and is essential to survival, the contribution of the γ-subunit to neuromuscular synaptogenesis is poorly understood. This lack of understanding arises, in part, from the focus of previous studies of $\gamma^{-/-}$, or γ^{ε} chimeric mice, which were limited to neonatal tissues (Koenen et al., 2005; Takahashi et al., 2002).

In the present study, we focused on the initial stages of neuromuscular synaptogenesis in $\gamma^{-/-}$ embryos. Specifically, we examined the timing and magnitude of AChR cluster formation and the patterning of motor nerves during development. Our results showed that the pre-patterned AChR clusters were completely absent during the initial stages of neuromuscular synaptogenesis in $\gamma^{-/-}$ embryos. Despite the absence of AChR clustering during these initial stages, MuSK, but not rapsyn, was clustered in $\gamma^{-/-}$ muscles. Furthermore, we observed profound changes in patterning of the motor innervation. That is, in contrast to the wild type in which presynaptic nerves innervate a narrow region of the muscle, presynaptic nerves in the $\gamma^{-/-}$ embryos were highly branched over a broad region of the muscle. In addition, the numbers of spinal motoneurons were markedly increased in the $\gamma^{-/-}$ embryos. Overall, these results demonstrate that the γ-subunit is required for the assembly of pre-patterned AChR clusters, which in turn play an essential role in determining the subsequent pattern of neuromuscular synaptogenesis.

MATERIALS AND METHODS

Animals

Takahashi et al. (Takahashi et al., 2002) have described the generation of mutant mice deficient in the gene encoding the AChR γ-subunit (*Chrng* – Mouse Genome Informatics); homozygote ($\gamma^{-/-}$) mice are perinatal lethal. We obtained heterozygote mice ($\gamma^{+/-}$) from the RIKEN BioResource Center (Tsukuba, Japan). To generate homozygous $\gamma^{-/-}$ embryos, heterozygotes were time-mated, and the day when a vaginal plug first appeared was designated as embryonic (E) day 0.5. After selected intervals of development, embryos were collected by Cesarean section of anesthetized pregnant mice. On average, 3-12 $\gamma^{-/-}$ embryos and a matching number of their wild-type littermate controls (+/+) were analyzed at each stage. All experimental protocols followed NIH Guidelines and were approved by the UT Southwestern Institutional Animal Care and Use Committee.

Immunocytochemistry

Muscle samples were fixed in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.3) at 4°C overnight prior to processing as wholemounts or frozen sections. The samples were blocked in dilution buffer (500 mM NaCl, 0.01 M phosphate buffer, 3% BSA and 0.01% thimerosal), and then incubated with primary antibody-neurofilament 150 (Chemicon, Temecula, CA), rapsyn (Affinity Bioreagents, Golden, CO), synaptophysin (Dako, Carpinteria, CA), synaptic vesicle protein 2 (SV2) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), MuSK (41101K) (Bowen et al., 1998), synaptotagmin 2 or syntaxin (gifts from Dr Thomas Sudhof, UT Southwestern Medical Center, Dallas, TX). Muscles were then incubated with fluorescein isothiocyanate-conjugated secondary antibodies and Texas Red-conjugated α-bungarotoxin (α-bgt) (10^{-8} M, Molecular Probes). Samples were then washed with PBS and mounted in 90/10 glycerol/PBS containing 1% N-propyl gallate. Fluorescent images were acquired using a Hamamatsu ORCA-285 camera or a Zeiss LSM 510 Meta confocal microscope. Quantification of fluorescence intensity and sizes of AChR clusters was made from confocal images acquired with identical, sub-saturating gains. The mean gray value (integrated density/total pixels), area, perimeter and Feret's diameter (the length of the greatest axis) were measured using NIH ImageJ.

AChE assay

Detection of AChE was based on the methods previously described (Enomoto et al., 1998). Briefly, diaphragm muscles were fixed with 2% PFA, rinsed in PBS and incubated in 0.2 mM ethopropazine, 4 mM acetylthiocholine iodine, 10 mM glycine, 2 mM cupric sulfate and 65 mM sodium acetate solution at pH 5.5, for 2-4 hours at 37°C. Staining for AChE was developed by incubating the whole-mount diaphragm for 2-5 minutes in sodium sulfide (1.25%, pH 6.0), followed by extensive washing. The diaphragms were then cleared with 50% glycerol in PBS and flat mounted onto a glass slide.

RT-PCR and quantitative real-time PCR

Total RNAs were isolated from de-skinned and eviscerated whole embryos at various stages using the TRI reagent (Molecular Research Center, Cincinnati, OH). The first-strand cDNA was synthesized by StrataScript reverse transcriptase (Stratagene, La Jolla, CA). The housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) served as an internal control. The following primers were used for PCR amplification of specific gene products: (1) AChR α-subunit primers, forward AAG CTA CTG TGA GAT CAT CGT CAC, reverse TGA CGA AGT GGT AGG TGA TGT CCA (product size: 244 bp) (Gattenlohner et al., 2002); (2) AChR ε-subunit primers, forward GGC AGT TTG GAG TGG CCT ACG ACA, reverse GCA GGA CGT TGA TAG AGA CCG TGC (product size: 489 bp) (Yumoto et al., 2005); GAPDH primers, forward TCA ACG GCA CAG TCA AGG CCG AGA, reverse ATG ACC TTG CCC ACA GCC TTG GCA GC (product size: 494 bp) (Cossins et al., 2004). Thirty-five cycles of PCR were performed for detection of the AChR ε-subunit expression.

Specific TaqMan probes were used for quantitative real-time PCR [Applied Biosystems (ABI), Foster City, CA]: AChR ε-subunit (assay ID, Mm00437411_m1) and phosphoglycerate kinase 1 (PGK1) (assay ID, Mm00435617_m1, for internal control), according to the ABI manuals. Briefly, total RNAs were isolated from forelimb muscles of three pairs of E18.5 wild-type and $\gamma^{-/-}$ embryos, and from wild-type adult muscles, which later served to calibrate the assay. The first-strand cDNA were synthesized using SuperScript III cDNA synthesis kit (Invitrogen Corporation, Carlsbad, CA). Each sample was assayed in triplicate reactions using TaqMan Universal PCR master mix. The amplification difference between AChR ε-subunit and PGK1 in each sample was calculated and then normalized to that of adult muscle using ΔΔCt (comparative Ct) method (Applied Biosystems, Foster City, CA).

Motoneuron counts

Quantification of motoneurons were carried out based on previously published methods (Buss et al., 2006; Clarke and Oppenheim, 1995). E18.5 embryos were fixed with 4% PFA, equilibrated with 30% sucrose, transversely sectioned (12 μm) and stained with Cresyl Violet. Motoneurons were identified by their characteristic size and shape, and their anatomical location in the ventral horn (Clarke and Oppenheim, 1995). Motoneurons in cervical segments (C3-C8) were counted, blind to genotype, in every ninth section, and total number of motoneurons within these segments was combined and then multiplied by 9 to generate total estimates of motoneurons (Clarke and Oppenheim, 1995).

Electrophysiology

Phrenic nerve-diaphragm muscle was dissected in normal rodent Ringer's solution (136.8 mM NaCl, 5 mM KCl, 12 mM $NaHCO_3$, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 11 mM d-glucose; pH 7.3) (Liley, 1956) and pinned to Sylgard coated dishes. End-plate potentials (EPPs) were evoked by supra threshold stimulation (2 V, 0.1 ms) of the nerve via a suction electrode connected to an extracellular stimulator (SD9, Grass-Telefactor, West Warwick, RI). To compare EPP amplitudes recorded from muscle fibers with different resting potentials, we normalized the EPP amplitude as described (Talbot et al., 2003), except we set the correction factor to 0.8 for mouse muscles (McLachlan and Martin, 1981), the reversal potential to 0 mV for the AChR gated conductance, and the common resting potential to –50 mV to reflect the low resting potentials frequently observed in embryonic muscles (Bennett and Pettigrew, 1974). EPPs that could not be separated from contaminating muscle action potentials were excluded from the quantification.

RESULTS

Absence of AChR clusters during initial stages of neuromuscular synaptogenesis in $\gamma^{-/-}$ embryos

To determine the role of AChR γ-subunit in neuromuscular synaptogenesis, we followed the $\gamma^{-/-}$ embryos during development from E13 to E18.5. These $\gamma^{-/-}$ embryos were invariably smaller than their wild-type littermates, and displayed characteristic hunchback and wrist drop phenotypes, similar to those observed in the choline acetyltransferase (ChAT)-null mutants (Brandon et al., 2003; Misgeld et al., 2002). These external phenotypes appeared as early as E15.5, although they became more apparent at later stages (E16.5-E18.5). Mutant embryos did not show any spontaneous motor activity, nor did they respond to a mild pinch of the tail or skin.

We first asked whether the γ-subunit is required for clustering of AChRs during the initial stages of neuromuscular synaptogenesis. To address this issue, we labeled whole-mount diaphragm muscles (E13-E15.5) with Texas Red-conjugated α-bgt. In wild-type diaphragm muscles, AChR clusters were first seen at E13 (Fig. 1A) – at this stage, they appeared as tiny and dim speckles concentrated along the central region of the muscle (arrowheads in Fig. 1A). As development proceeded, AChR clusters in wild-type muscles increased in size and fluorescence-labeling intensity (E14.5, Fig. 1B; E15.5, Fig. 1C). By contrast, AChR clusters were completely absent from the $\gamma^{-/-}$ diaphragm muscles; instead, diffused fluorescence was observed across the muscle surface (Fig. 1F-H), although the diffused fluorescence did appeared slightly more intense along the central regions, as illustrated by a line-scan analysis (see Fig. S1 in the supplementary material).

As different muscles vary in their onset of AChR clustering (Pun et al., 2002), we further examined a broad range of muscles that represent various segments of the anterior-posterior axis, including sternomastoid, triangularis sterni, intercostals, extensor digitorum longus (EDL) and soleus (Sol) muscles. Consistent with the diaphragm muscles, AChR clusters were absent from the other $\gamma^{-/-}$ muscles during E13-E15.5 (Fig. 1I,J and data not shown). These results demonstrated that the γ-subunit was required for the formation of pre-patterned AChR clusters.

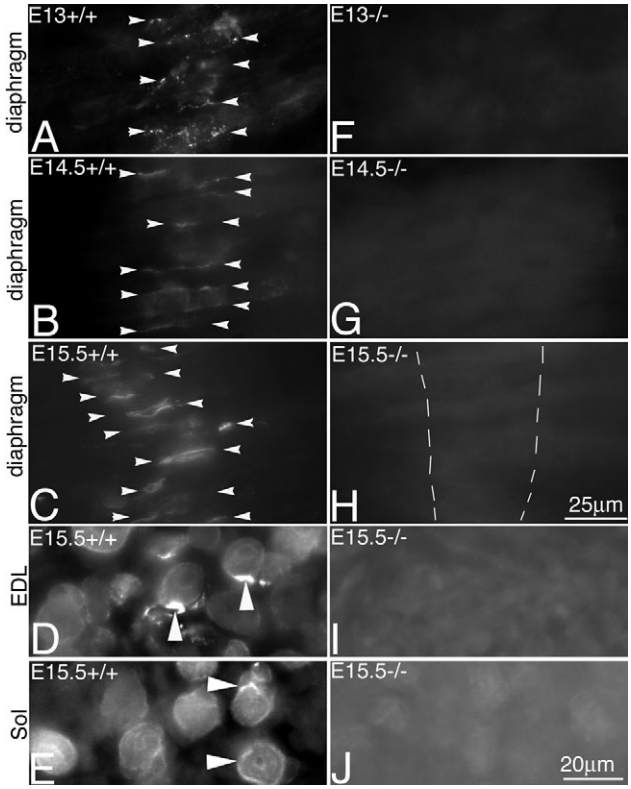

Fig. 1. Absence of AChR clusters during initial stages (E13-E15.5) of neuromuscular synaptogenesis in $\gamma^{-/-}$ embryos. Clustering of AChRs was detected by Texas Red-conjugated α-bungarotoxin. In whole-mount diaphragm muscles (**A-C**,**F-H**), AChR clusters were distributed along a central region of the muscle in the wild type (arrowheads in A-C), but were absent from the $\gamma^{-/-}$ muscles (F-H); instead, diffused fluorescence was observed on the surface of the muscles. Broken lines in H indicate the central region of the muscle. Similarly, AChR clusters were detected in EDL or soleus muscles in the wild-type (**D**,**E**), but not in the $\gamma^{-/-}$ muscles (**I**,**J**). Scale bars: 25 μm in A-C,F-H; 20 μm in D,E,I,J.

Delayed occurrence of AChR clusters in $\gamma^{-/-}$ muscles

Interestingly, AChR clusters appeared on the surface of $\gamma^{-/-}$ muscles at E16.5 (Fig. 2D), and remained throughout the subsequent stages (Fig. 2E,F). However, AChR clusters in $\gamma^{-/-}$ muscles were distributed across a much broader region, compared with those observed in wild-type muscles. For example, within the ventral quadrant of the diaphragm muscle, the average width of AChR cluster band in $\gamma^{-/-}$ was about twice that observed for wild types: 161.8±8.2 μm (n=3, number of embryos) in E16.5 $\gamma^{-/-}$ muscles, versus 77.7±15.6 μm (n=3) in E16.5 wild-type muscles; 152.6±21.2 μm (n=3) in E18.5 $\gamma^{-/-}$ muscles, versus 81.7±5.0 μm (n=3) in E18.5 wild-type muscles.

Furthermore, the individual AChR clusters in the $\gamma^{-/-}$ muscles were less intensely labeled by α-bgt and appeared larger in size than those in the wild-type muscles. For example, the mean gray value of the fluorescence intensity of individual AChR clusters in E18.5 $\gamma^{-/-}$ muscles was 68±8 (n=176 clusters, n=3 embryos), only about 55%

of the mean gray value measured in wild-type muscles (116±22, n=213, n=3). However, the average area, perimeter and Feret's diameter of AChR clusters in $\gamma^{-/-}$ muscles were 108±17 μm^2, 41±3 μm and 15±1 μm (n=176, n=3), respectively; by contrast, the same parameters measured in the wild-type muscles were 67±5 μm^2, 33±2 μm and 12±1 μm (n=213, n=3), respectively. In other words, the average area, perimeter and Feret's diameter of AChR clusters in $\gamma^{-/-}$ muscles were about 160±25%, 127±10% and 130±8%, respectively, of those observed in the wild-type muscles (Fig. 2G).

The delayed emergence of AChR clusters in the $\gamma^{-/-}$ embryos raises the following possibilities. First, in the absence of the γ-subunit, various combinations of the other AChR subunits, such as αβ, αβδ or $\alpha_2\beta\delta_2$, may assemble into functional receptors, although with reduced ligand-binding affinity (Kurosaki et al., 1987; Liu and Brehm, 1993; Sine and Claudio, 1991). Alternatively, the delayed appearance of AChR clusters may be due to expression of the AChR ε-subunit in embryonic muscles (Yumoto et al., 2005). Therefore, we performed RT-PCR to determine the expression of the ε-subunit. In parallel with ε-subunit expression, we also examined the expression of the AChR α-subunit and a 'housekeeping gene', glyceraldehyde phosphate dehydrogenase (GAPDH), as controls. Although the expression of the α-subunit and GAPDH was readily detectable in both wild-type and $\gamma^{-/-}$ muscles from E13.5 to E18.5, no ε-subunit expression was detected prior to E16.5. A low level of the ε-subunit expression first became detectable at E16.5 in both wild-type and $\gamma^{-/-}$ muscles (asterisk in Fig. 3), and the level was sharply increased at E18.5 in both wild-type and $\gamma^{-/-}$ muscles (** in Fig. 3). To further determine the level of ε-subunit expression, we carried out quantitative real-time PCR analysis. We detected a similar expression level of the ε-subunit gene in both wild-type and $\gamma^{-/-}$ muscles, which was ~3% of the expression observed for wild-type adult muscle (Fig. 3B). These results demonstrated that the emergence of AChR clusters at later stages in the $\gamma^{-/-}$ muscles was probably due to the expression of the ε-subunit.

To determine whether the ε-subunit was assembled into functional AChRs during embryonic stages, we measured EPPs in response to distinct toxins that specifically recognize the γ-AChRs [αA-conotoxin OIVB or αA-OIVB (Teichert et al., 2005)] and ε-AChRs [waglerin 1 (McArdle et al., 1999)]. As shown in Fig. 4, in normal Ringer's solution, the EPP amplitude in the $\gamma^{-/-}$ muscle were

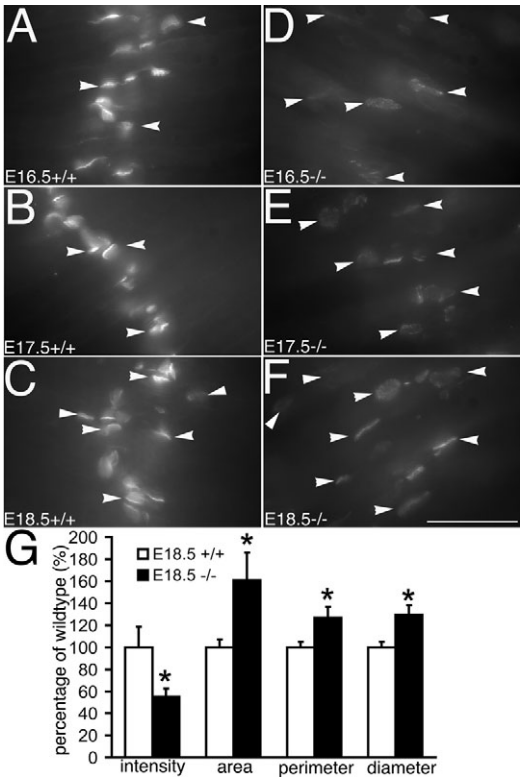

Fig. 2. Delayed occurrence of AChR clustering in the absence of the γ-subunit. (**A-F**) Whole-mount diaphragm muscles (E16.5-E18.5) labeled with Texas Red-conjugated α-bungarotoxin. AChR clusters were detected in the $\gamma^{-/-}$ muscles (arrowheads in D-F), although they were less numerous and appeared dimmer than those observed in the wild-type muscle (arrowheads in A-C). (**G**) Comparison of the fluorescence intensity (mean gray value), area, perimeter and Feret's diameter (the length of the greatest axis) of individual AChR clusters in the $\gamma^{-/-}$ muscles with those of the wild-type muscles. *P<0.05 (Student's t-test), Scale bar: 50 μm.

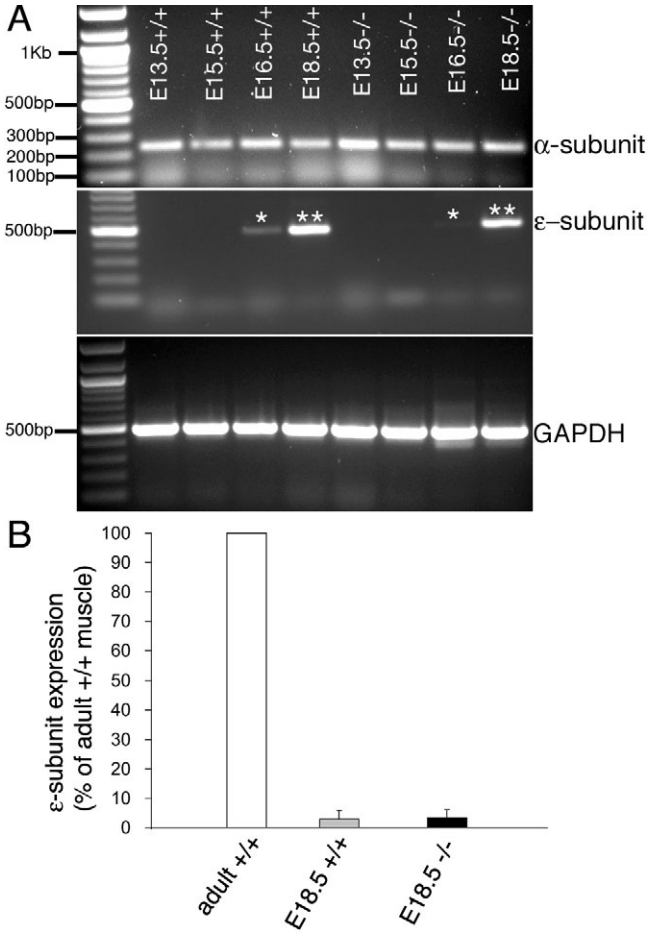

Fig. 3. Expression of the AChR ε-subunit in embryonic muscles. (**A**) RT-PCR analyses in wild-type and $\gamma^{-/-}$ embryos (E13.5, E15.5, E16.5 and E18.5). In both wild-type and $\gamma^{-/-}$ muscles, the expression of the α-subunit and GAPDH was readily detectable from E13.5 to E18.5, but the expression of ε-subunit was detectable only after E16.5 (*), and its level of expression was sharply increased at E18.5 (**). (**B**) Quantitative real-time PCR analysis using specific TaqMan probes for ε-subunit gene. The expression levels of ε-subunit gene in both wild-type and $\gamma^{-/-}$ muscles (E18.5) were similar (wild-type, 3.1±2.9%, n=3 embryos; $\gamma^{-/-}$, 3.4±2.7%, n=3 embryos), both at ~3% of level compared with the adult muscle (100%).

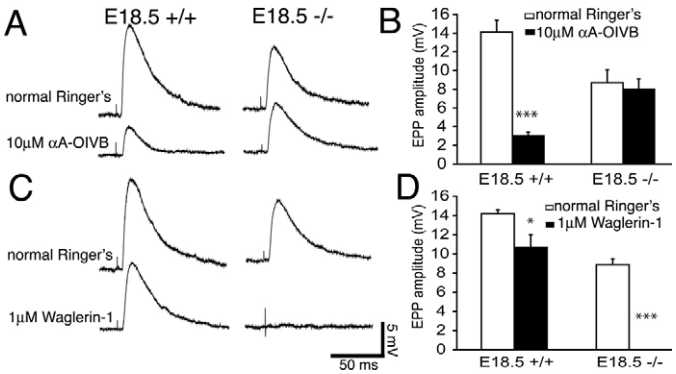

Fig. 4. Distinct pharmacological properties of EPPs recorded from wild-type and $\gamma^{-/-}$ muscles. (**A**) Sample EPP traces recorded from wild-type or $\gamma^{-/-}$ muscles in normal Ringer's, or in Ringer's with αA-conotoxin OIVB (10 μM). (**B**) Quantification of normalized EPP amplitudes. EPPs in wild-type muscles, but not $\gamma^{-/-}$ muscles, were sensitive to αA-conotoxin OIVB. (**C**) Sample EPP traces recorded from wild-type or $\gamma^{-/-}$ muscles in normal Ringer's, or in Ringer's with waglerin 1 (1 μM). (**D**) Quantification of normalized EPP amplitudes. Bath application of waglerin 1 (1 μM) in Ringer's completely blocked the EPPs in the $\gamma^{-/-}$ muscles, but was much less effective in the wild-type muscles. *P<0.05, ***P<0.001 (Student's t-test).

~37% smaller than that in the wild-type muscle; the average EPP amplitude was 14.2±0.8 mV (n=35, number of muscle fibers; n=8, number of embryos) in the wild-type and 8.9±0.5 mV (n=37, n=8) in the $\gamma^{-/-}$ muscles. The reduced EPP amplitude is consistent with the reduced fluorescence of the endplates labeled by α-bungarotoxin in the $\gamma^{-/-}$ muscles (Fig. 2). Application of the γ-AChR blocker αA-OIVB (10 μM) resulted in ~79% reduction of EPP amplitude in wild-type muscles: the average EPP amplitude declined to 3.0±0.4 mV (n=22, n=5) from the pre-toxin control value of 14.1±1.3 mV (n=16, n=5). By contrast, application of αA-OIVB had little effect on EPP amplitude in the $\gamma^{-/-}$ muscles; that is, mean EPP amplitude was 8.7±1.4 mV (n=17, n=4) and 8.0±1.1 mV (n=18, n=4) before and after treatment, respectively (Fig. 4A,B). Thus, $\gamma^{-/-}$ muscles were insensitive to the γ-AChR blocker.

By contrast, application of the ε-AChR blocker, waglerin 1 (1 μM), completely blocked the EPPs in the $\gamma^{-/-}$ muscles, but had much less effect on the wild-type muscles. That is, in the presence of waglerin 1 (1 μM), the average EPP amplitude was reduced to zero (n=24, n=5) in the $\gamma^{-/-}$ muscles, compared with the pre-waglerin 1 value of 8.9±0.6 mV (n=20, n=4) (P<0.001). Similar exposure of the wild-type muscle to waglerin 1 reduced the average EPP amplitude to 10.7±1.3 mV (n=13, n=3) from the pre-waglerin 1 value of 14.2±0.4 mV (n=19, n=3) (Fig. 4C,D). These electrophysiological results demonstrate that ε-subunits were assembled into functional AChRs in both wild-type and $\gamma^{-/-}$ muscles at E18.5 and that the postsynaptic response at the NMJ of the $\gamma^{-/-}$ muscles was mediated by ε-AChRs. By contrast, the postsynaptic response in E18.5 wild-type muscles was mediated by both the γ- and ε-AChRs, suggesting co-existence of both subtypes of AChRs at the same synaptic site at this stage (E18.5).

Aberrant pre-synaptic patterning in the absence of the γ-subunit

We next tested whether the development of presynaptic nerves was affected in $\gamma^{-/-}$ muscles. We used neurofilament or syntaxin antibodies to label pre-terminal nerves, and synaptophysin, synaptotagmin 2 or SV2 antibodies to label synaptic terminals. We found striking presynaptic defects in $\gamma^{-/-}$ muscles. As shown in Fig. 5, the nerves in the $\gamma^{-/-}$ embryos branched extensively and projected over a broad region of the muscle (Fig. 5B,D,F,H), whereas the nerves in wild-type embryos were nicely confined to the central region of the muscle (Fig. 5A,C,E,G). Increased nerve branching and extended projections in $\gamma^{-/-}$ embryos were also observed in limb muscles, for example, in tibialis anterior, EDL, medial and lateral gastrocnemius and Sol (see Fig. S2 in the supplementary material).

Interestingly, synaptic vesicle proteins, such as synaptophysin, synaptotagmin 2 or SV2, were seen accumulated in nerve terminals of both wild-type and $\gamma^{-/-}$ muscles. Preferential accumulation of synaptic vesicle proteins at nerve terminals was detectable as early as E15.5, when they appeared as numerous 'puncta', immunolabeled by either synaptophysin (Fig. 5E,F), synaptotagmin 2 (Fig. 5G,H, see also insets) or SV2 (data not shown). As development proceeded to subsequent stages (E16.5-E18.5), presynaptic nerve terminals became more intensely labeled by synaptic vesicle proteins (see Figs

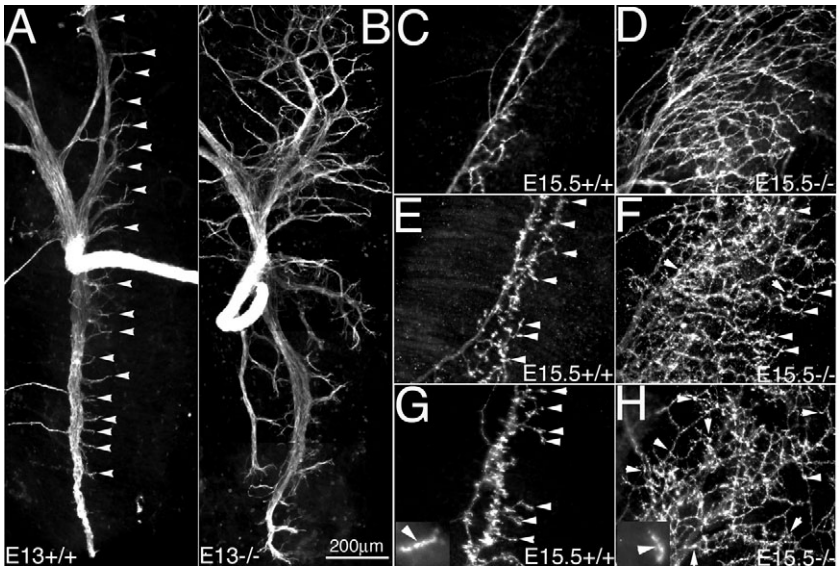

Fig. 5. Increased nerve branching and broadening of the innervation band in $\gamma^{-/-}$ muscles. Whole-mount diaphragm muscles were immunostained with antibodies against neurofilament (**A**,**B**), syntaxin (**C**,**D**), synaptophysin (**E**,**F**) or synaptotagmin 2 (Syt2) (**G**,**H**). (A,B) The hemi-diaphragm; (C-H) the dorsal quadrant diaphragm. In E13 wild-type muscle, collateral axons emanated from the nerve trunk in an orderly fashion and were mainly confined to the central region of the muscle (arrowheads in A); in E13 $\gamma^{-/-}$ muscle, noticeable increases of axons were observed and distributed across a broad region of the muscle (B). Similarly, at E15.5, intramuscular nerves were confined to the central region of wild-type muscle (C,E,G), but projected aberrantly to a broad region of the $\gamma^{-/-}$ muscles (D,F,H). Numerous puncta labeled by either synaptophysin or Syt2 antibodies at the nerve terminals are observed in both the wild-type and $\gamma^{-/-}$ muscles (arrowheads in E-H, also see inset in G,H). Scale bar: 200 μm.

S3 and S4 in the supplementary material, and Fig. 6). To determine the spatial relationship between AChR clusters and nerve terminals, we double-labeled diaphragm muscles with α-bgt and Syt2, and analyzed the number of AChR clusters and nerve terminals at both E16.5 and E18.5 stages. In E16.5 wild-type muscles, we identified 780 AChR clusters and 763 nerve terminals within the ventral quadrant diaphragm muscles from three embryos; there were slightly more AChR clusters than nerve terminals within the same region of the muscle. However, all nerve terminals were colocalized with AChR clusters: among 763 nerve terminals we observed, all of them were closely apposed by AChR clusters (see Fig. S3C, arrowheads in the supplementary material); a small fraction of AChR clusters remained aneural (around 2%, 17 out of 780 clusters were aneural) (see Fig. S3C, arrows in the supplementary material). In striking contrast, for E16.5 $\gamma^{-/-}$ muscles, there were more than twice as many nerve terminals as AChR clusters in the corresponding region. The majority of nerve terminals were not directly apposed by AChR clusters (supplementary material Fig. S3, arrows in F); among a total of 1183 nerve terminals examined, only 510 of them were apposed by AChR clusters (43%) (see Fig. S3F, arrowheads in the supplementary material). At E18.5, all nerve terminals in both wild-type and $\gamma^{-/-}$ muscles were directly apposed by AChR clusters (Fig. 6). We counted 267 neuromuscular synapses from the $\gamma^{-/-}$ and 505 neuromuscular synapses from the wild-type muscles; there was 100% co-localization of nerve terminals and AChR clusters in both genotypes.

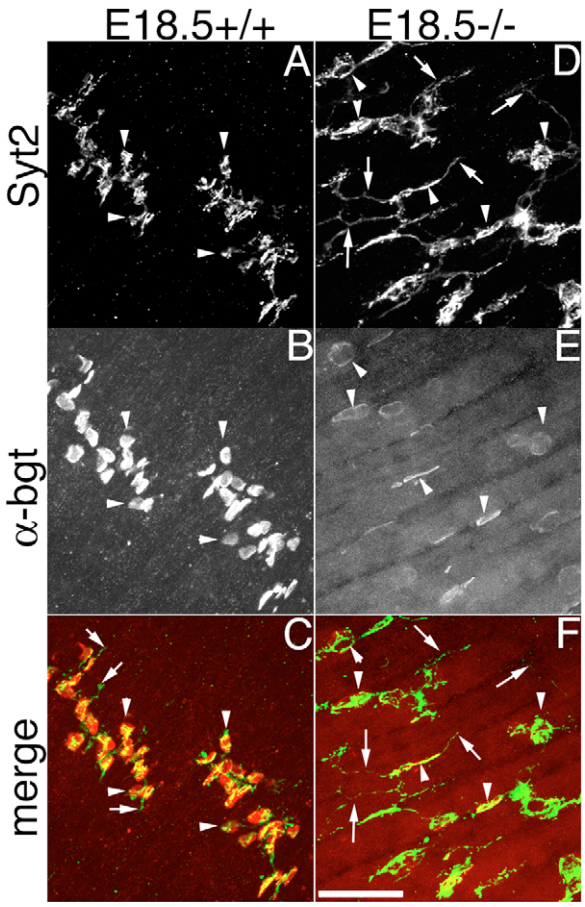

Fig. 6. Progressive accumulation of synaptic vesicle protein at the nerve terminals. Whole-mount diaphragm muscles (E18.5) were double-labeled with synaptotagmin 2 (Syt2) antibody (**A**,**D**) and Texas-Red-conjugated α-bgt (**B**,**E**). The merged images are shown in **C** and **F**. In both wild-type (A) and $\gamma^{-/-}$ muscles (D), individual nerve terminals (arrowheads in A,D) were heavily labeled by Syt2 antibody. AChR clusters are present in the end-plates of the $\gamma^{-/-}$ muscles (arrowheads in E), but they are less intensely labeled by α-bgt, compared with those observed in the wild-type muscles (arrowheads in B). Merged images show that nerve terminals are juxtaposed with AChR clusters in both $\gamma^{-/-}$ (arrowheads in F) and wild-type muscles (arrowheads in C); however, there were marked increases of nerve sprouting in the $\gamma^{-/-}$ muscles (arrows in D,F), compared with those in the wild-type muscle (arrows in C). Scale bar: 50 μm in A-F.

However, there were substantial differences in the developing presynaptic nerve terminals between the $\gamma^{-/-}$ and wild-type muscles. One of the striking differences was that the presynaptic nerves in the $\gamma^{-/-}$ muscles extended beyond the central region of the muscle so that numerous nerve sprouts projected towards the edge of the muscle (see Fig. S4B, arrows). By contrast, the nerves in the wild-type muscle were largely confined to the central region (see Fig. S4A, arrowheads in the supplementary material) and only a few nerve sprouts extended beyond the central region (see Fig. S4A, arrows in the supplementary material). The nerve sprouts in $\gamma^{-/-}$ muscles appeared to be extrasynaptic (Fig. 6F, arrows), as they extended beyond the synaptic sites (arrowheads in Fig. 6F). By contrast, in the wild-type muscle, nerve sprouting occurred to a much lesser extent (arrows in Fig. 6C). We quantified the difference between the wild-type and $\gamma^{-/-}$ muscles by measuring the width of AChR clusters and the width of innervation band within the ventral quadrant of the diaphragm muscle. The mean width of the innervation band in $\gamma^{-/-}$ muscles was 355.0±21.4 μm, more than twice that of the end-plate band (152.6±21.2 μm, n=3 embryos), whereas the mean width of the innervation band in the wild-type muscle was 84.6±8.5 μm, close to that of the end-plate band (81.7±5.0, n=3). Thus, both the innervation band and end-plate band were greatly expanded in the $\gamma^{-/-}$ muscles, compared with the wild-type muscles; however, the AChR clusters were always localized within the innervation band in both wild-type and $\gamma^{-/-}$ muscles.

Aberrant pattern of AChE distribution

AChE is a reliable marker for differentiated postsynaptic membrane (for a review, see Rotundo, 2003). We have previously observed that clustering of AChE occurs in aneural muscles independently of innervation, suggesting that AChE may also be prepatterned by mechanisms intrinsic to muscles (Lin et al., 2001). To determine whether AChE distribution depends on the presence of the pre-patterned AChR clusters, we carried out AChE staining in $\gamma^{-/-}$ muscles at E15.5, a stage when AChR clusters were absent (Fig. 1H). Our results showed that AChE clusters were present in $\gamma^{-/-}$ muscles (Fig. 7C), despite the total absence of AChR clustering this stage (Fig. 1H). However, AChE clusters were distributed over a broader surface of the $\gamma^{-/-}$ muscle (Fig. 7C) when compared with the wild-type muscle (Fig. 7A). The abnormally broad distribution of AChE clusters in the $\gamma^{-/-}$ muscles was also detected at E18.5 (Fig. 7D).

Clustering of MuSK in the absence of the γ-subunit

We next investigated mechanisms that might lead to the absence of pre-patterned AChRs in $\gamma^{-/-}$ muscles. The receptor tyrosine kinase MuSK is specifically expressed at postsynaptic sites (Glass et al., 1996; Jennings et al., 1993; Valenzuela et al., 1995). Therefore, we sought to determine whether the localization of MuSK was altered in $\gamma^{-/-}$ muscles. We examined whole-mount diaphragm muscles at

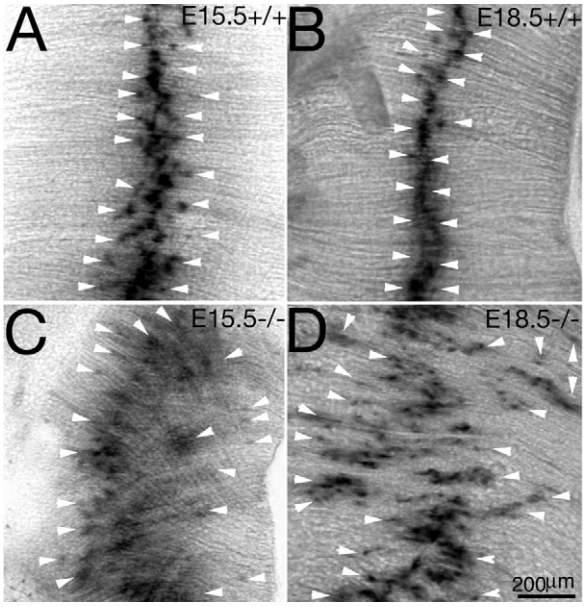

Fig. 7. Aberrant distribution of AChE clusters in $\gamma^{-/-}$ muscles. Whole-mount diaphragm muscles from the wild-type (**A**,**B**) and $\gamma^{-/-}$ embryos (**C**,**D**) were processed for AChE staining. While AChE clusters (arrowheads) were distributed along the central region of the wild-type muscles at E15.5 (A) and E18.5 (B), the AChE clusters were aberrantly distributed across a broad region in the $\gamma^{-/-}$ muscles at E15.5 (C) and E18.5 (D). Scale bar: 200 μm.

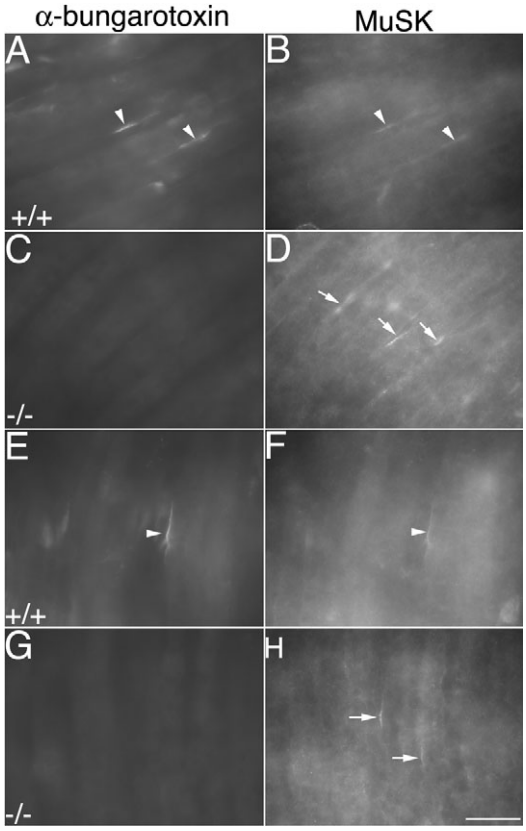

Fig. 8. Localization of MuSK in the absence of the γ-subunit. Whole-mount E15.5 diaphragm muscles from the ventral costal region (**A-D**) and sternal region (**E-H**) of wild-type (A-B,E-F) and $\gamma^{-/-}$ (C-D,G-H) embryos were doubly labeled with MuSK antibody and α-bungarotoxin. In wild-type muscles, MuSK staining (arrowheads in B and F) was detected at the central region of the muscle, corresponding to AChR clusters labeled by α-bungarotoxin (arrowheads in A,E). In the $\gamma^{-/-}$ muscles, MuSK staining was also seen at the central region of the muscle (arrows in D,H), despite the lack of AChR clustering (C,G). Scale bar: 10 μm.

E15.5, a stage when clustering of AChRs was absent from $\gamma^{-/-}$ muscles (Fig. 1). As shown in Fig. 8, MuSK was localized to the central region of the $\gamma^{-/-}$ muscle (arrows in Fig. 8D,H), despite the absence of AChR clusters (Fig. 8C,G). The localization of MuSK clusters in the $\gamma^{-/-}$ muscle was similar to that observed in the wild-type muscle (arrowheads in Fig. 8B,F), and consistent with the previously reported pattern of MuSK antibody staining in wild-type embryonic muscles (Bowen et al., 1998). Therefore, the phenotype developed in the $\gamma^{-/-}$ muscles is unlikely to be due to alternation of MuSK expression; instead, it probably resulted from the lack of the γ-subunit and consequently the absence of prepatterned AChRs (Fig. 1).

Abnormal localization of rapsyn in $\gamma^{-/-}$ muscles

Rapsyn is a cytoplasmic protein colocalized with AChRs in the postsynaptic muscle membrane (Froehner et al., 1981). Synaptic localization of rapsyn occurs at the initial stages of neuromuscular synaptogenesis (Noakes et al., 1993), and deletion of rapsyn leads to a total absence of AChR clustering (Gautam et al., 1995). Furthermore, rapsyn is absent from synaptic sites in AChR mutants of zebrafish (Ono et al., 2004; Ono et al., 2002). Because of its essential role in AChR clustering, we asked whether the localization of rapsyn was affected in $\gamma^{-/-}$ muscles. We carried out triple-immunostaining assay on muscle sections with antibodies against Syt2 (to label the nerve terminal) and rapsyn, as well as α-bgt (to label AChRs). In E15.5 wild-type muscles, as expected, rapsyn (Fig. 9C) was localized at synaptic sites marked by Syt2 antibodies (Fig. 9A) and α-bgt (Fig. 9B). However, in E15.5 $\gamma^{-/-}$ muscle, rapsyn staining was diffusely distributed across the entire sarcoplasm (Fig. 9F), with no specific localization at the synaptic sites marked by Syt2 antibodies (Fig. 9D). These results demonstrated that although rapsyn was expressed in $\gamma^{-/-}$ muscles, it failed to cluster at synaptic sites in the absence of the γ-subunit. Interestingly, synaptic localization of rapsyn was observed in E18.5 $\gamma^{-/-}$ muscle (Fig. 9L). This is probably due to the emergence of the ε-subunit in E18.5 $\gamma^{-/-}$ muscles.

Increased motoneuron survival in $\gamma^{-/-}$ embryos

Increased nerve branching in the $\gamma^{-/-}$ muscles (Fig. 5) raised a possibility there were increased motoneurons in the $\gamma^{-/-}$ embryos. Alternately, motoneuron number may remain unchanged, but motor axons may branch more extensively in the $\gamma^{-/-}$ embryos. We therefore compared motoneuron numbers between the wild type and $\gamma^{-/-}$ embryos. As shown in Fig. 10, motoneuron numbers in the $\gamma^{-/-}$ embryos were significantly increased. For example, within the cervical spinal segments (C3-C8), there was an ~65% increase of motoneuron number in the $\gamma^{-/-}$ (7473±283, n=3 embryos) compared with wild-type embryos (4539±247, n=3).

DISCUSSION

In the present study, we focused our studies on the initial stages of neuromuscular synaptogenesis (E13-E15.5) and showed that pre-patterned AChR clusters were completely absent in $\gamma^{-/-}$ muscles. AChR clusters emerged at later embryonic stages (E16.5-E18.5) owing to the expression of the ε-subunit. Thus, genetic ablation of

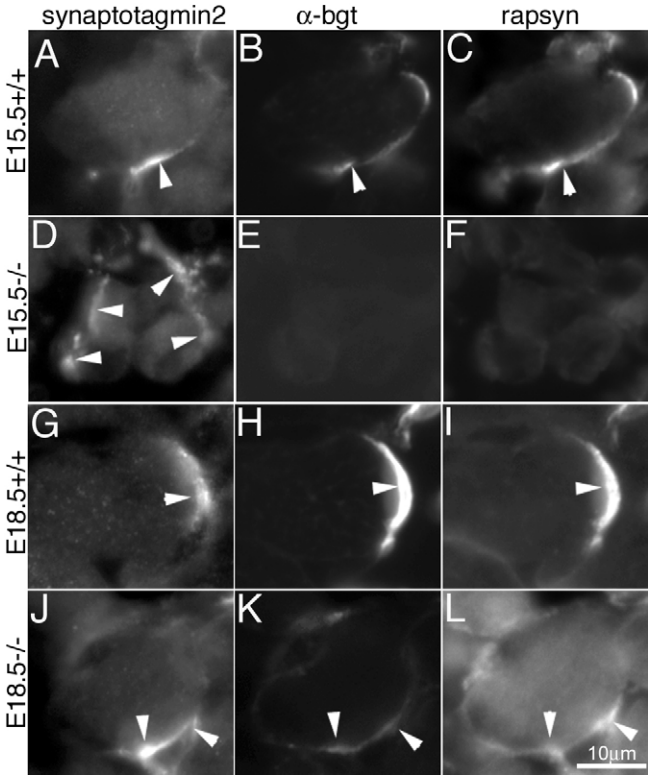

Fig. 9. Absence of rapsyn at synaptic sites in E15.5, but not E18.5, $\gamma^{-/-}$ muscles. Diaphragm muscle sections at E15.5 (**A-F**) and E18.5 (**G-L**) from wild-type (A-C,G-I) and $\gamma^{-/-}$ embryos (D-F,J-L) were triple-labeled with anti-Syt2 (A,D,G,J), α-bgt (B,E,H,K) and anti-rapsyn (C,F,I,L). At E15.5, rapsyn was highly localized to synaptic sites in wild-type muscles (arrowhead, C), but absent from synaptic sites in $\gamma^{-/-}$ muscles (F). There were also no AChR clusters (E) at synaptic sites (arrowhead in D) in the E15.5 $\gamma^{-/-}$ muscle. At E18.5, however, rapsyn (arrowheads) was localized to synaptic sites in both wild-type (I) and $\gamma^{-/-}$ muscles (L). Scale bar: 10 μm.

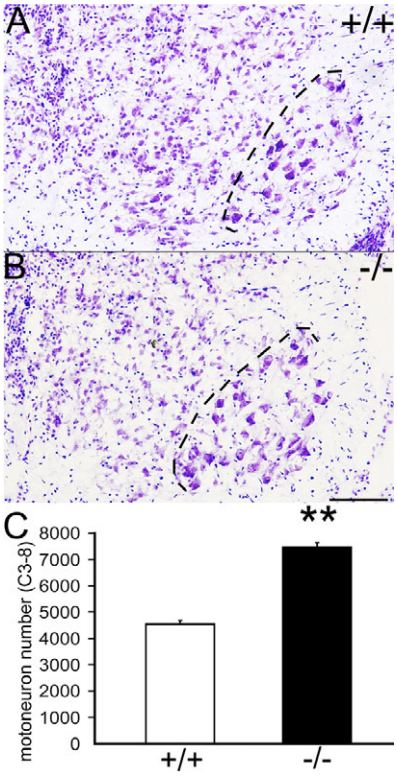

Fig. 10. Increased motoneuron survival in $\gamma^{-/-}$ embryos. Cross-sections of cervical spinal cords (C3-C8) from wild-type (**A**) and $\gamma^{-/-}$ (**B**) embryos (E18.5) were stained with Cresyl Violet. Motoneurons were identified in the ventral horn (within the region marked by broken lines) and their numbers were counted for each genotype. The total numbers of motoneurons from these segments (C3-C8) were presented in the bar graph (**C**). There were significantly more (65% increase) motoneurons in the $\gamma^{-/-}$ embryos (7473±283, n=3) than in the wild-type embryos (4539±247, n=3) (**P<0.001, Student's t-test). Scale bar: 100 μm.

the AChR γ-subunit specifically affected the formation of pre-patterned AChR clusters during the initial stages of neuromuscular synaptogenesis. The absence of pre-patterned AChR clusters in $\gamma^{-/-}$ muscles was associated with excessive nerve branching, increased motoneuron survival, as well as aberrant distribution of AChE and rapsyn. However, clustering of MuSK proceeded normally in $\gamma^{-/-}$ muscles.

The postsynaptic apparatus of the NMJ is assembled from large protein complexes that include AChRs, MuSK, rapsyn, utrophin, laminin, AChE, dystrobrevin and dystrophin-glycoprotein complex (DGC) (Bewick et al., 1996; Campbell, 1995; Ervasti and Campbell, 1991; Grady et al., 2000; Jacobson et al., 2001; Ohlendieck et al., 1991). These protein complexes interact intricately with each other, and altering one component may consequently affect the entire complex. For example, AChR clusters are closely associated with DGC, which in turn plays a crucial role in anchoring and stabilizing AChR clusters at the NMJ (Banks et al., 2003b; Henry and Campbell, 1996; Sunada and Campbell, 1995). Thus, it is possible that aberrant development of the NMJ in the $\gamma^{-/-}$ muscles may result from a broad disruption of postsynaptic proteins.

Our results demonstrated that MuSK, but not rapsyn, was clustered in the E15.5 $\gamma^{-/-}$ muscles, suggesting the clustering of MuSK may precede the clustering of AChRs. Interestingly, recent studies by Burden's group have shown that MuSK is prepatterned in developing muscles and dictates the formation of the NMJ (Kim and Burden, 2008). Thus, pre-patterning of MuSK may be one of the earliest events occurred during neuromuscular synaptogenesis. Activating MuSK at pre-patterned postsynaptic sites may then lead to the formation of pre-patterned AChR clusters, which may in turn sense the release of neurotransmitter from ingrowing axons (Chow and Poo, 1985; Hume et al., 1983; Young and Poo, 1983) and therefore establish communication with the nerve.

Clustering of rapsyn in the $\gamma^{-/-}$ muscles at E18.5, but not at E15.5, also suggests that synaptic targeting of rapsyn does appear to require the full complement of AChR subunits. Indeed, in vitro studies have shown that rapsyn is closely associated with various AChR subunits, including the β-subunit (Burden et al., 1983), the α-subunit (Maimone and Merlie, 1993), and the β-, γ- and δ-subunits (Huebsch and Maimone, 2003). In addition, rapsyn appears to associate with AChRs in an intracellular compartment prior to its insertion into the plasma membrane (Marchand et al., 2002; Moransard et al., 2003), suggesting that rapsyn and AChRs may arrive at the plasma membrane together. Furthermore, rapsyn clusters are absent from the NMJ in AChR δ-subunit mutants of zebrafish, demonstrating that AChRs are required for synaptic localization of rapsyn (Ono et al., 2004; Ono et al., 2002). Together, these observations support the hypothesis that the full complement of AChR subunits is required for targeting rapsyn to synaptic sites.

The delayed emergence of AChR clusters at E16.5 in $\gamma^{-/-}$ muscles was consistent with the onset of ε-subunit expression. Our RT-PCR and electrophysiology results demonstrate that the ε-subunit is expressed at late embryonic stages in both wild-type and $\gamma^{-/-}$ muscles. Indeed, a recent study shows that the expression of ε-subunit is detectable at E18 in leg muscles (Yumoto et al., 2005). However, immunostaining with anti-ε antibody shows that the epsilon subunit is only expressed after P0 (Missias et al., 1996). This discrepancy could be due to limited sensitivity of antibody immunostaining or lack of translation of the ε-subunit transcripts. The delayed AChR clusters appeared significantly larger in $\gamma^{-/-}$ muscles, compared with those observed in the wild-type muscles. Such increases in end-plate sizes are probably due to decreases in muscle activities in the $\gamma^{-/-}$ muscles. Consistent with this idea, similar increases in AChR cluster size were also observed in mutant embryos deficient in ChAT, which leads to a total blockade of ACh-mediated synaptic transmission (Brandon et al., 2003; Misgeld et al., 2002).

The delayed AChR clusters in $\gamma^{-/-}$ muscles could be nerve- or/and agrin-induced. Indeed, we observed 100% colocalization between the nerve terminals and AChR clusters at E18.5 in both wild-type and $\gamma^{-/-}$ muscles; there were no aneural AChR clusters at E18.5. The absence of aneural clusters at E18.5 could be due to dispersal effect of the ACh released from the nerve (Lin et al., 2005; Misgeld et al., 2005), or establishment of contacts by the newly arrived nerve terminals, or both. We cannot distinguish between these possibilities without using time-lapsed video-microscopy to follow the same nerve terminals/AChR clusters over time, as elegantly demonstrated in the zebrafish NMJ (Flanagan-Steet et al., 2005; Panzer et al., 2005; Panzer et al., 2006).

One of the hallmarks of presynaptic differentiation is progressive accumulation of synaptic vesicle proteins at the nerve terminal (Dahm and Landmesser, 1991; Lupa and Hall, 1989). Mechanisms underlying this process remain unclear. Our data suggest that the presence of pre-patterned AChR clusters is not required for initiating pre-synaptic differentiation. These results are consistent with studies in zebrafish NMJ previously reported: presynaptic nerve terminals develop normally in mutants lacking functional AChRs in zebrafish NMJ (Li et al., 2003; Ono et al., 2001; Panzer et al., 2006; Westerfield et al., 1990).

Previous studies have shown that motoneuron survival is markedly enhanced when ACh-mediated transmission is blocked either pharmacologically (Dahm and Landmesser, 1988; Hory-Lee and Frank, 1995; Oppenheim et al., 1989; Oppenheim et al., 2000; Pittman and Oppenheim, 1979; Pittman and Oppenheim, 1978) or genetically (Brandon et al., 2003; Misgeld et al., 2002). However, these approaches may unavoidably affect both the neuronal nicotinic AChRs in presynaptic cells and the muscle nicotinic AChRs in the postsynaptic cells. As the γ-subunit is expressed only in postsynaptic muscle cells, our results demonstrate blockade of ACh-mediated transmission specifically in postsynaptic muscle cells promotes motoneuron survival. Our results are consistent with previous reports demonstrating enhanced motoneuron survival in mutant mice lacking rapsyn, MuSK or agrin (Banks et al., 2001; Banks et al., 2003a; Terrado et al., 2001). Together, these studies suggest a postsynaptic mechanism regulates motoneuron survival during development.

We thank the RIKEN BioResource Center (Tsukuba, Japan) for maintaining and providing the AChR γ-subunit knockout mice, and Dr Atsushi Yoshiki at the RIKEN BioResource for coordinating the transferring of the mice. We are indebted to Drs Thomas Sudhof, Jane Johnson, Helmut Kramer, Paul Blount and Jonathan Terman for their critical comments on manuscript drafts, and to Drs William Betz, Mark Rich and Yoshie Sugiura for their valuable suggestions on electrophysiology. This work was supported by grants (to W.L.) from NIH/NINDS (NS055028), from Robert Packard Center for ALS Research at Johns Hopkins, from the Edward Mallinckrodt, Jr Foundation and from the Cain Foundation in Medical Research.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/11/1957/DC1

References

Anderson, M. J. and Cohen, M. W. (1977). Nerve-induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268**, 757-773.

Anderson, M. J., Cohen, M. W. and Zorychta, E. (1977). Effects of innervation on the distribution of acetylcholine receptors on cultured muscle cells. *J. Physiol.* **268**, 731-756.

Arber, S., Burden, S. J. and Harris, A. J. (2002). Patterning of skeletal muscle. *Curr. Opin. Neurobiol.* **12**, 100-103.

Banks, G. B., Chau, T. N., Bartlett, S. E. and Noakes, P. G. (2001). Promotion of motoneuron survival and branching in rapsyn-deficient mice. *J. Comp. Neurol.* **429**, 156-165.

Banks, G. B., Choy, P. T., Lavidis, N. A. and Noakes, P. G. (2003a). Neuromuscular synapses mediate motor axon branching and motoneuron survival during the embryonic period of programmed cell death. *Dev. Biol.* **257**, 71-84.

Banks, G. B., Fuhrer, C., Adams, M. E. and Froehner, S. C. (2003b). The postsynaptic submembrane machinery at the neuromuscular junction: Requirement for rapsyn and the utrophin/dystrophin-associated complex. *J. Neurocytol.* **32**, 709-726.

Bekoff, A. and Betz, W. J. (1976). Acetylcholine hot spots: development on myotubes cultured from aneural limb buds. *Science* **193**, 915-917.

Bennett, M. R. and Pettigrew, A. G. (1974). The formation of synapses in striated muscle during development. *J. Physiol.* **241**, 515-545.

Bewick, G. S., Young, C. and Slater, C. R. (1996). Spatial relationships of utrophin, dystrophin, beta-dystroglycan and beta-spectrin to acetylcholine receptor clusters during postnatal maturation of the rat neuromuscular junction. *J. Neurocytol.* **25**, 367-379.

Bezakova, G. and Ruegg, M. A. (2003). New insights into the roles of agrin. *Nat. Rev. Mol. Cell Biol.* **4**, 295-308.

Bowe, M. A. and Fallon, J. R. (1995). The role of agrin in synapse formation. *Annu. Rev. Neurosci.* **18**, 443-462.

Bowen, D. C., Park, J. S., Bodine, S., Stark, J. L., Valenzuela, D. M., Stitt, T. N., Yancopoulos, G. D., Lindsay, R. M., Glass, D. J. and DiStefano, P. S. (1998). Localization and regulation of MuSK at the neuromuscular junction. *Dev. Biol.* **199**, 309-319.

Braithwaite, A. W. and Harris, A. J. (1979). Neural influence on acetylcholine receptor clusters in embryonic development of skeletal muscles. *Nature* **279**, 549-551.

Brandon, E. P., Lin, W., D'Amour, K. A., Pizzo, D. P., Dominguez, B., Sugiura, Y., Thode, S., Ko, C. P., Thal, L. J., Gage, F. H. et al. (2003). Aberrant patterning of neuromuscular synapses in choline acetyltransferase-deficient mice. *J. Neurosci.* **23**, 539-549.

Burden, S. J. (2002). Building the vertebrate neuromuscular synapse. *J. Neurobiol.* **53**, 501-511.

Burden, S. J., DePalma, R. L. and Gottesman, G. S. (1983). Crosslinking of proteins in acetylcholine receptor-rich membranes: association between the beta-subunit and the 43 kd subsynaptic protein. *Cell* **35**, 687-692.

Buss, R. R., Gould, T. W., Ma, J., Vinsant, S., Prevette, D., Winseck, A., Toops, K. A., Hammarback, J. A., Smith, T. L. and Oppenheim, R. W. (2006). Neuromuscular development in the absence of programmed cell death: phenotypic alteration of motoneurons and muscle. *J. Neurosci.* **26**, 13413-13427.

Campbell, K. P. (1995). Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. *Cell* **80**, 675-679.

Changeux, J. P., Devillers-Thiery, A., Galzi, J. L. and Revah, F. (1992). The acetylcholine receptor: a model of an allosteric membrane protein mediating intercellular communication. *Ciba Found. Symp.* **164**, 66-89, discussion 87-97.

Chow, I. and Poo, M. M. (1985). Release of acetylcholine from embryonic neurons upon contact with muscle cell. *J. Neurosci.* **5**, 1076-1082.

Clarke, P. G. and Oppenheim, R. W. (1995). Neuron death in vertebrate development: in vitro methods. *Methods Cell Biol.* **46**, 277-321.

Cossins, J., Webster, R., Maxwell, S., Burke, G., Vincent, A. and Beeson, D. (2004). A mouse model of AChR deficiency syndrome with a phenotype reflecting the human condition. *Hum. Mol. Genet.* **13**, 2947-2957.

Creazzo, T. L. and Sohal, G. S. (1983). Neural control of embryonic acetylcholine receptor and skeletal muscle. *Cell Tissue Res.* **228**, 1-12.

Dahm, L. M. and Landmesser, L. T. (1988). The regulation of intramuscular nerve branching during normal development and following activity blockade. *Dev. Biol.* **130**, 621-644.

Dahm, L. M. and Landmesser, L. T. (1991). The regulation of synaptogenesis during normal development and following activity blockade. *J. Neurosci.* **11**, 238-255.
DeChiara, T. M., Bowen, D. C., Valenzuela, D. M., Simmons, M. V., Poueymirou, W. T., Thomas, S., Kinetz, E., Compton, D. L., Rojas, E., Park, J. S. et al. (1996). The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. *Cell* **85**, 501-512.
Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R. O., Snider, W. D., Johnson, E. M., Jr and Milbrandt, J. (1998). GFR alpha1-deficient mice have deficits in the enteric nervous system and kidneys. *Neuron* **21**, 317-324.
Ervasti, J. M. and Campbell, K. P. (1991). Membrane organization of the dystrophin-glycoprotein complex. *Cell* **66**, 1121-1131.
Ferns, M. and Carbonetto, S. (2001). Challenging the neurocentric view of neuromuscular synapse formation. *Neuron* **30**, 311-314.
Flanagan-Steet, H., Fox, M. A., Meyer, D. and Sanes, J. R. (2005). Neuromuscular synapses can form in vivo by incorporation of initially aneural postsynaptic specializations. *Development* **132**, 4471-4481.
Frank, E. and Fischbach, G. D. (1979). Early events in neuromuscular junction formation in vitro: induction of acetylcholine receptor clusters in the postsynaptic membrane and morphology of newly formed synapses. *J. Cell Biol.* **83**, 143-158.
Froehner, S. C., Gulbrandsen, V., Hyman, C., Jeng, A. Y., Neubig, R. R. and Cohen, J. B. (1981). Immunofluorescence localization at the mammalian neuromuscular junction of the Mr 43,000 protein of Torpedo postsynaptic membranes. *Proc. Natl. Acad. Sci. USA* **78**, 5230-5234.
Gattenlohner, S., Schneider, C., Thamer, C., Klein, R., Roggendorf, W., Gohlke, F., Niethammer, C., Czub, S., Vincent, A., Muller-Hermelink, H. K. et al. (2002). Expression of foetal type acetylcholine receptor is restricted to type 1 muscle fibres in human neuromuscular disorders. *Brain* **125**, 1309-1319.
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., Bowen, D. C., Stitt, T. N., Radziejewski, C., Bruno, J., Ryan, T. E., Gies, D. R., Shah, S., Mattsson, K., Burden, S. J. et al. (1996). Agrin acts via a MuSK receptor complex. *Cell* **85**, 513-523.
Goda, Y. and Davis, G. W. (2003). Mechanisms of synapse assembly and disassembly. *Neuron* **40**, 243-264.
Grady, R. M., Zhou, H., Cunningham, J. M., Henry, M. D., Campbell, K. P. and Sanes, J. R. (2000). Maturation and maintenance of the neuromuscular synapse: genetic evidence for roles of the dystrophin-glycoprotein complex. *Neuron* **25**, 279-293.
Henry, M. D. and Campbell, K. P. (1996). Dystroglycan: an extracellular matrix receptor linked to the cytoskeleton. *Curr. Opin. Cell Biol.* **8**, 625-631.
Hoffmann, K., Muller, J. S., Stricker, S., Megarbane, A., Rajab, A., Lindner, T. H., Cohen, M., Chouery, E., Adaimy, L., Ghanem, I. et al. (2006). Escobar syndrome is a prenatal myasthenia caused by disruption of the acetylcholine receptor fetal gamma subunit. *Am. J. Hum. Genet.* **79**, 303-312.
Hory-Lee, F. and Frank, E. (1995). The nicotinic blocking agents d-tubocurare and alpha-bungarotoxin save motoneurons from naturally occurring death in the absence of neuromuscular blockade. *J. Neurosci.* **15**, 6453-6460.
Huebsch, K. A. and Maimone, M. M. (2003). Rapsyn-mediated clustering of acetylcholine receptor subunits requires the major cytoplasmic loop of the receptor subunits. *J. Neurobiol.* **54**, 486-501.
Hume, R. I., Role, L. W. and Fischbach, G. D. (1983). Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. *Nature* **305**, 632-634.
Jacobson, C., Cote, P. D., Rossi, S. G., Rotundo, R. L. and Carbonetto, S. (2001). The dystroglycan complex is necessary for stabilization of acetylcholine receptor clusters at neuromuscular junctions and formation of the synaptic basement membrane. *J. Cell Biol.* **152**, 435-450.
Jennings, C. G., Dyer, S. M. and Burden, S. J. (1993). Muscle-specific trk-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases. *Proc. Natl. Acad. Sci. USA* **90**, 2895-2899.
Kim, N. and Burden, S. J. (2008). MuSK controls where motor axons grow and form synapses. *Nat. Neurosci.* **11**, 19-27.
Koenen, M., Peter, C., Villarroel, A., Witzemann, V. and Sakmann, B. (2005). Acetylcholine receptor channel subtype directs the innervation pattern of skeletal muscle. *EMBO Rep.* **6**, 570-576.
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.
Li, W., Ono, F. and Brehm, P. (2003). Optical measurements of presynaptic release in mutant zebrafish lacking postsynaptic receptors. *J. Neurosci.* **23**, 10467-10474.
Liley, A. W. (1956). An investigation of spontaneous activity at the neuromuscular junction of the rat. *J. Physiol.* **132**, 650-666.
Lin, W., Burgess, R. W., Dominguez, B., Pfaff, S. L., Sanes, J. R. and Lee, K. F. (2001). Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. *Nature* **410**, 1057-1064.
Lin, W., Dominguez, B., Yang, J., Aryal, P., Brandon, E. P., Gage, F. H. and Lee, K.-F. (2005). Neurotransmitter acetylcholine negatively regulates neuromuscular synapse formation by a Cdk5-dependent mechanism. *Neuron* **46**, 569-579.
Liu, Y. and Brehm, P. (1993). Expression of subunit-omitted mouse nicotinic acetylcholine receptors in Xenopus laevis oocytes. *J. Physiol.* **470**, 349-363.
Lupa, M. T. and Hall, Z. W. (1989). Progressive restriction of synaptic vesicle protein to the nerve terminal during development of the neuromuscular junction. *J. Neurosci.* **9**, 3937-3945.
Maimone, M. M. and Merlie, J. P. (1993). Interaction of the 43 kd postsynaptic protein with all subunits of the muscle nicotinic acetylcholine receptor. *Neuron* **11**, 53-66.
Marchand, S., Devillers-Thiery, A., Pons, S., Changeux, J. P. and Cartaud, J. (2002). Rapsyn escorts the nicotinic acetylcholine receptor along the exocytic pathway via association with lipid rafts. *J. Neurosci.* **22**, 8891-8901.
McArdle, J. J., Lentz, T. L., Witzemann, V., Schwarz, H., Weinstein, S. A. and Schmidt, J. J. (1999). Waglerin-1 selectively blocks the epsilon form of the muscle nicotinic acetylcholine receptor. *J. Pharmacol. Exp. Ther.* **289**, 543-550.
McLachlan, E. M. and Martin, A. R. (1981). Non-linear summation of end-plate potentials in the frog and mouse. *J. Physiol.* **311**, 307-324.
McMahan, U. J. (1990). The agrin hypothesis. *Cold Spring Harb. Symp. Quant. Biol.* **55**, 407-418.
Misgeld, T., Burgess, R. W., Lewis, R. M., Cunningham, J. M., Lichtman, J. W. and Sanes, J. R. (2002). Roles of neurotransmitter in synapse formation: development of neuromuscular junctions lacking choline acetyltransferase. *Neuron* **36**, 635-648.
Misgeld, T., Kummer, T. T., Lichtman, J. W. and Sanes, J. R. (2005). Agrin promotes synaptic differentiation by counteracting an inhibitory effect of neurotransmitter. *Proc. Natl. Acad. Sci. USA* **102**, 11088-11093.
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., Chu, G. C., Klocke, B. J., Sanes, J. R. and Merlie, J. P. (1996). Maturation of the acetylcholine receptor in skeletal muscle: regulation of the AChR gamma-to-epsilon switch. *Dev. Biol.* **179**, 223-238.
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.
Moransard, M., Borges, L. S., Willmann, R., Marangi, P. A., Brenner, H. R., Ferns, M. J. and Fuhrer, C. (2003). Agrin regulates rapsyn interaction with surface acetylcholine receptors, and this underlies cytoskeletal anchoring and clustering. *J. Biol. Chem.* **278**, 7350-7359.
Morgan, N. V., Brueton, L. A., Cox, P., Greally, M. T., Tolmie, J., Pasha, S., Aligianis, I. A., van Bokhoven, H., Marton, T., Al-Gazali, L. et al. (2006). Mutations in the embryonal subunit of the acetylcholine receptor (CHRNG) cause lethal and Escobar variants of multiple pterygium syndrome. *Am. J. Hum. Genet.* **79**, 390-395.
Nitkin, R. M., Smith, M. A., Magill, C., Fallon, J. R., Yao, Y. M., Wallace, B. G. and McMahan, U. J. (1987). Identification of agrin, a synaptic organizing protein from Torpedo electric organ. *J. Cell Biol.* **105**, 2471-2478.
Noakes, P. G., Phillips, W. D., Hanley, T. A., Sanes, J. R. and Merlie, J. P. (1993). 43K protein and acetylcholine receptors colocalize during the initial stages of neuromuscular synapse formation in vivo. *Dev. Biol.* **155**, 275-280.
Ohlendieck, K., Ervasti, J. M., Matsumura, K., Kahl, S. D., Leveille, C. J. and Campbell, K. P. (1991). Dystrophin-related protein is localized to neuromuscular junctions of adult skeletal muscle. *Neuron* **7**, 499-508.
Ono, F., Higashijima, S., Shcherbatko, A., Fetcho, J. R. and Brehm, P. (2001). Paralytic zebrafish lacking acetylcholine receptors fail to localize rapsyn clusters to the synapse. *J. Neurosci.* **21**, 5439-5448.
Ono, F., Shcherbatko, A., Higashijima, S., Mandel, G. and Brehm, P. (2002). The Zebrafish motility mutant twitch once reveals new roles for rapsyn in synaptic function. *J. Neurosci.* **22**, 6491-6498.
Ono, F., Mandel, G. and Brehm, P. (2004). Acetylcholine receptors direct rapsyn clusters to the neuromuscular synapse in zebrafish. *J. Neurosci.* **24**, 5475-5481.
Oppenheim, R. W., Bursztajn, S. and Prevette, D. (1989). Cell death of motoneurons in the chick embryo spinal cord. XI. Acetylcholine receptors and synaptogenesis in skeletal muscle following the reduction of motoneuron death by neuromuscular blockade. *Development* **107**, 331-341.
Oppenheim, R. W., Prevette, D., D'Costa, A., Wang, S., Houenou, L. J. and McIntosh, J. M. (2000). Reduction of neuromuscular activity is required for the rescue of motoneurons from naturally occurring cell death by nicotinic-blocking agents. *J. Neurosci.* **20**, 6117-6124.

Panzer, J. A., Gibbs, S. M., Dosch, R., Wagner, D., Mullins, M. C., Granato, M. and Balice-Gordon, R. J. (2005). Neuromuscular synaptogenesis in wild-type and mutant zebrafish. *Dev. Biol.* **285**, 340-357.

Panzer, J. A., Song, Y. and Balice-Gordon, R. J. (2006). In vivo imaging of preferential motor axon outgrowth to and synaptogenesis at prepatterned acetylcholine receptor clusters in embryonic zebrafish skeletal muscle. *J. Neurosci.* **26**, 934-947.

Pittman, R. H. and Oppenheim, R. W. (1978). Neuromuscular blockade increases motoneurone survival during normal cell death in the chick embryo. *Nature* **271**, 364-366.

Pittman, R. and Oppenheim, R. W. (1979). Cell death of motoneurons in the chick embryo spinal cord. IV. Evidence that a functional neuromuscular interaction is involved in the regulation of naturally occurring cell death and the stabilization of synapses. *J. Comp. Neurol.* **187**, 425-446.

Pun, S., Sigrist, M., Santos, A. F., Ruegg, M. A., Sanes, J. R., Jessell, T. M., Arber, S. and Caroni, P. (2002). An intrinsic distinction in neuromuscular junction assembly and maintenance in different skeletal muscles. *Neuron* **34**, 357-370.

Rotundo, R. L. (2003). Expression and localization of acetylcholinesterase at the neuromuscular junction. *J. Neurocytol.* **32**, 743-766.

Rupp, F., Payan, D. G., Magill-Solc, C., Cowan, D. M. and Scheller, R. H. (1991). Structure and expression of a rat agrin. *Neuron* **6**, 811-823.

Sanes, J. R. and Lichtman, J. W. (2001). Induction, assembly, maturation and maintenance of a postsynaptic apparatus. *Nat. Rev. Neurosci.* **2**, 791-805.

Sine, S. M. and Claudio, T. (1991). Gamma- and delta-subunits regulate the affinity and the cooperativity of ligand binding to the acetylcholine receptor. *J. Biol. Chem.* **266**, 19369-19377.

Sunada, Y. and Campbell, K. P. (1995). Dystrophin-glycoprotein complex: molecular organization and critical roles in skeletal muscle. *Curr. Opin. Neurol.* **8**, 379-384.

Takahashi, M., Kubo, T., Mizoguchi, A., Carlson, C. G., Endo, K. and Ohnishi, K. (2002). Spontaneous muscle action potentials fail to develop without fetal-type acetylcholine receptors. *EMBO Rep.* **3**, 674-681.

Talbot, J. D., David, G. and Barrett, E. F. (2003). Inhibition of mitochondrial Ca^{2+} uptake affects phasic release from motor terminals differently depending on external [Ca^{2+}]. *J. Neurophysiol.* **90**, 491-502.

Teichert, R. W., Rivier, J., Torres, J., Dykert, J., Miller, C. and Olivera, B. M. (2005). A uniquely selective inhibitor of the mammalian fetal neuromuscular nicotinic acetylcholine receptor. *J. Neurosci.* **25**, 732-736.

Terrado, J., Burgess, R. W., DeChiara, T., Yancopoulos, G., Sanes, J. R. and Kato, A. C. (2001). Motoneuron survival is enhanced in the absence of neuromuscular junction formation in embryos. *J. Neurosci.* **21**, 3144-3150.

Valenzuela, D. M., Stitt, T. N., DiStefano, P. S., Rojas, E., Mattsson, K., Compton, D. L., Nunez, L., Park, J. S., Stark, J. L., Gies, D. R. et al. (1995). Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. *Neuron* **15**, 573-584.

Westerfield, M., Liu, D. W., Kimmel, C. B. and Walker, C. (1990). Pathfinding and synapse formation in a zebrafish mutant lacking functional acetylcholine receptors. *Neuron* **4**, 867-874.

Witzemann, V., Schwarz, H., Koenen, M., Berberich, C., Villarroel, A., Wernig, A., Brenner, H. R. and Sakmann, B. (1996). Acetylcholine receptor epsilon-subunit deletion causes muscle weakness and atrophy in juvenile and adult mice. *Proc. Natl. Acad. Sci. USA* **93**, 13286-13291.

Yang, X., Li, W., Prescott, E. D., Burden, S. J. and Wang, J. C. (2000). DNA topoisomerase IIbeta and neural development. *Science* **287**, 131-134.

Yang, X., Arber, S., William, C., Li, L., Tanabe, Y., Jessell, T. M., Birchmeier, C. and Burden, S. J. (2001). Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. *Neuron* **30**, 399-410.

Young, S. H. and Poo, M. M. (1983). Spontaneous release of transmitter from growth cones of embryonic neurones. *Nature* **305**, 634-637.

Yumoto, N., Wakatsuki, S. and Sehara-Fujisawa, A. (2005). The acetylcholine receptor gamma-to-epsilon switch occurs in individual endplates. *Biochem. Biophys. Res. Commun.* **331**, 1522-1527.