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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.

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