# The Development and Molecular Characterization of Muscle Spindles from Wildtype and Mutant Mice

Dissertation

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**Für meine Familie**

**For my family**

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Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbstständig und ohne unerlaubte Hilfe angefertigt wurde.

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## Abstract

Muscle spindles are complex stretch-sensitive mechanoreceptors that consist of 4-12 specialized muscle fibers. These intrafusal muscle fibers are innervated in the central (equatorial) region by an afferent sensory axon and in both peripheral (polar) regions by efferent γ-motoneurons. Until now little is known about muscle spindle development at the molecular level, especially about the development of cholinergic specializations. My study shows that nicotinic acetylcholine receptors (AChR) are concentrated at the γ-motoneuron endplate as well as in the equatorial region. Moreover, enzymes required for the synthesis and removal of acetylcholine, including choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), as well as vesicular acetylcholine transporter (VAChT) and the AChR-associated protein rapsyn are all concentrated at the polar γ-motoneuron endplate and (with the exception of AChE) also at the equatorial region. Finally, the presynaptic protein bassoon, involved in synaptic vesicle exocytosis, is also present at the γ-motoneuron endplate and at the annulospiral sensory nerve ending.

During postnatal development, the AChR subunit composition at the γ-motoneuron endplate changes from the γ-subunit containing fetal AChR to the ε-subunit containing adult AChR. This is similar to the postnatal change at the neuromuscular junction. In the equatorial region the ε-subunit expression starts around postnatal week two; however the γ-subunit persists in the central region despite the onset of the ε-subunit expression. Therefore, the γ- and ε-subunits are simultaneously present in the equatorial region. This result was confirmed using a mouse line in which the AChR γ-subunit was genetically labelled by green fluorescence protein (GFP). In this mouse, the GFP-labelled AChR γ-subunits are concentrated at the contact site of the intrafusal fiber with the sensory nerve ending. This result indicates different AChR maturation occurs within two areas of the same intrafusal fiber.

I also show that agrin and the agrin receptor complex (consisting of LRP4 and MuSK) are present in muscle spindles in the region of the sensory and motor innervation. Moreover, agrin, MuSK, and LRP4 are expressed by proprioceptive neurons in dorsal root ganglia but only agrin and LRP4 were detected in the cell body of γ-motoneurons in the spinal cord. In mice with a targeted deletion of agrin, AChR aggregates are absent from the polar region and γ-motoneuron endplates do not form. By contrast, AChR aggregates remain detectable in the central part of intrafusal

fibers. Moreover, muscle-specific re-expression of mini-agrin is sufficient to restore the formation of synaptic specializations at γ-motoneuron endplates. These results show an unusual AChR maturation at the annulospiral endings and confirm that agrin is a major determinant for the formation of γ-motoneuron endplates. Agrin on the other hand appears dispensable for the aggregation of AChRs in the central region of intrafusal fibers.

## Zusammenfassung

Muskelspindeln sind komplexe, dehnungsempfindliche Mechanorezeptoren, die aus vier bis zwölf spezialisierten Muskelfasern bestehen. Diese intrafusalen Muskelfasern werden im zentralen (äquatorialen) Bereich durch afferente Axone und in beiden peripheren (polaren) Regionen von efferenten γ-Motoneuronen innerviert. Über die Entwicklung von Muskelspindeln auf molekularer Ebene ist kaum etwas bekannt, vor allem was cholinerge Spezialisierung angeht. In meiner Doktorarbeit konnte ich zeigen, dass nikotinische Azetylcholinrezeptoren (AChR) an der neuromuskulären Endplatte von γ-Motoneuronen sowie in der äquatorialen Region konzentriert sind. Auch die Enzyme, die für die Synthese, den Transport und den Abbau von Acetylcholin verantwortlich sind, (Cholinazetyltransferase (ChAT), Azetylcholinesterase (AChE) und vesikuläre Azetylcholintransporter (VAChT) wurden in der polaren und der äquatorialen Region gefunden. Diese Ergebnisse zeigen, dass sowohl die sensorischen afferenten- als auch die motorischen efferenten Neurone im Bereich des Kontaktes mit den Intrafusalfasern cholinerg spezialisiert sind.

Während der postnatalen Entwicklung der neuromuskulären Endplatte verändert sich die Zusammensetzung der AChR Untereinheiten. Aus den γ-Untereinheit enthaltenden embryonalen AChRen werden ε-Untereinheit enthaltende adulte AChRen gebildet. Vergleichbare postnatale Veränderungen findet man auch an der neuromuskulären Endplatte der Extrafusalfasern. Im Gegensatz zu diesen Synapsen bleibt in den Intrafusalfasern die Expression der γ-Untereinheit im zentralen Bereich der Nervenfaser neben der Expression der ε-Untereinheit während der postnatalen Entwicklung erhalten. Diese fehlende gamma-zu-epsilon-Umschaltung wurde mit Hilfe von transgenen Mäusen bestätigt, bei denen die AChR γ-Untereinheiten mittels GFP genetisch markiert waren. Diese Ergebnisse zeigen, dass AChR γ-Untereinheiten in adulten intrafusalen Fasern dort konzentriert sind, wo sie Kontakt zu sensorischen Nervenendigungen haben.

Agrin und der Agrin Rezeptor-Komplex - bestehend aus MuSK und LRP4 (LDL Rezeptor-beziehend Protein) - konnten in Muskelspindeln im Bereich der sensorischen und motorischen Innervation nachgewiesen werden. Außerdem sind

Agrin und sein Rezeptor-Komplex in propriozeptiven Neuronen der Spinalganglien exprimiert, während nur Agrin und LRP4 in γ-Motoneuronen im Rückenmark zu finden sind. In Agrin knock-out Mäusen ist keine AChR Aggregation in der Polarregion zu finden, was zu Defekten in der Ausbildung der gamma-Motoeneuronen Endplatten führt. Im Gegensatz dazu sind die AChR Aggregate im zentralen Teil der intrafusalen Fasern nicht betroffen. Muskelspezifische Überexpression von Mini-Agrin reicht aus, um die Bildung von synaptischen Spezialisierungen in den Endplatten von γ-Motoneuronen wiederherzustellen.

Diese Ergebnisse zeigen eine ungewöhnliche AChR Reifung an den annulospiralen sensorischen Endigungen und bestätigen, dass Agrin, ein essenzieller Faktor für die Bildung der Endplatten von γ-Motoneuronen ist, während er nicht notwendig für die Aggregation von AChRs in der zentralen Region von intrafusalen Fasern zu sein scheint.

# 1. Introduction

Everyone is familiar with getting information from one's eyes, ears, nose, hands or tongue. Have you ever thought about obtaining input from a huge organ of our body – your skeletal muscles? The term proprioception was used to describe such a sense. The prefix proprio- comes from Latin proprius, meaning "one's own". Proprioception describes the continuous information flow to the brain about the relative positions of limbs and parts of the body as well as changes of their position. The main proprioceptive sensors are the muscle spindles, localized inside the skeletal muscles. Muscle spindles inform the brain about the location of the extremities in space and about the stretch generated in each muscle. Muscle spindles are sensitive to changes in muscle length and to the speed of these changes. In contrast, Golgi tendon organs are sensitive primarily to muscle tension.

The German neurologist M. H. Romberg first detailed the loss of proprioception in a patient with tabes dorsalis, a demyelinating type of syphilis (Romberg and Sieveking, 1853). He asked the patient with ataxia (loss of motor coordination) to stand and close his eyes. The patient then experienced complete loss of balance, suggesting a sensory abnormality, due to lack of his proprioceptors. This test was then named the "Romberg sign", and is now one of the most commonly used tests to diagnose proprioceptive abnormalities. So far only a few cases have been described with total proprioceptive defects (Cole and Sedgwick, 1992; Sacks, 1998). However, neuropathologic complications, like loss of sensory neurons and impairment of proprioception, have been reported in several diseases, such as infective disease leprosy (Khadilkar et al., 2008) and syphilis, degenerative disease like Parkinson's disease (Proske and Gandevia, 2012), as well as metabolic diseases including diabetes (Swash and Fox, 1974). In all cases the consequences are severe, demonstrating the importance of proprioception for everyday life.

## 1.1 Structure of muscle spindles

Muscle spindles were firstly characterized as sense organs by Sherrington in 1894 (Sherrington, 1894) as being approximately 6-10 mm long structures, which localize within skeletal muscle tissue. They consist of 4 to 12 specialized skeletal muscle fibers called intrafusal fibers. Intrafusal fibers are arranged in parallel with the force-producing extrafusal skeletal muscle (Gandevia and Burke, 2004) but do not generate significant force (see Figure 1A).

Using morphological criteria, intrafusal fibers can be subdivided into two different types: nuclear chain fibers, where the nuclei form a single chain along the central part of the fiber; and nuclear bag fibers, in which the nuclei are clustered in the equatorial region (Figure 1B). Both fiber types are surrounded by a connective tissue capsule. The nuclear chain fibers run entirely within this capsule and their diameter is around 10-12µm. The nuclear bag fibers are larger fibers with diameters of 20-25µm that extend beyond the capsule. Due to the high density of nuclei in the equatorial region, most of the contractile apparatus is replaced and only a circumferential ring of sarcomere is left in the subsarcolemmal region. The function of the accumulation of nuclei is still unclear. Based on the specificity of the myosin ATPase, nuclear bag fibers can be classified into bag1 and bag2 fibers (Banks et al., 1977; Ovalle, 1972). The bag2 fiber is always the largest in the capsule and therefore determines the overall size of muscle spindles. The number of intrafusal fibers varies considerably among species and muscles. For example, muscle spindles in the posterior cricoarytenoid muscle of marmoset contain only one intrafusal fiber per spindle (Desaki and Nishida, 2006). In mammals one muscle spindle usually consists of at least one bag1, one bag2 and more than four nuclear chain fibers.

Two connective tissue capsules enclose the entire intrafusal muscle fibers, nerves and blood supply, except in the polar region (Hunt, 1990). In the equatorial region, the outer capsule is enlarged and the inner capsule surrounds the intrafusal fibers. Between the outer and inner capsule, the capsule space is formed and filled with hyaluronate (Fukami, 1986). The outer capsule consists of a multilayer of cells covered by a basement membrane (Nakajima et al., 1968). Like the blood-brain-barrier, this membrane and the associated cells form a selectively permeable filter (Dow et al., 1980; Ovalle, 1976). The components of basement membranes belong to four protein families: collagen, nidogen, laminin and heparan sulfate proteoglycans. Each family has several isoforms (Timpl and Dziadek, 1986). The inner capsule cells form several layers around each intrafusal fibers (Adal and Cheng, 1980). The links between inner and outer capsule cells provide a network, which compartmentalizes the capsular space into small partitions (Adal and Cheng, 1980). Sherrington suggested that the capsular space might be confluent with the lymphatic system (Sherrington, 1894), however, further evidence for this remains to be demonstrated. Overall, the function of the capsule is still unknown.

Intrafusal muscle fibers are innervated by both sensory- and motoneurons. Afferent innervation of muscle spindles arises from 2 types of sensory neurons: Type Ia and type II afferents (Hunt, 1990). Both Ia and II afferent nerves are myelinated and have a diameter of 13-20µm (Purves et al., 2012). Fast Ia afferent nerve fibers (with 50-80m/s conduction velocity) innervate bag1, bag2 and chain fibers (Banks et al., 1982) and form the primary sensory nerve endings (Banks, 1986), which are coiled around the central (equatorial) region of the intrafusal fibers and have been named "annulospiral endings". In contrast, slow type II afferent sensory endings (with 30-70m/s conduction velocity) only innervate bag2 and chain fibers and form secondary sensory nerve endings (Banks et al., 1982). Ruffini described these nerve endings as "flower spray" endings (Ruffini, 1898). The secondary nerve endings are always localized in direct vicinity of the primary sensory nerve endings, close to the polar region (Barker, 1974). In my study about sensory nerve endings, I focused on the annulospiral endings, since there is no molecular marker available to identify the "flower spray" endings.

The cell bodies of the proprioceptive neurons constitute a minor fraction of all neurons in the dorsal root ganglion (DRG) and can be selectively labelled by antibodies against parvalbumin and the TrkC neurotrophin receptor (Arber et al., 2000; Hippenmeyer et al., 2002; Honda, 1995).

At both polar regions, intrafusal fibers are innervated by γ-motoneurons (Hunt and Kuffler, 1951a). The cell bodies of the efferent γ-motoneurons are localised in the ventral horn of the spinal cord (Ashrafi et al., 2012; Friese et al., 2009; Shneider et al., 2009). According to their morphology, the motor endings were classified into plate endings and trail endings; the former ones being usually found on bag fibers and the latter being found on chain fibers (Banks, 1981; Banks et al., 1985). Bag1 fibers are always innervated by dynamic axons, which are sensitive to the changes in muscle length; whereas Bag2 and chain fibers are always innervated by static axons, which are sensitive to the change of the muscle tone (Bessou and Pages, 1975; Boyd et al., 1977). Additionally, Arbuthnott et al. (1982) showed that morphologically the γ-motoneuron endplates also have junction folds in addition to AChR aggregates and a basement membrane in the synaptic cleft, similar to the neuromuscular junction (NMJ) (Arbuthnott et al., 1982).

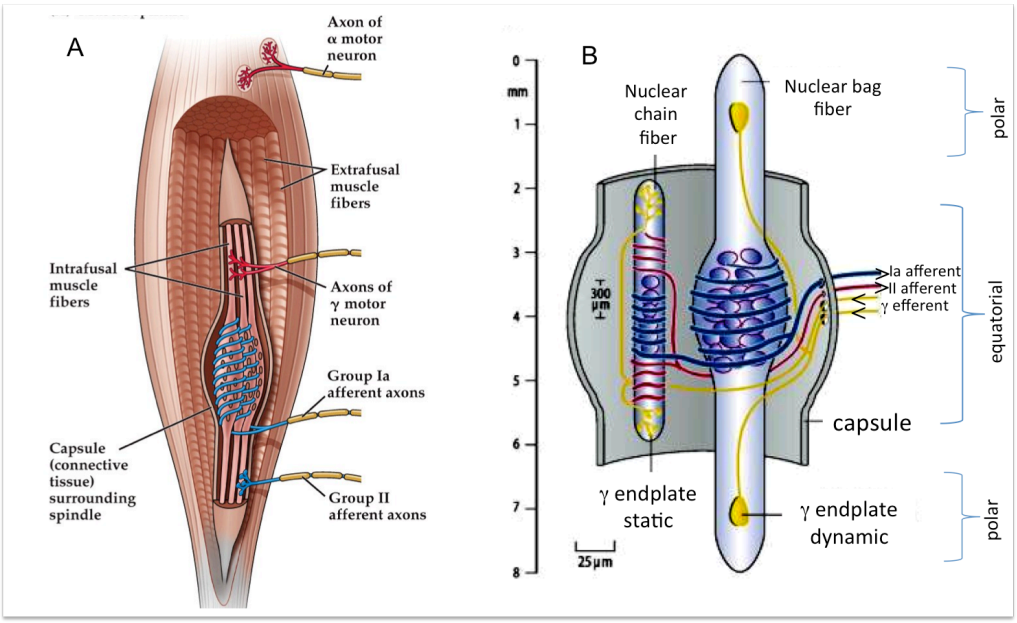



Figure 1. Structure of muscle spindles.

(A). Intrafusal fibers are found in parallel to extrafusal fibers. They are surrounded by a connective tissue capsule. Adapted from Purves et al. (2012). (B) Two types of intrafusal fibers can be distinguished: nuclear bag and nuclear chain fibers. Intrafusal fibers are innervated by efferent motoneurons in both polar regions. In the equatorial region the nuclear bag fibers are innervated by Ia proprioceptive sensory nerve endings and the nuclear chain fibers are innervated by both Ia and II sensory nerves. Adapted from Schmidt et al. (2007).

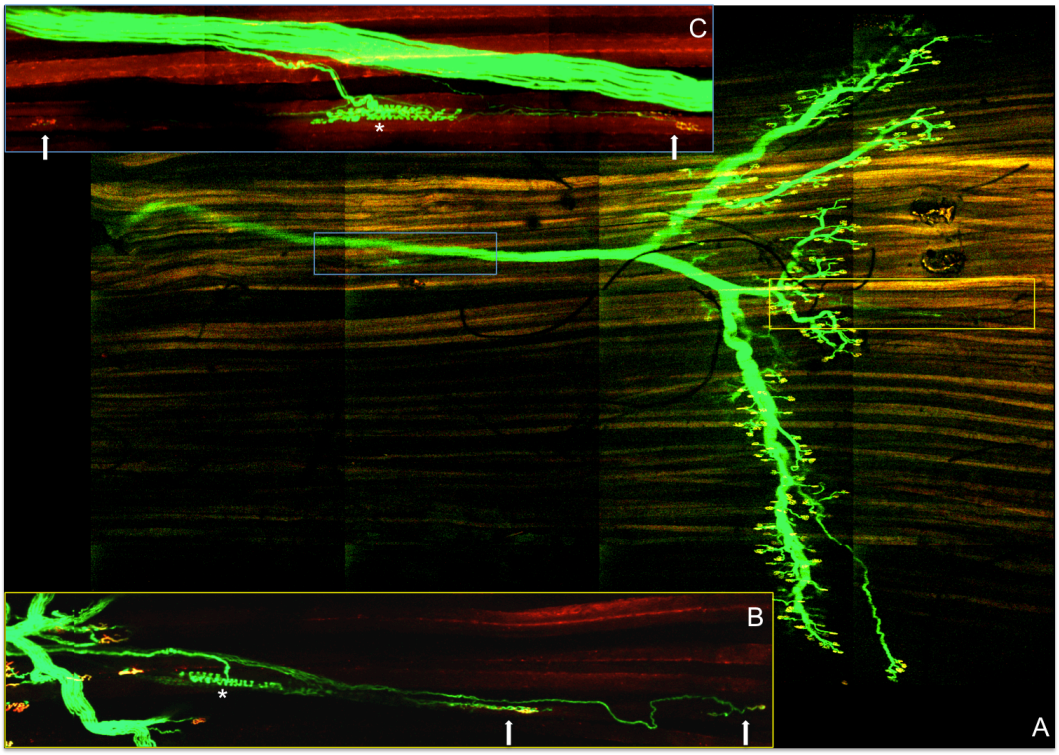



Figure 2. Distribution of muscle spindles in skeletal muscle.

Confocal image of a whole-mount mouse ETA muscle (*musculus epitrochleoanconeus*). The yellow fluorescence protein YFP (in green) labels all moto- and sensory neurons and Alexa 594-conjugated alpha-bungarotoxin (α-BTX, red channel) labels AChRs. In the whole ETA muscle only two muscle spindles can be detected (labelled by yellow and blue boxes in A). (B and C) Arrows show the endplate of γ-motoneurons at the polar region. Asterisks indicate the annulospiral sensory nerve endings.

## 1.2 **Functions of muscle spindles**

Muscle spindles serve as proprioceptors and have two main physiological functions: one associated with conscious motor control from the brain and the other with unconscious reflex control from the spinal cord (Purves et al., 2012).

The muscle spindle is responsible for detecting changes in muscle length and muscle tension. When a muscle is stretched, tension is placed on the intrafusal fibers, which activates unknown mechanically-gated ion channels (Proske and Gandevia, 2012). The presumable influx of $Na^+$ and possibly $Ca^{2+}$ leads to depolarization of the nerve ending and the formation of a receptor potential (Shepherd, 1983). This then induces the formation of action potentials where the frequency is proportional to the amount of stretch (static) and to the change of stretch over time (dynamic). Information about the stretch is sent through Clark's column (lower body) or directly (upper body) to higher centres of the brain (cerebellum and cortex), where proprioceptive information is integrated and the appropriate motor response is generated (Burke, 1980). Information about movement control from higher centres of the brain is sent through descending pathway (corticospinal tract and cerebellospinal tract) to the relevant skeletal muscles.

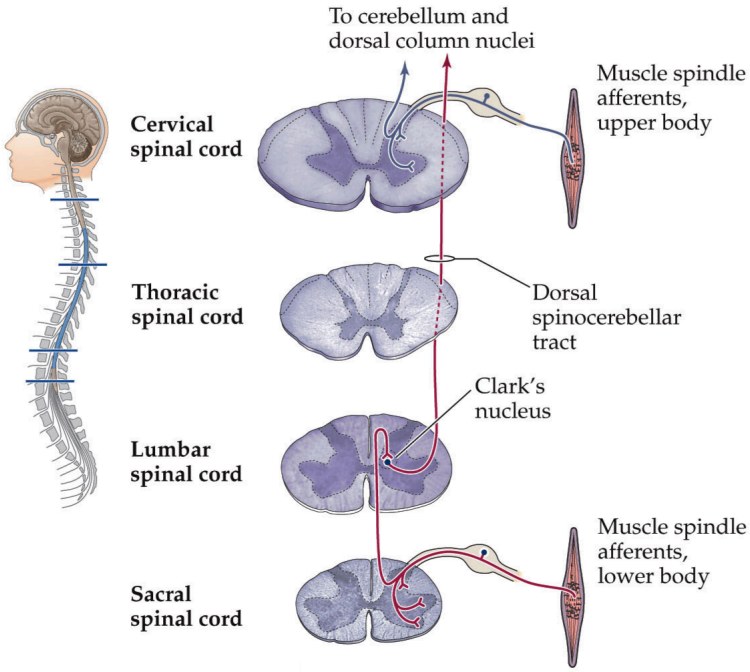



Figure 3. The ascending proprioceptive pathway for the lower and upper body.

Proprioceptive afferents from muscle spindles in the lower part of the body project to the dorsal and ventral horn of the relevant spinal cord level and project firstly to Clark's nucleus. Neurons in Clark's nuclei then transmit the information through the spinocerebellar tract to the cerebellum and to the dorsal column nuclei. Proprioceptive afferents from muscle spindles in the upper body also project to dorsal and ventral horn, and project directly to higher centres of the brain, without processing in Clark's nucleus (Purves et al., 2012).

Muscle spindles are part of the monosynaptic stretch reflex (myotatic reflex), which is a muscle contraction response to stretch within the same muscle. The monosynaptic stretch reflex is involved in the adjustment of posture and balance by regulating the muscle tone. The whole reflex arc consists merely of five components: sensor (muscle spindles), sensory afferent nerve, synaptic relay station in the spinal cord, motor efferent nerve, and effector (the relevant skeletal muscle; Figure 4A). The information regarding muscle stretch coming from muscle spindles is sent via sensory neurons to the spinal cord. The sensory nerve cell body is localized in the dorsal root ganglion (DRG). Its axon projects into the ventral horn of the spinal cord, where the motoneuron cell bodies are localized. There the only central synapse of the entire reflex is formed between proprioceptive neurons and the dendrites of α-motoneurons. Proprioceptive information is then transported by motoneurons to their neuromuscular junctions to control muscle movement and muscle tone of the homonymous muscle

(Figure 4A-C). At the same time, Ia afferent sensory nerves also activate interneurons, which inhibit the motoneurons innervating the antagonist muscles. The entire reflex response is rapid and without conscious control of the brain (Liddell and Sherrington, 1924). Under the control of lower centres in the spinal cord, the stretch reflex arc acts as a negative feedback loop to keep muscle length constant by regulating the muscle tone (Figure 4D).

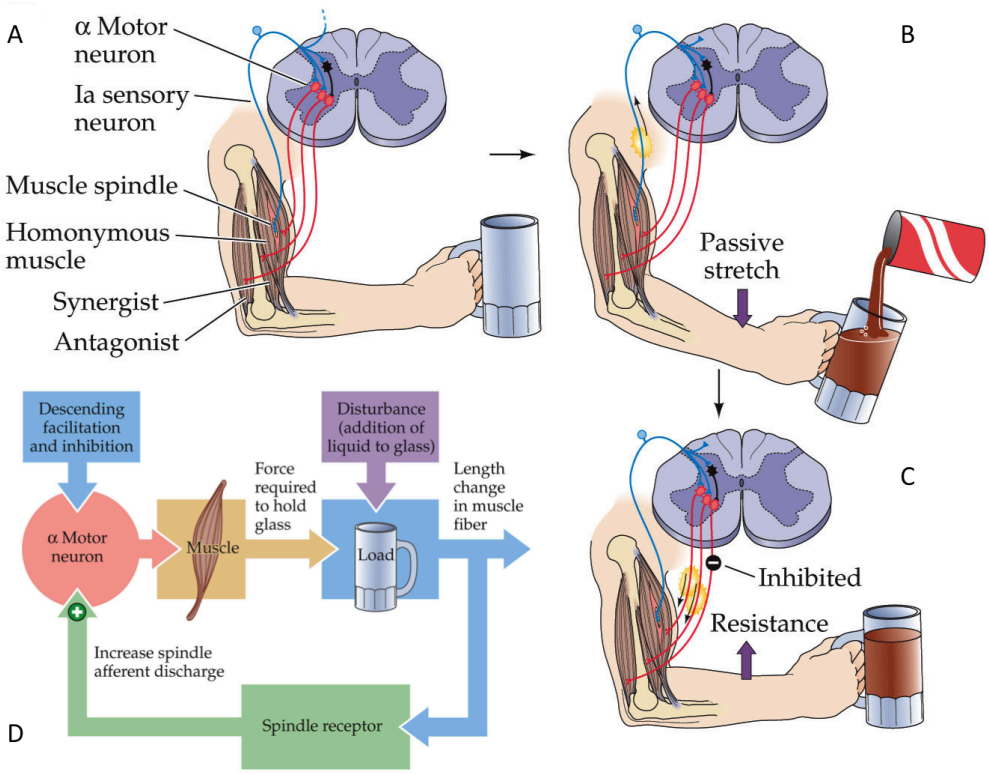



Figure 4. The monosynaptic stretch reflex and its function.

(A) The monosynaptic stretch reflex arc consists of muscle spindles, sensory neurons, spinal cord, α-motoneurons and homonymous skeletal muscles. (B and C) Passive stretch of muscle leads to the activation of Ia sensory nerve and active the motoneurons, which innervate synergistic muscles. Meantime, the Ia sensory nerve also activates inhibitory interneurons, which in turn inhibit the motoneurons, innervating antagonist muscles. (D) The monosynaptic stretch reflex acts as a negative feedback loop in regulating the length and tension of muscle (Purves et al., 2012).

While these findings explain the function of the sensory innervations of muscle spindles, the role of γ-motoneurons was unknown for a long time. In 1951 Hunt and Kuffler first described a short silent period of sensory nerve ending recording during the contraction of extrafusal fibers (Hunt and Kuffler, 1951a). They asked how the

activity of muscle spindles is kept constant even during contraction (where the stretch of the muscle fiber is absent). They subsequently discovered that γ-motoneurons control the sensitivity of muscle spindles by regulating the length of intrafusal fibers. When extrafusal fibers are stretched, intrafusal fibers are stretched simultaneously and information about stretch is transmitted by sensory nerve from muscle spindles to the spinal cord and to the motor centre in the brain. When extrafusal fibers contract, the length of extrafusal muscle fibers becomes shorter. This leads to passive loss in tension in the intrafusal fibers. The loose intrafusal fiber cannot response to stretch. The activation of γ-motoneurons causes the contraction of intrafusal fiber during the contraction of extrafusal fibers. Therefore, the co-activation of α- and γ-motoneurons maintains the tension of intrafusal fiber during the contraction of extrafusal muscle fibers. This ensures the sensitivity of the muscle spindle during all phases of muscle movement and tension regulation (Figure 5).

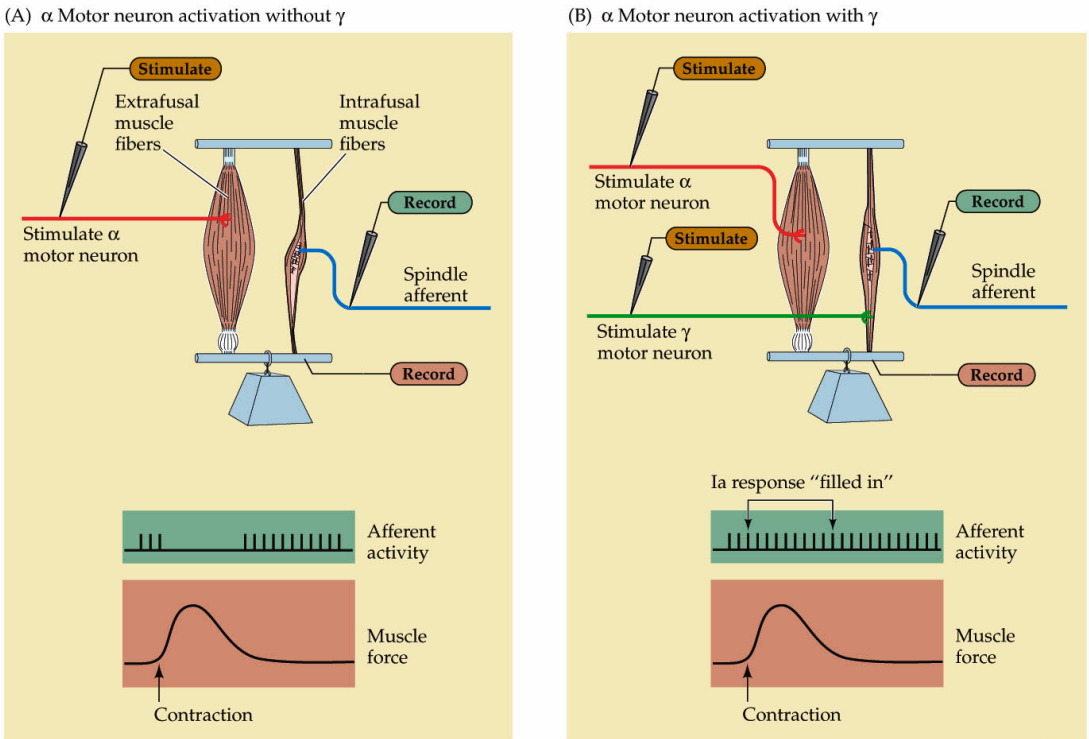



Figure 5. Schematic description of the role of γ-motoneuron in regulating muscle spindles during the contraction of skeletal muscle.

(A) When α-motoneurons are activated without the stimulation of γ-motoneurons, the response of Ia sensory afferent decreases and the intrafusal fiber is loose. (B) When γ -motoneurons are co-activated with α-motoneurons, Ia sensory afferent nerves remain activate during the contraction of the extrafusal fibers. Thus, the α- and γ-

motoneuron-coactivation ensures the activity of muscle spindles during all phases of contraction (Purves et al., 2012).

## 1.3 Development of muscle spindles in mice

At the beginning of myogenesis, myoblasts start to fuse and form primary myotubes (Williams and Goldspink, 1971). Both intrafusal and extrafusal muscle fibers are derived from these primary myotubes. At around E11 - E14 the initial contact between intrafusal fiber and sensory neuron is established (Chen et al., 2003). Between E15 and E17 the primary myotubes, which are contacted by sensory nerve endings, differentiate into intrafusal fibers (Soukup et al., 1995). The only currently available marker for this differentiation process is Egr-3, a zinc-finger transcription factor of the early growth response family (Tourtellotte et al., 2001). Later, additional molecular mechanisms contribute to muscle spindle development: the sensory nerve endings release neuregulin-1, which binds to the ErbB2/3 (erythroblastic leukemia viral oncogene homologue) receptors in the muscle fiber plasma membrane. This binding triggers an intracellular signalling pathway, leading to the final differentiation of intrafusal fibers as well as to their survival (Hippenmeyer et al., 2002)(Figure 6). Accordingly, adult ErbB2 knockout mice have no muscle spindles, causing ataxia and a waddling gait (Andrechek et al., 2002; Leu, 2003). Elimination of Ig-neuregulin-1, an isoform of neuregulin expressed in proprioceptive neurons, results in defects in muscle spindle development (Hippenmeyer et al., 2002). Moreover, the activity of neuregulin-1 relies on its cleavage by the protease β-secretase (Bace1). *Bace1* mutant mice show similar defects in motor coordination and lack muscle spindles at adult stages, indicating that Bace1 and neuregulin-1 regulate the formation and the maintenance of muscle spindle cooperatively (Cheret et al., 2013). On the other hand, intrafusal fibers provide neurotrophin-3 (NT3) to proprioceptive sensory neurons to ensure their survival during the time of naturally occurring cell death via the NT3 receptor TrkC. Accordingly, proprioceptive neurons are TrkC-positive neurons within the dorsal root ganglia (Hasegawa and Wang, 2008). Mice that lack NT3 or its TrkC receptor have no proprioceptive neurons in the DRG and these animals also do not develop functional muscle spindles (Ernfors et al., 1994; Klein et al., 1994). A functional afferent sensory innervation of intrafusal fibers therefore appears necessary for normal muscle spindle development. In contrast, selective deletion of γ-

motoneurons in mice shows no obvious phenotypes and muscle spindles develop normally (Shneider et al., 2009).

During the formation of muscle spindles the bag2 fiber differentiates first, followed by the bag1 fiber and finally the chain fibers (Milburn, 1984; Zelena, 1976; Zelena and Soukup, 1973). The molecular signals that lead to the differential formation of the different intrafusal fibers are unknown.

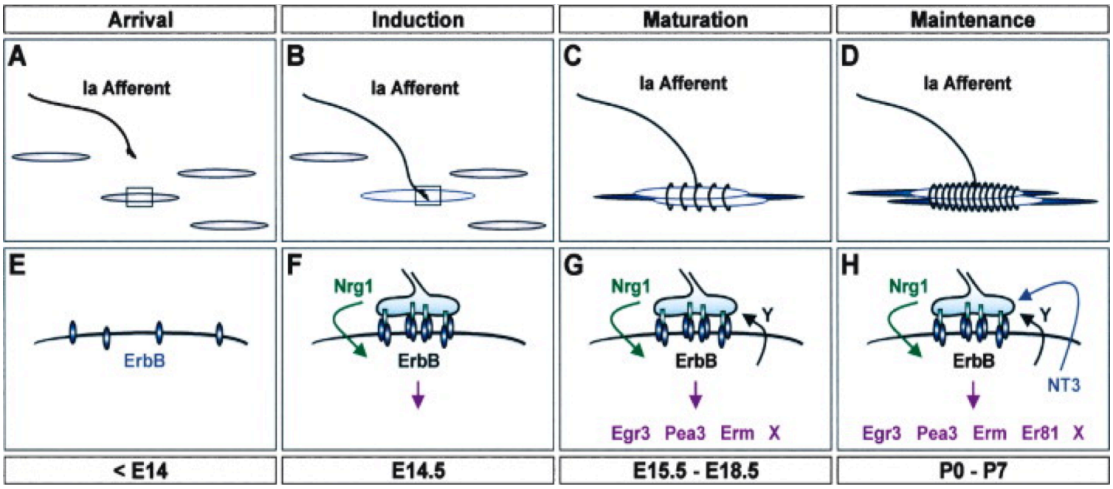



Figure 6. Development of muscle spindles.

Panels A-D show the development of an undifferentiated myotube into the fully differentiated intrafusal fibers. Panels E-H show the main molecular interactions involved in the differentiation process. (A and E) Before the arrival of Ia sensory fibers the primary myotubes that differentiate into intrafusal fibers are morphologically and molecularly similar to myotubes differentiating into extrafusal fibers. In the myofiber plasma membrane, ErbB receptors are diffusely distributed. (B and F) As soon as Ia sensory neurons contact the primary myotubes, Nrg1 is released by the Ia sensory afferent neurons, binds and activates ErbB receptors. This leads to the clustering of ErbB receptors and to the initiation of intrafusal fiber differentiation. (C and G) Subsequently, the Ia sensory nerve ending starts to branch (C). Nrg1-ErbB receptor signalling activates downstream transcription factors, such as Egr3, Pea3, Erm and possibly other yet unknown factors X (G). An unknown retrograde signal Y influences the branching and refinement of Ia sensory nerve endings (D and H). During the first postnatal week the Ia neuron starts to form the typical annulospiral endings (D). Further developed intrafusal fibers start to express intrafusal fiber-specific molecules, including NT-3 and Er81 (H). NT-3 acts retrogradely on the Ia afferent nerve and affects the survival of proprioceptive sensory neurons. Adapted from Hippenmeyer et al. (2002).

## 1.4 Synaptogenesis at the NMJ

Chemical synapses enable the transfer of information between neurons and between neurons and non-neuronal cells. During synaptogenesis, the pre- and post-synaptic membranes differentiate in direct apposition to each other. The presynapse develops many organelles and molecules, required for the synthesis, packaging and release of neurotransmitters. The postsynaptic site develops aggregated receptors and associated molecules to ensure the rapid and efficient response to the neurotransmitter as well as the termination of neurotransmission (Sanes and Lichtman, 1999). Due to its large size and its experimental accessibility, the neuromuscular junction is the best-characterized synapse in the entire nervous system.

During development of the NMJ, several key processes are required: one is the aggregation of acetylcholine receptors and the acquisition of cholinergic synaptic specializations at the contact site between muscle fiber and motoneuron. Another one is the molecular switch in the subunit composition of the AChR.

### 1.4.1 Cholinergic specialization at the NMJ

Acetycholine (ACh) was the first neurotransmitter identified (Loewi, 1921). Otto Loewi was awarded the Nobel Prize in Physiology or Medicine in 1936 for this discovery. Later research has shown that ACh is the excitatory neurotransmitter at neuromuscular junctions in all mammalian skeletal muscles (Campbell and Reece, 2002). For the synthesis of ACh, choline acetyltransferase (ChAT) is the key enzyme. ChAT is synthesized in the soma of motoneurons. It is then transported to the nerve terminal. The function of ChAT is to transfer acetyl-groups from acetyl-CoA to choline, leading to the formation of the neurotransmitter acetylcholine. Mice lacking ChAT show excessive nerve branching, a broader endplate band but normal AChR clusters. However, due to impaired cholinergic neurotransmission these mice die at birth (Brandon et al., 2003; Misgeld et al., 2002). After the synthesis ACh is concentrated into the presynaptic vesicles by the vesicular acetylcholine transporter (VAChT), where they are stored until release (Usdin et al., 1995).

In mammals the VAChT gene is contained entirely in the first intron of the ChAT gene (Cervini et al., 1995; Erickson et al., 1994). VAChT mRNA (Schafer et al., 1994) and VAChT immunoreactivity (Arvidsson et al., 1997) are detected in rat PNS (peripheral nervous system) and CNS (central nervous system) tissue. The VAChT mRNA shows a similar distribution pattern to cholinergic neurons. Mice with

deficient in VAChT primarily demonstrate deficient neurotransmission at the NMJ leading to symptoms reminiscent of myasthenia gravis (Prado et al., 2006).

After ACh binding to the postsynaptic AChRs, the ligand-gated channels open and a postsynaptic receptor potential is triggered, which leads to the formation of an action potential, which finally initiates muscle contraction via calcium influx from the sarcoplasmatic reticulum. Synaptic transmission is terminated by the rapid hydrolysis via acetylcholine esterase (AChE). The AChE present at NMJ is anchored by a collagen-like tail (ColQ) within the basement membrane of the synaptic cleft (Feng et al., 1999).

### 1.4.2 Regulation of the γ- to ε-subunit change.

The nicotinic AChRs (nAChRs) of NMJs consist of five subunits, two α, one β, one δ and either a γ- or a ε-subunit. During the first postnatal weeks the fetal form of the AChR ($\alpha_2\beta\gamma\delta$), containing a γ-subunit, is replaced by the adult form of AChR ($\alpha_2\beta\delta\varepsilon$), containing a ε-subunit (Missias et al., 1996). A reverse γ-to-ε-switch is observed after denervation (Missias et al., 1996).

The fetal form and the adult form of AChRs differ in their ion conductance and their channel open time (Mishina et al., 1986). The average conductance changes from about 40 pS in the fetal AChR to 60 pS in the adult AChRs. Especially the $Ca^{2+}$ conductance increases almost 3-fold during the postnatal development to adult AChRs (Villarroel and Sakmann, 1996). The average open time changes from 11 ms to 6 ms from the fetal to adult AChR (Mishina et al., 1986). Effects of point mutations in M1-M4 segments of γ- and ε-subunits indicate that the major amino acids which effect ion conductance are localized in the M2 transmembrane domain of each subunit (Herlitze et al., 1996). The key difference is that alanine/threonine in the M2 domain and lysine in the region flanking the M2 domain in the γ-subunits are replaced by the charged polar amino acid serine/isoleucine and glutamine in the ε-subunits (Herlitze et al., 1996).

## 1.5 **The Agrin/MuSK/LRP4 signalling pathway**

The molecular mechanisms underlying the formation, maintenance and regeneration of all synaptic specializations have been thoroughly investigated (Sanes and Lichtman, 1999). Any genetic and acquired deficiency of the molecules which are associated with the development of NMJs (e.g. agrin, MuSK, LRP4, Dok7, rapsyn)

leads to diseases. Genetic mutation can lead to congenital myasthenic syndrome, and acquired deficiency results in myasthenia gravis (Punga and Ruegg, 2012). Numerous studies over the past 30 years have demonstrated that the agrin/MuSK/LRP4 signalling pathway is necessary and sufficient for the formation, maintenance and regeneration of all synaptic specialization of the neuromuscular junction in vertebrates (Wu et al., 2010). As I have investigated the role of these molecules during muscle spindle development, I want to briefly introduce these synaptogenic molecules in the following paragraphs.

Agrin is a heparan sulfate proteoglycan, initially isolated from the basement membrane fractions of the synapse-rich electric organ of the marine ray *Torpedo californica* (Godfrey et al., 1984). It plays a central role in the formation and maintenance of the neuromuscular junction and directs postsynaptic specialization. The core protein of agrin is about 220 kDa in size. There are three laminin G-like domains at the C-terminal. The G1 and G2 domains of agrin can bind with the α-subunit of dystroglycan, the core protein of the dystrophin-associated glycoprotein complex (DGC). The G3 domain of agrin is mainly involved in the aggregation of AChR at NMJs (Figure 7).

At the N-terminus, alternative first exon usage results in the synthesis of either a soluble and secreted basement membrane-associated NtA-form of agrin, or in the synthesis of a transmembrane form of agrin (TM-agrin). Multiple isoforms of agrin are also generated by alternative splicing at the C-terminal end. These isoforms differ in their distribution and function. Depending on the number of inserted amino acids at the splicing sites of the C-terminal region, agrin has different isoforms with 0, 8, 11 or 19 amino acids inserts (Ruegg et al., 1992). This splice site is called “B” in chick or “z” in rodents. Isoforms with inserts (agrin B/z+ isoforms) are at least 10000-fold more active in AChR clustering and MuSK activation than isoforms lacking these inserts (agrin B/z0 isoforms; Gesemann et al., 1995; Ruegg et al., 1992). All motoneurons, myotubes and Schwann cells can express inactive isoforms of agrin (Fallon and Gelfman, 1989), whereas motoneurons and Schwann cells can secret active isoforms (Magill-Solc and McMahan, 1988; Yang et al., 2001a). In addition to muscle tissue, agrin is widely expressed in non-neural tissues, such as kidney glomerulus, blood vessels and the immune system.

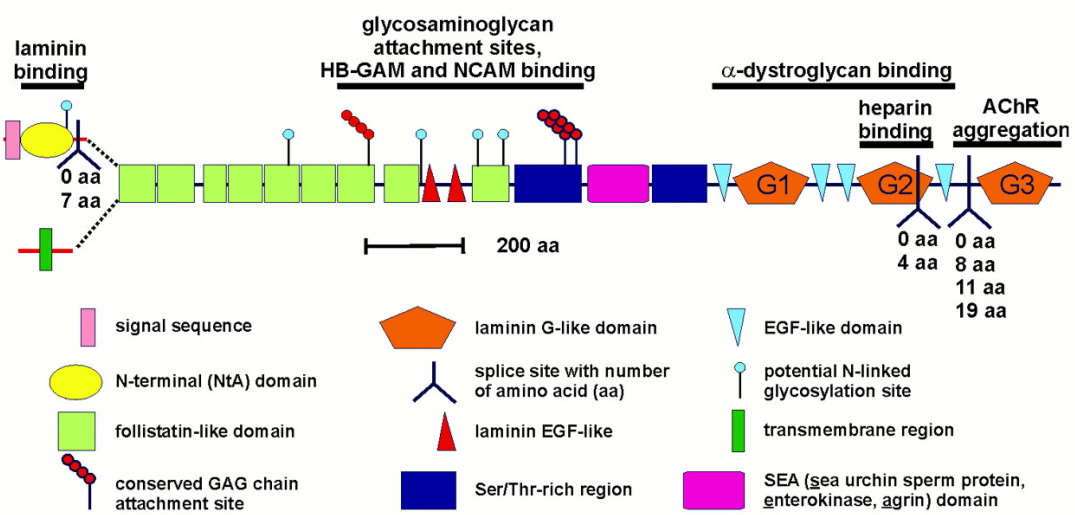



Figure 7. The domain structure of agrin predicted by the cDNA.

The protein domains of agrin are schematically represented by different boxes. At the N-terminus, secreted and transmembrane forms of agrin can be distinguished. Glycosaminoglycan attachment sites localize in the central region. Heparin and dystroglycan binding, and AChR activation sites are present in the C-terminal region. Alternative splicing sites are labelled and include the number of amino acids (Kröger and Schröder, 2002).

### 1.5.1 Functions of agrin

Previous studies showed that agrin induces the aggregation of AChRs and about 3-dozen other molecules in mouse skeletal muscle fibers (Burgess et al., 1999; Cohen et al., 1997) and in cultured muscle cells (Burgess et al., 1999; Godfrey et al., 1984). In skeletal muscle of agrin-deficient mice, the number, size and density of AChRs is dramatically reduced (Gautam et al., 1996; Lin et al., 2001). Moreover, loss of agrin from adult neuromuscular junctions leads to degeneration of these muscle fibers (Samuel et al., 2012). These results suggest that agrin is essential for the formation, stabilization and maintenance of AChR aggregates at NMJs. In addition to the aggregation of AChRs, agrin also regulates other aspects of postsynaptic differentiation, including aggregation of nuclei, synapse-specific gene expression, aggregation of AChE and more than 30 additional molecules, as well as the formation of junction folds (Kröger and Schröder, 2002). Moreover, agrin is required for the γ- to ε- subunits switch of AChRs (Jones et al., 1996; Rimer et al., 1997); binds to LRP4 and activates the intracellular signalling cascade by activation of the muscle-specific tyrosine kinase MuSK.

The transmembrane form of agrin (TM-agrin) is widely expressed in the developing CNS (Burgess et al., 1999; Annies et al., 2006; Annies and Kröger, 2002; McCroskery et al., 2006). In the adult mouse brain, TM-agrin keeps a high expression level in the regions where synaptogenesis remains active, including the lateral ventricle of the cortex, hippocampus (O'Connor et al., 1994) and olfactory bulb (Burk et al., 2012). However, the functions of agrin in the CNS are not fully understood.

### 1.5.2 The agrin receptor complex: MuSK and LRP4

The formation, maintenance and regeneration of the NMJ is mediated by the binding of agrin to a transmembrane protein complex, consisting of a tetramer of two proteins: the tyrosine kinase MuSK (muscle-specific kinase) (DeChiara et al., 1996) and the agrin binding component of the complex: the transmembrane protein LRP4 (Kim et al., 2008; Zhang et al., 2008).

MuSK is a tyrosine kinase with many substrates and was initially identified in the *Torpedo* electric organ (Jennings and Burden, 1993) and later found concentrated in the postsynaptic membrane at NMJs (Valenzuela et al., 1995). MuSK was initially identified as being required for NMJ formation (DeChiara et al., 1996; Gautam et al., 1999) and was soon considered to be part of the receptor of agrin – although it does not directly interact with agrin (Glass et al., 1996). Similar to agrin null mice, mice lacking MuSK have no AChR clusters and die perinatally, indicating that MuSK is required for the formation of NMJs (DeChiara et al., 1996; Lin et al., 2001; Yang et al., 2001b). For MuSK activation, dimerization must occur. Agrin induces this dimerization (Hopf and Hoch, 1998) and the autophosphorylation of MuSK (Glass et al., 1996). MuSK then mediates the postsynaptic differentiation via a complex signalling cascade which is only partially understood (Wu et al., 2010).

Although agrin and MuSK play an essential role in the formation and stabilization of NMJs, these two proteins do not interact directly (Glass et al., 1996). Instead, the agrin-binding part of the agrin receptor complex is a transmembrane protein, named low-density lipoprotein receptor-related protein 4 (LRP4) (Kim et al., 2008; Zhang et al., 2008). LRP4 is a member of the LDL receptor family. It contains an extracellular N-terminal region, a transmembrane domain, and a short intercellular C-terminal region without an identifiable catalytic domain (Johnson et al., 2005; Lu et al., 2007; Tian et al., 2006; Yamaguchi et al., 2006). The extracellular domain is sufficient to mediate NMJ formation (Gomez and Burden, 2011). LRP4 is also required for the

development of lung, kidney, limbs and ectodermal organs (Johnson et al., 2005; Simon-Chazottes et al., 2006; Weatherbee et al., 2006). Mutations in LRP4 lead to a failure of neuromuscular junctions formation (Weatherbee et al., 2006), abnormal development of the apical ectodermal ridge and polysyndactyly (Johnson et al., 2005) and limb development deficiency (Weatherbee et al., 2006). Moreover, anti-LRP4 autoantibodies have been shown to cause a reduction of AChRs at NMJs, leading to myasthenia gravis (Pevzner et al., 2012).

LRP4 is expressed in myotubes and is concentrated at NMJs (Zhang et al., 2008). In LRP4 -/- mice the formation of the NMJ failed, similarly as the mice lacking agrin and MuSK (Weatherbee et al., 2006). This result demonstrates that LRP4 is required for the formation of NMJs. In addition to myotubes, motoneurons apparently also express LRP4 (Wu et al., 2012). Muscle LRP4 acts in cis, and neuronal LRP4 in trans, to activate MuSK signalling (Wu et al., 2012). Taken together, these results show that LPR4 is necessary and sufficient for the agrin-induced MuSK activation and subsequent NMJ formation.

# 2 Aims of study

Muscle spindles are the most important proprioceptors in the human body. At the end of the 19th century and during the whole 20th century, scientists focused on the anatomy, the structure and the physiological function of muscle spindles leading to our current understanding of the function of muscle spindles in proprioception. However, the development of muscle spindles is only partially understood and the molecular regulators for many aspects of muscle spindle differentiation have not been identified.

In my thesis I have addressed the following questions:

1) What are the molecular components of the muscle spindle extracellular matrix?
2) How does the γ-motoneuron endplate in the polar regions develop and are the molecular determinants at this synapse similar to those described at the neuromuscular junction?
3) What are the molecular synapse-like specializations at the sensory nerve terminal and how do they develop?
4) Where are AChRs localized and how does the AChR subunit composition mature during muscle spindle development?
5) Are agrin and its receptor complex expressed in muscle spindles and what role do they play during development?

# 3 Materials and Methods

## 3.1 Chemicals

| Chemical | Producer/Manufacturer | Catalog - Nr. |
|---|---|---|
| Alexa Fluor® 594 conjugated α-bungarotoxin | Invitrogen | B13423 |
| Albumin Fraction V | Carl Roth GmbH + Co. KG | 8076.2 |
| Ampicilin | Gibco | 11593-019 |
| B-27® Supplements | Gibco | 17504-044 |
| Chromalaun, Chromkaliumsulphate $Cr^{3+}K^{+}(SO_4^{2-})$*12 $H_2O$ | Carl Roth GmbH + Co. KG | 4389.1 |
| Collagenase Type I, *Clostridium histolyticum* | Calbiochem | 24153 |
| D (+)-Saccharose | Carl Roth GmbH + Co. KG | 4621.1 |
| DAPI | Carl Roth GmbH + Co. KG | 6335.1 |
| Di-Sodium hydrogen phosphate dehydrate Na2HPO4 | Carl Roth GmbH + Co. KG | 4984.2 |
| Deoxynucleotide solution mix, dNTP 10mM | Biolabs | N0447s |
| Ethanol ROTIPURAN® min. 99.8% | Carl Roth GmbH + Co. KG | 9065.3 |
| Ethanol min. 99.8%, denatured | Carl Roth GmbH + Co. KG | K928.3 |
| Ethanol, 70% denatured | Carl Roth GmbH + Co. KG | T913 |
| DMEM/F12 medium | Gibco by Life Technologies® | 21331-020 |
| foetal bovine serum, FBS | PAA | A11-151 |
| Formaldehyde | Carl Roth GmbH + Co. KG | 2541 |
| Gelatine | Carl Roth GmbH + Co. KG | 4274.1 |
| Glycerin ROTIPURAN® | Carl Roth GmbH + Co. KG | 3783.1 |
| Glycin PUFFERAN® | Carl Roth GmbH + Co. KG | 3908.5 |
| HBSS | Gibco | 24020-091 |
| HCL ROTIPURAN® | Carl Roth GmbH + Co. KG | P074.1 |
| HEPES | Carl Roth GmbH + Co. KG | 9105.2 |

| | | |
|---|---|---|
| Ketaminhydrochlorid (Ketavet) 100 mg/mL | Pfizer | D3821-07 |
| L-Glutamate, 100x | Gibco | 25030-024 |
| Neurobasal medium | Gibco | 21003-049 |
| Neurotrophin-3 | R&D system | 267-N3-005/CF |
| NGF | R&D system | |
| Potassium chloride KCl | Carl Roth GmbH + Co. KG | 6781.3 |
| Potassium dihydrogen phosphate KH2PO4 | Carl Roth GmbH + Co. KG | 3904.1 |
| Proteinase K | Carl Roth GmbH + Co. KG | 7528.2 |
| Saline (0.9 % NaCl Solution) | Braun | 9511083 |
| Sodium Chloride NaCl | Carl Roth GmbH + Co KG | 9265.1 |
| Streptomycinsulfat | Carl Roth GmbH + Co KG | HP66 |
| Taq polymerase | NEB | |
| Tissue Tec® 4583 O.C.T.™ Compound | Sakura Finetek Europe B.V. | |
| TRIS PUFFERAN® | Carl Roth GmbH + Co. KG | 4855.2 |
| Triton® X-100 | Sigma Aldrich | 9002-93-1 |
| 2.5% Typsin, 10x | Invitrogen | 15090-046 |
| Rompun, 2 % Xylazinehydrochlorid | Bayer | KP07TPA |
| Laminin | Becton Dickinson | 354232 |
| PeqGOLD Universal Agarose | Peqlab | 35-1010 |
| Mowiol 4-88 | Carl Roth GmbH + Co. KG | 713.2 |
| Roti®-Liquid Barrier Markers | Carl Roth GmbH + Co. KG | AN92.1 |
| Goat serum | PAA | B11-035 |

## 3.2 **Antibodies**

### 3.2.1 Primary antibodies

| **Antigen** | **Host** | **Source (Cat.Nr)** | **Dilution** |
|---|---|---|---|
| AChE | rb | Claire Legay, Paris (Marsh et al., 1984) | 1:1000 |
| AChRε H-160 | rb | Santa Cruz sc-13999 | 1:50 |

| | | | |
|---|---|---|---|
| AChRε M-20 | goat | Santa Cruz sc-1455 | 1:200 |
| AChRγ | goat | Santa Cruz sc-1453 | 1:200 |
| Agrin (ms 204) | rb | Prof.M. Ruegg, Basel | 1:500 |
| Bassoon (SAP7f-GST) | rb | Dr. Wilko D. Altrock, Magdeburg | 1:1000 |
| Beta3-tubulin | ms IgG2b | Sigma T8660 | 1:1000 |
| ChAT | goat | Abcam AB144p (purified) | 1:100 |
| Collagen IV | rb | Millipore AB756p | 1:40 |
| Laminin γ1 chain Mab (3E10) | rat | Dr. Sorokin | undiluted |
| Laminin β2 mouse | rb | Dr. Sorokin | 1:500 |
| Laminin-1 α1 β1 γ1 mouse | rb | Dr. Sorokin | 1:1000 |
| LRP4 ext. | rb | Atlas HPA011934 | 1:200 |
| LRP4 int. | rb | Sigma Aldrich HPA012300 | 1:200 |
| Musk | rb | M. Ruegg 2008 | 1:400 |
| NeuN | ms | Millipore | 1:300 |
| Neurofilament heavy | chick | Millipore | 1:2000 |
| Neurofilament heavy | rb | Sigma Aldrich N4142 | 1:500 |
| Nidogen | rb | Dr.R.Timpl, Munich,Germany | 1:1000 |
| Parvalbumin | gt | Swant PVG 214 | 1:5000 |
| Rapsyn | rb | Lifespan Biosciences, Biozol | 1:200 |
| S100 | rb | Sigma S8193 /S2644 | 1:100 |
| Synaptophysin | rb | DAKO A0010, | 1:200 |
| VAchT | goat | Millipore AB1578 | 1:2000 |
| VGluT1 (c terminus) | gp | Millipore AB5905 | 1:250000 |

### 3.2.2 Secondary antibodies

| **Species specificity** | **Fluorescence tag** | **Source (Cat.Nr)** | **Dilution** |
|---|---|---|---|
| donkey anti goat IgG | Alexa Fluor® 488 | Invitrogen A11055 | 1:1000 |
| donkey anti goat IgG | Cy3 | Dianova (706-165-148) | 1:1000 |

| | | | |
|---|---|---|---|
| donkey anti guinea pig IgG | Cy3 | Dianova (706-166-148) | 1:1000 |
| donkey anti guinea pig IgG | Cy5 | Dianova (706-175-148) | 1:1000 |
| donkey anti guinea pig IgG | DyLight 649 | Millipore (AP1493SD) | 1:1000 |
| donkey anti maus IgG | Alexa Fluor® 594 | Invitrogen A21203 | 1:1000 |
| donkey anti maus IgG | Cy5 | Dianova (706-175-148) | 1:1000 |
| donkey anti rabbit IgG | Cy3 | Jackson (711-165-152) | 1:1000 |
| donkey anti rat IgG | Alexa Fluor® 488 | Invitrogen A21208 | 1:1000 |
| goat anti guinea pig IgG | Alexa Fluor® 488 | Invitrogen A11073 | 1:1000 |
| goat anti guinea pig IgG | Alexa Fluor® 488 | Mobitec | 1:1000 |
| goat anti maus IgG | Alexa Fluor® 488 | Molec.Probes (A11029) | 1:1000 |
| goat anti maus IgG | Alexa Fluor® 488 | Invitrogen | 1:1000 |
| goat anti maus IgG | Alexa Fluor® 647 | Invitrogen A21236 | 1:1000 |
| goat anti maus IgG1 | Alexa Fluor® 488 | Invitrogen A21121 | 1:1000 |
| goat anti maus IgG1 | Alexa Fluor® 594 | Invitrogen A21125 | 1:1000 |
| goat anti maus IgG2b | Alexa Fluor® 488 | Invitrogen A21203 | 1:1000 |
| goat anti rabbit IgG | Alexa Fluor® 488 | Invitrogen A21141 | 1:1000 |
| goat anti rabbit IgG | Alexa Fluor® 647 | Invitrogen A21245 | 1:1000 |
| goat anti rabbit IgG | Alexa Fluor® 594 | Invitrogen A11037 | 1:1000 |

## 3.3 **Methods**

### 3.3.1 Molecular methods

#### 3.3.1.1 DNA isolation and genotyping

To each mouse tail, 190µl of tail lysis solution (50mM EDTA, 50mM Tris-HCl, 0.5% SDS, pH 8.0) and 7.5 µl Proteinase K (10mg/ml) were added and incubated at 54°C overnight. After cooling of the lysed tail samples and centrifugation (12.000rpm for 10min at RT), a 1:10 dilution of each tail in water was subjected to PCR analysis. To identify the Thy1-YFP transgene, primers eYFP F1 and eYFP R4 were used.
eYFP F1 : 5‘ – ATC TTC TTC AAG GAC GAC GGC AAC TAC AAG - 3‘
eYFP R4: 5‘ - AGA GTG ATC CCG GCG GCG GTC ACG AAC TCC – 3‘
The product size of Thy1-YFP transgene PCR product is about 400bp.

Reactions for PCR were prepared as Table 3. PCR was performed with following conditions: denaturation 98°C, annealing 60°C and extension 72°C.

Table 1. PCR reactions.

| | |
|---|---|
| 10-100 ng | DNA template |
| 1 µl | Polymerase (1 U) |
| 5 µl | 10x reaction buffer |
| 1 µl | 10 mM dNTP (Desoxy-Nucleotide-Triphosphate)-Mix: (dATP, dTTP, dGTP, dCTP) |
| 2 µl | sense-Primer, 10 pM |
| 2 µl | anti-sense-Primer, 10 pM |
| add 50 µl | $H_2O$ |

3.3.1.2 Agarose gel electrophoresis of DNA

The PCR products were separated on 2% agarose gels. Agarose was dissolved in 1x TAE buffer and boiled in the microwave until the agarose was completely solubilised. The agarose was then cooled down to around 50°C ethidiumbromide (0.5 µg/ml) was added and the agarose was poured into the gel apparatus. Samples were loaded into the gel slots. Electrophoresis was carried out in 1x TAE buffer at 100 to 130 volts for 30min. The DNA bands were visualised under UV-light and documented with a camera.

3.3.2 Cell and tissue culture methods

3.3.2.1 Coating of cover slips

Cover slips (diameter 12mm, Roth) were first washed in acetone and further washed for in 70% ethanol containing 0.01 M HCl, each step for one hour at 4 °C. Cover slips were then washed one hour in 100% ethanol and dried at room temperature. Subsequently, cover slips were sterilized through baking for 2h at 180°C. One sterilized cover slips were placed in each well of a 24-well plate and moistened with PBS. PBS was replace by 1% poly-D-lysine (PDL, Sigma P0899) in PBS and slips were incubated for 2h at 37°C. Afterwards the cover slips were washed four times with autoclaved Millipore water. Plates were dried for 2h under a laminar flow hood and then stored at 4°C until use (not more than one week).

Laminin (Becton Dickinson) was diluted in HBSS (Hanks' Balanced Salt solution) with the end concentration 10µg/ml and added to each PDL-coated cover slip on 24-wells plate. The plate was incubated at 37°C for four hours before plating neurons.

#### 3.3.2.2 Dissociated dorsal root ganglion neuron culture

Timed pregnant mice (E14.5) were anesthetized with CO2 and sacrificed by cervical dislocation. Embryos were isolated by caesarean section and immediately placed into ice cold Hanks balanced salt medium (HBSS) supplemented with 10 mM HEPES. Dorsal root ganglions were dissected from the embryonic spinal cord and pooled in ice cold HBSS containing 10 mM HEPES and spun down at 1500 rpm for 5 minutes. After removing the supernatant medium, the pellet consisting of DRG neurons was dissociated with collagenase (final concentration 5 µg/µl) and trypsin (final concentration 0.5%) at 37°C for 15 min. Proteolytic enzymes were partially inactivated by adding F12 medium containing 10% fetal bovine serum (FBS). Cells were again centrifuged at 1500 rpm for 5 minutes and resuspended in fresh DRG culture medium (1x B27, 2mM L-glutamine, 1U ampicilin, 1µg/ml streptomycin, 10 ng/ml NT-3, 10 ng/ml NGF in neurobasal medium). Cells were counted using a "Neubauer" microscope counting chamber and the neurons were plated on PDL-laminin coated cover slips at a density of 2- 5 x 104 cells/well. After 16 days cells were fixed in 4% PFA and stained.

### 3.3.3 *In vivo* methods

#### 3.3.3.1 Animals

All mice were bred in the animal facility of the Ludwig-Maximilians-University Munich, at Schillerstrasse 44, 80336 Munich. The day of the vaginal plug was considered as embryonic day (E) 0. The day of birth was considered as postnatal (P) day 0. Animals were kept in a 12h day-night cycle. All experimental procedures were performed in accordance with guidelines of the European Union and the government of Upper Bavaria.

##### 3.3.3.1.1 Thy1-YFP 16 mice

We used Thy1-YFP-16 transgenic mouse line (Feng et al., 2000) provided by Prof. Dr. Martin Kerschensteiner (Ludwig-Maximilians-University, Munich) for identification of muscle spindle neuronal innervation. Thy1 (Thymocyte differentiation antigen 1, CD90), a cell surface glycoprotein is a member of the

immunoglobulin superfamily. The Thy1-YFP16 line had strong expression of YFP in almost all motor and sensory axons (>80%), retinal ganglion neurons and dorsal root ganglion neurons (Feng et al., 2000). The Thy1-YFP 16 mice were used as homozygous animals.

#### 3.3.3.1.2 AChR$^{\gamma\text{-GFP}/\gamma\text{-GFP}}$ knock-in mice

AChR$^{\gamma\text{-GFP}/\gamma\text{-GFP}}$ knock-in mice were provided by Prof. Dr. Veit Witzemann (Max-Plank-Institute for Medical Research, Heidelberg). In this mouse line the AChR γ-subunit gene was labelled by GFP in the intracellular domain between transmembrane domains M3 and M4 of AChR (Yampolsky et al., 2008). Although the expression level of the γ-subunit decreased after GFP-labelling, the development of pre- and postsynaptic formation was normally and there were not motor deficits reported (Gensler et al., 2001; Yampolsky et al., 2008). Hind limb muscle of P0 mice was used to confirm the expression of the GFP protein, conjugated with the AChR γ- subunit in NMJs and muscle spindle. Adult (1 year old) muscle tissue was used to test if AChR γ-subunit was present in the equatorial region of muscle spindles.

#### 3.3.3.1.3 Agrin knock-out and mini-Agrin rescue mouse

We used agrin-deficient mice and miniagrin rescue mice provided by Dr. Shuo Lin (Biocenter of Basel University, Schwitzerland, Lin et al., 2001). Miniagrin is a miniaturized form of neural agrin containing the 8-amino acid insert at the B/z splice site. This miniagrin, including the laminin-binding domain and acetylcholine receptor-aggregation domain, is sufficient to induce postsynaptic differentiation in muscle fibers (Lin et al., 2008; Meier et al., 1998). Under the control of the promoter of muscle creatine kinase (MCK), the cDNAs encoding chick miniagrin (c-mag$_{B8}$) was expressed in agrin deficient mouse line. The reexpression of miniagrin started at embryonic day 13.5 and could rescue the perinatal lethality of agrin deficient mice. In order to use control embryos with the same age, we used tissue of both agrin deficient mice and miniagrin rescue mice from embryonic day 18.5 for our experiments. To obtain these mouse lines, heterozygous agrin deficient mice (agrn+/-) were mated with agrin rescue mice (agrn-/-, Tc-magB/z8). In order to visualize motor- and sensory nerve heterozygous agrin deficient mice and agrin rescue mice were crossed

with Thy1-YFP mice. Control animals were littermates with a different genotype from the same cage. Muscle from hind limbs was used for all experiments.

#### 3.3.3.2 Anaesthesia, fixation and perfusion of animals

For perfusion animals were anesthetized by intraperitoneal injection of Ketamine (Ketamine hydrochloride 100µg per gram of bodyweight) and Rompun (Xylazinhydrochloride, 20 µg per g of bodyweight) with insulin syringes (0.3mm/ 30G). A lethal dose was required to ensure that mice are sedated during perfusion. To ensure the mouse was properly sedated after injection, pinching the toes to judge the level of response to a painful stimulus was required.

Once the mouse was sedated and without pain response, it was placed on a styrofoam plate with its back site down. The four limbs were spread as much as possible and paws were secured with very thin syringe needles. The abdomen was washed with ethanol and opened at the level of the diaphragm. The abdomen was cut through the ribs. The chest was opened until the heart was easy to access. The needle was placed into the left ventricle; PBS buffer was pumped with speed 3 ml/min. Then the right atrium was cut immediately and the mice were perfused with PBS for two minutes. Perfusion was monitored and considered good since the colour of liver became brighter (around 2 minutes). Then PBS was changed to 4% PFA, which was perfused for 17 to 20 min for adult mice and 10 min for P15-P30 mice.

#### 3.3.3.3 Tissue fixation and sectioning for light microscopy

Young animals between P0 and P15 could not be fixed by perfusion and instead were decapitated. Accordingly I did not dissect individual muscles but instead their hind limbs were skinned, dissected and fixated by immersion in 4 % PFA for 1 h to 2 h at room temperature (RT).

Pregnant mice (E18.5) were anesthetized with $CO_2$ and sacrificed by cervical dislocation. Embryos were collected in cold PBS, washed to remove blood and transferred immediately into 4% PFA solution. Embryos were fixed overnight at 4°C. To avoid damages due to water crystals, the hind limbs were incubated in the cryoprotection medium, 30 % sucrose solution in PBS, at 4 °C.

M. quadriceps (Q) (from P0 to P30 animals), m. soleus (SOL), m. tibialis anterior (TA) and m. extensor digitorum longus (EDL) were carefully dissected from fixed adult hind limbs. In case of embryonic muscle tissue, whole hind limbs were dissected. Muscle or hind limbs were quickly embedded in TissueTec® on dry ice. In

order to increase the chances of finding muscle spindles, reduce cutting time and save antibodies, two or four sections of muscles or entire hind limbs were embedded in the same TissueTec® block and as many tissue sections as possible were collected on one glass slide. Cryosections were obtained with a Leica CM3050S cryostat at -20 °C. Muscle tissue was serially cut into a 10 µm (for SOL, TA, EDL)/ 30 µm (for Q and hind limbs) thick longitudinal sections. The slices were collected on coated glass slides and stored at -20 °C before using.

### 3.3.4 Immunohistochemistry

Immediately before use glass slides were dried 30 min at room temperature and surrounded with water resistant barrier liquid. TissueTec® was washed away with PBS and unspecific binding sites were blocked by incubation with blocking solution (1% BSA, 0.5% Triton X-100 in PBS) for 30 min at RT. Sections were incubated with primary antibodies (diluted in blocking solution, details see 6.3.1.) over night at 4 °C. After three washings with PBS, 10 min each, the slides were incubated with the secondary antibodies, diluted in blocking buffer for 90 min at RT. Thereafter sections were washed with PBS and the nuclei were labelled by incubation with DAPI (4', 6' Diamidino-2-phenylindole, 2 ng/µl) for 10 min at RT. After destaining DAPI, the sections were mounted with Mowiol, and stored at - 4 °C before analysis.

To ensure the specificity of primary antibody and to rule out unspecific binding of secondary antibody, positive and negative control staining were always run in parallel.

For whole mounts, soleus muscles were removed from the fixed hind limbs of Thy1-YFP mice and pinned on a Sylgard dish filled with PBS. Alexa594-conjugated α-BTX (2 µg/ml in PBS) was injected into the muscle and incubated for 30 min at RT. AChRs were labelled with α-BTX in PBS for half an hour at RT. The soleus muscle was cut longitudinally into bundles as thin as possible and the sections were incubated in permeabilization solution (1% Triton-X100 in PBS) at 4°C for four hours. To visualize the neurons and the NMJs, neurofilament heavy (NFH, Sigma N4142, 1:2000) and synaptophysin (SYN, DAKO A0010, 1:200) were applied in blocking buffer, 4°C overnight. After repeated washing steps, at least three times at least four hours with PBS, samples were incubated in goat anti rabbit antibody (1:1000) two hours 4°C. Wash again at least four hours with PBS and mount with Mowiol.

ETA muscles were dissected from fixed forelimb of Thy1-YFP mice. Since the ETA muscle is very thin, permeabilization was not necessary. After incubation in Alexa594-conjugated α-BTX for half an hour, the tissue could be mounted directly.

Cell cultures on coverslips were fixed in 4 % PFA for 10 min. Fixed cells were blocked in 2% BSA, 0.5 % Triton-X100 in PBS for 1 h prior to staining. Primary and secondary antibody and DAPI staining were performed identical as described for the cryosections. After staining coverslips were mounted with Mowiol on glasses slides.

### 3.3.5 Electron microscopy

For electron microscopy, mice were killed and transcardially perfused using PBS. Soleus muscle was dissected immediately and fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer overnight. The tissue was washed with 0.1M cacodylate buffer for 90 min and then incubated in osmium tetroxid in 0.1M cacodylate buffer for 2-5 hours. The tissue was washed again with 0.1M cacodylate buffer for 90 min and dehydrated in increasing alcohol concentrations ranging from 30% to 100% and finally in propylene oxide. The tissue was infiltrated with Epon overnight and polymerised in and polymerization oven at 60°C for 48 hours. The embedded tissue block was then cut using a Reichert Jung Ultracut E Ultramikrotom into semi-thin sections (0.5 µm) and stained with colour solution (1% toluidine blue, 1% azure II, 1% methylene blue and 1% borax in aqua dest.) to increase the contrast in light microscopy. Muscle spindles were identified by light microscopy and the blocks with muscle spindles were then cut to ultra-thin sections (60 nm thick) with a diamond knife. Ultra-thin sections were transferred to grids and contrasted with 1% uranyl-acetate and 0.4% lead citrate buffer.

### 3.3.6 Image processing

IHC stainings were analysed using a Zeiss (LSM710) confocal laser-scanning microscope. Pictures were taken using 25x (LD LCI Plan-Apochromat 25x/0.8 Imm Korr DIC), 40x (LD C-Apochromat 40x/1.1 W Korr M27) or 63x (LCI Plan-Neofluar 63x/1.3 Imm Korr Ph3 M27) lens. Stacks of 5-15 optical sections with 1-2 µm interval and plane pictures as well as partial scans were taken. The resolution of the pictures was 512x512 or 1024x1024 pixel. Laser power levels, scanning speed, and the confocal pinhole size were kept constant between experimental and control specimens. Data were saved as LSM format so that all information about scanning

was maintained. For further processing, pictures were exported in TIFF format. Digital processing of entire images, including adjustment of brightness and contrast was performed using the ZEN2009 software.

To identify muscle spindles among a mass of muscle fibers, at least two of the following conditions had to be positive. (1) Morphology of annulospiral ending labelled by Thy1-YFP or NFH/SYN; (2) aggregated nuclei stained by DAPI; (3) VGluT1-positive sensory nerve ending; (4) the diameter of intrafusal fiber being about half of the diameter of extrafusal fiber.

For electron microscopy, a Zeiss transmission electron microscope (EM 902A) was used for final investigation with 80 kV at magnifications from 3,000 to 140,000. Digital image acquisition was performed by a Morada slow-scan-CCD camera connected to a PC using the ITEM® 5.0 software (Soft-imaging-systems, Münster, Germany); images were stored as uncompressed TIFF files in 16 bit gray scale and further processed using Adobe® Photoshop® CS5.

### 3.3.7 Data analysis

Analysis of the formation of γ-motorneuron endplates of muscle spindles in E18.5 wildtype, agrin knock out and mini-agrin rescue mouse embryos from the same littermates were carried out on single confocal optical sections. To quantify the results, I determined the average distance between the central sensory ending and the γ-motoneuron innervation in wildtype and mutant mice. In agreement with a previous study (Kucera et al., 1988) we found that in E18.5 wildtype mice all γ-motoneuron endplates had formed at a distance of ~200 μm from the central part of the sensory innervation. I therefore determined in all three mice strains the number of AChR aggregates colocalizing with a γ-motoneuron on intrafusal fibers at a distance between 100 and 300 μm from the center of the sensory ending. Only those intrafusal fibers for which it was possible to unambiguously follow an individual γ-motoneuron from the central VGluT1-positive area to the distal-most tip were scored. While at least one AChR aggregate colocalizing with a motorneuron was consistently detected in intrafusal fibers from wildtype and from agrin rescue mice, no AChR aggregates were observed colocalizing with a γ-motoneuron in agrin -/- mice.

To compare in detail the morphological differences between the equatorial region of muscle spindles from wildtype and from agrin knockout mice, maximium intensity projections of the stack of optical sections were generated using the ZEN2009

software. The density of aggregated nuclei and the branches of sensory nerve endings in these maximium intensity projection pictures were counted and the number of nuclei and branch points per area was determined using the NIH public domain Java image processing program software package ImageJ (http://rsbweb.nih.gov/ij).

For the quantification of bassoon immunoreactivity, maximium intensity projections of the stacks of optical sections were analysed. The number of bassoon puncta per area was determined using ImageJ and the diameter of bassoon punta were counted using the ZEN2009 software.

Statistical significance was tested using student's t-test. P-values less than 0.05 were considered significant.

# 4 Results

## 4.1 Distribution of extracellular matrix proteins in muscle spindles

Intrafusal fibers are surrounded by an inner and an outer capsule. Both capsules contain a basement membrane, which has been identified by electron microscopy (Patten and Ovalle, 1991) and is indicated by arrows in Figure 8. According to the position of the basement membrane, they were classified as internal capsule basement membrane und external capsule basement membrane (green line in Figure 9C and D). The internal capsule basement membrane was classified as BM surrounding sensory nerve ending (red line) and BM, directly contacting the intrafusal fiber (blue line in Figure 9C and D). However the molecular composition of these basement membranes is not known. To investigate the presence of individual ECM proteins, I used antibodies against the main extracellular matrix components (i.e. collagen IV, nidogen, various laminin isoforms).

At postnatal day 10, the annulospiral nerve endings have developed and muscle spindles are functional. Collagen IV, the main structural component of basement membranes, was identified in the inner and outer capsules (Figure 9G).

In the extracellular matrix both collagen IV and laminin are linked to each other by a glycoprotein named nidogen, also known as entactin (Carlin et al., 1981; Kimura et al., 1998). Two isoforms of nidogen have been identified - nidogen I and nidogen II (Kohfeldt et al., 1998; Timpl et al., 1983). To investigate the distribution of nidogen in muscle spindles, an antibody reacting with both nidogen isoforms was used. Nidogen was present in the basement membrane covering the sensory nerve as well as in the inner and outer capsule in the equatorial region. At the polar region, nidogen was concentrated at the endplates of γ-motoneurons.

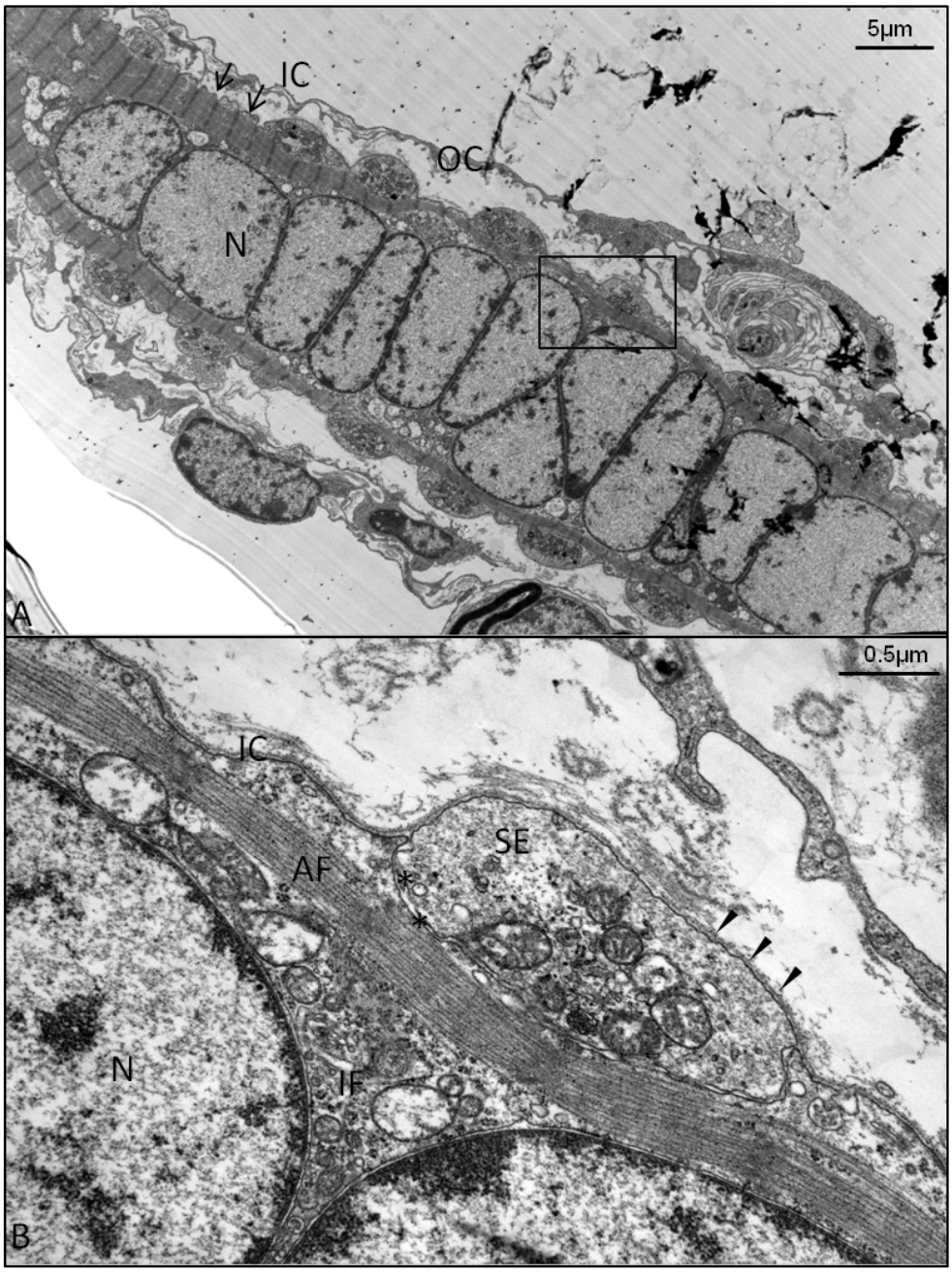



Figure 8. Electron micrograph of muscle spidle.

(A). An electron micrograph of the muscle spindle equatorial region from the longitudinal section of mouse soleus muscle. Nuclei (N) are highly aggregated in the equatorial region. The intrafusal fiber is surrounded by an inner capsule (IC) and an outer capsule (OC). (B). Magnified pictures of the black box in (A). A basement membrane (arrowhead) covers the sensory nerve ending (SE), whereas at the contact site between the sensory nerve ending and intrafusal fiber (asterik) no basement membrane is observed. Between the aggregated nuclei and the cell membrane of intrafusal fiber a thin bundle of actin filament (AF) is present.

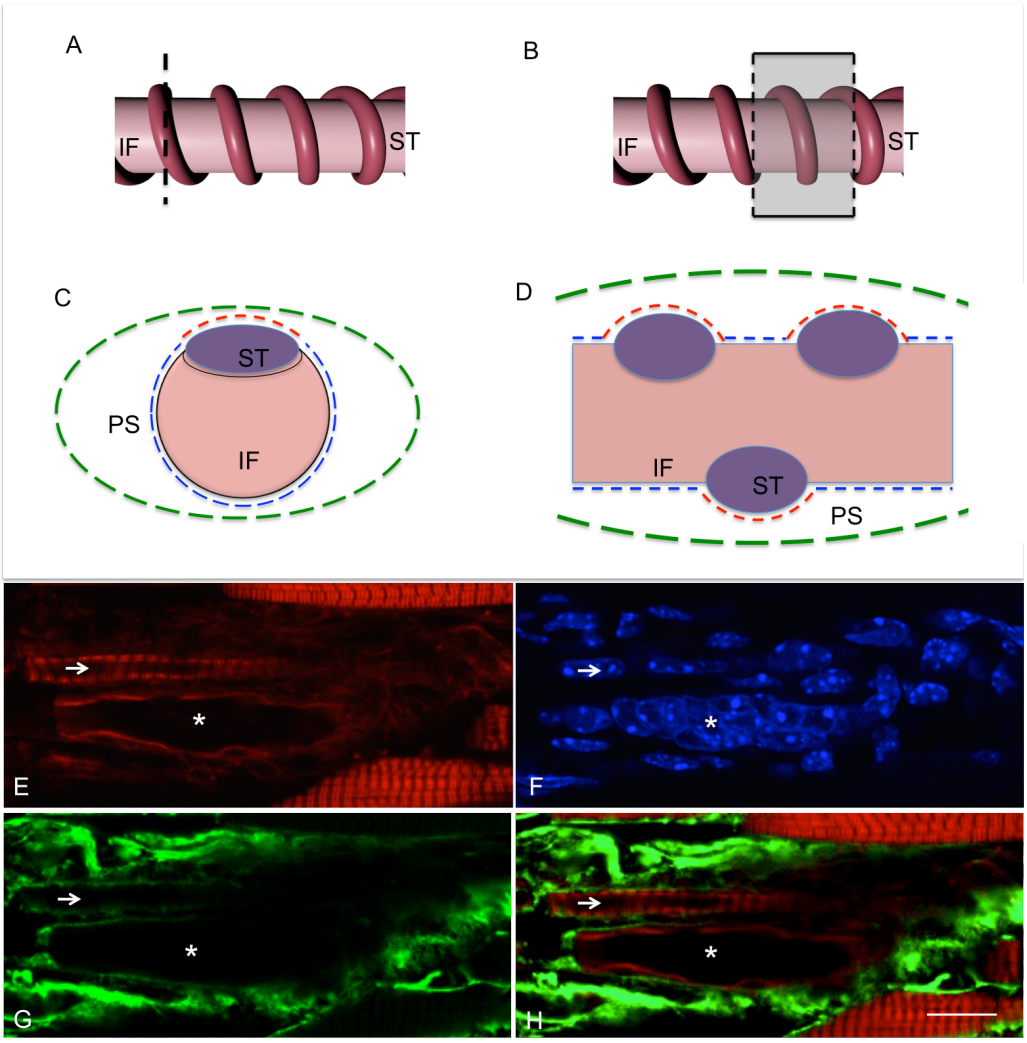



Figure 9. Distribution of Collagen IV in the equatorial region of muscle spindles.

(A and B). Schematic illustration of the annulospiral sensory nerve ending. In panel A dashed line indicates the transverse section of an intrafusal fiber. In panel B the grey plate shows the longitudinal section of an intrafusal fiber. (C and D) Schematic representation of the basement membrane in both the transverse (C) and the longitudinal (D) sections of muscle spindle. The red lines show the BM covering the sensory nerve ending (defined as BM on sensory sector by Maier and Mayne, 1993). The blue lines show the BM directly contacting the intrafusal fiber, where sensory nerve is absent (also called as BM on non-sensory sector by Maier and Mayne). The green lines indicate the outer capsule surrounding intrafusal fibers. IF, intrafusal fiber; SE, sensory ending; PS, perifibral sheath. (D). Alexa 594-conjugated phalloidin was used to illustrate the distribution of F-actin in muscle fibers. The arrow indicates a nuclear chain fiber labelled by phalloidin with a few sarcomeres. The asterisk indicates a nuclear bag fiber, where actin is present only beneath the sarcomere membrane. (E) DAPI-labelled nuclei. In the nuclear chain fiber the nuclei are aligned one after another (arrow), whereas in the nuclear bag fiber nuclei are aggregated (asterisk). (F) Collagen IV is present in the inner capsule, which surrounds intrafusal fiber tightly and in the outer capsule. Panel G shows a merge picture of panels D and F, to illustrate the relative position of intrafusal fiber and capsules.

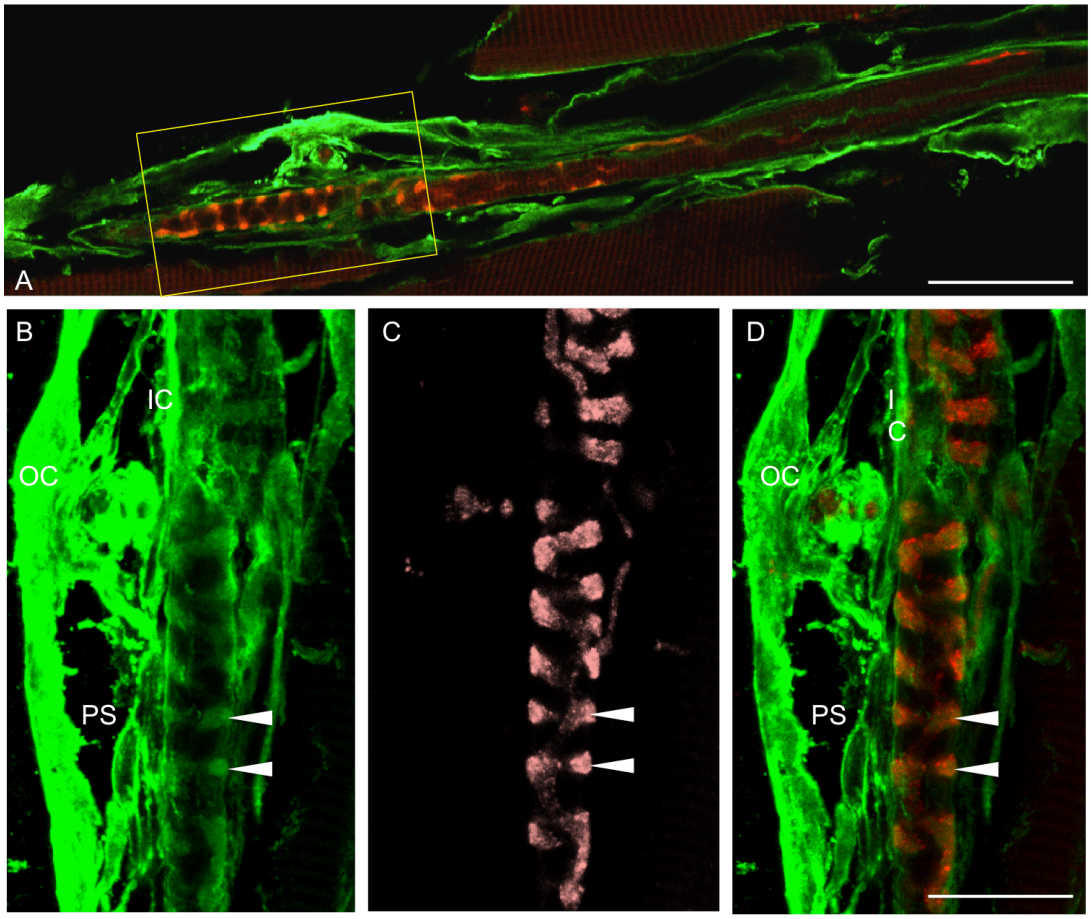



Figure 10. Distribution of nidogen in muscle spindles.

(A). Longitudinal overview of a muscle spindle. (B-D). Close-up view of the region squared in (A). (B) Nidogen in green is present in the BM covering the sensory nerve (arrowhead), the inner capsule (IC), the outer capsule (OC) and the connective tissue in the peripheral space (PS). The BM covering on the sensory nerve ending (arrowhead in panel B) is precisely co-localizing with VGluT1 labelled sensory nerve ending (arrowhead in panel C).

Previous studies have analysed the distribution of laminin immunoreacitivity in muscle spindles and reported a presence of laminin at both the sensory sector and the non-sensory sector (Maier and Mayne, 1993). However, the distribution of the various laminin isoforms has not been investigated. This appears particularly important, since different laminin isoforms have specific distribution patterns at the NMJs and affect the development and the maintenance of this particular type of synapse (Nishimune et al., 2008; Noakes et al., 1995b; Samuel et al., 2012). Laminins are heterotrimers consisting of α, β and γ subunits. I therefore investigated if different laminin subunits have also a specific distribution in muscle spindles. To address this question, the distribution of pan-laminin, the γ-subunit and the β2-subunit (also called as S-laminin, synaptic isoform of laminin) was investigated. I detected the γ1-subunit in the inner

and outer capsule, but not in the basement membrane surrounding the sensory nerve endings (red line in Figure 9C and D). Similar to its distribution at the NMJ, the β2-subunit was concentrated at the endplate of γ-motoneurons in the polar region of the muscle spindle where it colocalized with α-bungarotoxin (α-BTX). At the equatorial region of muscle spindles the immunoreactivity of laminin β2-subunit was present at the outer capsule and at the basement membrane surrounding sensory nerve ending. These results show that at the equatorial region β2-subunit is close to the sensory-myo-junction and the γ1-isoform is present outside of the sensory-myo-junction.

The proteoglycan agrin was detected at the outer capsule and at the basement membrane surrounding sensory nerve endings. More results about agrin are described in the chapter 4.5 - 4.6.

The distribution of the ECM molecules investigated in my study is summarized in Figure 12 and Table 2.

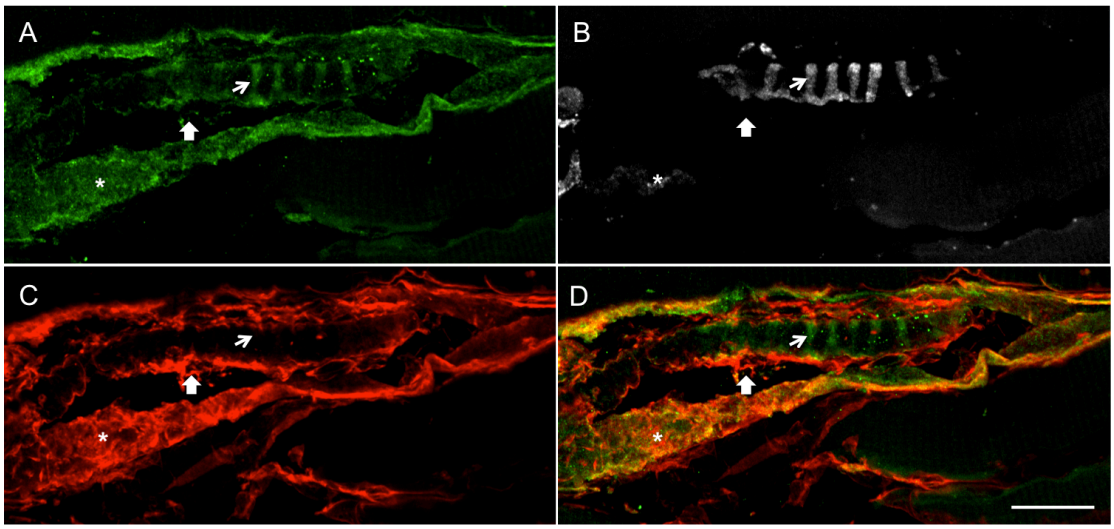



Figure 11. Different distribution of laminin isoforms at the equatorial region of muscle spindles.

(A) Laminin β2 chain labels clearly the BM surrounding the sensory nerve ending (thin arrow) and the outer capsule (asterisk). (B) VGluT1 labels the sensory nerve ending. (C) Laminin γ1 chain labels the inner (bold arrow) and outer capsule (asterisk). (D) Merge picture of (B) and (C). Scale bar 20µm.

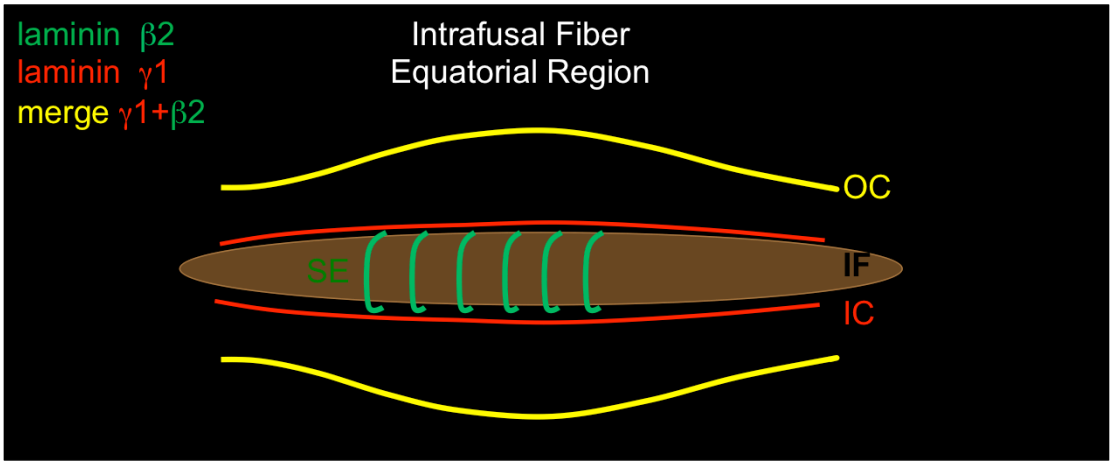



Figure 12. Schematic representation of the laminin isoform distribution in the basement membrane of the central part of muscle spindles.

Laminin γ1 and β2 chains all distribute in the outer capsule. In the inner capsule only γ1 subunit is detectable and on the BM covering the sensory nerve ending β2 chain is specifically present.

Table 2. Distribution of tested molecules in the equatorial region of muscle spindles.

| | Outer capsule | Inner capsule | Sensory ending |
|---|---|---|---|
| **Laminin γ 1** | + | + | - |
| **Laminin β 2** | + | - | + |
| **Nidogen** | + | + | + |
| **Collagen IV** | + | + | - |
| **Agrin** | + | - | + |
| **AChE** | - | + | - |

## 4.2 Cholinergic specializations in adult muscle spindles

Previous studies have shown that AChRs are aggregated at NMJs and in muscle spindles (Arber et al., 2000; Arbuthnott et al., 1982; Engel et al., 1977) but their precise distribution was never investigated. To confirm and extend these results, I labelled muscle spindles in m. quadriceps with fluorescence-conjugated α-BTX in postnatal and adult C57BL/6J mouse. I observed that aggregated AChRs were present not only at the NMJ and at the endplate of γ-motoneurons, but also at the equatorial region of muscle spindles (Figure 13 D').

In order to further analyse the cholinergic specializations in muscle spindles, I investigated the distribution of several molecules that are markers for cholinergic

synapses, including choline acetyltransferase (ChAT), the enzyme, which synthesizes acetylcholine, acetylcholineesterase (AChE), the enzyme, which degrades acetylcholine, as well as the vesicular ACh transproter (VAChT), which loads ACh into synaptic vesicles (Arvidsson et al., 1997). At the neuromuscular junction ChAT and VAChT are present in the presynapse and AChE is concentrated in the synaptic cleft, where it binds to the basement membrane. Consistent with the concentration of the AChR in muscle spindles I detected these three molecules at the endplate of γ-motoneurons where they colocalized with α-BTX labelled AChRs; at the equatorial region the immunoreactivity of ChAT and VAChT colocalized with VGluT1-labelled sensory nerve endings. AChE immunoreactivity in contrast was absent from the sensory nerve endings. VGluT1 is a marker for sensory nerve endings (Wu et al., 2004), but the neuron is not necessarily a glutamergic one (El Mestikawy et al., 2011; Figure 13).

Moreover, rapsyn (receptor-associated protein at the synapse) a 43 kDa cytoplasmic protein, which links AChRs to the subsarcolemmal cytoskeleton, was also aggregated in the equatorial region and at the endplate of γ-motoneurons (Figure 14).

In summary, the endplates of γ-motoneurons as well as the area of contact between sensory neuron and intrafusal fibers show specializations reminiscent of cholinergic synapses, including a high concentration of AChR, VAChT, ChAT and rapsyn. This confirms that γ-motoneurons form a regular cholinergic synapse at the polar region of intrafusal fibers. My results also indicate the possibility of a cholinergic synaptic transmission at the equatorial region.

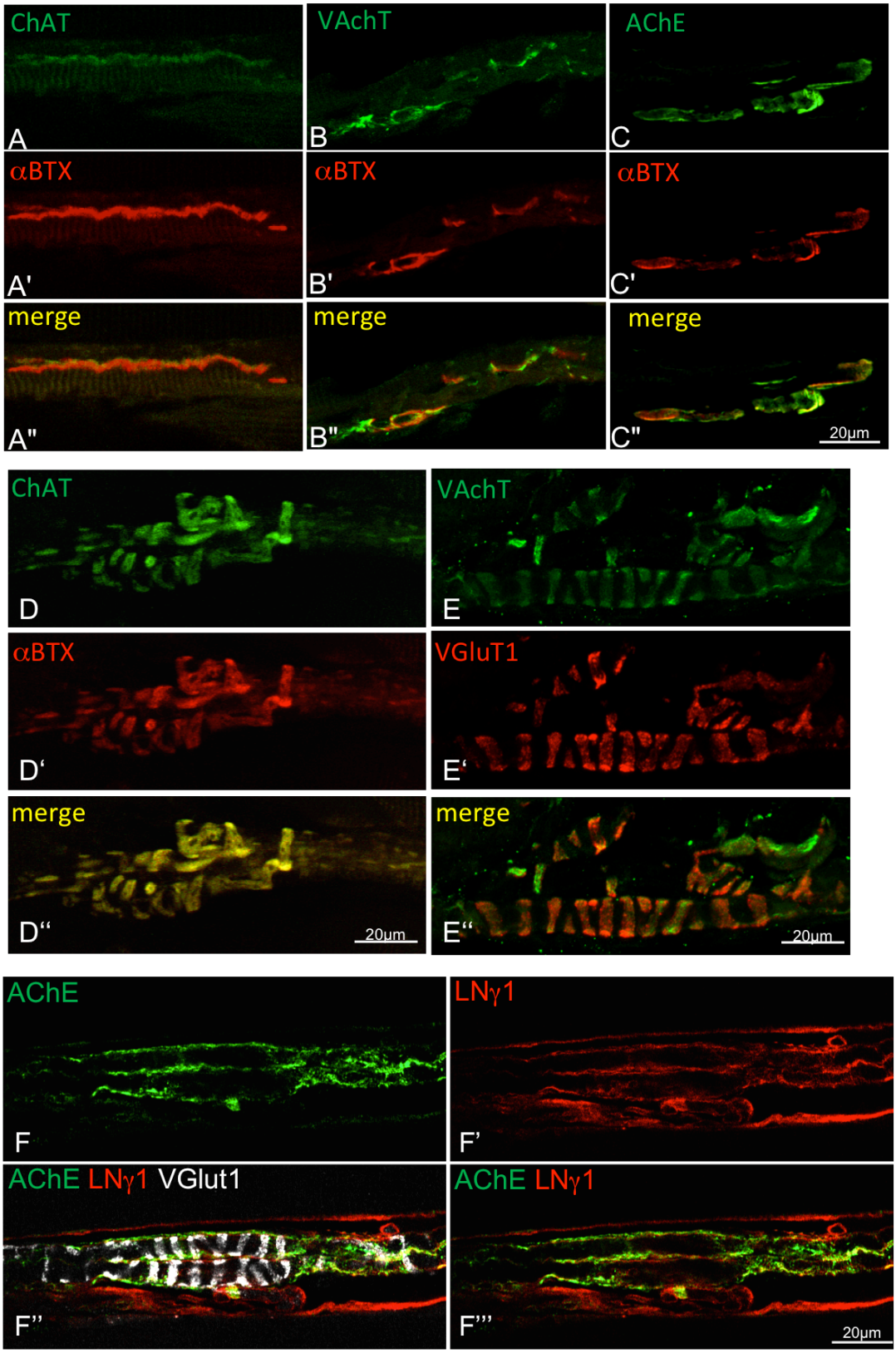



Figure 13. Cholinergic specializations in muscle spindles.

(A-C) The endplates of γ-motoneurons were labelled by antibodies against choline acetyltransferase (A); against vesicular acetylcholine transporter (B) and against acetylcholinesterase (C). (A'-C') α-bungarotoxin (α-BTX) labelled AChR was

aggregated at the endplates of γ- motoneurons. Merged pictures are shown in A''-C''. (D-E) The equatorial regions of muscle spindles were labelled by ChAT and VAChT and the immunoreactivity colocalized with the α-BTX- (D') or VGluT1-positive (E') sensory nerve endings. (F) AChE was present in the equatorial region, but did not colocalize with VGluT1 (F''), Instead, AChE immunoreactivity could be detected in the inner capsule, labelled by antibodies against the laminin-γ1 chain.

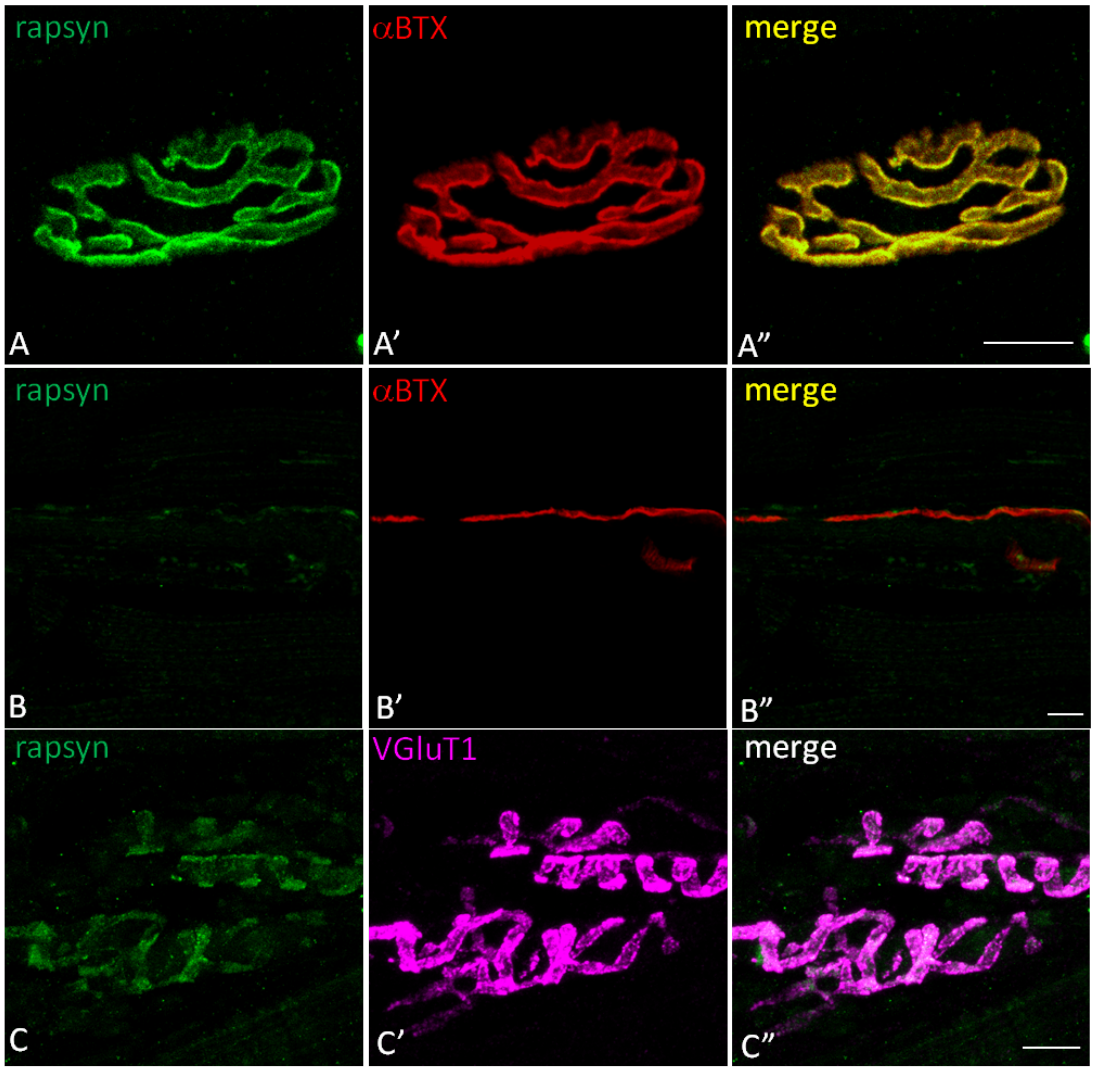



Figure 14. Rapsyn is concentrated at the equatorial region and the γ-motoneuron endplate of muscle spindles.

(A-A', B-B') Rapsyn staining is colocalized with aBTX stained NMJ and the γ-motoneurons endplate. (C-C') Rapsyn immunoreactivity was detectable at the sensory nerve ending, which is labelled by VGluT1. Scale bar 10μm.

### 4.3 **Active zones at the endplates of alpha- and gamma- motoneurons**

The presence of AChE, VChAT and of the AChR in the central region suggested the possibility of cholinergic synaptic transmission between sensory neuron and intrafusal fiber. To further investigate this hypothesis, I analysed the presence of

molecules involved in synaptic vesicle exocytosis in the central part of muscle spindles. The active zone is a specialized region of presynaptic membrane, which contains electron-dense material (Couteaux and Pecot-Dechavassine, 1970; Harlow et al., 2001; Hirokawa and Heuser, 1982; Nagwaney et al., 2009). Active zones are the sites of preferential synaptic vesicle release. In rodents several components of the active zone have been identified, including bassoon and piccolo, Rim, Munc13, CAST/Erc2 and voltage-gated calcium channels (Chen et al., 2011; tom Dieck et al., 1998).

To investigate if active zone-like structures are present in the presynaptic site of the endplate of γ-motoneurons and the sensory nerve ending, immunohistochemistry was performed using an antibody against bassoon. At the NMJ the bassoon staining showed a punctate pattern (Chen et al., 2011). Likewise, I detected a punctate staining pattern of bassoon immunoreactivity at the endplates of γ-motoneurons (Figure 15B, B'). Interestingly, bassoon immunoreactivity had a punctate pattern also at the sensory nerve terminal (Figure 15C, C'). These results indicate that release sites are present at the polar region and at the equatorial region of muscle spindles, supporting the possibility of cholinergic synaptic transmission at the sensory endings.

To compare active zones at the sensory nerve ending and at the γ-motoneurons endplate, I determined the diameter and the density of bassoon puncta at both places and compared the density with that at the neuromuscular junction. My results show that at the endplate of γ-motoneurons the number of active zones ($2.7/\mu m^2$) is similar as that at NMJ ($2.4/\mu m^2$). The density of bassoon puncta at sensory nerve endings, in contrast, was considerably lower compared to the density at the NMJ and the endplates of γ-motoneurons ($1.4/\mu m^2$; Figure 15D). The diameter of the bassoon puncta at NMJs (0.4 μm) and at endplates of γ-motoneurons (0.39 μm) was almost identical, whereas it was slightly but not significantly smaller at sensory nerve endings (0.35 μm; Figure 15E). These results demonstrate that sensory nerve terminals contain bassoon puncta with a similar size but a lower density compared to the NMJ and the γ-motoneuron endplate, suggesting the presence of a specialized cytomatrix reminiscent of a presynaptic terminal at the sensory nerve endings.

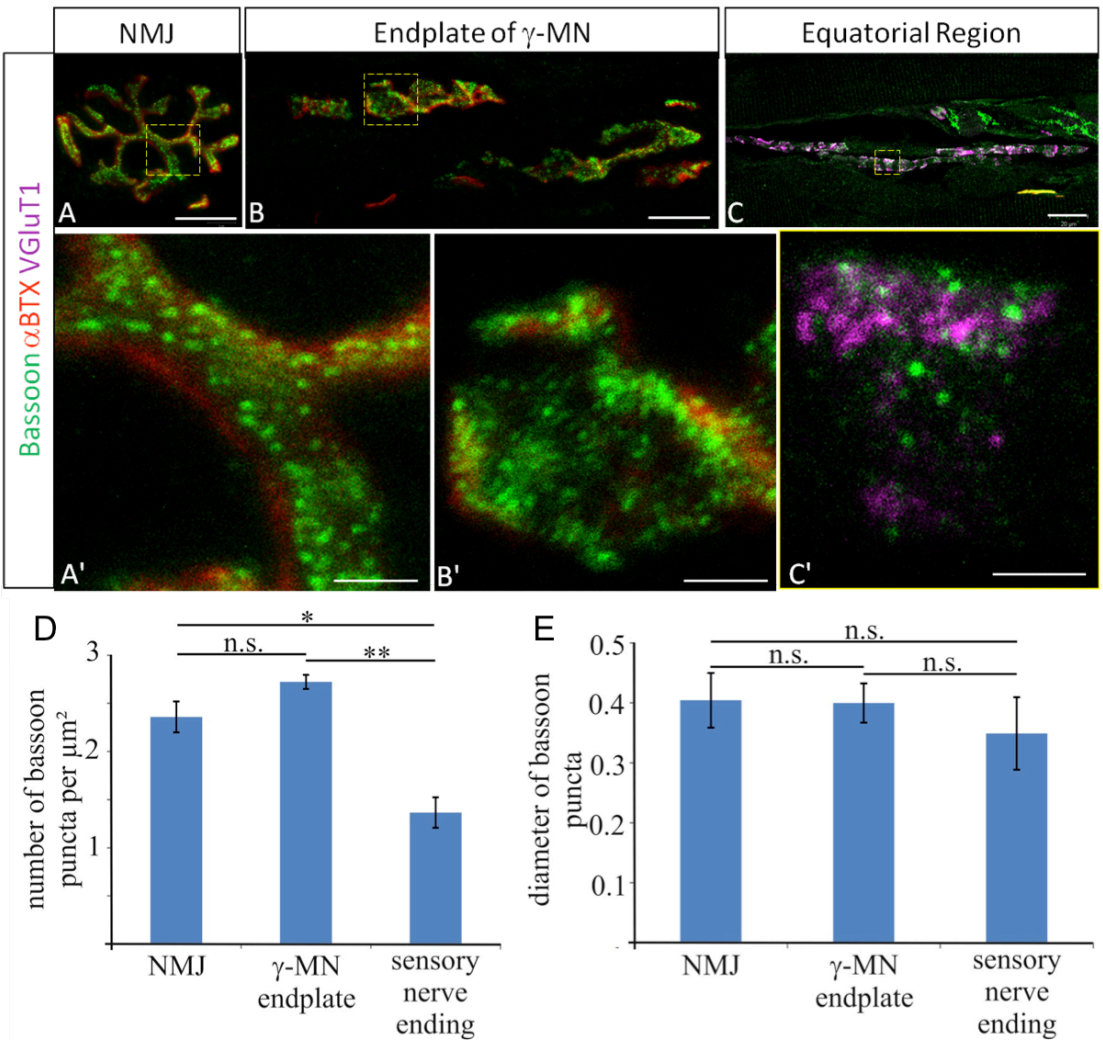



Figure 15. Punctate bassoon-immunoreactivity is concentrated at the neuromuscular junction, the γ-motoneuron endplates and sensory nerve endings.

(A, B, C) show the punctate pattern of Bassoon staining at the NMJ, γ-motoneuron endplates and sensory nerve endings. Scale bar 20µm. (A', B', C') Higher magnification of the areas indicated in A, B and C. Scale bar 2µm. (D) Quantification of the density of bassoon puncta at NMJs, γ-motoneuron endplates and sensory nerve endings of adult quadriceps muscle. (E) Quantified analysis of the diameter of bassoon puncta Data are shown as mean ±SEM; N=4. * $p<0.05$, **$p<0.01$; student's t-test.

## 4.4 Development of AChR subunit composition in muscle spindles

My previous results demonstrated the presence of the molecular machinery required for cholinergic synaptic transmission at the contact site between the sensory nerve endings at the intrafusal muscle fiber. To further analyse the cholinergic specializations at this site, I addressed if AChRs undergo a postnatal maturation similar to its maturation at the NMJ by determing the γ- to ε-subunit switch of AChRs during postnatal muscle tissue development.

To this end I performed immunohistochemistry using antibodies that specifically recognize the AChR γ-subunit and the ε-subunit. At the NMJ, the γ-subunit of the AChR is present during pre- and postnatal development, until P14, but is absent from adult NMJs (Missias et al., 1996). In contrast, the ε-subunit is not present during early postnatal NMJ development but appears after P10, replacing the γ-subunit. This γ-to-ε switch transforms the fetal AChR with a long open time and a large conductance to the adult type of AChR, containing the ε-subunit. Using the γ- and ε-subunit specific antibodies, I confirmed the subunit switch at the γ-motoneuron endplate, i.e. a downregulation of the γ-subunit with a concomitant upregulation of the ε-subunit between P7 and P14. Likewise, I detected the γ-subunit at early postnatal stages at the central region of intrafusal fibers (Figure 16A, B). In contrast however to the NMJ and the γ-motoneuron endplate, at the sensory nerve ending of intrafusal fibers, the γ-subunit was still detectable at adult stages (Figure 16C, D) despite the onset of expression of the ε-subunit around postnatal week two. This indicates the simultaneous presence of fetal and of adult AChRs at the central region of adult intrafusal fibers (Figure 16A, B).

To independently confirm these results, I investigated the AChR subunit switch using a mouse line, in which the γ-subunit was genetically labelled with GFP (Yampolsky et al., 2007). In the quadriceps muscle of newborn transgenic mice, the GFP signal could be directly detected by confocal microscope at the NMJ, the endplate of γ-motoneurons and at the equatorial region of muscle spindles (data not shown but see Yampolsky et al., 2007). As expected, no GFP signal was observed at the adult NMJs and only very weak signal could be detected at the endplate of γ-motoneurons (data not shown). Most importantly γ-subunit-specific GFP labelling was prominent at the equatorial region of adult muscle spindles and this signal was co-distributed with VGluT1-stained sensory nerve endings (Figure 17E).

These two independent experiments led to the same conclusion: a γ- to ε-subunit switch of AChR occurs in muscle spindles at the γ-motoneurons endplate and at neuromuscular junctions. In contrast, at the equatorial region in the adult muscle spindles, ε-subunits start to be expressed during postnatal development. However, the γ-subunit expression is not concomitantly downregulated but remained present. This

leads to the simultaneous presence of the fetal and the adult AChR at the equatorial region of muscle spindles. These results are summarized in Table 3.

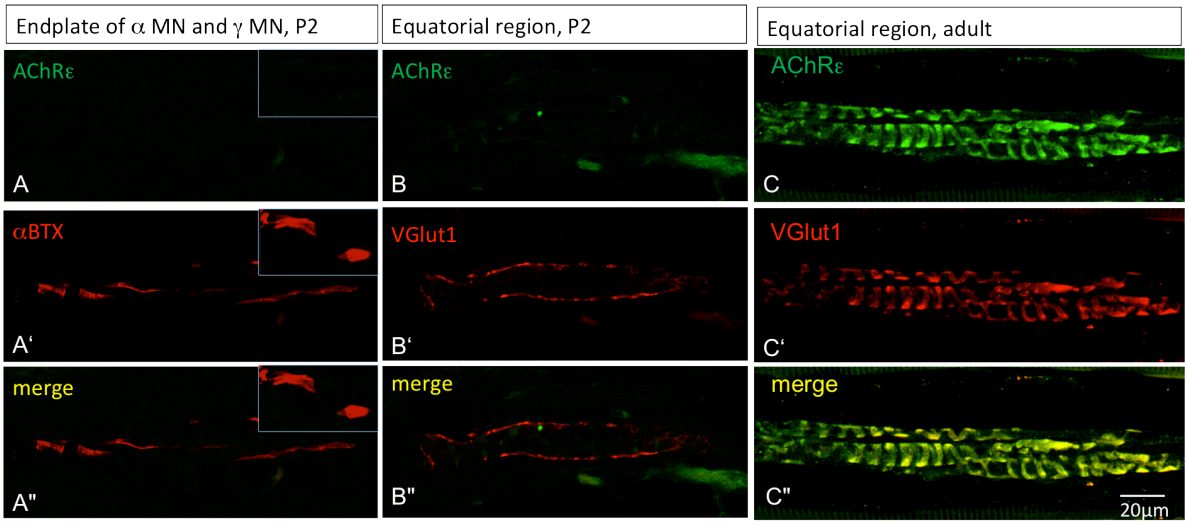
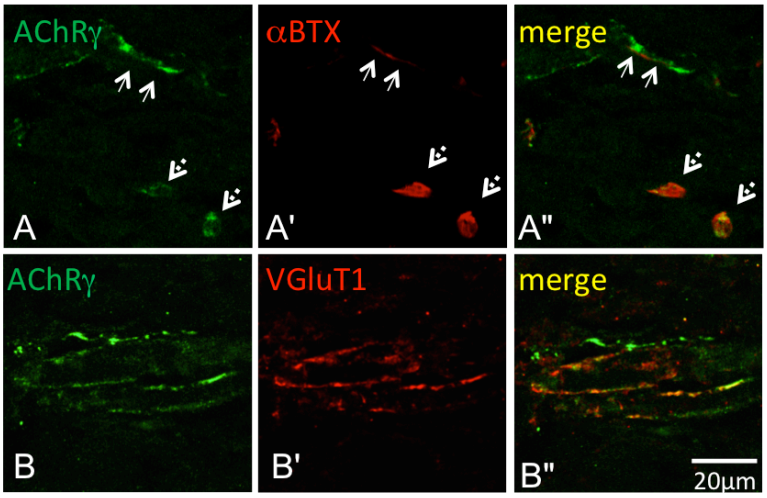



Figure 16. The distribution of AChR epsilon subunit in postnatal and alult skeltal muscle.

(A) AChR ε-subunits are absent at P2 endplates of γ-motoneurons and NMJs (squares), where they are indicated by α-BTX staining (A', A''). (B) AChR ε-subunits are also absent in the equatorial region, where the sensory nerve ending is stained with anti-VGluT1 antibody (B, B'') in P2 mice. (C) In adult mouse muscle, the ε-subunit of AChRs is present at NMJs, γ-motoneuron endplates (data not shown) and in the equatorial region (C, C', C'').



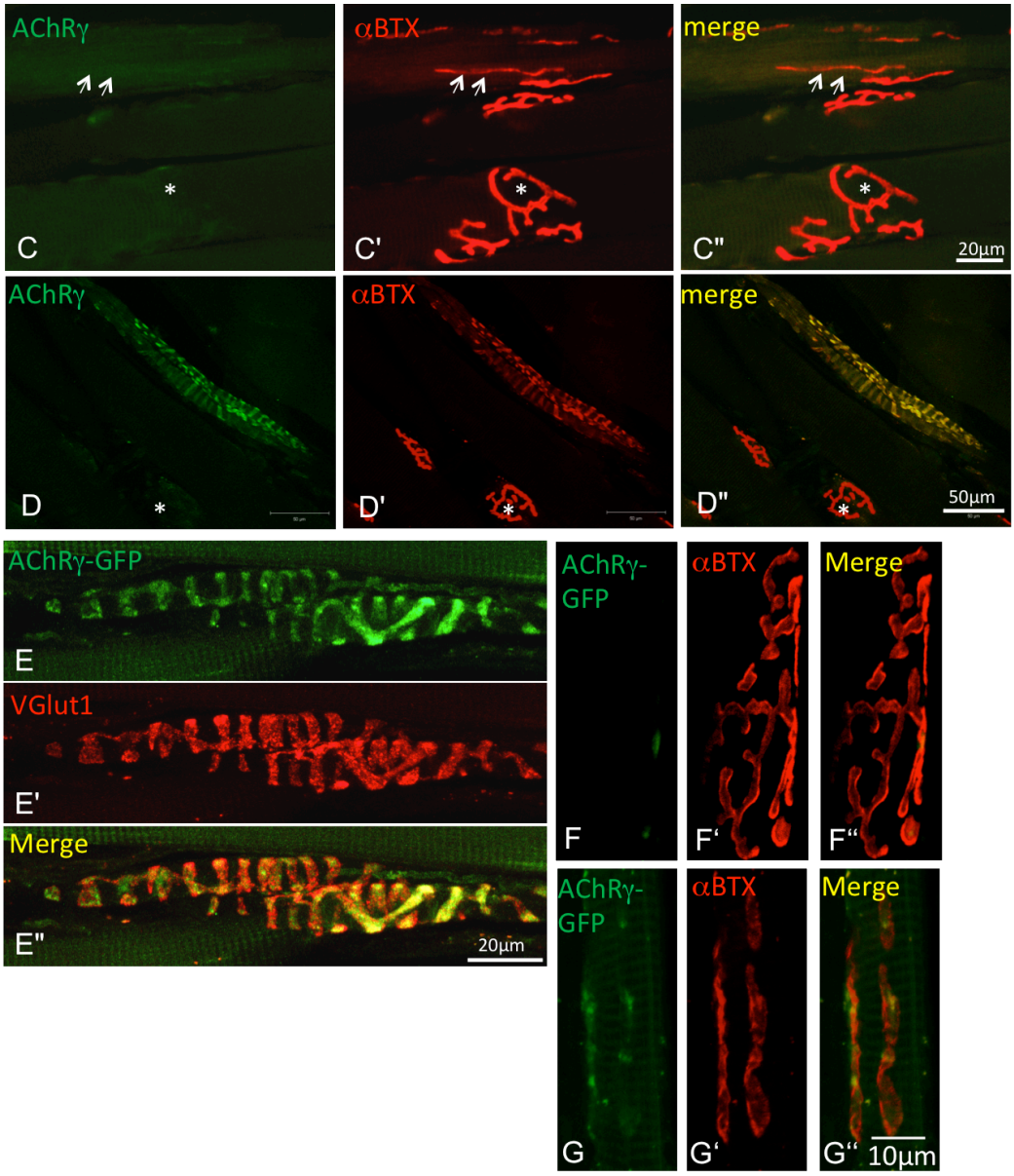



Figure 17. The distribution of the AChR γ-subunit in postnatal and adult skeltal muscle.

(A) AChR γ-subunits are concentrated at P2 endplates of γ-motoneurons and NMJs (squares), where they colocalized with α-BTX staining (A', A''). (B) AChR γ-subunits were concentrated in the equatorial region and colocalized with VGluT1-labeled sensory nerve endings (B, B'') in P2 mice. (D) In adult mouse muscle the γ-subunit of AChRs was undetectable at NMJs (asterisk) and at endplates of γ-motoneurons (arrow). (E) At the equatorial region of adult muscle spindles, the AChR γ-subunits were present and colocalized with α-BTX. (C) Confirmation of the presence of the γ-subunit at adult annulospiral endings using a mouse line, in which the γ- subunits of the AChRs were genetically labelled with GFP. AChRγ-GFP labelling was present in the equatorial region and co-localized with VGluT1-labelled sensory nerve ending. As negative control, no AChRγ-GFP signal was detectable at adult NMJs (F-F"), however, faint GPF signal was present at the γ-motoneuron endplates.

Table 3. Expression of the alpha- and gamma-subunits in postnatal and adult muscle spidles.

| | postnatal stages | adult stages |
|---|---|---|
| **sensory nerve ending** | γ subunit | ε subunit<br>γ subunit |
| **γ-motoneuron endplate** | γ subunit | ε subunit |
| **α-motoneuron endplate (NMJ)** | γ subunit | ε subunit |

### 4.5 The role of agrin and its receptor complex during muscle spindle development

Agrin has been well characterized for its role during formation, maintenance and regeneration of the cholinergic specializations at the NMJ. Agrin is the key synaptogenic molecule and is responsible for formation of most if not all pre- and postsynaptic specialization. Agrin is also required for the AChR γ- to ε-subunit switch at the NMJ (Jones et al., 1996; Rimer et al., 1997). To address if agrin also plays a role in the γ- to ε-subunit switch of AChRs in muscle spindles, we investigate agrin and its receptor complex, including MuSK and LRP4.

As a first step, I investigated the distribution of agrin and of its receptor complex, MuSK and LRP4, in adult muscle spindles, using antibodies against these proteins. The immunohistochemical analysis showed that agrin, MuSK and LRP4 were concentrated at the endplates of γ-motoneurons (Figure 18A-I). Agrin, MuSK and LRP4 were also present at the equatorial region, where they codistributed with VGluT1-labelled sensory nerve endings (Figure 18J-R). In addition, agrin could be detected in the region surrounding the intrafusal fiber, most likely representing the connective tissue capsule (Figure 18K). These results show the presence of agrin and its receptor complex at the sites of cholinergic specializations in muscle spindles.

In order to analyse if the immunoreactivity in the central region of muscle spindles and at the γ-motoneuron endplate was derived from an expression of agrin and its receptor complex by the intrafusal muscle fibers or by the neuron, I determined the

presence of these molecules in the cell bodies of proprioceptive neurons and in γ-motoneuron cell bodies.

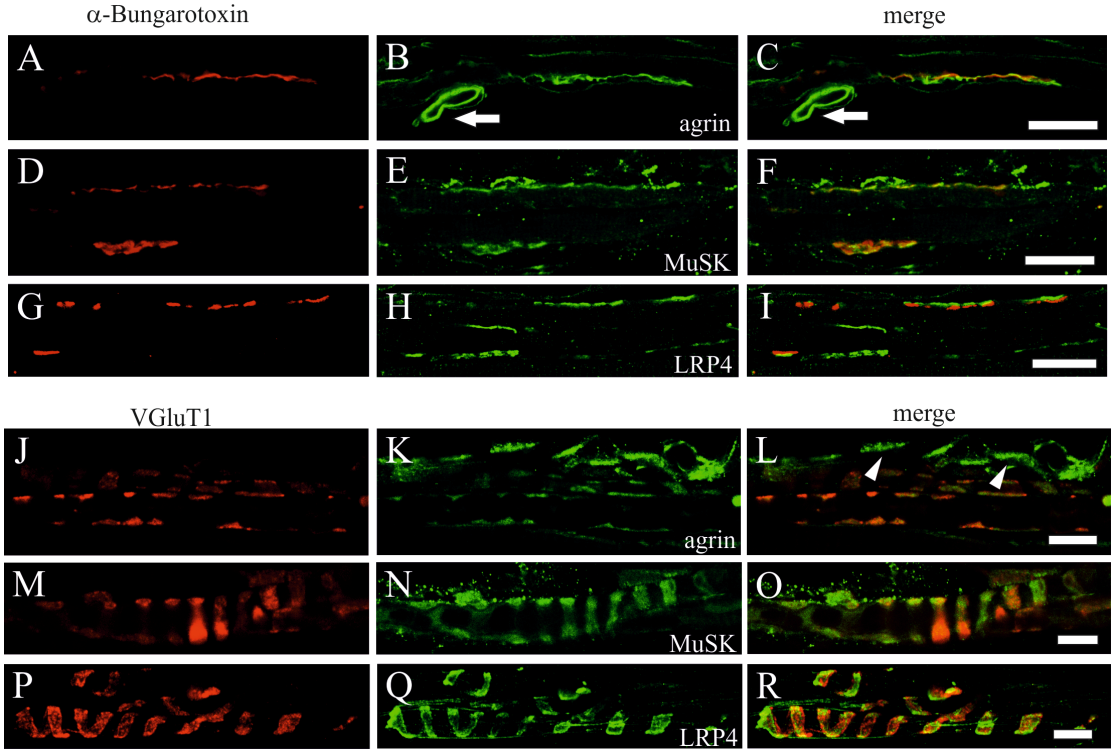
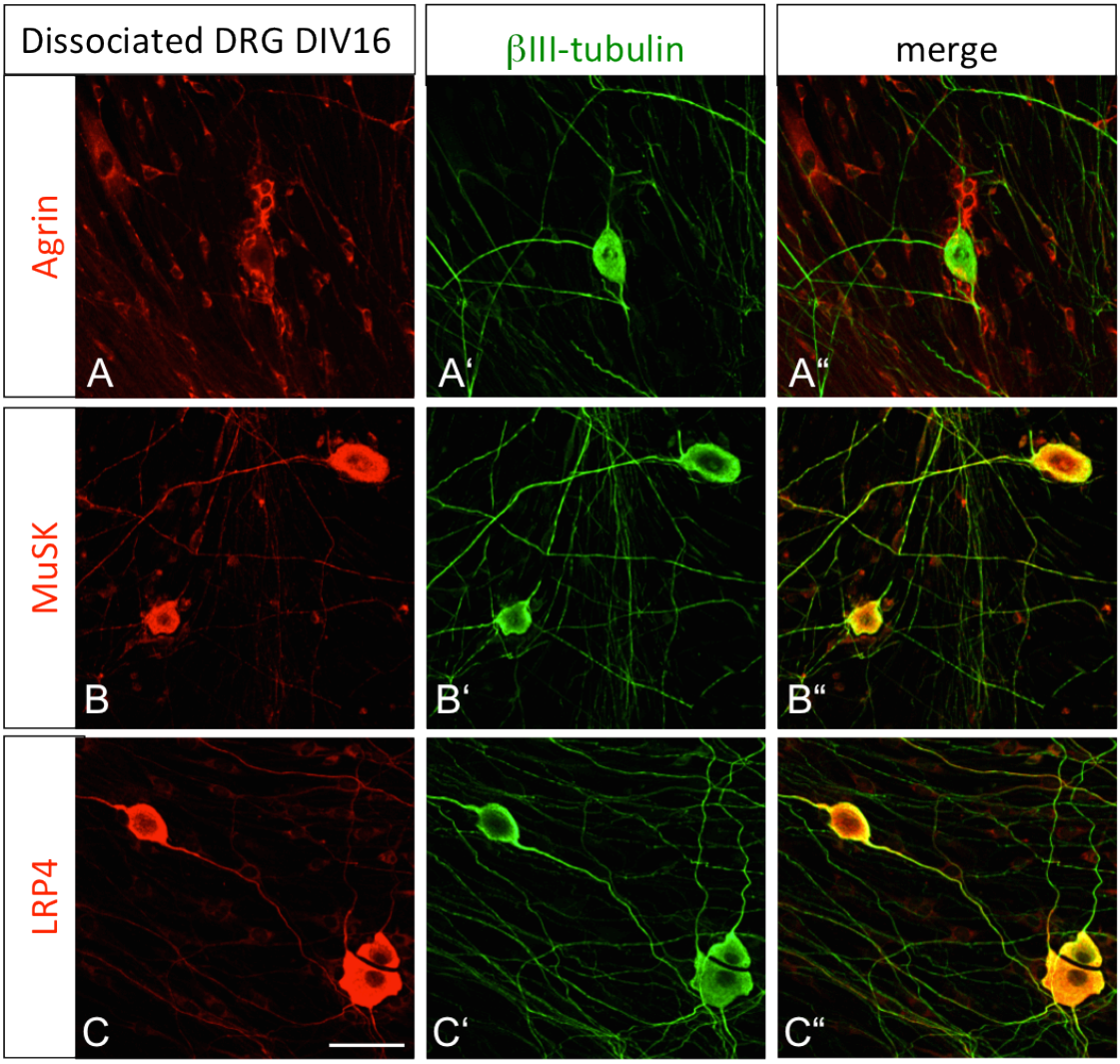



Figure 18. Distribution of agrin, MuSK and LRP4 in adult muscle spindles.

Agrin and its receptor complex consisting of the signal transducing tyrosine kinase MuSK and the agrin-binding protein LRP4 are present at the polar region (A-I) and equatorial region (J-R). Immunoreactivity for agrin (B,K), MuSK (E,N) and LRP4 (H,Q) was concentrated at the endplates of γ-motoneurons, where it colocalized with α–BTX-labelled AChRs (A,D,G), and at adult annulospiral endings marked with anti-VGluT1 antibodies (J,M,P). Agrin immunoreactivity was additionally associated with several non-neuronal structures including blood vessels (marked by an arrow in panel B and C), and the connective tissue outer capsule of muscle spindles (labeled by arrowheads in panel L). Scale bar A-I: 20 µm; J-R: 10 µm

To this end I first investigate if dorsal root ganglion neurons express agrin, MuSK and LRP4 in dissociated DRG culture (Malin et al., 2007). DRG neurons were cultures *in vitro* with a selective culture medium containing NT-3 (Neurotrophin-3) and NGF (nerve growth factor; Friedel et al., 1997). In this medium TrkA positive cutaneous neurons and TrkC positive proprioceptive neurons do not survive (Friedel et al., 1997). The cells were fixed after 16 days in culture and IHC staining was performed using antibodies against βIII-tubulin. These neurons, representing mainly proprioceptive neurons, were positive for agrin, MuSK and LRP4 (Figure 19A-C), suggesting the expression of these proteins by proprioceptive neurons.

I next addressed if agrin and its receptor complex are also expressed by sensory neurons in the DRG *in vivo*. To distinguish proprioceptive neurons from other DRG neurons, I used antibodies against parvalbumin, which has been shown to selectively label proprioceptive neurons (Celio, 1990), which represent a minor fraction of all DRG neurons. As shown in (Figure 19D-F), agrin, MuSK and LRP4 immunoreactivity was colocalized with parvalbumin-labelled proprioceptive neurons. These results indicate that agrin and its receptor complex, MuSK and LRP4, are expressed by proprioceptive neurons. In addition, agrin was present in connective tissue around neurons whereas MuSK and LRP4 were exclusively detected in neurons. These results suggest that the immunoreactivity for agrin and its receptor complex detected in muscle spindles is at least in part derived from the sensory neurons.



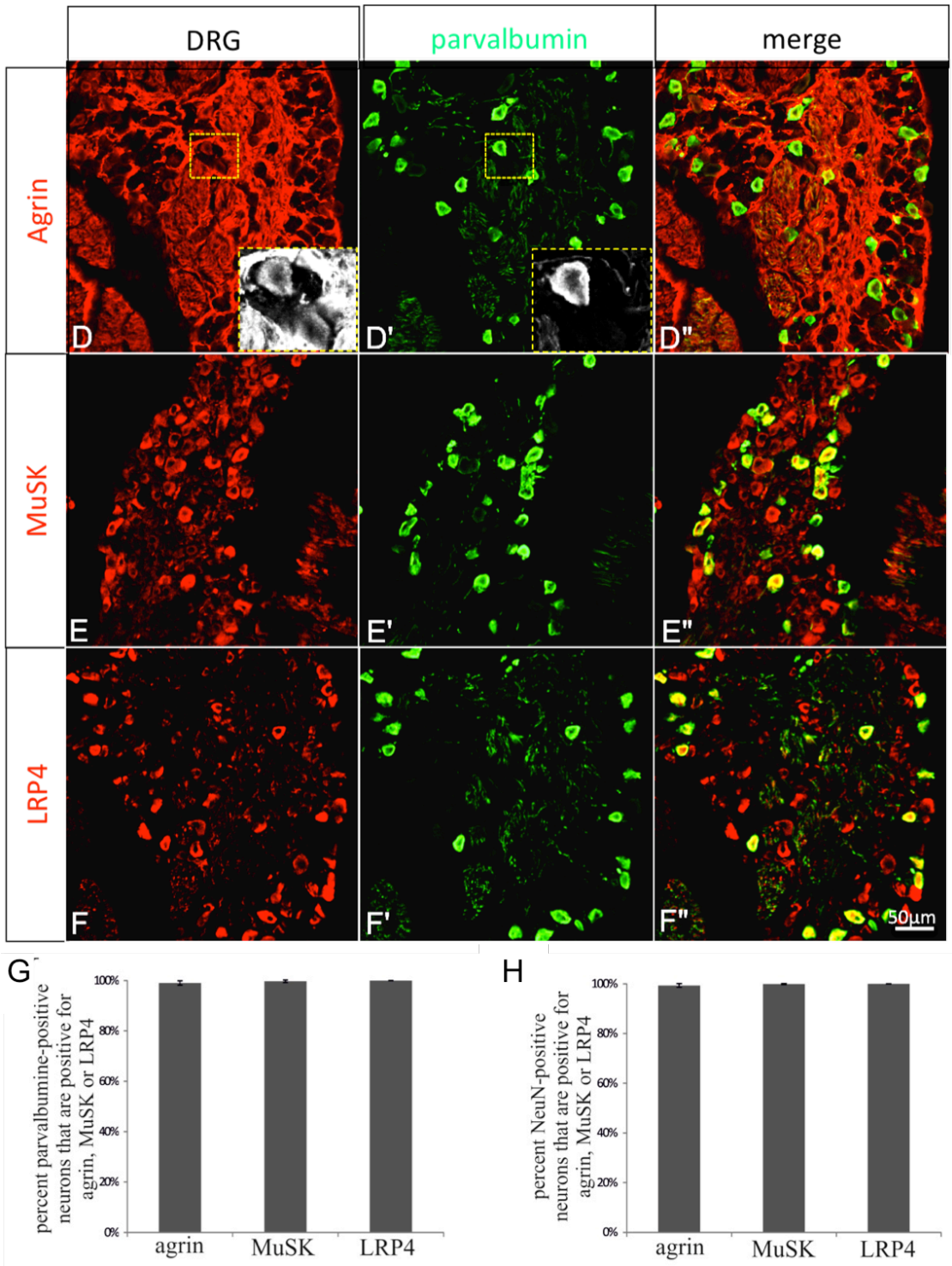



Figure 19. Distribution of agrin, MuSK and LRP in DRG neurons.

(A-C) In dissociated DRG neuronal culture, agrin and its receptor complex (consisting of the signal transducing tyrosine kinase MuSK and the agrin-binding protein LRP4) are present in β-III tubulin labelled DRG neurons (A'-C', A''-C''). Scale bar 50µm. (D-F) In cryosections of mouse DRG, parvalbumin-positive proprioceptive sensory neurons (D'-F') express agrin, MuSK and LRP4 (D-F). Agrin is also present in other cells within the DRG, including glial cells and connective tissue. Higher magnifications of the boxed areas are shown in the respective inserts. Scale bar 50µm. (G) Quantification of the percentage of agrin, MuSK and LRP4

positive comparing with parvalbumin positive DRG cells. The results demonstrate that all parvalbumin positive proprioceptive nerons express agrin, MuSK and LRP4. (H) Identification of DRG cells, labelled by NeuN. All DRG neurons (NeuN positive) express agrin, MuSK and LRP4. Quantification are showed as mean ±SEM; N=3.

To address if γ-motoneurons have similar contribution for the formation of the γ-motoneuron endplate as α-motoneurons at NMJ, I further compared the distribution of agrin/MuSK/LRR4 in α- and γ-motoneurons in the ventral horn of the spinal cord (Figure 20A,E,I). Gamma-motoneuron cell bodies lie in direct vicinity to the cell bodies of α-motoneurons in the ventral horn of the spinal cord. They can however be distinguished from α-motoneurons by their smaller cell body cross-sectional area of around 230 μm², whereas the cell body cross-sectional area of α-motoneurons is bigger, i.e. about 770 μm² (Friese et al., 2009). To identify α- and γ-motoneurons, two additional markers were applied in IHC, ChAT and Err3. ChAT is expressed in both α- and γ-motoneurons (Shneider et al., 2009); whereas the Zinc-finger transcription factor Err3 is expressed at high levels only in γ- motoneurons and at only very low levels in α-motoneurons (Friese et al., 2009). The IHC results revealed that agrin and LRP4 are expressed in bigger α-motoneurons, and also detected in the smaller ChAT positive, Err3 positive γ-motoneurons. In contrast, MuSK was present neither in α- nor in γ-motoneurons. These results show that at least part of the immunoreactivity for agrin and LRP4 detected at the endplate of γ-motoneurons is due to the expression of both proteins by γ-motoneurons.

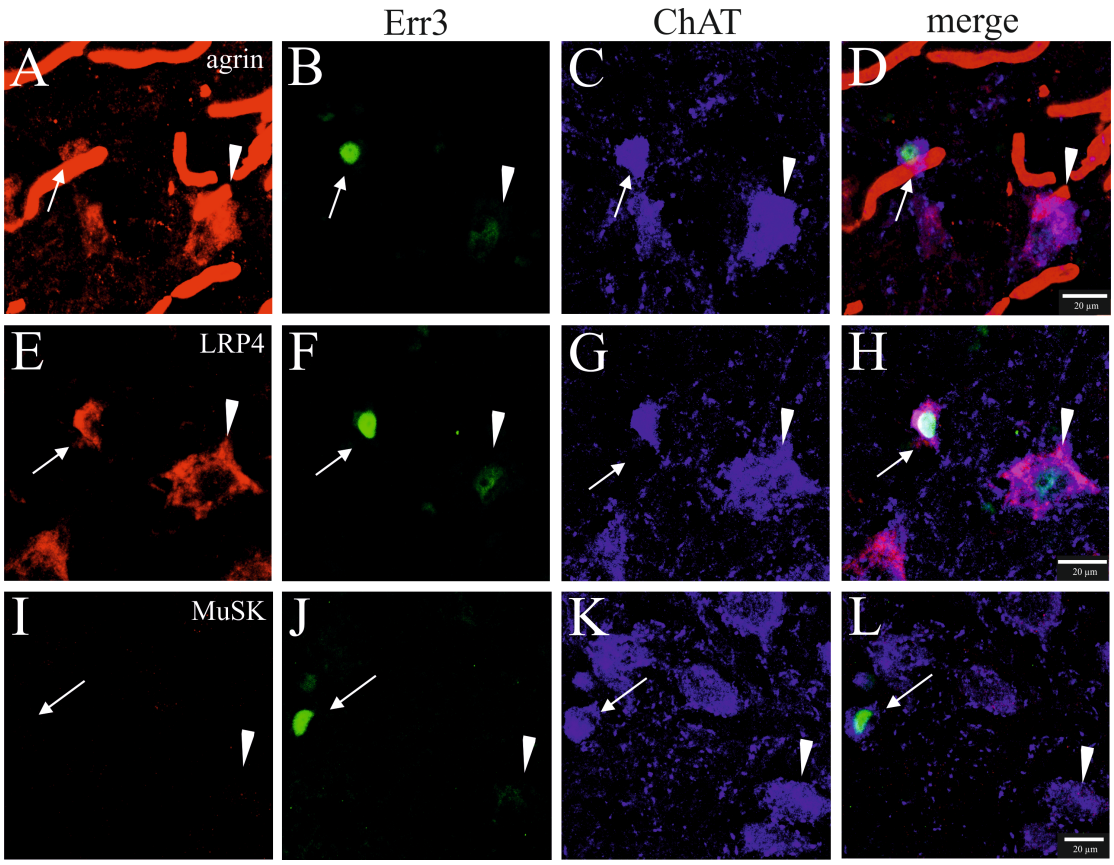



Figure 20. Agrin and LRP4 but not MuSK were expressed in the adult ventral horn motoneurons.

Ventral horn motoneurons in the cryosection of adult mouse spinal cord (2 months old) were labelled by ChAT (C,G,K), Err3 (B,F,J) and agrin(A), MuSK(E), LRP4(I). The merged pictures (D,H,L) showed that agrin and LRP4 were expressed in Err3/ChAT double positive small neruons (γ-motoneurons) and MuSK immunoreactivity was absent in γ-motoneurons. Scale bar 20 µm.

## 4.6 **The role of agrin during the development of muscle spindles**

To investigate the function of agrin during the development of muscle spindles, agrin knockout mice were analysed (Lin et al., 2001). In agrin knockout mice, NMJs do not form and the mice die perinatally due to respiratory muscle failure (Gautam et al., 1996).

### 4.6.1 Morphological alterations of agrin mutant muscle spindles

To investigate the function of agrin during muscle spindle development, I analysed E18.5 quadriceps muscles from wildtype and knockout mice. The embryonic tissue had to be used due to the perinatal lethality of agrin-deficient mice. At this stage, muscle spindles are functional and the stretch reflex is present (Chen et al., 2003; Kozeka and Ontell, 1981; Maeda et al., 1985). However, morphologically, muscle

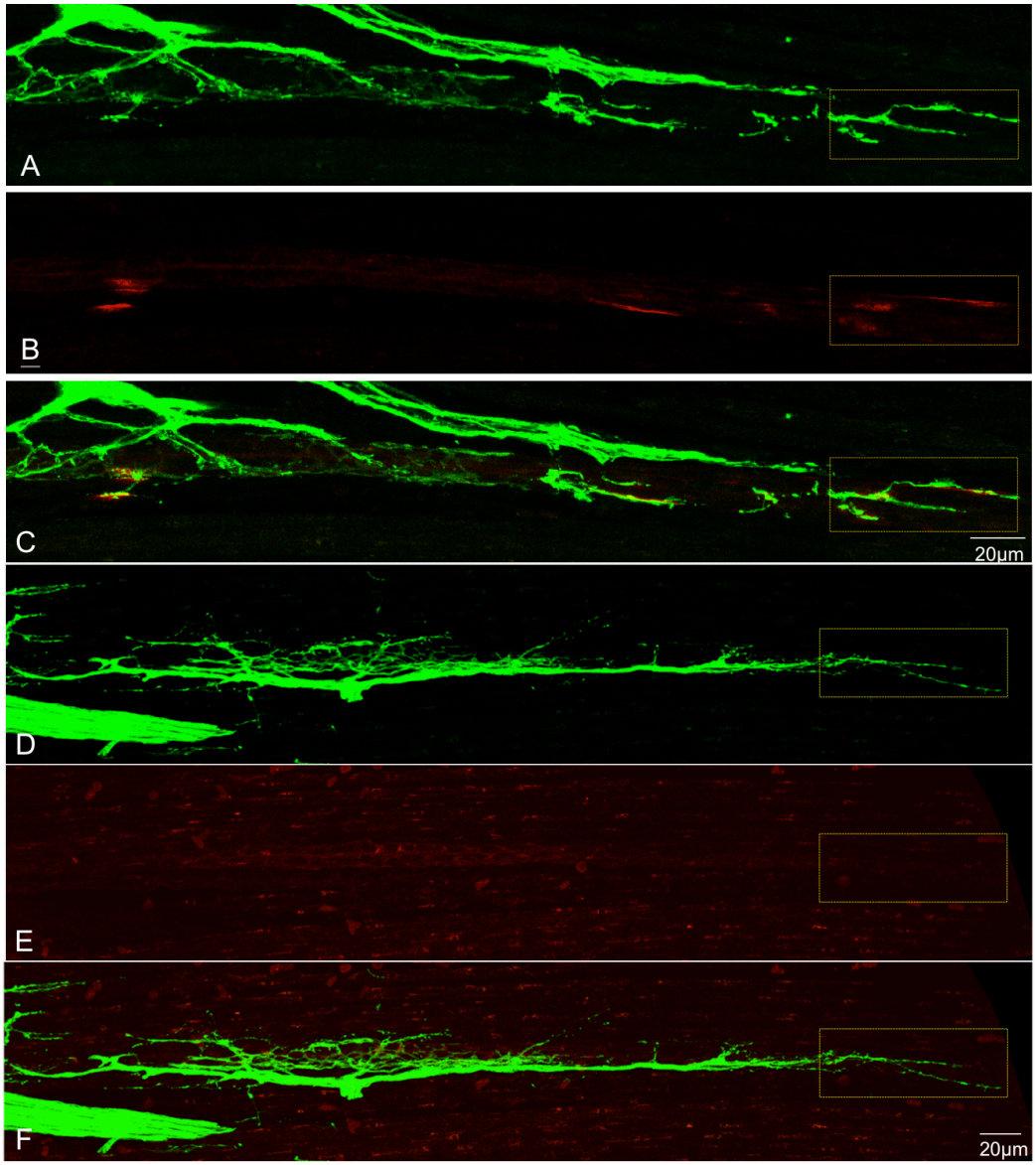
spindles are still immature and the sensory nerve had formed a “spider-web-like” network on intrafusal fibers (Figure 21A), which will develop into annulospiral sensory nerve endings at later stages (Maeda et al., 1985). In agrin knockout mice, intrafusal fibers could be detected and nuclei were aggregated at the equatorial region. Using antibodies against NFH/SYN and VGluT1 (which both label the sensory nerve endings), immature muscle spindles were identified and the intrafusal fibers were surrounded by branched nerve terminals similar to what has been observed in wild-type mice (Figure 21D).

To more precisely compare the sensory nerve endings from agrin wildtype and knockout mice, the density of nuclei and the density of branchpoints in the equatorial region were quantified (Figure 22). The quantification showed that despite the absence of agrin, nuclei were still aggregated in the central region and the branching pattern of sensory nerve endings could develop normally.

While in the equatorial region the muscle spindle morphology of wildtype and agrin knockout mice were similar if not identical, at the polar region a notable difference was observed. In wildtype mice the endplate of γ-motoneurons was formed, with its typical long and thin morphology. In agrin knockout mice, however, we found that even though the motor nerve still extended to the polar region, there were no aggregates of AChRs colocalizing with γ-motoneurons. These results demonstrate that agrin is required for the aggregation of AChRs at the endplate of γ-motoneurons.

The perinatal lethality of agrin knockout mice can be rescued by the muscle-specific reexpression of a mini-agrin construct (Lin et al., 2008). Mini-agrin contains the main functional domains of agrin: the N-teminal laminin-binding domain and the C-terminal LRP4-binding and MuSK activating domain. Additionally, the construct contains also a myc-tag, allowing the distinction of mini-agrin from endogenous agrin using an antibody against the tag.

To address the question if mini-agrin can also be re-expressed in intrafusal fiber, IHC using antibody against myc-tag was performed. The staining showed that mini-agrin/myc-tag is present at both extrafusal fiber and intrafusal muscle fiber (data not shown).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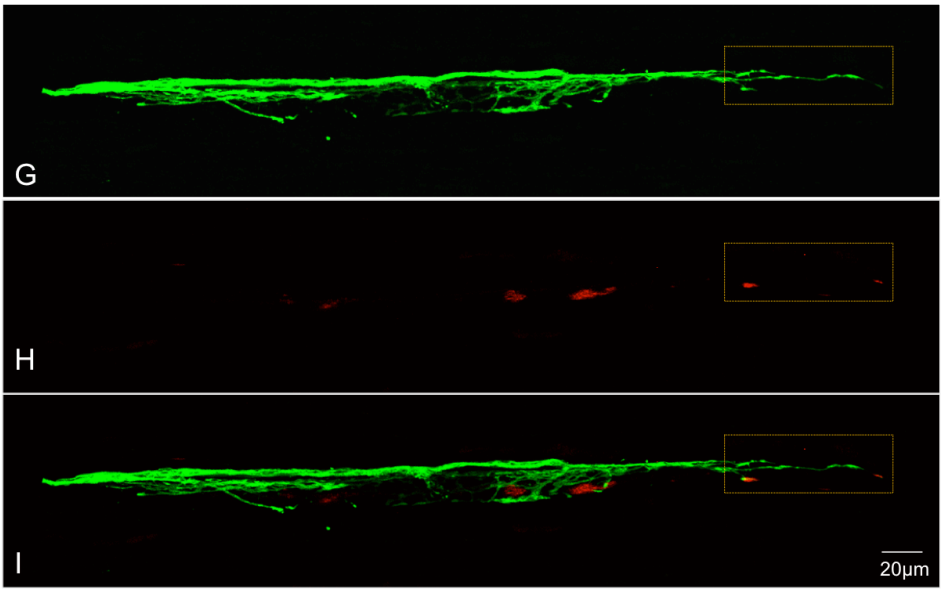
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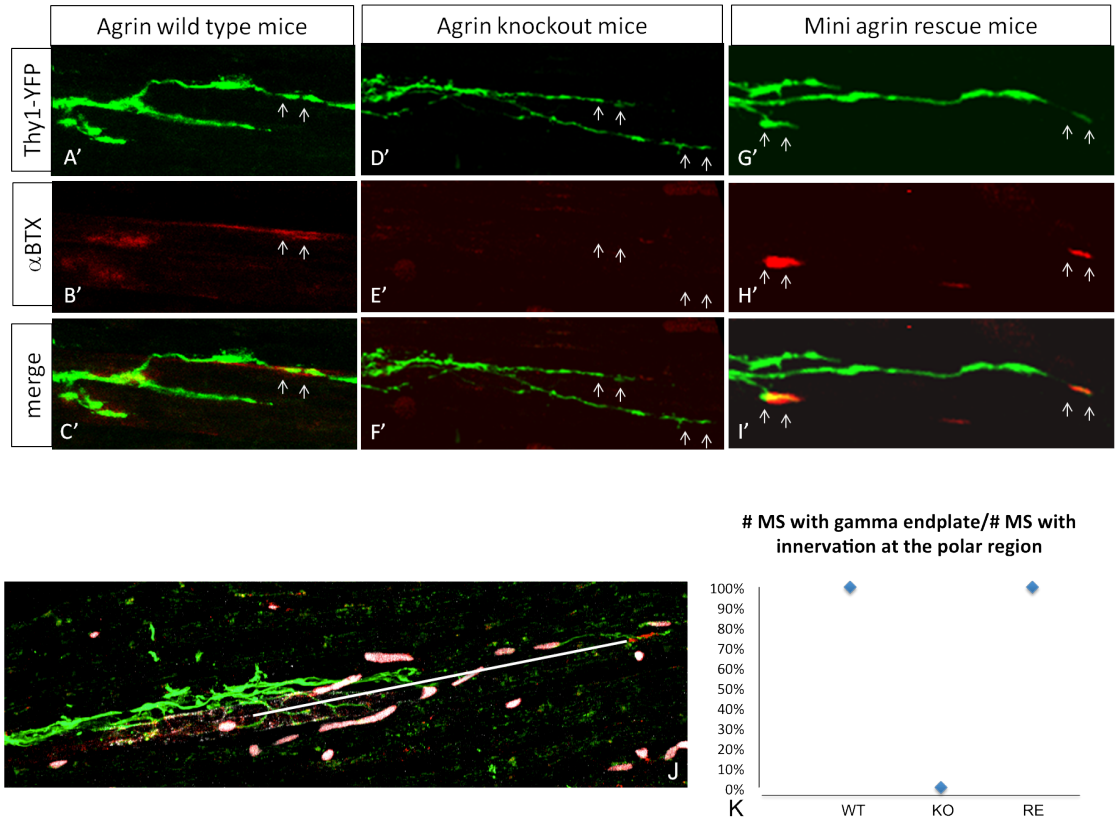


Figure 21. Muscle spindles in wildtype, agrin knockout and miniagrinrescue mice.

Confocal images from E18.5 mouse muscle spindles of wildtype (A-C), agrin knockout (D-F) and miniagrin c-magB8 rescue mice (G-I). Sensory and motoneurons were visualized by genetically labelled Thy1-YFP (A,D,G). αBTX labelled AChRs are shown in red (B,E,H). Axons from γ-motoneurons extended from the equatorial region along the length of the intrafusal fiber to both polar regions (areas are indicated by squares in panels A-I). High magnifications of the termination sites of γ-motoneurons are shown in panels A'-I'. (A-C, A'-C') In the muscle spindles in E18.5 wildtype mouse embryos AChRs were precisely colocalized with Thy1-YFP labelled motoneuron terminals. (D-F, D'-F') In E18.5 agrin knockout mouse embryos, the γ-motoneurons still extended along the surface of intrafusal fibers to the polar region, but no co-localizing AChR aggregates could be detected. (G-I, G'-I') In E18.5 mini-agrin rescue mouse embryos, muscle-specific reexpression of a short version of the agrin cDNA rescued the formation of γ-motoneuron endplates. (J) The results in (A-I) were quantified by counting the number of AChR aggregates (labelled by α-BTX) colocalizing with γ-motoneurons at a distance between 100 and 300 µm from the center of the annulospiral endings (distance between the center of equatorial region and the γ-motoneuron endplate is indicated by the white line). Only those γ-motoneurons were analysed which could be unambiguously followed for 300 µm distally. This analysis showed (K) no AChR aggregates colocalizing with a γ-motorneurons in agrin -/- mice (N=10). In contrast, in all cases investigates from wildtype (N=10) and agrin rescue mice (N=6) we detected AChR aggregates colocalizing with γ-motorneurons.

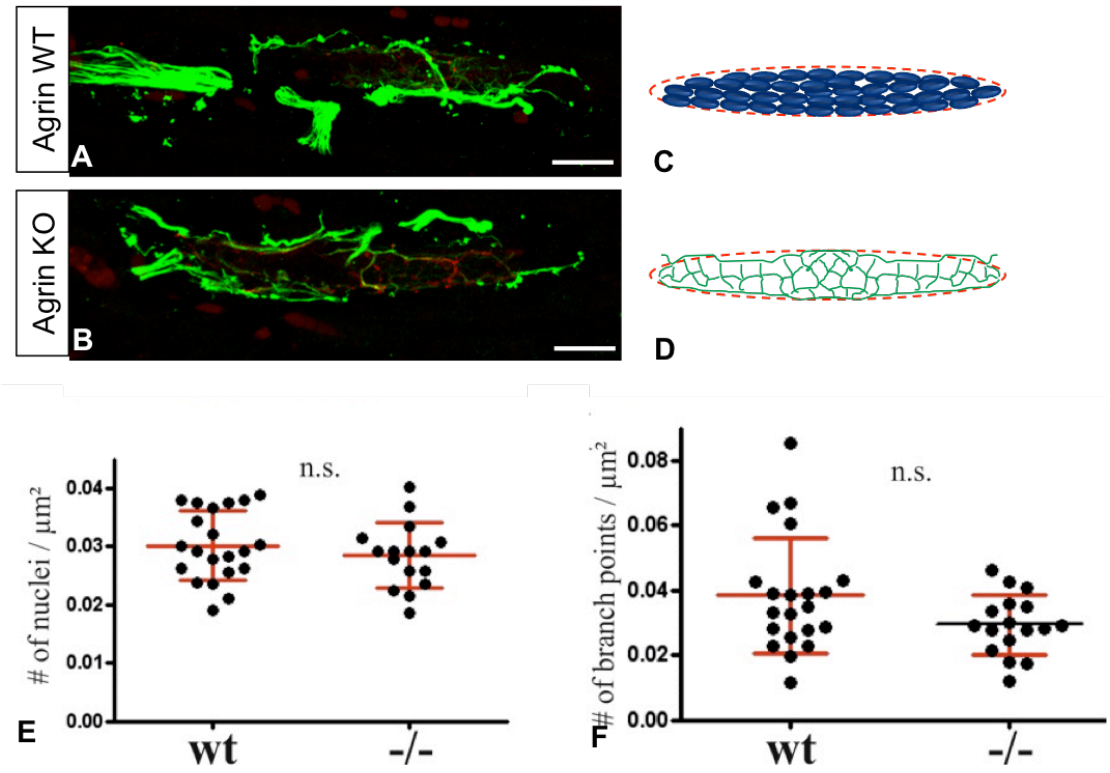


Figure 22. Morphological analysis of the equatorial region in wildtype and agrin knockout muscle spindle.

(A and B) Confocal images of high magnification of the equatorial region of muscle spindles in wildtype (A) and agrin knockout (B) mice embryos. Green colour shows transgenic GFP labelled sensory nerve ending; red colour indicates VGluT1 immunoreactivity. (C and D) schematic representation of the equatorial region. The red line indicates the extent of intrafusal fibers. The blue ovals indicate nuclei (C); the green lines show the branches of sensory nerve endings. (E and F) the density of nuclei and the density of the branch points in the equatorial region were quantified in wildtype and agrin knockout mice embryos. Each dot in panels E and F represents a single muscle spindle. The red lines represent the mean ± STDEV of 3 mice. No significant difference for both parameters between WT and knockout animals was observed. Scale bar in panels A and B: 20 µm.

Reexpression of mini-agrin in intrafusal fibers rescued the formation of the endplate of γ-motoneurons. Analysis of muscle spindles from agrin-rescue mice revealed that agrin was the key molecule, playing the role for the aggregation of AChR at the γ-motoneurons endplate.

To further confirm these results, a quantitative analysis of the γ-motoneuron endplates was performed. We firstly defined that the aggregation of AChR always localizes 110-300µm from the center of the intrafusal fiber of E18.5 wildtype mouse hind limb muscles (Figure 21J). The central point of aggregated nuclei was defined as

the centre of intrafusal fiber. In this area single motor axons spreading to the polar region were observed and the number of AChR aggregates was identified. We found that in wildtype (n=10) and mini-agrin rescue mice (n=6) the AChR aggregates were 100% colocalized with an axon from a motoneuron, whereas in agrin knockout mice (n=10) no colocalization of AChR aggregagtes with a motoneuron was detected.

Collectively these results suggest that agrin is required for the aggregation of AChRs at the endplate of γ -motoneurons.

### 4.6.2 Identification of "pre- and postsynaptic" molecular characteristics at sensory nerve endings in muscle spindles of agrin-deficient mice

At the morphological level no obvious difference of the equatorial region between wildtype and agrin knockout mice was observed. To investigate the effect of agrin deficiency at the equatorial region during the development of muscle spindle at the molecular level, we determined if the "presynaptic" and "postsynaptic" characteristics in the sensory-muscle junction were altered. To this end the presynaptic molecule bassoon and the postsynaptic AChRs were analysed. In E18.5 wildtype mice bassoon immunoreactivity showed a punctate pattern at the equatorial region of muscle spindles (Figure 23A-C), which was colocalized with AChRs. To our surprise, in agrin knockout mice AChR staining was still present and colocalized with punctate bassoon immunoreactivity (Figure 23D-E).

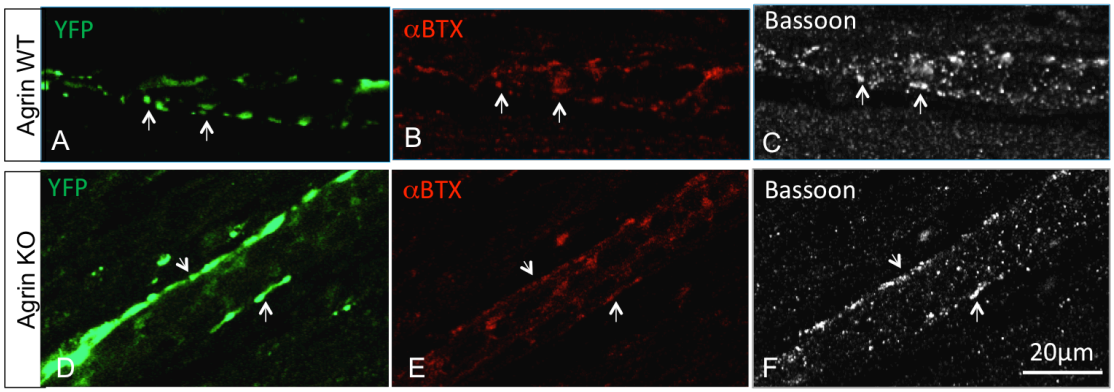


Figure 23. Distribution of the presynaptic marker Bassoon in the equatorial region of muscle spindles.

Confocal images of Bassoon immunoreactivity in the equatorial region of agrin wildtype and knockout muscle spindles. In muscle spindle of wildtype embryos (A-C) as well as muscle spindle of agrin knock out moue embryos, Thy1-YFP transgenically labelled sensory nerve terminal (A) and α-BTX stained AChRs (B). Puncta-

distributed Bassoon labelling is colocalized with YFP and aBTX labelled sensory nerve ending (arrow heads).

In order to confirm the presence of AChR at the annulospiral sensory nerve endings of agrin knockout mice, antibodies against the γ-subunit of AChR, were used to stain muscle spindles from wildtype and agrin knockout mice. The results (Figure 24) showed that in agrin wildtype and agrin knockout muscle spindles the γ-subunit was co-distributed with the α-BTX labelled α-subunit.

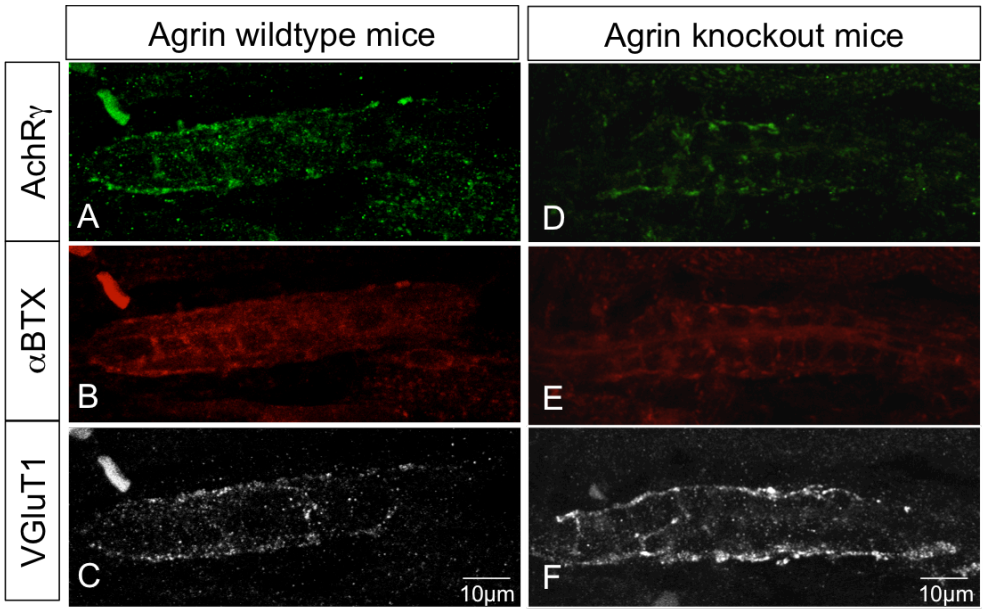


Figure 24. The distribution of AChR gamma subunit in the muscle spindle equatorial region.

(A-C) AChR α-subunit is codistributed with γ-subunit in the equatorial region of wildtype muscle spindles. (D-F) show at the equatorial region of agrin knockout muscle spindles the immonoreactivity of AChR γ-subunit antibody staining (D) is co-distributed with αBTX labelled AChR α-subunit (E). VGluT1 staining (C, F) indicated the range of equatorial sensory nerve ending.

In summary, these results show that deficiency of agrin affects neither the expression of AChRs nor the distribution of active zone-specific proteins.

# 5 Discussion

My work provides insights into how cholinergic synapse-like specializations in muscle spindles develop and which role agrin plays during this process. The starting point of my work was to describe the distribution of several molecules, which are associated with synapse-like specializations and with their formation. My work is based on the knowledge derived from the formation and maintenance of NMJs; the best characterized synapse in the entire nervous system. The NMJ is the communication site between an α-motoneuron and an extrafusal muscle fiber. At this particular synapse, which is the prototype of a cholinergic synapse, many molecules are specifically concentrated in the region of neuron to muscle contact. In my work I characterized the distribution of these molecules in developing and adult muscle spindles. I also investigated the γ- to ε-subunit switch of the AChR in the polar and in the equatorial regions. Finally, I analysed the molecular mechanisms that underlie the formation of these synapse-like specializations during muscle spindle development. My findings can be summarized as follows:

1. the composition of the extracellular matrix in muscle spindles is not uniform, but instead specific ECM proteins have unique distributions;
2. sensory- and motoneurons are specialized in the region of contact with the intrafusal muscle fiber, and contain high concentrations of AChRs, rapsyn and other markers of cholinergic synapses;
3. the sensory nerve terminal contains molecules indicative of a presynaptic specialization and vesicle exocytosis;
4. at γ-motoneuron endplates AChRs undergo a complete postnatal γ-to-ε subunit switch, similar to the postnatal development of the NMJ;
5. at the sensory nerve endings the γ-subunit of AChRs is present during embryonic and early postnatal development and continues to be expressed in adult spindles despite the postnatal increase in ε-subunit expression, leading to the simultaneous presence of fetal- and adult-type AChRs at the adult annulospiral sensory nerve endings;
6. agrin, MuSK and LRP4 are expressed by proprioceptive neurons *in vitro* and in parvalbumin-positive DRG neurons *in vivo*;
7. agrin, LRP4 but not MuSK are expressed by γ-motoneurons in the ventral horn of the spinal cord;

8. agrin is required for the formation of the endplate of γ-motoneurons but not for the aggregation of AChRs at sensory nerve terminals;
9. muscle-specific reexpression of agrin rescues the formation of γ-motoneuron endplates in muscle spindles.

In the following paragraphs I will discuss the most relevant issues regarding these findings.

## 5.1 Basement membrane in muscle spindles

The basement membrane is an important component of skeletal muscles. Several lines of evidence have shown that the basement membrane not only provides a scaffold for extracellular matrix proteins (Paulsson, 1992), maintains muscle integrity, promotes myogenesis and muscle development (Campbell and Stull, 2003), but also other additional instructive functions e.g. laminin is required for the myelination of Schwann cells (McKee et al., 2012). These functions are reflected by the composition of the basement membrane at the neuromuscular junction. Only specific isoforms of ECM proteins, including collagens, laminins, nidogens and proteoglycans are specifically concentrated at NMJs (Fox et al., 2008; Gautam et al., 1995; Noakes et al., 1995; Sanes et al., 1990). These NMJ-specific isoforms are collagen IV (α3, α4, α5) and $(\alpha5)_2\,\alpha6$ (Fox et al., 2007; Sanes et al., 1990); laminin β2 (Noakes et al., 1995), nidogen-2 (Fox et al., 2008) and agrin (Gautam et al., 1995). Mice lacking these molecules show specific abnormalities in the formation and maintenance of synaptic specializations. For example, nerve-derived agrin is required for the clustering of AChRs, for the pre- and postsynaptic differentiation, and maintenance of NMJs (Gautam et al., 1995; Samuel et al., 2012); muscle derived laminin β2 regulates the formation of the motor nerve ending. Mice lacking laminin β2 show loss of active zones, dispersal of synaptic vesicles, intrusion of Schwann cells into the synaptic cleft and less junctional folds (Noakes et al., 1995). Nidogen-2 mutant mice show immature or fragmented NMJs (Fox et al., 2008). Comparing the distribution and function of molecules in the synaptic BM of NMJs, I hypothesize that: (1) the components of extracellular molecules might also specifically distribute in the BM of muscle spindles; (2) in addition to structural components in the basement membrane, these proteins may have specific functions during the development of muscle spindles.

To test the hypothesis that the ECM components have specific distributions in muscle spindles, and to test which function agrin might have, I first investigated the presence of extracellular matrix molecules in muscle spindle longitudinal sections. In the outer capsule Col IV, nidogen, laminin γ1 and β2 chains and agrin are concentrated; in the inner capsule Col IV, nidogen, laminin γ1 and AChE were detectable. Interestingly, in the BM, which covered the sensory nerve ending, only nidogen, laminin β2 and agrin were present. This distribution is similar to the specific presence of ECM protein isoforms at NMJs. Laminin β2 and agrin play essential role in the formation and maintenance of NMJs. I therefore hypothesized that laminin β2 and agrin might be involved in the formation of annulospiral sensory nerve endings and the regulation of the synapse-like „pre-synaptic" differentiation. Moreover, electron microscopic investigations clearly showed that the BM covers the sensory nerve ending but does not extend into the space between the sensory nerve ending and intrafusal muscle fiber. These results open another possible function of EMC protein isoforms, i.e. that they might be involved in the contact between the sensory nerve ending and terminal Schwann cells. Due to the limited specificity of the anti-nidogen antibodies, the nidogen-1 and -2 isoforms could not be distinguished. More specific antibodies should be applied in the experiments. My results reveal the special localization of ECM protein isoforms and indicate these molecules may have some special function for the development or function of muscle spindles.

## 5.2 **Cholinergic specialization in muscle spindles**

Previous studies (Arber et al., 2000; Hippenmeyer et al., 2002) and the data I collected, confirm the presence of AChRs at the equatorial region of muscle spindles. 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. After all – the annulospiral sensory nerve ending is not a synapse but instead its function is to reliably detect stretch and send signals regarding the change of muscle length to the CNS. The question therefore arises as to what the function of the cholinergic molecules at this localization is. One possibility is that they might represent a synapse. This hypothesis is 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 a synaptic vesicles. Some of the vesicles contain glutamate, which is thought to modulate spindle sensitivity via presynaptic 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 able to release vesicles, which contain acetylcholine.

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 required for 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 the sensory nerve terminal with the intrafusal fiber is much bigger compared to the area of nerve-muscle contact at neuromuscular junctions. This suggests a function for vesicle exocytosis in the entire region of intrafusal muscle fiber sensory nerve contact region.

With the limited resolution of the confocal microscope, 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 widths. However, the presence of a reporter gene expressed under the control of regulatory elements from the α- and ε-subunit AChR gene in nuclei at the central region of intrafusal muscle fibers (Sanes et al., 1991) strongly suggests that the AChRs are expressed by intrafusal fibers and concentrated in their plasma membrane at the contact site to the sensory nerve terminal. Likewise, the presence of bassoon immunoreactivity in parvalbumin-positive proprioceptive neurons (data not shown) as well as in F11 cells (a cell line derived from DRG neurons; Goswami et al., 2010) and the expression of

bassoon mRNA in DRGs are detected by *in situ* hybridization (Diez-Roux et al., 2011)(http://www.eurexpress.org/). These strongly suggest 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 directly opposite the AChRs localized in the intrafusal muscle fiber plasma membrane.

In contrast to other markers for cholinergic synapses, we were unable to detect 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 (Gossrau and Grozdanovic, 1997; Schober and Thomas, 1978). 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).

Due to the unusual localization of AChE the question arises, how is acetylcholine degraded in the equatorial region after putative synaptic transmission? In order to address this question, I compared the distribution of acetylcholinesterase among equatorial regions of the muscle spindle with NMJs (Figure 25). At the NMJ, basal lamina-associated AChE rapidly degrades ACh molecules in the synaptic cleft (Vigny et al., 1983) and terminates the synaptic transmission (Byrne, 1997). The degradation of ACh at the endplate of γ-motoneurons is similar as that at NMJs. In contrast, in muscle spindles ACh is localized in the gap between the sensory nerve ending and intrafusal fiber, whereas AChE is present at a certain distance, possibly in the basement membrane covering on the sensory nerve ending. The long distance between ACh and AChE suggests that ACh cannot be degraded rapidly, so that AChR might be continuously active. Therefore, ACh in the equatorial region might be

degraded by AChE slowly and this might be involved in the specific functions of AChRs at the equatorial region.

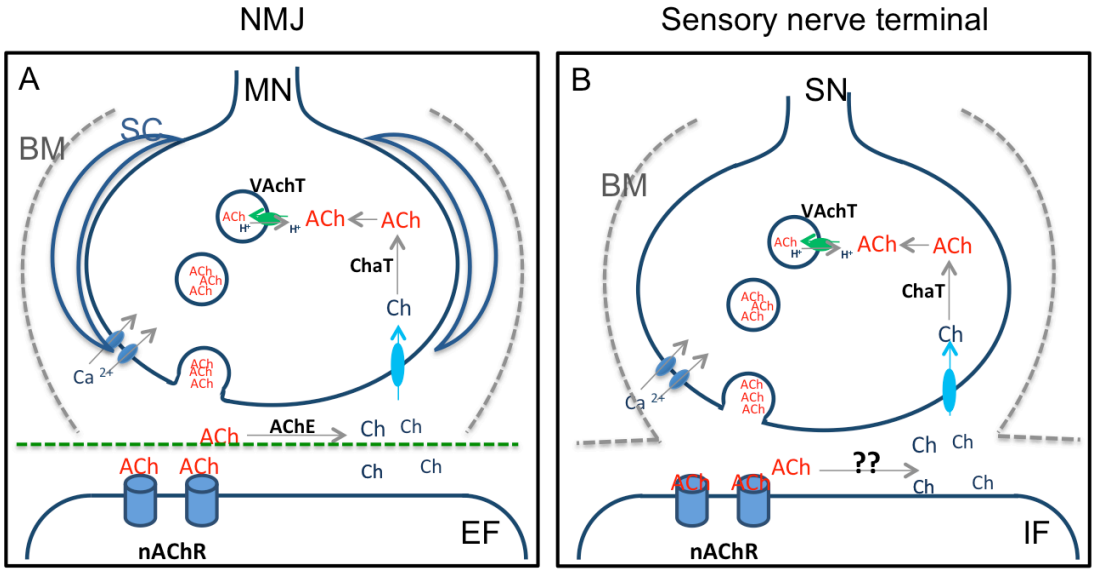


Figure 25. Schematic representation of potential cholinergic transmission at the NMJ and at the annulospiral sensory nerve ending.

(A) Cholinergic transmission in NMJ. Molecules VAChT and ChAT are present in the presynaptic terminal. Nicotinic AChR is localized in the postsynaptic membrane of muscle fibers. The basement membrane is present in the syanaptic cleft (30nm wide) (Hunt and Kuffler, 1951b; Purves et al., 2012) between motor nerve ending and the extrafusal skeletal muscle fibers (green dashed line) and covering on terminal Schwann cells (grey dashed line). AChE is anchored in the basement membrane, where AChE degrades acetylcholine to choline and acetate. Choline is uptaken by the presynaptic transporter and acetate is diluted by the surrounded medium (Byrne, 1997). (B) In the sensory nerve terminal of muscle spindles, VAChT and ChAT are present in the sensory nerve terminal. No basement membrane was detected in the gap (20nm wide) between sensory nerve ending and intrafusal fibers (Zelena, 1994). Nicotinic AChR is present in the intrafusal fiber membrane. AChE is detected in the basement membrane, which covers the sensory nerve ending. MN, motor nerve; EF, extrafusal muscle fiber; Pre, presynaptic neuron; Post, postsynaptic neuron; SN, sensory nerve terminal; IF, intrafusal muscle fiber; BM, basement membrane; SW, Schwann cell.

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 in response to the application of ACh or succinylcholine (Carr and Proske, 1996). The balance of evidence suggests that the excitatory effects of the cholinergic agonists are the result

of intrafusal muscle fiber contracture (mediated by AChRs at γ-motoneuron endplates in the polar region). However, another possibility suggested by the present study is a direct action of ACh released from sensory nerve terminals on spindle sensory endings (for a detailed discussion see Carr and Proske, 1996). Our results showing the 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, suggest 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 favour 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 (Bernheim et al., 1996; Chen et al., 1990; Cull-Candy et al., 1982; Fertuck and Salpeter, 1976). Clearly further studies are needed to clarify the role of the AChRs in muscle spindle function.

### 5.3 **The development of AChR ---- Gamma to epsilon subunits switch**

The γ- to ε-subunit switch of the AChRs at NMJs takes place during the first two postnatal weeks, resulting in the replacement of low conductance ion channels by high conductance ion channels (Missias et al., 1996; Yampolsky et al., 2008). In my study I find that the γ- to ε- subunit switch also occurs in muscle spindles. At the endplate of γ-motoneurons, the ε-subunit expression starts around postnatal week two and in the meantime the expression of γ-subunits is downregulated. This shows a similar AChR maturation at the NMJ and at the endplate of γ-motoneurons. However, within the same intrafusal fiber, AChRs at the equatorial region show a different developmental maturation: at postnatal week two, the ε-subunits start to become expressed, but the downregulation of the AChR γ-subunit does not occur. Instead its expression continues despite the onset of the ε-subunit upregulation. This raises several questions. Among others: What regulates the subunit switch at the γ-motoneuron endplate and are the mechanisms identical to those characterized in detail at the NMJ? Why is the AChR γ-subunit necessary in adult muscle spindles? The role of muscle activity, neural innervation and myogenesis factors during down-regulation

of γ-subunit gene and up-regulation of ε-subunit will be discussed in the following paragraphs.

At the NMJ, the ε-subunit gene is almost exclusively transcribed by synaptic nuclei, suggesting that the expression of the ε-subunit is under the control of factors released by the α-motoneuron (Brenner et al., 1994; Brenner et al., 1990). Rimer et al (1997) have shown that in denervated rat soleus muscle after induction with neural agrin cDNA, an ectopic postsynaptic-like apparatus can be formed with 30% ε-subunits and 70% γ-subunits. After reinnervation, despite the absence of apposed axon terminals, γ-subunits switch to ε-subunits gradually in the extrajuctional postsynaptic-like apparatus (Rimer et al., 1997). This observation indicates that agrin is the only nerve-derived factor required for this switch. We assume that in the endplate of γ-motoneurons, agrin may similarly play the main role regulating the γ- to ε-subunit switch. Consistent with this hypothesis, I showed that agrin is expressed in the cell bodies of γ-motoneurons in the ventral horn of the spinal cord. Furthermore, an absence of agrin AChRs results in a lack of aggregated AChRs at the endplates of γ-motoneurons and at NMJs. Considering the high similarity of the α- and γ-motoneurons I speculate that agrin might play the same role in regulating the γ- to ε-subunits switch in the endplate of γ-motoneurons as at NMJs.

Why is there no down-regulation of the AChR γ-subunit in the central region of muscle fibers? Similar to the distribution of agrin and of its receptor complex, I demonstrate that agrin is expressed in the cell bodies of parvalbumin-positive proprioceptive dorsal root ganglion neurons. However at the equatorial region, absence of agrin apparently has no effect on the expression of the AChR γ-subunit. Instead the AChR γ-subunit remains present in the equatorial region of the muscle spindle despite the onset of the ε-subunit expression. This result is incompatible with a role for agrin during the onset of expression of the ε-subunit, and down-regulation of the γ-subunit. Instead my results suggest that factors other than agrin might be involved in the regulation of subunit expression and AChR maturation. One possibility is the electrical activity of muscle fibers, which will be discussed in the following section.

The γ-subunit is transiently expressed during the development of NMJs and down regulated by muscle activity (Goldman et al., 1988; Witzemann et al., 1991). Kues

and colleagues have shown that inhibition of muscle activity by blocking the nerve conduction with tetrodotoxin (TTX) leads to a smaller increase in γ-subunit mRNA levels at extrasynaptic regions (Kues et al., 1995). The depolarization of NMJ leads to the calcium efflux from the sarcoplasmic reticulum and influx through the sarcolemma membrane. The dramatic increase of calcium levels in the cytoplasm activates protein kinase C (PKC) (Huang et al., 1992; Klarsfeld et al., 1989), that further activates muscle contraction and represses AChR γ-subunit gene expression (Adams and Goldman, 1998; Huang et al., 1994; Sanes and Lichtman, 1999). Calcium concentration might be one mechanism by which the expression of the AChR γ-subunit gene can be down regulated. Can this mechanism be applied to the central region of muscle spindles? On the one hand, most sarcomeres are replaced by aggregated nuclei in the equatorial region of the intrafusal fiber, suggesting that the central part of the intrafusal fibers cannot contract and the main source of calcium is absent. In addition, due to the highly aggregated nuclei in the equatorial region, the central part of muscle spindles might be electrically isolated (Hunt and Louis, 1990). Therefore γ-motoneuron-induced action potentials might not be propagated from the polar region to the equatorial region. This electrical isolation in combination with the absence of the sarcoplasmic reticulum and the low concentration of AChRs in the muscle fiber plasma membrane, as well as the low concentration of AChR-containing vesicles, might be responsible for the absence of γ-subunit down-regulation. On the other hand, the presence of the fetal type AChR with a longer open time might facilitate the response to prolonged stimulation in the adult intrafusal fiber. Interestingly, the only mature innervated muscle identified so far to maintain a high expression of the fetal-type of AChR into adulthood due to ongoing transcription of the γ-subunit 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 lower 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.

## 5.4 The role of Agrin-MuSK-LRP4 signalling in the development of muscle spindles

### 5.4.1 Expression of agrin and its receptors in γ-motoneurons and DRG neurons

Although MuSK was initially identified as a muscle-specific kinase, recent studies have shown a relatively widespread distribution of the MuSK protein and its RNA in many tissues. For example MuSK has been identified in the neural tube, eye vesicles and spleen of *Xenopus* (Fu et al., 1999); the brain and liver of chicken (Ip et al., 2000); and the brain (especially in neurons) in mouse, as well liver, lung, heart and spleen (Garcia-Osta et al., 2006). Likewise, LRP4 is present in cortical neurons (Tian et al., 2006) and in non-neural tissues such as lung, kidney, limbs and ectodermal organs (Johnson et al., 2005; Simon-Chazottes et al., 2006; Weatherbee et al., 2006). My results demonstrate for the first time the presence of agrin and its receptor complex, MuSK and LRP4, in dorsal root ganglion neurons. The presence of agrin in DRG confirms previous in situ hybridization data, demonstrating that agrin isoforms are present in the ventral region of the spinal cord and in the dorsal root ganglion (Ma et al., 1994, 1995). It is tempting to speculate that agrin and its receptor complex are transported along the sensory nerve to the annulospiral sensory ending similar to the transport of agrin to the NMJ along the axon of α-motoneurons (Magill-Solc et al., 1990). This might explain the immunoreactivity in the equatorial region of intrafusal fibers with antibodies against these proteins where they colocalize with markers for the sensory nerve ending. However, it cannot be ruled out that intrafusal muscle fibers also express these proteins. This would be very similar to the NMJ where it has been shown that agrin is expressed by muscle fibers (Lieth et al., 1992) as well as by the motoneurons (Magill-Solc and McMahan, 1988, 1990), although different isoforms are synthesized by both tissues (Gesemann et al., 1995; Ruegg et al., 1992). Likewise, LRP4 has been shown to be expressed by skeletal muscle fibers and by α-motoneurons (Wu et al., 2012). What is the function of agrin, MuSK and LRP4 in DRG neurons and their sensory nerve endings? Agrin has been shown to promote the

attachment of DRG neurons when it is used as a substrate to inhibit neurite outgrowth of DRG neurons *in vitro* (Baerwald-de la Torre et al., 2004; Chang et al., 1997). Therefore, one possible role of agrin could be to regulate the formation of the annuospiral nerve endings. Morphologic assays using NFH and Thy1-YFP immunoreactivity show a branched sensory nerve network, demonstrating that in the agrin -/- mice embryos nuclei in the equatorial region aggregate normally. The morphology of the sensory nerve ending has no obvious detectable differences compared with wildtype mice. This indicates that axon guidance and branching are not affected by the absence of agrin. However, due to the perinatal lethality of agrin -/- mice, my analysis is restricted to late embryonic stages and I cannot exclude a role for agrin during later postnatal stages.

α-motoneurons have a large cell body, innervate force-producing extrafusal fibers and receive direct Ia-derived proprioceptive sensory input, whereas γ-motoneurons have a small cell body, innervate intrafusal fibers in muscle spindles and lack direct proprioceptive input (Burke et al., 1977; Eccles et al., 1960; Eccles et al., 1957; Enjin et al., 2012; Friese et al., 2009). γ-motoneurons express a high level of the transcription factor Err3 (Friese et al., 2009). I found both small HB9-positive-motoneurons and high level-Err3-expressing cells are agrin and LRP4 positive, but MuSK negative. This result indicates that γ-motoneurons express agrin and LRP4 just like α-motoneurons, whereas MuSK is absent from these neurons. This suggests that the MuSK immunoreactivity observed in the equatorial region of intrafusal fibers is derived from intrafusal fiber-expressed MuSK. In agrin knockout mice, γ-motoneurons do not express agrin. In these mice, I found that although motor axons can spread along intrafusal fiber surface to both polar regions, no bassoon immunoreactivity and AChR aggregation were observed, suggesting that neither γ-motoneuron endplates nor NMJs can form. Moreover, reexpression of the c-mag$_{B8}$ mini-agrin construct under the creatine kinase promoter in skeletal muscle was not only sufficient to rescue the NMJ formation and perinatal lethality (Lin et al., 2008), but also restores the formation of the endplate of γ-motoneurons. This result indicates that agrin plays an important role in regulating synaptogenesis at γ-motoneuron endplates, further supporting the similarities between these two cholinergic synapses.

# 6 Appendix

## 6.1 List of abbrevations

| | |
|---|---|
| NMJ | Neuromuscular Juntion |
| α-BTX | alpha-Bungarotoxin |
| Ach | acetylcholine |
| AChE | acetylcholineasterase |
| AChR | acetylcholine receptor |
| Apc | adenomatous polyposis coli |
| ATPase | adenylpyrophosphatase |
| BM | basement membrane |
| ChAT | choline acetyltransferase |
| CK2 | Casein kinase 2 |
| CLASP2 | Cytoplasmic linker associated protein 2 |
| CLIP-170 | Cytoplasmic linker proteins 170, Restin |
| CNS | central nervous system |
| ColQ | collagen-like “tail” |
| DGC | dystrophin-associated glycoprotein complex |
| DOK7 | docking protein 7 |
| DRG | dorsal root ganglion |
| E11 | embryonic day 11 |
| EGF | Epidermal Growth Factor |
| Egr3 | early growth response 3 |
| Er81 | ETS transcription factor ER81 |
| ErbB | erythroblastic leukemia viral oncogene homologue |
| Err3 | estrogen-related receptor gamm |
| ETA | Musculus epitrochleoanconeus |
| GFAP | Glial fibrillary acidic protein |
| GFP | green fluorescent protein |
| GSK3 | glycogen synthase kinase-3 |
| GTO | Golgi Tendon Organ |
| HB-GAM | heparin-binding growth-associated protein |
| LRP4 | low density lipoprotein receptor-related protein 4 |
| mRNA | messenger RNA |

| | |
|---|---|
| ms | microsecond |
| MuSK | Muscle-Specific Kinase |
| MyoD | Myogener Faktor 3, Myf-3 |
| nAChRs | nicotinic AChRs |
| NCAM | neural cell adhesion molecule |
| NGF | nerve growth factor |
| NFH | neurofilament heavy |
| Nrg | neuregulin |
| NT-3 | neurotrophin-3 |
| NtA | N-terminal |
| P5 | postnatal day 5 |
| PCA | posterior cricoarytenoid muscle |
| Pdzrn3 | PDZ domain-containing RING finger protein 3 |
| pea3 | pea3-type ETS-domain transcription factor |
| PI3-K | phosphatidylinositol kinase |
| PNS | peripheral nervous system |
| pS | pico Siemens |
| rapsyn | 43 kDa receptor-associated protein of the synapse |
| RATL | Rapsyn-Associated Transmembrane Linker |
| RNA | Ribonucleic acid |
| siRNA | Small interfering RNA |
| SYN | synaptophysin |
| TM-agrin | trans-membrane agrin |
| TTX | Tetrodotoxin |
| VAChT | vesicular acetylcholine transporter |
| VGluT1 | vesicular glutamate transporter 1 |

## 6.2 List of Figures

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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 the polar region 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 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.

Key words: muscle spindle; synaptogenesis; acetylcholine receptor; bassoon; annulospiral ending, γ-motoneuron; proprioception

## 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 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 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 ($AChR^{\gamma\text{-}GFP/\gamma GFP}$) 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 0 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 immunofluorescense 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 (tom 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-immunofluorescent analyses. No staining was observed when primary antibodies or Alexa-conjugated α-bungarotoxin were omitted. The nuclei were routinely stained using DAPI (Roth, Karlsruhe, Germany) at a concentration of 2µg/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. Figure 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.

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

acetyltransferase (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 Figure 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 might be associated with the plasma membrane or with the basal lamina surrounding each intrafusal fiber. 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 (Figure 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; Chen et al., 2012). Interestingly, we also detected punctate bassoon immunoreactivity clustered in the equatorial region of intrafusal fibers where it partially colocalized 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 Figure 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 bassoon 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. 3 G). 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 lower 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 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. 3H). 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 spindle (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 AChRs 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 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 gene in nuclei at the central region of intrafusal muscle fibers (Sanes et al., 1991) strongly suggests that the AChRs are expressed by intrafusal fibers and concentrated in their plasma membrane

at the contact site to the sensory neuron. Likewise, the presence of bassoon immunoreactivity in parvalbumine-positive proprioceptive neurons (see Fig. S1) as well as in F11 cells (a cell line derived from DRG neurons; Goswami et al., 2010) and 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 directly 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 a synaptic vesicles. At least some of the vesicles contain glutamate which is thought to modulate spindle sensitivity via presynaptic 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 required for 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 much bigger 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 suggested by the present study is a direct action of ACh released from sensory nerve terminals on spindle sensory endings (for a detailed discussion see Carr and Proske, 1996). Our results showing the 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 (slow twitch), quadriceps (fast twitch) and extensor digitorum longus (fast twitch), 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-subunit in the central region of adult intrafusal muscle fibers, strongly suggesting the simultaneous presence of fetal and the 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 by 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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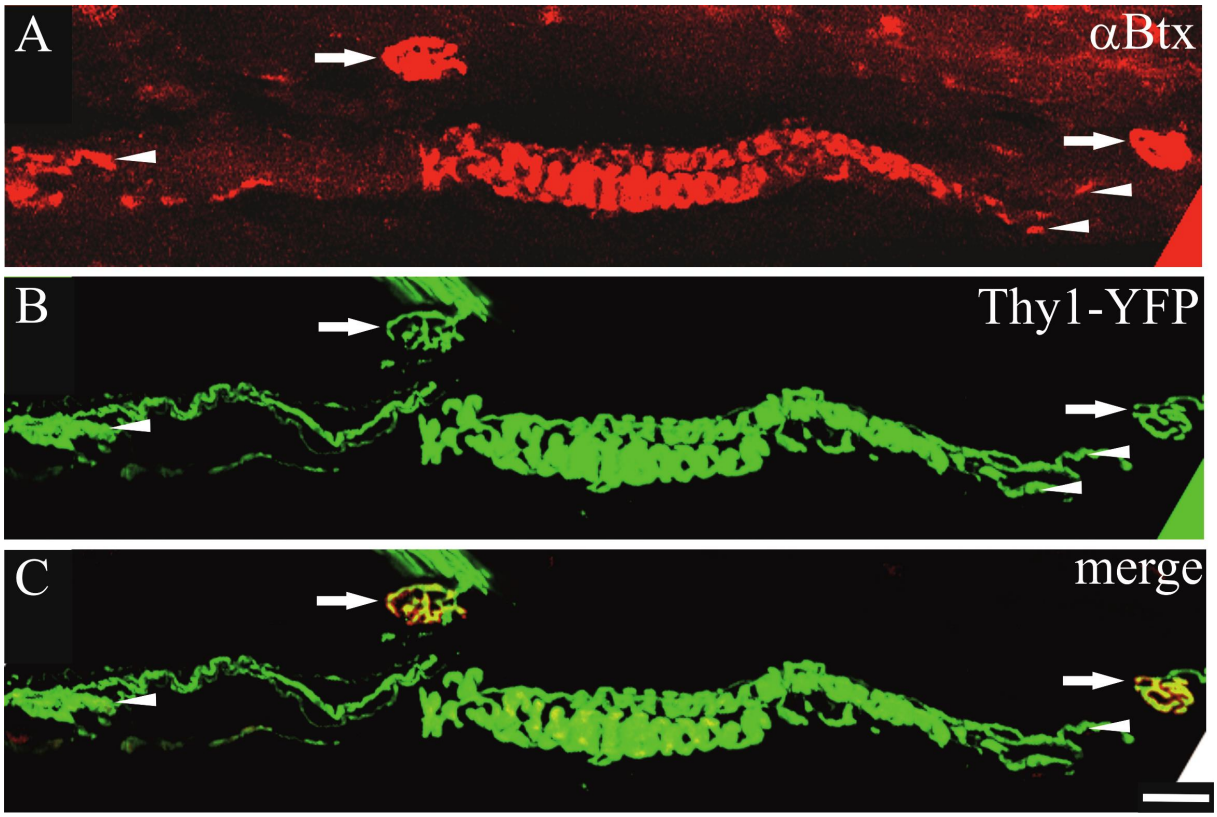
**Figure Legends:**

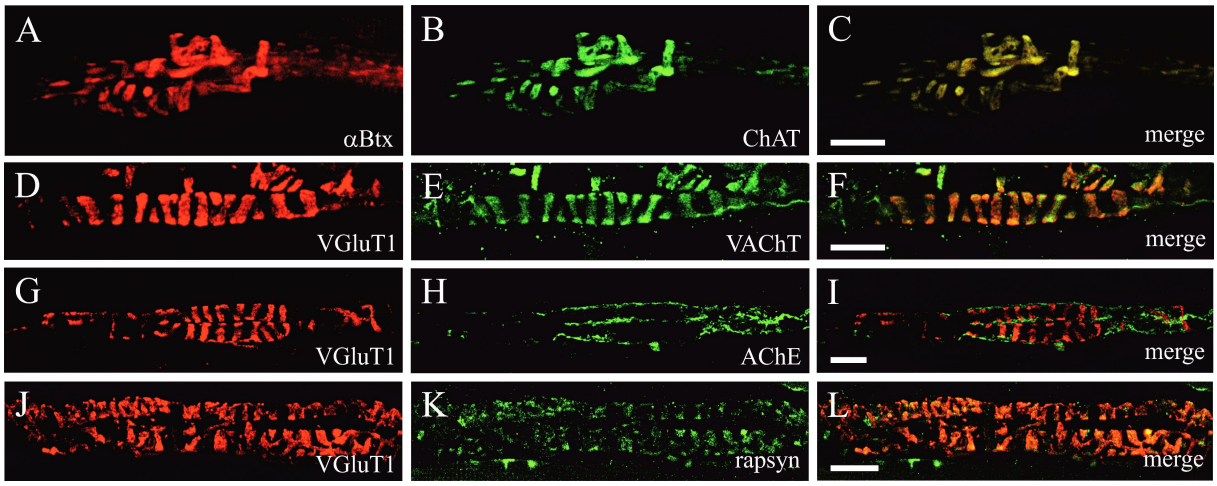
**Figure 1:**




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 the AChRs with α-bungarotoxin (αBtx, A). The sensory- and motoneurons were genetically labelled 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

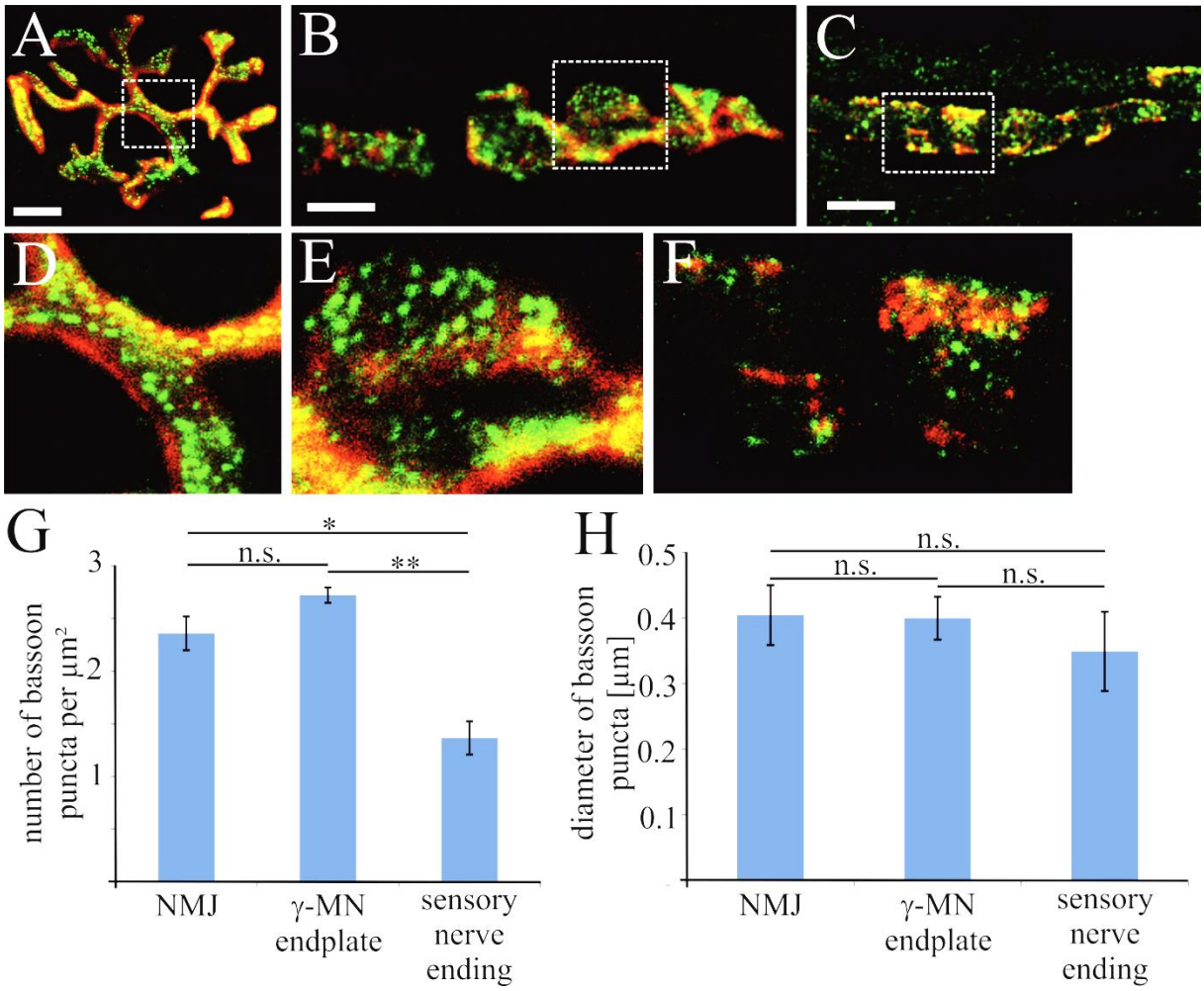
**Figure 2:**




The contact site between sensory nerve terminal and intrafusal fiber 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

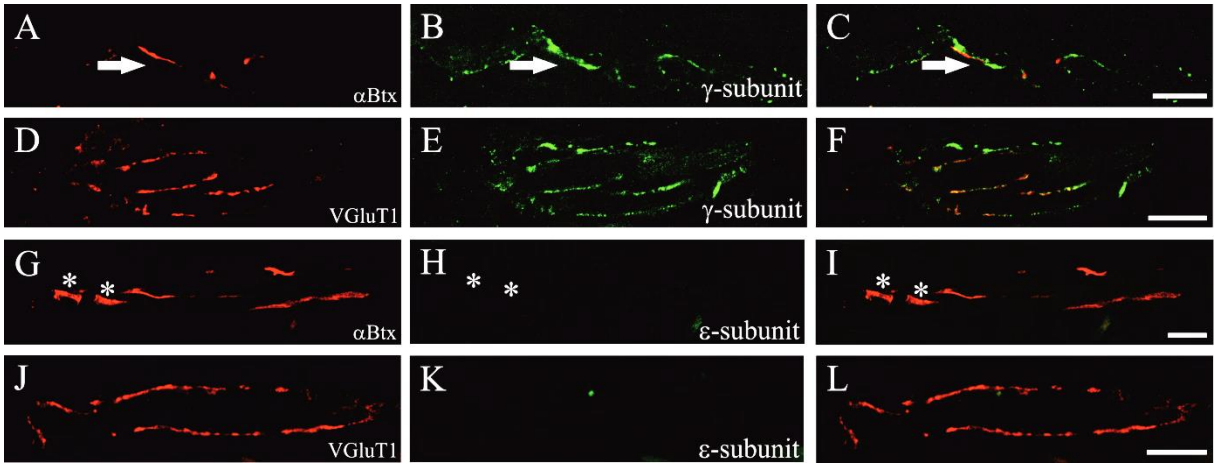
**Figure 3:**




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 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 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

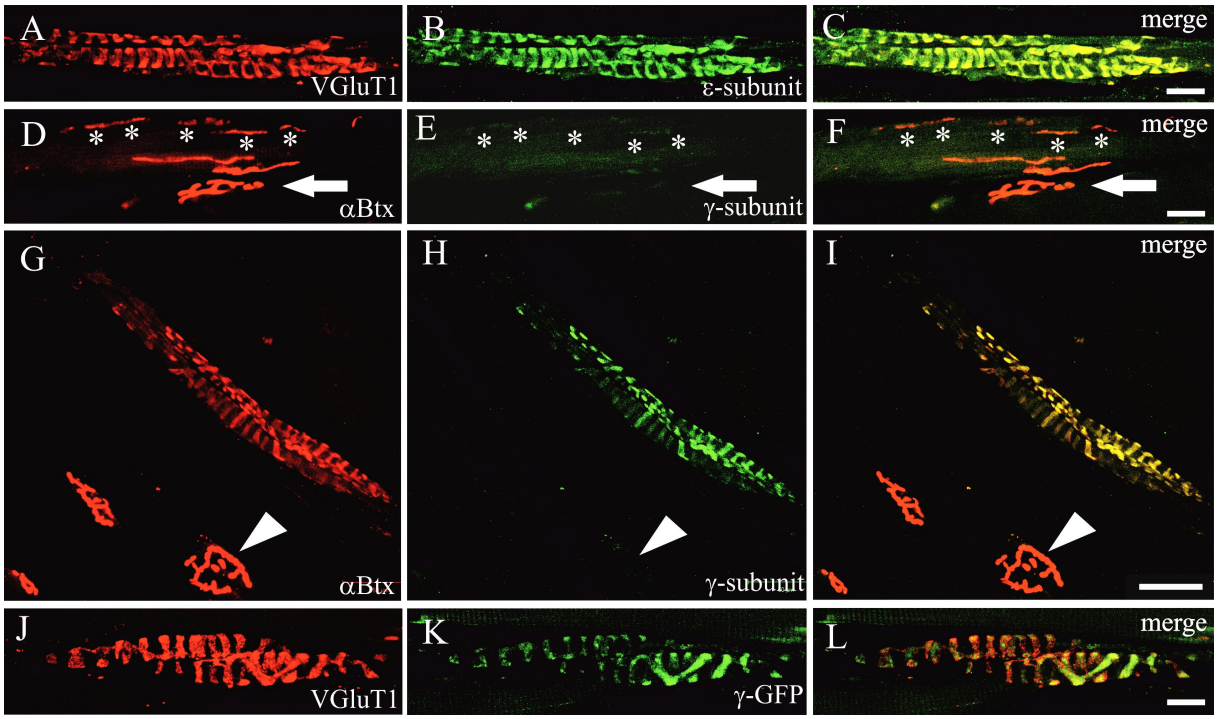
**Figure 4:**




Acetylcholine receptors in the equatorial and polar regions of postnatal muscle spindles contain the γ-subunit but lack the ε-subunit. The polar region (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

**Figure 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 ε-subunit of the AChR was 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 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

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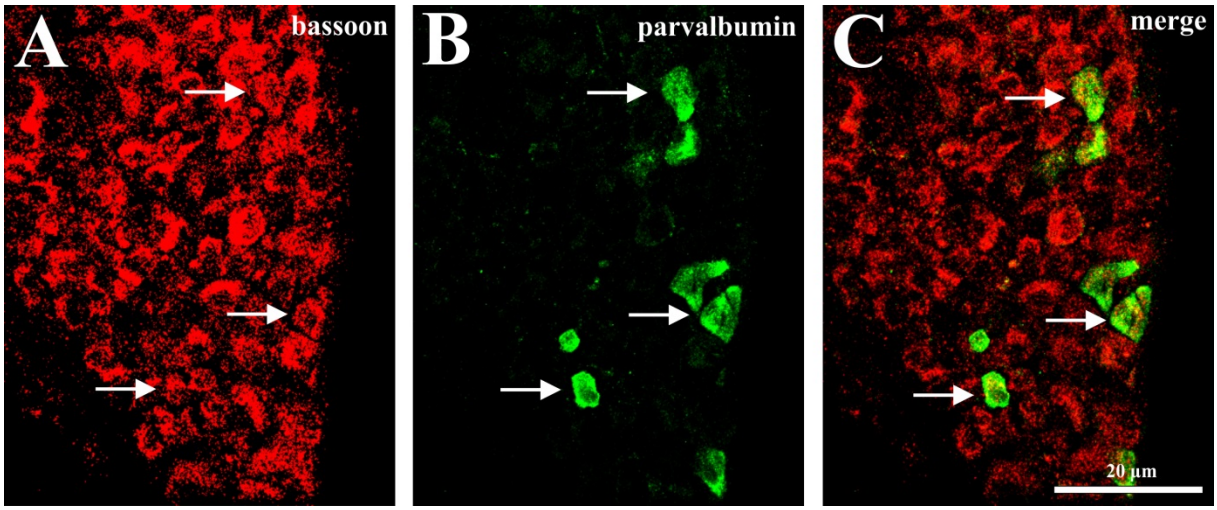
**Supplementary Figure S1:**




Bassoon is present in proprioceptive DRG neurons.
Dorsal root ganglia were labeled with anti-bassoon (A) and with anti-parvalbumin antibodies (B), which selectively stains proprioceptive neurons. The sections were prepared from the lumbar region of newborn mice, since the parvalbumin antibody does not react with adult DRG tissue. Maximum intensity projections are shown. The anti-bassoon antibodies label most if not all neuronal cell bodies in the DRG and are also found in parvalbumin-positive proprioceptive neurons (C).

# Curriculum Vitae

**Personal Data**

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| *Name:* | Yina Zhang |
| *Birth date and place:* | 13. Jan. 1980, Datong, Shanxi , PR. China |
| *Nationality:* | Chinese |

**Academic Training**

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|---|---|
| *May, 2009 – Apr, 2014* | PhD thesis; title: "The Development and Molecular Characterization of Muscle Spindles from Wildtype and Mutant Mice"<br>Supervisor: Prof. Dr. Stephan Kröger |
| *May, 2008 - Mar, 2009* | Master's Degree in Biology (grade: "very good")<br>Project title: "The changes of the hippocampal neural cells in mice mutant with conditional inactivation of *B-RAF* Genes."<br>Supervisor: Dr. Rudolf Götz and Prof. Dr. Ulf Rapp, Institute for Medical Radiology and Research at the Julius-Maximilians-University Würzburg |
| *Oct, 2004 - May, 2008* | Undergraduate studies in Biology<br>Julius-Maximilians-University Würzburg |
| *Jun, 2004 - Sep, 2004* | Learning German and passed the DSH exam at Julius-Maximilians- University Würzburg |
| *Okt, 2003 - Mai, 2004* | Learning German at the Euro School in Würzburg |
| *Sep, 1998 - Jun, 2003* | Bachelor's Degree in Human medicine<br>ChangZhi Medical College, China |

***Conferences***

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|---|---|
| *May, 2013* | The XIV International Symposium on Cholinergic Mechanisms, HangZhou, China |

***Awards***

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| *May, 2013* | Best poster awarded at the XIV International |

Symposium on Cholinergic Mechanisms, HangZhou, China

***Publications***

- Pfeiffer V, Götz R, Xiang C, Camarero G, Braun A, **Zhang Y**, Blum R, Heinsen H, Nieswandt B, Rapp UR. (2013) Ablation of BRaf Impairs Neuronal Differentiation in the Postnatal Hippocampus and Cerebellum. PLoS ONE 8(3): e58259.
- Gasperi C, Melms A, Schoser B, **Zhang Y**, Meltoranta J, Risson V, Schaeffer L, Schalke B, and Kröger S. (2014) "Anti-Agrin Autoantibodies in Myasthenia gravis" Neurology 82(22): 1976-1983.
- **Zhang Y**, Wesolowska M, Karakatsani A, Wizemann V, Kröger S. "Cholinergic Specialization at Developing Murine Muscle Spindles". (Manuscript under rerevision by Developmental Biology)
- **Zhang Y**, Lin S, Karakatsani A, Rüegg M, Kröger S. "Differential Regulation of AChR Clustering in the Polar and Equatorial Region of Murine Muscle Spindles". (Manuscript under rerevision by The European Journal of Neuroscience)