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Formation of cholinergic synapse-like specializations at developing murine muscle spindles

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ABSTRACT

Muscle spindles are complex stretch-sensitive mechanoreceptors. They consist of specialized skeletal muscle fibers, called intrafusal fibers, which are innervated in the central (equatorial) region by afferent sensory axons and in both polar regions by efferent γ-motoneurons. We show that AChRs are concentrated at the γ-motoneuron endplate as well as in the equatorial region where they colocalize with the sensory nerve ending. In addition to the AChRs, the contact site between sensory nerve ending and intrafusal muscle fiber contains a high concentration of choline acetyltransferase, vesicular acetylcholine transporter and the AChR-associated protein rapsyn. Moreover, bassoon, a component of the presynaptic cytomatrix involved in synaptic vesicle exocytosis, is present in γ-motoneuron endplates but also in the sensory nerve terminal. Finally, we demonstrate that during postnatal development of the γ-motoneuron endplate, the AChR subunit stoichiometry changes from the γ-subunit-containing fetal AChRs to the ε-subunit-containing adult AChRs, similar and approximately in parallel to the postnatal subunit maturation at the neuromuscular junction. In contrast, despite the onset of ε-subunit expression during postnatal development the γ-subunit remains detectable in the equatorial region by subunit-specific antibodies as well as by analysis of muscle spindles from mice with genetically-labeled AChR γ-subunits. These results demonstrate an unusual maturation of the AChR subunit composition at the annulospiral endings and suggest that in addition to the recently described glutamatergic secretory system, the sensory nerve terminals are also specialized for cholinergic synaptic transmission, synaptic vesicle storage and exocytosis.

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Introduction

Proprioception and the control of movement require muscle spindles, mechanosensors that are sensitive to local changes in muscle fiber length (Proske and Gandevia, 2012). Muscle spindles are rare, but can be found in virtually all skeletal muscles. They consist of 3–10 specialized small encapsulated muscle fibers (intrafusal fibers) distributed throughout the muscle in parallel with extrafusal fibers (Hunt, 1990; Proske, 1997). Intrafusal muscle fibers are innervated by sensory- and motor neurons (Banks, 1994). In their central (equatorial) part, intrafusal muscle fibers are in direct contact with afferent proprioceptive sensory neurons, termed "type Ia afferents" and "type II afferents" according to their axonal conduction velocity. Type Ia afferents form so called annulospiral sensory nerve endings whereas type II axon terminals flank these primary endings (Schroder et al., 1989). The cell bodies of these pseudounipolar sensory neurons constitute a minor fraction of all neurons in the dorsal root ganglion (DRG; Arber et al., 2000; Hippenmeyer et al., 2002) that can be selectively labeled by antibodies against parvalbumin (Honda, 1995). The annulospiral endings are the main stretch-sensitive units and in the spinal cord the axons of these proprioceptive neurons make precise excitatory monosynaptic connections with the α-motoneurons, which innervate the homonymous target muscle (Mears and Frank, 1997; Kanning et al., 2010; Wang et al., 2012).

In addition to the afferent sensory neurons, mammalian intrafusal muscle fibers are innervated by efferent γ-motoneurons (Hunt and Kuffler, 1951) as well as (to a lesser extent) by collaterals of α-motoneurons (called β-motoneurons; Bessou et al., 1965). Gamma-motoneurons have their cell bodies in the ventral horn of the spinal cord among those of α-motoneurons (Friese et al., 2009; Shneider et al., 2009; Ashrafi et al., 2012). Axons of γ-motoneurons enter the spindle and penetrate the connective tissue capsule together with the sensory fibers in the central region of the

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spindle but innervate intrafusal muscle fibers at both ends (polar regions) where they form cholinergic synapses that appear in some aspects similar to the neuromuscular junction formed by α-motoneurons on extrafusal muscle fibers (Arbuthnott et al., 1982). The function of γ-motoneurons is to regulate muscle spindle sensitivity to stretch. Gamma-motoneuron-induced contraction of the polar regions of intrafusal fibers maintains tension in the equatorial region during muscle contraction (Kuffler et al., 1951; Hunt and Kuffler, 1954). This allows the control of the mechanical sensitivity of spindles over a wide range of lengths and velocities (Hulliger, 1984).

The differentiation of muscle spindles in rodents begins during embryonic development but the maturation continues into postnatal life (Kozeka and Ontell, 1981; Kucera and Walro, 1994; Maier, 1997). Muscle spindle development and the establishment of the monosynaptic stretch reflex require the exchange of factors between neurons and intrafusal muscle fibers (for review see Chen et al., 2003). Absence or loss of function of these factors usually results in degeneration of muscle spindles and loss of motor control (Ladle et al., 2007; Cheret et al., 2013). While the role of these factors during muscle spindle development have been relatively well characterized, the formation of synapse-like specializations at γ-motoneuron endplates or the development of the contact site between sensory neuron and intrafusal fibers in the central (equatorial) part of muscle spindles, have not been analyzed at the molecular level. In particular, the development of cholinergic synapse-like specializations in muscle spindles has not been studied, despite an important role of acetylcholine in adult muscle spindles (reviewed by Carr and Proske, 1996). In this study we report the concentration of AChRs in intrafusal fibers at all sites of innervation, i.e. at the equatorial annulospiral sensory nerve endings as well as at the γ-motoneuron endplates. Moreover, we show that both types of nerve-to-muscle contact sites contain a high concentration of proteins characteristic of cholinergic synapses, including the vesicular acetylcholine transporter, choline acetyltransferase and the AChR-associated protein rapsyn. We also find immunoreactivity in the central part of intrafusal fibers for the active zone-specific presynaptic cytomatrix protein bassoon. Finally we show that AChRs at γ-motoneuron endplates undergo a γ-to-ε subunit switch during early postnatal development reminiscent of the fetal to adult conversion of AChRs at the neuromuscular junction. In contrast, γ- and ε-subunits are simultaneously present in AChRs at the adult sensory nerve ending. These results demonstrate an unexpected cholinergic specialization at the equatorial region and a difference in the AChR subunit maturation between the equatorial and the polar region at intrafusal fibers of developing murine muscle spindles.

Materials and methods

Mice

Use and care of animals was approved by German authorities and according to national law (TierSchG§7). Mice were kept in sterile cages and deeply anesthetized using xylazine (Bayer AG, Leverkusen, Germany) and ketamine (Pfizer, Berlin, Germany). Animals were transcardially perfused with PBS followed by 4% paraformaldehyde for 18 min and the muscles (soleus, quadriceps and extensor digitorum longus) were dissected. We did not observe any principal difference in muscle spindle development in different muscles. Muscle spindles were investigated either in C57BL/6 wildtype mice or in the Thy1-YFP16 mouse line which expresses the YFP in all motor and sensory axons, retinal ganglion cells and dorsal root ganglion neurons (Feng et al., 2000). Unless stated otherwise, adult mice refer to three month old animals.

Mice in which the γ-subunit of the AChR was fused to the humanized green fluorescent protein ($AChR^{\gamma-GFP/\gamma GFP}$) have been described in detail previously (Gensler et al., 2001; Yampolsky et al., 2008). These mice express a γ-subunit-GFP fusion protein that forms functionally intact GFP-labeled AChR receptor pentamers, which are correctly targeted to the postsynaptic membrane. Although the AChR expression level is decreased after GFP-labeling, the development of pre- and postsynaptic specializations at the neuromuscular junction is normal and the mice are healthy and display no obvious phenotypic difference to wild-type mice (Gensler et al., 2001; Yampolsky et al., 2008). Two adult (1-year old) and 3 postnatal day 1 mice were used in this study.

Immunohistochemistry

After fixation and dissection, muscles were either sectioned into 10–30 μm thick longitudinal sections using a cryostat or processed as whole mounts. Care was taken to section parallel to the muscle spindle longitudinal axis in order to completely reconstruct individual intrafusal fibers, including both polar regions, during the confocal microscopic analysis. Indirect immunofluorescence staining using various antibodies (see below) was performed as described (Tsen et al., 1995). Results were obtained from at least 5 different sections from 3 muscles derived from at least 3 animals. We observed no difference between male and female mice. The following antibodies were used: rabbit anti-AChE (Marsh et al., 1984; Cartaud et al., 2004), goat anti-AChR ε-subunit (Santa Cruz), goat anti-AChR γ-subunit (Santa Cruz), rabbit anti-rapsyn antibodies (BIOZOL, Eching, Germany; Choi et al., 2012), goat anti-parvalbumin (SWANT, Basel, Switzerland), goat anti-vesicular acetylcholine transporter (VAChT; Millipore, Schwalbach, Germany); goat anti-choline acetylcholintransferase (ChAT; Abcam, Cambridge, UK), rabbit anti-bassoon (Dieck et al., 2005; Jastrow et al., 2006), and guinea pig anti-vesicular glutamate transporter 1 (VGluT1; Millipore, Darmstadt, Germany). AChRs were visualized using Alexa594-conjugated α-bungarotoxin (α-Btx; Life Technologies, Darmstadt, Germany) at a concentration of 2 μg/ml. Within muscle spindles, the proprioceptive sensory neuron terminals were visualized either via anti-VGluT1 immunoreactivity (Wu et al., 2004) or genetically via Thy1-YFP expression (Feng et al., 2000). Primary antibodies were detected using the appropriate Alexa488-conjugated goat anti-rabbit, donkey anti-goat, or donkey anti-guinea pig secondary antibody. Each of the anti-goat, anti-guinea pig, and anti-rabbit secondary antibody was preabsorbed against IgGs of the other two species, eliminating crossreactivity in double-immunofluorescence analyses. No staining was observed when the primary antibodies or the Alexa-conjugated α-bungarotoxin were omitted. The nuclei were routinely stained using DAPI (Roth, Karlsruhe, Germany) at a concentration of 2 μg/ml.

After staining the sections were embedded in Mowiol mounting medium (Roth, Karlsruhe, Germany) and analyzed using a Zeiss LSM 710 laser scanning confocal microscope. Sequentially scanned confocal Z-stacks of whole muscle spindles were obtained using 1 μm optical sections and compiled using the ZEN2009 software (Zeiss, Oberkochen, Germany). Laser power levels, photomultiplier gain levels, scanning speed, and the confocal pinhole size were kept constant within experimental and control specimens. Digital processing of entire images, including adjustment of brightness and contrast, was performed using Photoshop CS3 (Adobe, Munich, Germany).

Quantification of bassoon immunoreactivity

The number and size of the bassoon puncta at neuromuscular junctions, endplates of γ-motoneurons, and at sensory nerve

terminals was quantified in a total of 3 C57/BL6 7-week-old wildtype mice. The puncta were randomly selected by eye from the Z-stacks of scans from whole muscle spindles. We restricted our analysis to the area overlaying α-bungarotoxin-labeled AChR clusters or VGluT1-labeled sensory nerve terminals, respectively. The density of bassoon puncta was manually determined in maximum intensity projection images from confocal optical sections in 11 muscle spindles from 3 mice using the NIH public domain Java image processing program software package ImageJ (http://rsbweb.nih.gov/ij) and expressed in number of puncta per μm². The diameter of randomly selected bassoon puncta was determined manually in the same 3 animals by analyzing maximum intensity projections from high-resolution confocal scans from 11 muscle spindles and 15 neuromuscular junctions in the direct vicinity of the spindles using the ZEN2009 software (Zeiss). In total 100 bassoon puncta from neuromuscular junctions, 103 puncta from γ-motoneuron endplates and 116 puncta from sensory nerve endings were analyzed.

Results

As a first step to characterize cholinergic synapse-like specializations in muscle spindles, we determined the distribution of AChRs using fluorescently labeled α-bungarotoxin (α-Btx) in Thy1-YFP mice. These mice express the YFP-gene under the control of the Thy1 promotor (Feng et al., 2000) and precisely label all sensory and motor neurons. Fig. 1 shows the distribution of AChRs in an adult soleus muscle spindle together with its innervation by YFP-labeled sensory and motor neurons. In agreement with previous publications (Arber et al., 2000; Hippenmeyer et al., 2002), aggregates of AChRs were observed in the polar regions (arrowheads in Fig. 1) as well as in the central area where the sensory nerve forms the annulospiral ending around the intrafusal muscle fibers (arrows in Fig. 1). In the central region, AChRs are precisely codistributed with the sensory nerve ending, demonstrating an aggregation of AChRs at the contact site between sensory neuron and intrafusal fiber. These results show that Thy1-YFP mice are well suited to investigate the sensory and motor innervation of muscle spindles and that AChRs are subcellularly concentrated at the motor- as well as at the sensory neuro-muscular contact sites.

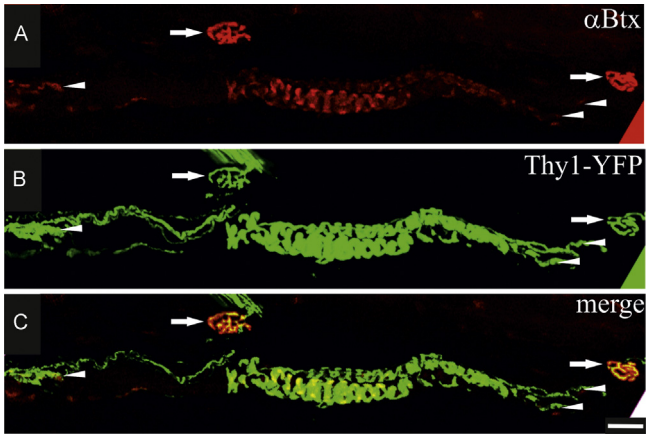

Fig. 1. AChRs are concentrated at γ-motoneuron endplates and in the equatorial region of intrafusal muscle fibers. A confocal z-stack of an adult soleus muscle spindle is shown after labeling AChRs with α-bungarotoxin (αBtx, A). The sensory- and motoneurons were genetically labeled by Thy1-YFP expression (B). AChRs are aggregated in the polar region of intrafusal fibers where they colocalized with the endplate of the γ-motoneuron (indicated by arrowheads in all panels) and in the central (equatorial) region at the contact site between the intrafusal fiber and the sensory nerve terminal. Two neuromuscular junctions on extrafusal fibers are indicated by large arrows. Scale bar in C: 20 μm.

To confirm the presence of cholinergic specializations at annulospiral endings we investigated the distribution of other markers for cholinergic synapses. To this end we analyzed the distribution of the vesicular acetylcholine transporter (VAChT; responsible for loading of acetylcholine into synaptic vesicles), choline acetyl transferase (ChAT; the key enzyme for acetylcholine synthesis), acetylcholine esterase (AChE; the key enzyme for acetylcholine breakdown), and the AChR-associated protein rapsyn (which forms a stoichiometric complex with AChRs and localizes the receptor to the postsynaptic membrane) at sensory nerve endings (Fig. 2). These proteins have previously been shown to be concentrated at the adult neuromuscular junction (NMJ) which therefore served as an internal control. As shown in Fig. 2, VAChT, ChAT and rapsyn were concentrated in the central region of muscle spindles at the contact site between the sensory nerve terminal (selectively labeled by antibodies against VGluT1; Wu et al., 2004) and intrafusal muscle fiber. In contrast, AChE immunoreactivity was not clustered at the sensory nerve endings, but was detected around the entire intrafusal fiber (Fig. 2(G), (H), (I)). Double-labeling of muscle spindles with anti-laminin and anti-AChE antibodies showed a colocalization of both proteins (data not shown), suggesting that AChE is associated with the plasma membrane or with the basal lamina surrounding intrafusal fibers. Collectively, these results indicate that the contact site between sensory nerve terminal and intrafusal muscle fiber contains a number of molecules indicative of cholinergic synapse-like specializations.

The previous results had revealed a concentration of molecules indicative of cholinergic synaptic specializations at the contact site between intrafusal fiber and sensory- and motoneurons, respectively. To investigate whether these sites also contain molecules involved in presynaptic vesicle exocytosis, we analyzed the distribution of bassoon, a component of the presynaptic active zone cytomatrix of the neuromuscular junction and of synapses in the CNS (Juranek et al., 2006; Chen et al., 2012; Gundelfinger and Fejtova, 2012). Bassoon immunoreactivity at the NMJ and at γ-motoneuron endplates was punctate (Fig. 3(A)–(E)). At the NMJ these puncta colocalized with voltage-dependent calcium channels and correspond to a concentration of bassoon at presynaptic active zones (Chen et al., 2011, 2012). Interestingly, we also detected punctate bassoon immunoreactivity clustered in the equatorial region of intrafusal fibers where it partially overlapped with anti-VGluT1 immunoreactivity (Fig. 3(C), (F)), indicating the presence of active zone-like structures also in the region of proprioceptive sensory neuron terminals. Staining of postnatal DRGs with antibodies against parvalbumin and bassoon demonstrates an expression of bassoon in proprioceptive neurons (see Supplementary Fig. S1). Although we cannot exclude a transport of bassoon selectively to synapses in the spinal cord, this result is consistent with the hypothesis that the bassoon immunoreactivity observe in the central region of intrafusal fibers is due to the presence of the protein in the sensory nerve terminal.

The bassoon puncta within the three different myo-neuronal contact sites were evenly distributed. To further characterize the bassoon puncta, we determined their density as well as their diameter. We detected similar densities of bassoon puncta at NMJs and at γ-motoneuron endplates (2.4 and 2.7 puncta per μm² for NMJs and γ-motoneuron endplates, respectively; Fig. 3G). These values agree well with the previously determined density of 2.6 puncta per μm² at the adult NMJ (Chen et al., 2012). In contrast, a significantly smaller number of bassoon puncta was detected at sensory nerve endings (1.4 puncta per μm²; Fig. 3(G)), suggesting that the density of bassoon-immunopositive active zone-like structures in sensory nerve endings is considerably lower compared to NMJs and to γ-motoneuron endplates. We also determined the diameter of the bassoon puncta to investigate if the

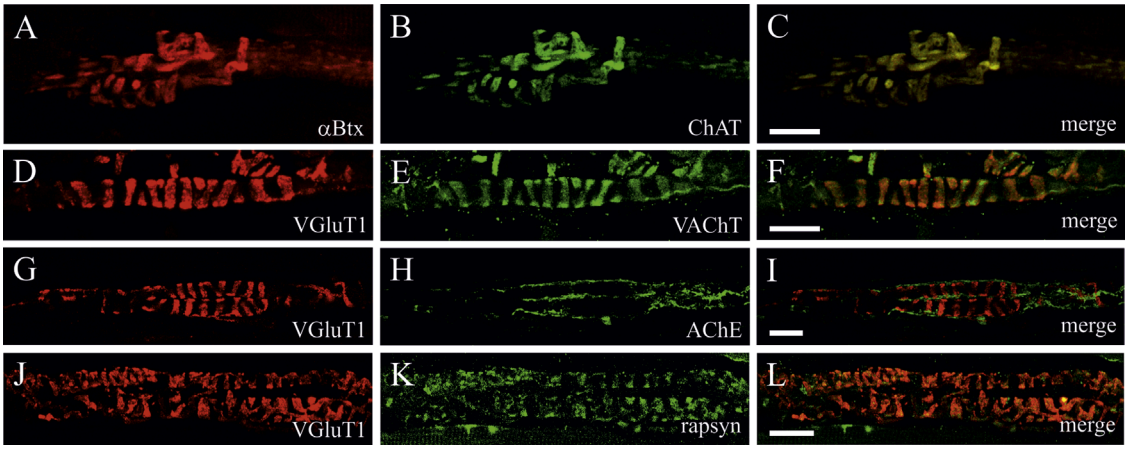

Fig. 2. The contact site between sensory nerve terminals and intrafusal fibers contains cholinergic synapse-like specializations. The distribution of choline acetyltransferase (ChAT, B), vesicular cholinacetyltransporter (VChAT, E), acetylcholine esterase (AChE, H) and the acetylcholine receptor-associated protein rapsyn (K) was analyzed in the equatorial region of intrafusal fibers. The sensory nerve terminal was visualized using α-bungarotoxin (αBtx, panel A) or antibodies against the vesicular glutamate transporter-1 (VGluT1, panels D,G,J). The merged pictures (panels C,F,I,L) demonstrate that with the exception of AChE, all proteins were concentrated at the contact site between sensory nerve terminal and intrafusal muscle fiber. In contrast, AChE immunoreactivity surrounded the intrafusal fibers and was apparently not concentrated in the contact region between sensory neuron and intrafusal muscle fiber (panel I). Scale bar in C,F,I,L: 20 μm.

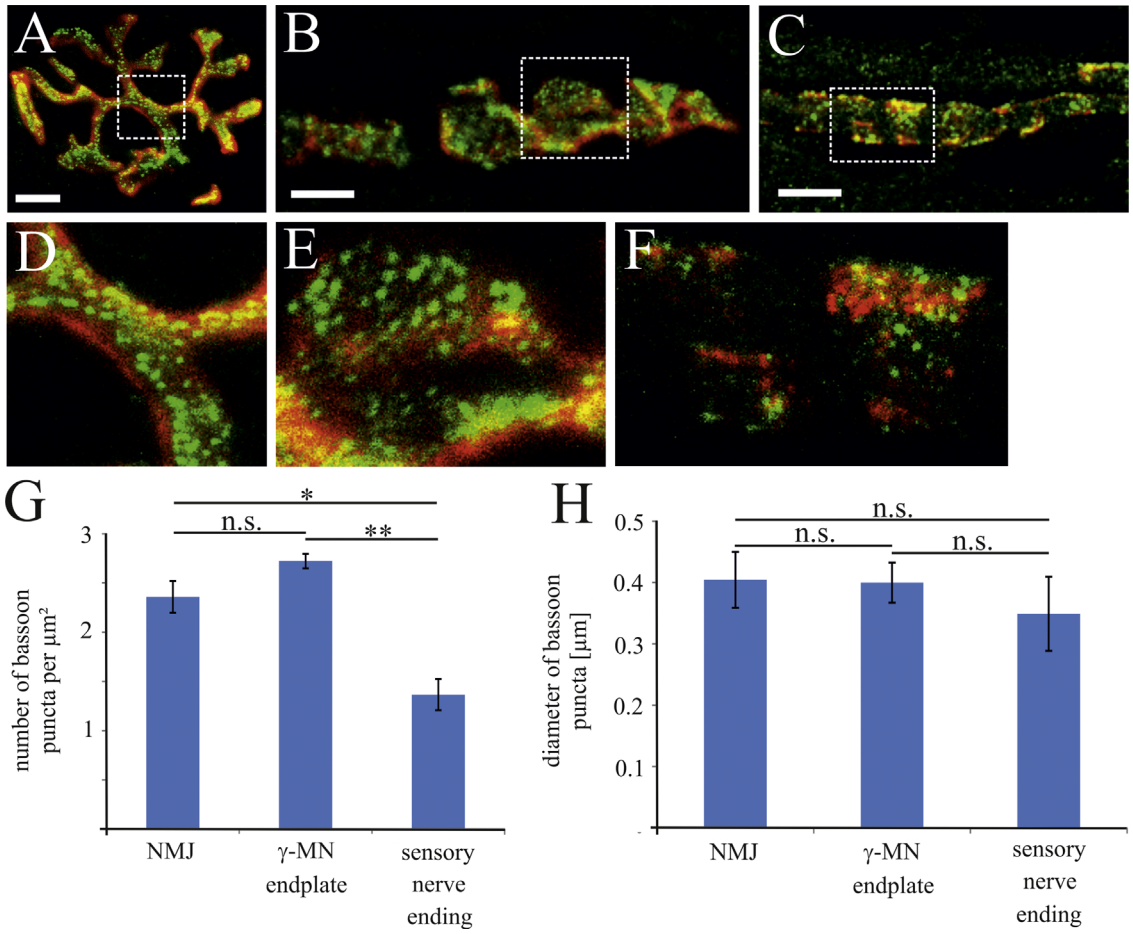

Fig. 3. Punctate bassoon-immunoreactivity is concentrated at the neuromuscular junction, the γ-motoneuron endplate and at sensory nerve terminals. The presynaptic active zone-specific protein bassoon was visualized by fluorescent immunohistochemistry using an anti-bassoon antibody (green channel in panels A–F). AChRs were labeled using the Alexa594-conjugated α-bungarotoxin (red channel in panels A,B,D,E) and the sensory nerve terminal was stained with anti-VGluT1 antibodies (red channel in panels C and F). Panels A–C show maximum intensity projections of serial confocal sections of a NMJ, a γ-motoneuron endplate and of a sensory nerve terminal, respectively. Panels D–F show high magnifications of the regions indicated by white dashed boxes in panels A–C. Note the punctate distribution of bassoon at the neuromuscular junction (panels A,D), at the γ-motoneuron endplate (panels B,E) and at the sensory nerve terminal (panels C,F). The density of bassoon puncta was similar at the NMJ and at the γ-motoneuron endplate, but considerably lower at the sensory nerve ending (G). The diameter of the bassoon puncta was not statistically different (n.s.) at the NMJ, γ-motoneuron endplates and sensory nerve endings (H). The error bars in G and H represent the mean +/− SEM with $N=3$. Scale bar in A,B: 5 μm; C: 10 μm.

active zone-like structures have a different size in the three types of nerve endings. The diameter of the bassoon puncta at the NMJ, the γ-motoneuron endplate and the sensory nerve terminal was not significantly different (0.40, 0.39 and 0.35 μm for the NMJ, the γ-motoneuron endplate and the sensory nerve terminal, respectively; Fig. 3(H)). These results demonstrate bassoon puncta in the equatorial region of intrafusal fibers with a similar size but a lower density compared to the NMJ and to the γ-motoneuron endplate, suggesting the presence of a specialized cytomatrix reminiscent of presynaptic terminals in the region of sensory nerve endings.

During the first 2 weeks of postnatal development of the neuromuscular junction the adult type AChR (containing the ε-subunit) gradually replaces the fetal, γ-subunit-containing AChR (Missias et al., 1996). This γ-to-ε switch transforms a receptor with a long mean open time and low conductance to a receptor with short open time, larger conductance, decreased inactivation time and a 3-fold increase in calcium conductance (Brenner and Sakmann, 1978; Mishina et al., 1986; Villarroel and Sakmann, 1996). We determined whether a similar γ-to-ε switch occurs in muscle spindles using subunit-specific antibodies. To this end, we first analyzed muscle spindles from early postnatal stages (P2-P5). At this age, murine intrafusal muscle fibers have developed and the muscle spindles respond to stretch (Kozeka and Ontell, 1981; Maeda et al., 1985; Chen et al., 2003). However, the sensory endings are morphologically immature and the adult annulospiral structure has not yet completely differentiated (Maeda et al., 1985; Kucera et al., 1988). Instead the sensory nerve terminals have formed a dense "spider-web-like" network which will subsequently transform into the adult annulospiral endings (Maeda et al., 1985). We detected the AChR γ-subunit at γ-motoneuron endplates, at neuromuscular junctions, and at annulospiral endings in early postnatal muscle spindles (Fig. 4(A)–(F)). In contrast, the expression of the AChR ε-subunit was below detectable levels at γ-motoneuron endplates and at sensory nerve terminals as well as at the NMJ (Fig. 4(G)–(L) and data not shown). This further confirms the presence of AChRs at the contact sites between intrafusal muscle fiber and sensory- as well as γ-motoneurons. Moreover, our results demonstrate that at both sites, the fetal AChR is expressed during early postnatal stages.

We next investigated if the fetal AChRs are replaced by the adult-type of AChRs during subsequent development by analyzing the expression of the AChR ε-subunit. Antibodies specifically detecting the ε-subunit stained adult (three month old) α- and γ-motoneuron endplates (data not shown) as well as at the adult annulospiral sensory nerve endings (Fig. 5(A)–(C)), consistent with the presence of the adult-type AChR at these sites. Interestingly, while the γ-subunit was apparently absent from adult NMJs and from adult γ-motoneuron endplates (Fig. 5(D)–(F) and data not shown), it remained detectable at the equatorial region of adult muscle spindles (Fig. 5(G)–(I)), demonstrating the persistent expression of the γ-subunit in the central part of intrafusal muscle fibers. These results indicate the simultaneous presence of the fetal and adult type of AChR at adult annulospiral sensory nerve endings.

To independently confirm the presence of the fetal type of AChR at adult sensory endings, we investigated muscle spindles from mice in which the γ-subunit of the AChR was genetically labeled using GFP (Gensler et al., 2001; Yampolsky et al., 2008). As expected, the AChR γ-subunit was detectable at P0 NMJs, γ-motoneuron endplates and at the contact site between intrafusal fiber and sensory neuron (data not shown). In adult muscle spindles from these mice, however, the AChR γ-subunit was absent at γ-motoneuron endplates and neuromuscular junctions but remained detectable at annulospiral endings (Fig. 5(J)–(L); Yampolsky et al., 2008), confirming our previous results using subunit-specific antibodies. In summary, our results demonstrate the subcellular concentration of AChRs at sites of sensory and motor innervation of intrafusal fibers and show a complete postnatal replacement of the γ- by the ε-subunit at the γ-motoneuron endplates and a continuous presence of the γ-subunit despite expression of the ε-subunit at adult annulospiral sensory nerve endings. Thus, the maturation of the fetal to adult AChR is fundamentally different in the central and the polar regions of intrafusal fibers.

Discussion

The development of muscle spindles includes the establishment of cholinergic synapses between γ-motoneurons and intrafusal fibers as well as the formation of specialized sensory nerve endings necessary for the reliable and sensitive detection of

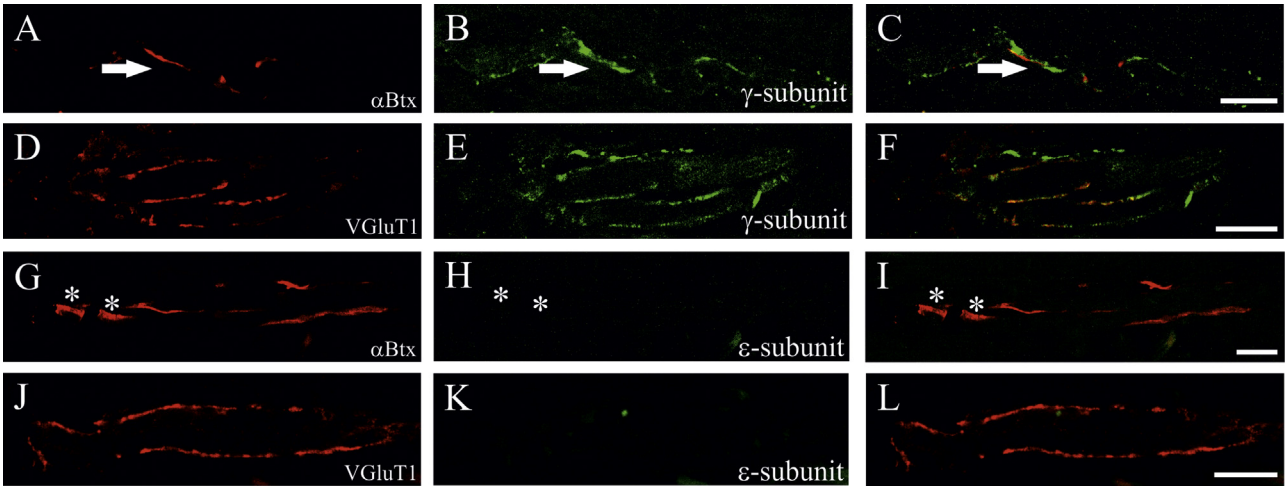

Fig. 4. Acetylcholine receptors in the equatorial and polar regions of postnatal muscle spindles contain the γ-subunit but lack the ε-subunit. The polar regions (A–C and G–I) of muscle spindles were analyzed using Alexa594-conjugated α-bungarotoxin to label the γ-motoneuron endplate and antibodies against the AChR γ-subunit (panel B,E). Muscle spindles were from postnatal day 5 (panels A–F) or postnatal day 2 (panels G–L) mice. The equatorial region of the same muscle fibers were labeled by antibodies against VGluT1 (panels D,J) and against the ε-subunit (panel H,K). The merged pictures (panels C,F,I,L) show that the AChR γ-subunit was concentrated at γ-motoneuron endplates (C) where it colocalized with α-bungarotoxin-labeled AChRs and at the equatorial region of intrafusal fibers where it precisely colocalized with VGluT1 (F). In contrast, the ε-subunit was absent from early postnatal γ-motoneuron endplates (labeled by asterisks in panels G,H,I) as well as from the equatorial region (L). Scale bar: C,F,I, L: 20 μm.

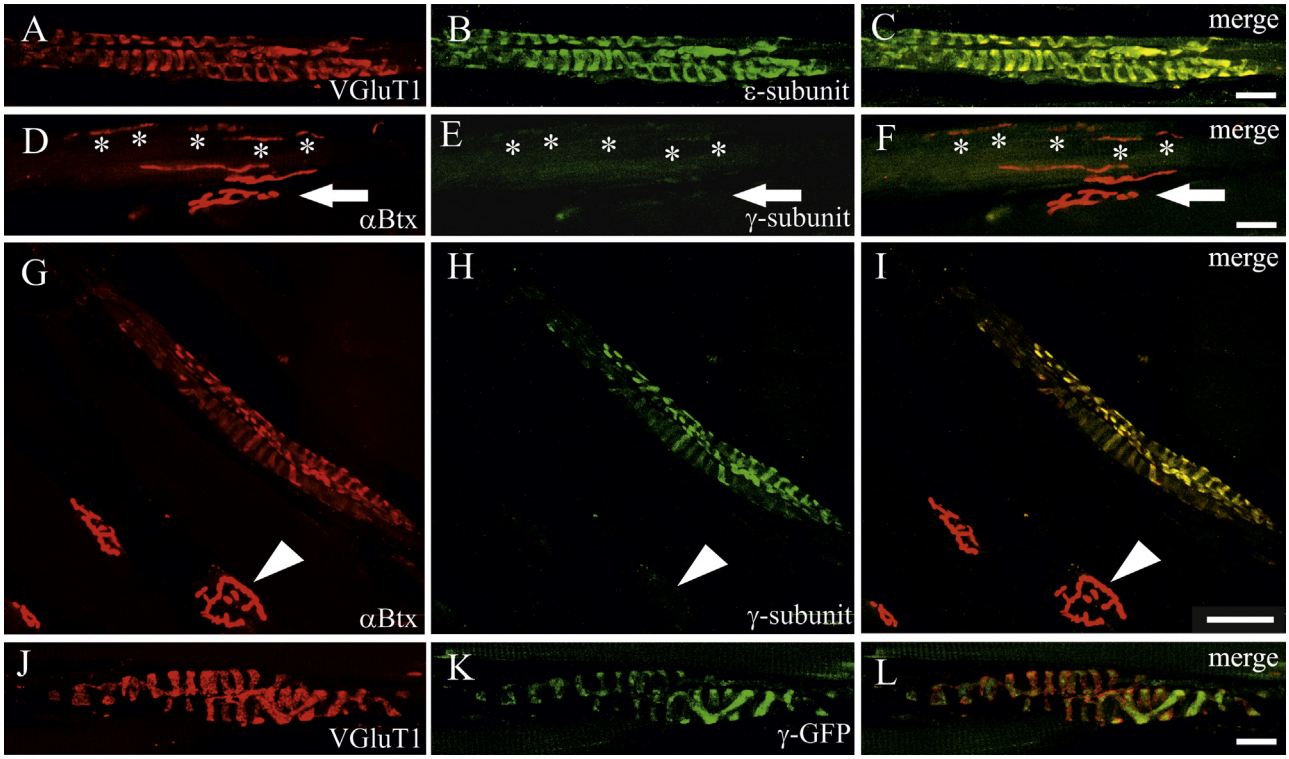

Fig. 5. The AChR γ- and ε-subunits are simultaneously expressed in the equatorial region of adult muscle spindles. The polar and equatorial regions of adult (2 month old) intrafusal fibers were marked by Alexa594-conjugated α-bungarotoxin (αBtx) and VGluT1, respectively, and by anti-ε- (B) and anti-γ-subunit-specific antibodies (E,H). The AChR ε-subunits were concentrated at sensory nerve terminals and precisely colocalized with the VGluT1 immunoreactivity (A–C). The γ-subunit was not detectable at AChR aggregates of adult γ-motoneuron endplates (asterisks in panels D–F) and at neighboring adult neuromuscular junctions (arrows in panels D–F). In contrast, the γ-subunit remained detectable at the equatorial region of intrafusal fibers (panels G–H) but was conspicuously absent from neighboring neuromuscular junctions (arrowheads in panels G–I). Analysis of intrafusal muscle fibers from 2 one year old $AChR^{\gamma GFP/\gamma GFP}$ mice confirmed the expression of the γ-subunit at the equatorial region of adult muscle spindles (K) where it colocalized with the sensory nerve terminal marker VGluT1 (J,L). All panels show maximum intensity projections of serial confocal optical sections. Scale bar C,F,L: 20 μm; I: 50 μm.

muscle fiber length and changes thereof (Maier, 1997). In this study we show (1) that sensory- and motoneurons are specialized in the region of contact to the intrafusal muscle fiber, containing high concentrations of AChRs, rapsyn and other markers of cholinergic synapses, (2) that the equatorial region of intrafusal fibers contains molecules indicative of a presynaptic specialization and vesicle exocytosis, (3) that at the γ-motoneuron endplate AChRs undergo a complete postnatal γ-to-ε switch in their subunit composition, similar to the postnatal AChR maturation at the NMJ, and (4) that at the sensory nerve endings the AChR γ-subunits are present during embryonic and early postnatal development and continue to be expressed in adult spindles despite the postnatal increase in ε-subunit expression, leading to the simultaneous presence of fetal- and adult-type of AChRs at the adult annulospiral sensory nerve endings. Thus, both types of nerve-muscle contacts sites differ in their development with respect to the maturation of the AChR subunit composition.

We were unable to determine the exact subcellular localization of the AChRs and of bassoon at the contact site between sensory nerve terminal and intrafusal muscle fiber because both cells are only separated by a narrow cleft of approximately 20 nm width, making it impossible by confocal microscopy to distinguish an association of the immunoreactivity with the muscle fiber membrane from a localization in the sensory nerve terminal. However, the presence of a reporter gene expressed under the control of regulatory elements from the α- and ε-subunit AChR genes in nuclei at the central region of intrafusal muscle fibers (Sanes et al., 1991) demonstrates that AChRs are expressed by intrafusal fibers, strongly suggesting that they are concentrated in their plasma membrane at the contact site to the sensory neuron. Likewise, the presence of bassoon immunoreactivity in parvalbumin-positive DRG proprioceptive neurons (see Fig. S1), as well as in the DRG neuron-derived F11 cell line (Goswami et al., 2010) together with the expression of bassoon mRNA in DRGs as detected by in situ hybridization (Diez-Roux et al., 2011; http://www.eurexpress.org/) strongly suggests an association of the bassoon immunoreactivity with the sensory nerve terminal. In summary these results are consistent with the hypothesis that bassoon is concentrated in the sensory nerve terminal opposite to the AChRs localized in the intrafusal muscle fiber plasma membrane.

The presence of proteins indicative of a presynaptic cholinergic synapse, including VAChT, ChAT or bassoon, at the contact site of the sensory nerve terminal is unexpected, since this structure is not a synapse but instead specialized for the sensitive and reliable detection of muscle stretch. However, our results are in agreement with previous studies that demonstrated the presence of 50 nm diameter clear synaptic-like vesicles which undergo calcium- and activity-dependent exo- and endocytosis in sensory nerve terminals of adult muscle spindles (Bewick et al., 2005). Moreover, synapsin I and synaptophysin have previously been detected in proprioceptive nerve endings (De Camilli et al., 1988; Simon et al., 2010), suggesting the presence of synaptic vesicles. At least some of the vesicles contain glutamate which is thought to modulate spindle sensitivity via neuron-associated metabotropic glutamate receptors (Bewick et al., 2005). Our results extend these studies by demonstrating the presence in sensory nerve terminals of key enzymes involved in acetylcholine synthesis and in concentrating acetylcholine in vesicles. The presence of these proteins suggests the possibility that the sensory nerve terminal might be capable of releasing acetylcholine-containing vesicles.

We did not observe differences in the size of bassoon puncta at the neuromuscular junction and at the endplate of γ-motoneurons, respectively, compared to sensory nerve terminals. However, the density of the puncta was smaller in sensory nerve terminals compared to γ-motoneuron endplates and NMJs. This difference in density might reflect a difference in the number of vesicle release sites. It is conceivable that the number of released vesicles at sensory nerve terminals is low, since a frequent exocytosis of synapse-like vesicles is not needed for the main function of the sensory nerve terminal, i.e. the detection of muscle stretch. Moreover, although the density of the bassoon puncta was lower in sensory nerve terminals compared to NMJs, the total number is likely to be higher, since the contact area of sensory nerve terminal to intrafusal fiber is larger compared to the area of nerve-muscle contact at neuromuscular junctions. This suggests a function of the vesicle exocytosis in the entire area of intrafusal muscle fiber sensory nerve contact region.

In contrast to other markers for cholinergic synapses, we were unable to detected AChE directly at sensory nerve terminals. Instead we found AChE staining at γ-motoneuron endplates as well as low intensity AChE immunoreactivity associated with the intrafusal muscle fiber surface. This is consistent with previous results showing the absence of AChE reaction product from the cleft between sensory nerve ending and intrafusal fiber and a low concentration of AChE in the equatorial region in cross sections from rat muscle spindles (Schober and Thomas, 1978; Gossrau and Grozdanovic, 1997). The resolution of confocal microscopy did not permit a precise subcellular localization and, therefore, we were unable to determine the structures AChE was associated with in the equatorial region of muscle spindles. Since the antiserum used in our study detected the collagen-tail forms as well as the membrane-associated forms of AChE and since the anti-AChE and anti-laminin immunoreactivity colocalized, it is possible that AChE is associated with the surface of intrafusal fibers and/or with the collagenous inner- and/or outer capsules, as has been shown in the polar region of muscle spindles (Schober and Thomas, 1978).

The function of AChRs and of the other proteins indicative of cholinergic synapse-like specializations at sensory nerve endings is currently unclear. Several studies have investigated the effect of ACh or succinylcholine at muscle spindles and have recorded a change in action potential frequency of the sensory nerve (for review see Carr and Proske, 1996). The balance of evidence suggests that the excitatory effects of the cholinergic agonists are the result of intrafusal muscle fiber contractures (mediated by AChRs at γ-motoneuron endplates in the polar region). However, another possibility supported by our study is a direct action of ACh released from sensory nerve terminals on intrafusal fibers (for a detailed discussion see Carr and Proske, 1996). Our results showing a concentration of AChRs and associated proteins together with the key enzymes for acetylcholine synthesis and ACh uptake into synaptic vesicles in the equatorial region of intrafusal fibers suggests that acetylcholine is synthesized and stored in vesicles by the sensory nerve terminal. This opens the possibility that ACh released from the sensory terminal might activate intrafusal fiber-associated AChRs in the equatorial region. While our studies favor a synapse-like role of AChRs at sensory nerve terminals, it remains possible that the AChRs have an (unknown) non-synaptic role, similar to the AChRs and associated proteins present at the myotendinous junctions (Fertuck and Salpeter, 1976; Cull-Candy et al., 1982; Chen et al., 1990; Bernheim et al., 1996). Clearly further studies are needed to clarify the role of the AChRs in muscle spindle function.

At γ-motoneuron endplates we observed a γ-to-ε subunit switch that appeared similar to the switch described at neuromuscular junctions (Missias et al., 1996; Yampolsky et al., 2008). We did not systematically investigate the detailed time course of the AChR maturation at γ-motoneuron endplates in different muscles, since even at the NMJ the time-course of the channel conversion is rather heterogeneous (Yampolsky et al., 2008). However, the switch in muscle spindles occurred within the first 2 postnatal weeks, around the same time as at neuromuscular junctions. Moreover, we observed an overall similar time course of AChR subunit switch in muscle spindles from several muscles, including soleus (primarily slow twitch fibers), quadriceps (primarily fast twitch fibers) and extensor digitorum longus (primarily fast twitch fibers), suggesting that the γ-to-ε switch in muscle spindles does not heavily depend on the preferential fiber type composition of the muscle (data not shown).

Our results reveal a simultaneous expression of the AChR γ- and ε-subunits in the central region of adult intrafusal muscle fibers, strongly suggesting the simultaneous presence of fetal and adult type of AChRs. The simultaneous expression of the γ- and ε-subunits in the equatorial part of intrafusal muscle fibers demonstrates that the up-regulation of the ε-subunit expression does not depend on the down-regulation of the AChR γ-subunit gene expression. Interestingly, the only mature innervated muscle to maintain a high expression of the fetal-type of AChR into adulthood due to ongoing transcription of the γ-subunit identified so far is a small subpopulation of multiply innervated fibers (MIFs) in extraocular muscles (Horton et al., 1993; Kaminski et al., 1996; Missias et al., 1996). It has been suggested that in these slow tonic muscles the γ-to-ε switch does not occur because of the special type of nerve stimulation it receives (Missias et al., 1996). MIFs develop tonic contractions and relax and contract more slowly than twitch fibers, but they do not generate action potentials (Kaminski et al., 1996). The longer open time and lesser resistance to desensitization of the embryonic type of AChR would allow muscle fibers expressing the γ-subunit to respond better to repeated or prolonged stimulation. Thus, the presence of fetal AChRs might be advantageous for the high neuronal firing frequencies observed in extraocular muscles. Whether this hypothesis also applies to muscle spindles and what the functional consequences of a persistent presence of a fetal-type of AChR at annulospiral sensory endings are, remains to be tested. It also remains to be determined why the γ-subunit expression is not down-regulated in the equatorial region of muscle spindles. At the neuromuscular junction the expression of the fetal AChR is thought to be locally down-regulated after innervation by reducing the expression of the γ-subunit gene due to nerve-induced electrical muscle activity (Goldman et al., 1988; Witzemann et al., 1991) and due to an unidentified nerve-derived neurotrophic signal (Kues et al., 1995). One possibility why the expression of the γ-subunit gene is not reduced in the equatorial part of muscle spindles might therefore be the electrical isolation of this region and the absence of a neuron-derived neurotrophic signal. Motoneuron-induced action potentials are not propagated from the polar to the equatorial region of intrafusal fibers (Hunt, 1990) and, thus, any signal downstream of these action potentials would not reach the equatorial region of intrafusal fibers. In any case, muscle spindles differ from multiple innervated extraocular muscle fibers in that a γ-to-ε switch occurs in the polar regions but not in the central equatorial region, thus, defining two subcellular areas within the same intrafusal fiber with different AChR maturation progression.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.07.011.

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