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Genetic evidence for the role of muscle-specific acetylcholine receptors in the formation of neuromuscular synapses

A Dissertation submitted in partial satisfaction of the

requirements for the degree of Doctor of Philosophy

in

Biology

by

Mahru C. An

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2007

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2007

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ABSTRACT OF THE DISSERTATION

Genetic evidence for the role of muscle-specific acetylcholine receptors in the formation of neuromuscular synapses

by

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Doctor of Philosophy in Biology

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Synaptic specialization requires interactions between presynaptic postsynaptic, and glial partners. The development of mouse neuromuscular junctions is pre-patterned within a narrow central band of muscle, where motor axons undergo two aspects of presynaptic differentiation: regulated branching and specialization of nerve terminals. Evidence suggests that acetylcholine (ACh) inhibits nerve branching and presynaptic specialization, yet it is unclear whether the inhibition is mediated through postsynaptic or non-postsynaptic AChRs. Postsynaptic AChRs may play a role in pre-patterning the synapses and in eliciting a retrograde signal to induce presynaptic specialization. Here we have used genetic methods to address these issues. We report that clustering of postsynaptic AChRs is absent in muscle AChRα1 mutant mice. Like mice deficient in ACh biosynthesis, presynaptic axons are highly branched with synaptic sites distributed in a broader muscle territory, suggesting that

postsynaptic AChRs are involved in pre-patterning synapses and mediates ACh inhibition of nerve branching. Surprisingly, presynaptic specialization is present in the absence of postsynaptic AChR clusters. Additional evidence supports that ACh inhibits specialization of nerve terminals through non-postsynaptic AChRs. These results suggest that ACh negatively regulates these two aspects of presynaptic differentiation via distinct mechanisms. Also presented here, through data obtained from mice that lack Schwann cells due to a loss of erbB2, are preliminary evidence for a possible role of Schwann cells in the timing of presynaptic development, in part of an ongoing project to determine the role of Schwann cells in NMJ development.

Chapter 1:

GENERAL INTRODUCTION

The mammalian central nervous system is an evolutionary phenomenon that is built on the functional networking of billions of neurons. This networking would not be possible without a highly efficient, highly reproducible mechanism by which neuronal cells form connections to communicate with each other. This communication occurs through a refined network of synapses, which are formed, modulated, and repatterned, through a self-regulated process of cell-cell signaling. It is important to better understand the process by which the formation, maturation, and regulation of synapses takes place, so that the deeper questions concerning neural development, function, and regeneration can be better studied. This dissertation is a result of my efforts to contribute to the understanding of synapses and synapse formation.

Synapse formation

The synapse is a highly specialized structure that allows for a neuronal cell to signal with other neuronal cells as well as certain non-neuronal cells such as those in glands or muscle. In the central nervous system (CNS), a synapse typically comprises the axon terminal of one neuron, apposed to the dendrite of another neuron. Signaling between the two cells occurs through the controlled release of neurotransmitter from the preterminal cell membrane,

which upon binding to corresponding receptors on the postsynaptic cell membrane, triggers an electrochemical change in the postsynaptic cell. This event represents the controlled passage of information, or potential, from one cell to another, and in complex systems such as the mammalian brain, occurs in concert with trillions of other such connections. The intricate network of synapses that make up the human adult brain control activities such as vision, memory, learning, and consciousness. Paramount in the development of these processes is the ability of synapses to be dynamic in nature, which is evident in mechanisms of synaptic plasticity such as long-term potentiation, and there is increasing evidence for the involvement of synapse disassembly and reformation in mechanisms of activity-dependent plasticity. Furthermore, understanding the process of synaptogenesis can further aid us in our effort to realize effective treatments for diseases in which loss of neuronal function, due to injury or degeneration, must be must be restored. However, many of these questions are by nature difficult to study, due to the complex nature of the system in which they exist. Thus, it is important to provide a simple framework of knowledge from which to better understand the process of synapse formation.

The Neuromuscular Junction

One of the most well studied synapses has been the vertebrate neuromuscular junction (NMJ). The NMJ is a specific type of synapse that is

formed at the junctional interface between motor nerve terminal and muscle fiber in the vertebrate peripheral nervous system (PNS). While there are inherent difficulties in studying the formation of central synapses, due to the difficulty of experimentally manipulating the developing CNS in vivo, development of NMJ synapses are a more accessible and structurally simple system to study, allowing the analysis of discrete factors such as size, shape, and molecular composition at a high spatial and temporal resolution (Burden, 1998; Witzemann, 2006). Despite differences in complexity between NMJ and CNS, there are similarities in synaptic structure and function, suggesting that mechanisms of synapse formation may be conserved between the two. For example, the concentration of postsynaptic neurotransmitter receptors, and the presence of active zones, which are hallmarks of NMJ synapses can be found at certain CNS synapses. Also, organizers of NMJ synaptogenesis such as agrin and neuregulin (NRG) to be discussed in further detail below, are expressed in the CNS, suggesting that they may play similar roles in the formation of some CNS synapses (Burden, 1998). Thus NMJs have emerged as one of the most effective model systems for studying the development and formation of synapses.

As typified by synapses, the functionality of the NMJ stems from the highly specialized nature of the three elements involved – the presynaptic motor nerve terminal, postsynaptic muscle fiber, and perisynaptic Schwann cell, are specialized components of nerve, muscle, and glial cells respectively.

Each specialized cellular component plays a role in not only the functionality of the mature synapse, but also in providing signals which mediate the overall development, maturation, and maintenance of synaptic structure and function.

The motor nerve terminal is a specialized portion of the presynaptic axon with large numbers of synaptic vesicles containing the neurotransmitter acetylcholine (ACh), which is also found in the cholinergic neurons of the CNS. Mitochondria, which are necessary for the energy that is required for neurotransmitter synthesis and release, are correspondingly localized in greater number at synaptic sites (Burden, 1998; Sanes and Lichtman, 1999). The terminal is characterized by its polarized accumulation of vesicles towards the membrane that is adjacent to the postsynaptic cell. On this membrane are further dense patches of vesicles adjacent to the presynaptic membrane, at what are called active zones, where the vesicles are adhered to the membrane, or docked, and ready to fuse with the membrane. When an action potential reaches the nerve terminal, activation of calcium channels results in calcium influx, and the resulting rise in local calcium concentration triggers the fusion of the synaptic vesicle membrane with the plasma membrane of the nerve terminal. Fusion of synaptic vesicles results in exocytotic release of their contents into the synaptic cleft, which is the narrow space between the presynaptic and postsynaptic membranes (Burden, 1998; Hughes et al., 2006; Sanes and Lichtman, 1999). Specialization of the presynaptic terminal in this

manner allows for the rapid release of large amounts of neurotransmitter into the synaptic cleft by the nerve in response to stimulation.

Correspondingly, the postsynaptic muscle fiber is specialized to maximize its response to any release of neurotransmitter into the synaptic cleft. This is achieved in large part by the extremely high concentration of acetylcholine receptors (AChRs) found specifically in the membrane regions that appose nerve terminals. High receptor density is enhanced by a structural element: postjunctional folds in the membrane that probably serve to increase surface area and enhance efficiency of signaling (Burden, 1998; Sanes and Lichtman, 1999). Proteins associated with the signaling of AChRs, as well as various cytoskeletal elements are also concentrated at postsynaptic sites. It is thought that cytoskeletal proteins may be involved not only in the generation and maintenance of the folds, but also in the maintenance of high synaptic AChR density at concentrations 1,000 fold higher than at extrasynaptic sites (Burden, 1998).

In the synaptic cleft between the two membranes, there is a basal lamina generally associated with muscle membranes that is also thought to serve a role in the maintenance and function of neuromuscular synapses, as well as possibly in their development. In addition to other laminar proteins, the basal lamina of the NMJ contains a collagen-tailed form of the enzyme acetylcholinesterase (AChE), which serves to catalyze the hydrolysis of excess ACh in the cleft, a function that is essential for allowing the synapse to return to

resting state for the next nerve impulse. Some other components of the basal lamina include, collagen IV, laminin, heparan sulfate proteoglycans, and also the signaling molecules agrin and neuregulin, which play important roles in the formation and maintenance of NMJ synapses (Burden, 1998).

Motor nerves are associated with a type of glial cell called a Schwann cell, which is essential for motor nerve function and development. Schwann cells primarily serve the function of providing myelin insulation to axons in the vertebrate peripheral nervous system. This myelination is essential for the functional efficiency of conduction along those axons. Glial cells are also found associated with central synapses and are thought to play auxiliary roles in regulating the synaptic environment. At the NMJ, a type of Schwann cell called the perisynaptic Schwann cell (PSC) serves a similar function, insulating the synaptic environment and providing trophic support. These PSCs are derived from a common precursor to myelinating Schwann cells, however, they are genetically and structurally distinct, performing specialized functions at the neuromuscular synapse (Auld and Robitaille, 2003; Burden, 1998; Feng et al., 2005; Jessen and Mirsky, 1997).

The high specialization of the components of the neuromuscular synapse is essential for its efficient function as a mediator of cell-cell communication. The formation and differentiation of these synaptic units must be a highly refined and reproducible process in order for large-scale synaptic development and structuring to occur. Studies over the past several decades

into the mechanism of neuromuscular synapse formation have led us to understand that NMJ development is a result of the combined input of nerve, muscle, and Schwann cell. A better knowledge of the sequence of signals and events that occur during NMJ development will lead us to a better understanding the complex process of synaptogenesis. The following sections outline in detail the signals and events occurring during mammalian embryonic development that lead to the formation of a mature NMJ.

Development of the mammalian NMJ

Synapses of the mammalian neuromuscular junction are patterned along the muscle fibers in a directed manner. In adult muscles, synapses are formed, or patterned, specifically along a central region of the muscle. The directed patterning of synapses along this defined end-plate band, and the discouragement of synapse formation in non-central regions is a complex process mediated by the continued signaling that occurs between the postsynaptic membrane and preterminal partners, during the development, maturation and maintenance of the NMJ.

Postsynaptic differentiation and AChR clustering

Muscle fibers are formed from the fusing of multiple muscle stem cells, or myoblasts. The resulting multinucleated myotube begins to express the AChR subunit genes at a much higher level than found in the original myoblast,

and assembles the translated subunits to form functional receptors, which are shuttled to the plasma membrane at an initial uniform density of ~1000/μm^2 (Salpeter and Loring, 1985; Sanes and Lichtman, 1999). One of the major events in the differentiation of the muscle fiber undergoing postsynaptic differentiation membrane involves the clustering of these AChRs to concentrations of >10,000/μm^2 at areas of intended synaptic specificity, coupled with the decrease of AChR concentrations at extra-synaptic regions to ~10/μm^2 (Salpeter and Loring, 1985; Sanes and Lichtman, 1999). This synaptic redistribution is facilitated by the direct clustering of diffuse AChRs in the membrane, the transcriptional activation of AChR subunit genes at synaptic nuclei, and the transcriptional repression at extra-synaptic nuclei, in a process that is initially muscle intrinsic, but later mediated by signals from the incoming nerve terminal (Lin et al., 2001; Witzemann, 2006).

AChR clustering is the aspect of mammalian neuromuscular development that has been studied in the most detail, as discussed in the following sections, primarily in experiments using cultured myotubes using fluorescently tagged α-bungarotoxin to bind to and visualize the presence of highly accumulated levels of AChR, but also through the study of in vivo models of neuromuscular development in rat, mouse, and chick. Three decades of research have contributed to the discovery of a host of proteins that have been found either to associate with, or to be involved with the clustering of AChRs at the postsynaptic membrane. In order to completely understand

the dynamic nature of synaptic development, it is likely necessary that we understand the full extent to which these proteins, which include agrin, MuSK, rapsyn, AchE, ErbB2, and NRG, act as mediators of synaptic development at the NMJ.

Agrin and postsynaptic differentiation

Agrin is a ~200kDa protein that was isolated by McMahan and colleagues from the exracellular matrix of *Torpedo* electric organ, a densely innervated tissue that is homologous to skeletal muscle (Burden, 1998; McMahan, 1990). It contains four epidermal growth factor (EGF)-like repeats and three laminin G domain homologous regions contained in the carboxy-terminal region, which are thought to be involved with inducing AChR clusters (Burden, 1998). Differential splicing results in the presence of various isoforms of agrin that are expressed in different cells, including neurons and skeletal muscle cells (Ferns et al., 1992; Ruegg et al., 1992; Willmann and Fuhrer, 2002). The neuronal form of agrin appears to be the most active in clustering AChRs. Agrin is synthesized and released by motor neurons into the synaptic basal lamina, where it binds to with high affinity to laminin through its amino-terminus, and exists in high concentrations (Cohen and Godfrey, 1992; McMahan, 1990; Reist et al., 1992).

Agrin was found to aggregate AChRs in myotubes in culture, and muscle fibers overexpressing agrin induced AChR aggregation in apposition to

agrin patches (Cohen et al., 1997; Jones et al., 1996; Meier et al., 1997; Rimer et al., 1997; Yang et al., 2001), while antibodies against agrin were shown to block AChR clustering of nerve-muscle synapses in cell culture (Reist et al., 1992). This was further supported by in vivo data that showed a lack of normal synapses in mice lacking agrin (Gautam et al., 1996). Thus, agrin is necessary and sufficient for induction of AChR clustering. Furthermore, distribution of other AChR associated proteins, including AchE, rapsyn, utrophin, NRG, and NRG receptors, is regulated by agrin as well (Apel et al., 1995; Burden, 1998; McMahan, 1990; Meier et al., 1997). Thus it is thought that agrin released from the nerve terminal plays an important role in the induction and maintenance of AChR clustering in the muscle membrane.

While the full nature of the mechanism through which agrin causes AChRs to cluster is unclear, evidence strongly suggests a postsynaptic receptor complex-mediated mechanism of agrin signaling (Burden, 1998; Sanes and Lichtman, 1999; Willmann and Fuhrer, 2002). In this model, agrin would bind to and activate a receptor complex on the postsynaptic membrane, initiating a signaling cascade, and through the collaborative action of other AChR-associated proteins, causes the clustering of AChRs on the postsynaptic membrane proximal to the site of agrin binding.

MuSK

One molecule that has been suggested to be involved with this putative receptor complex is a muscle-specific receptor tyrosine kinase (RTK) called MuSK. Receptor tyrosine kinases are transmembrane receptors with intrinsic tyrosine kinase activity that have been shown to be involved in many biological response pathways. The muscle-specific kinase MuSK is a RTK that is specific to the skeletal muscle lineage. Musk is highly expressed in muscle, and in the absence of innervation, is uniformly distributed, however upon innervation becomes highly localized with AChRs at motor end-plates (Burden, 1998; Willmann and Fuhrer, 2002). MuSK was first linked with agrin when it was shown that mice lacking MuSK expression lacked normal neuromuscular synapses, similarly to agrin mutant mice, but more severe (DeChiara et al., 1996; Gautam et al., 1996). Several lines of evidence suggest that MuSK is required for agrin-mediated signaling. Agrin induces the rapid tyrosine phosphorylation of MuSK, and in culture neither MuSK mutant myotubes nor myotubes expressing a dominant-negative form of MuSK are able to form clusters in response to agrin, while a soluble extracellular MuSK fragment also inhibits agrin-induced clustering (Burden, 1998; Glass and Yancopoulos, 1997). MuSK undergoes autophosphorylation in response to agrin stimulation, and mutational analysis shows that some of the tyrosine residues found on MuSK's cytoplasmic domain are necessary for agrin mediated AChR clustering. Furthermore, MuSK's kinase activity is necessary for AChR

clustering activity. However, while agrin can be chemically cross-linked to MuSK, MuSK cannot directly bind directly to agrin, thus it is thought that another component is necessary for the receptor complex to be fully functional, which is consistent with other RTKs that require co-receptors to function. This component is often called MASC, for myotube-associated specificity complex (Burden, 1998; Glass and Yancopoulos, 1997; Willmann and Fuhrer, 2002).

Downstream of MuSK

Rapsyn is a 43 kDa protein that was also found in *Torpedo* synaptic membranes (Burden et al., 1983; Porter and Froehner, 1983; Sobel et al., 1978). Like MuSK, rapsyn is co-localized with AChRs at motor endplates (Burden et al., 1983). Rapsyn mutant mice lack normal clustering of many postsynaptic proteins, including AChRs, NRG receptors, utrophin, and dystroglycan (Gautam et al., 1995). However MuSK is localized to synaptic sites in rapsyn mutant mice, suggesting that MuSK can cluster to synaptic sites independent of rapsyn. In contrast, rapsyn cannot form clusters independent of AChRs in myotubes, requiring some association with AChRs to form aggregates (Marangi et al., 2001). Thus AChRs may not simply be passively recruited to the synapse, but may play an active role in their organization. In fact, AChRs and rapsyn are found in a 1:1 stoichiometry at adult NMJs, while MuSK is much less abundant than either AChRs or rapsyn (LaRochelle and Froehner, 1986; LaRochelle and Froehner, 1987). This, along with MuSK's

non-reliance upon rapsyn, suggests a model in which two independent complexes exist: MuSK forms a preassembled signaling complex that is able to aggregate independently of rapsyn or AChRs, while AChRs and rapsyn form a separate preassembled signaling complex. In fact, it has been shown that MuSK and AChR may affect different populations of Src-related kinases, which react differently in response to agrin. This leads to a possible mechanism of stepwise assembly, where MuSK-associated complex acts as a primary scaffold that is clustered by agrin, and follows by recruiting AChR complexes through interaction with rapsyn (Mittaud et al., 2001; Mohamed et al., 2001; Willmann and Fuhrer, 2002).

NRG signaling and synapse-specific transcription

While agrin-mediated signaling acts to reorganize proteins at the muscle membrane, a second signaling pathway regulates postsynaptic differentiation by controlling synaptic gene expression. Functional AChRs at NMJs are multi-subunit receptors composed of α, β, δ, and γ or ε subunits (Green and Millar, 1995). The level of AChR subunit at synaptic sites is regulated through a process in which their gene expression is selectively enhanced at subsynaptic nuclei, and repressed at extrasynaptic nuclei. This process is regulated in part by neuregulin(NRG)-1, a member of the neuregulin gene family first isolated from chick brain as AChR-inducing activity (ARIA), for its ability to induce AChR gene transcription in cultured muscle cells (Chu et al., 1995; Gundersen

et al., 1993). Furthermore, AChR density is reduced at the endplates of heterozygous mice lacking one copy of NRG-1 (Sandrock et al., 1997). NRG-1 is a growth factor known to act by binding and activating the epidermal growth factor (EGF) receptor family of erbB receptor tyrosine kinases. NRG is concentrated at synaptic sites, while both ErbB2 and ErbB4 are concentrated at postsynaptic NMJs. Both muscle and motor nerve express different isoforms of NRG, while different erbB receptors are expressed in muscle and Schwann cell (erbB2/4 in muscle, and 2/3 in Schwann cells). Thus it is thought that NRG-1 acts by binding and activating a combination of erbB2 and erbB4 expressed by muscle and concentrated at the NMJ (Schaeffer et al., 2001; Si et al., 1998). Subsequent to this, multiple signaling cascades, likely involving activation of Ras, Raf, ERK, JNK, and the transcription factors c-Fos and c-Jun, are activated to converge on the regulation of the transcription of AChR subunits (Altiok et al., 1997; Si et al., 1996; Tansey et al., 1996; Won et al., 1999).

Nerve-independent postsynaptic differentiation

The knowledge of the existence of nerve-secreted factors such as agrin and neuregulin that make such clear and directing impacts upon postsynaptic specialization naturally led to the idea that clustering of AChRs is in large part due to the direction of the motor nerve. However, recent genetic studies have challenged this neurocentric view, suggesting that postsynaptic differentiation

is pre-patterned in the muscle independent of nerve derived signals. Lin and colleagues showed that postsynaptic differentiation can be initiated by mechanisms that are agrin independent, and nerve independent (Lin et al., 2001). Furthermore, postsynaptic AChR clusters form at the central band of muscle by a mechanism that is intrinsic to the muscle. Thus initiation of neuromuscular synaptogenesis begins as a muscle-intrinsic mechanism, and subsequent signals between nerve, muscle, and Schwann cell serve to further refine the formation and patterning of synapses. It follows then, that presynaptic development is likely mediated in part by signals from the postsynaptic target.

Presynaptic differentiation

Motoneurons of the mammalian NMJ originate from the ventral portion of the neural tube, and are guided through ventral roots or cranial nerves to skeletal muscle fibers, where they form synapses with newly formed muscle fibers. Motor axons do not branch until reaching the muscle fiber, where they branch numerous times, innervating tens to hundreds of muscle fibers (Landmesser, 1992; Leber et al., 1990; Lewis et al., 1981; Sanes and Lichtman, 1999). Synaptic transmission commences immediately upon contact with the myotube, although initially at a very low rate, due to the relative low density of the immature specializations of pre- and postsynapstic structures (Kullberg et al., 1977; Nakajima et al., 1980). Furthermore, it is known that the

extending growth cone is able to release neurotransmitter even before making contact with the muscle fiber (Hume et al., 1983; Young and Poo, 1983). Subsequent to initial contact, the nerve terminal undergoes synaptic specialization, with the dramatic increase in synaptic vesicle number, and the formation of vesicle rich active zones. Also the terminal becomes polarized, and loses cytoskeletal elements normally found in the axon, while volume and area of the synapse are increased.

Several lines of evidence suggest that target-derived factors are likely to organize presynaptic differentiation. The postsynaptic muscle forms AChR clusters at the central band of muscle in a nerve-independent, muscle-intrinsic manner, such that AChR aggregates exist initially on muscle as aneural clusters clearly before the formation presynaptic specializations by the nerve (Lin et al., 2001). Conversely, the nerve only forms presynaptic specializations at sites of contact with muscle fibers (Lupa et al., 1990). The mechanism by which nerve terminals are induced to differentiate is not well understood. Recent evidence suggests laminin β2, a component of the basal lamina in the synaptic cleft, plays a role in organizing nerve terminal differentiation. Genetic studies in mice have shown that mice lacking laminin β2 show marked defects in maturation of motor nerve terminals (Noakes et al., 1995). Further studies suggest that laminin acts by interacting with calcium channels, which leads to clustering of channels, and organization of active zones (Nishimune et al., 2004).

Schwann cells and NMJ development

Schwann cells originate primarily from the neural crest, and are inherent to the correct development of the PNS, migrating with developing motor axons from the spinal nerve to reside adjacent to both peripheral nerve axons and synapses, where they play important roles (Jessen et al., 1994; Mirsky and Jessen, 1996). In addition to their function in provide an insulating myelination along the length of the axon, Schwann cells support the development and maintenance of the nerve by providing trophic factors such as nerve growth factor (NGF) (Bandtlow et al., 1987), brain derived neurotrophic factor (BDNF) (Acheson et al., 1991; Meyer et al., 1992), and ciliary neurotrophic factor (CNTF) (Friedman et al., 1992; Rende et al., 1992). Schwann cells are also important for fostering the process of central axonal regeneration, assisting in the removal of myelin debris, and lining the basal lamina of the original nerve (Xu et al., 1995). PSCs found at neuromuscular synapses are genetically and functionally different from myelinating Schwann cells, and do not myelinate the nerve terminal, however they express proteins typical of myelinating Schwann cell such as the myelin protein P0, myelin associated protein, and galactocereboside (Georgiou and Charlton, 1999). PSCs are closely apposed to the nerve terminal and their release sites, and are likely to play roles in synaptic transmission. In contrast to myelinating Schwann cells, they express a large number of ion channels and neurotransmitter receptors such as L-type

calcium channels, muscarinic, and purinergic receptors (Georgiou and Charlton, 1999; Jahromi et al., 1992; Robitaille et al., 1996; Robitaille et al., 1997), and have been found to respond to ACh and ATP by an increase in intracellular calcium. Thus, PSCs can sense synaptic activity, and may be able to modulate synaptic activity to regulate synaptic transmission, through Ca^{2+} dependent, or G-protein dependent signaling pathways (Bourque and Robitaille, 1998; Feng et al., 2005; Robitaille, 1998). Many of these factors would support a role for PSCs in maintenance and development of neuromuscular synapses as well. PSC gene expression is acutely responsive to changes that affect nerve terminals (Georgiou and Charlton, 1999; Woolf et al., 1992), and nerves may be similarly attuned to changes in PSC state. Nerve retraction occurs one week after ablation of PSCs by complement mediated cell lysis in adult frog NMJs, while nerve in mice, retraction and death of motoneurons results seemingly from the loss of Schwann cells in ErbB2 or ErbB3 deficient mice. Schwann cells, however, are not necessary for navigation of motor axons to their target muscles in these mice, as the nerve is present at early stages of development and NMJs are transiently formed, but are not maintained. Thus, while PSCs may play a role in the maintenance and maturation of neuromuscular synapses, they do not seem to be required for initiation of synapse formation.

Models for studying the mammalian NMJ

In studying the mammalian NMJ, it is of special importance to choose the most appropriate model system for the question to be addressed. Much of the work that has led to our understanding of the signaling mechanisms governing neuromuscular differentiation have been performed in cell culture experiments, through the use of cell lines or primary cultures. While these types of systems allow an immense flexibility in the type of questions that can be addressed, they are ultimately unable to completely duplicate or represent the in vivo environment in which these events actually occur. Advances in our understanding of, and ability to modify individual mouse gene expression, have led to a recent explosion of transgenic mouse model systems, and the widespread use of mouse genetics as a tool for studying protein function in vivo.

In mouse, a variety of options are available in choosing a model system for studying mouse neuromuscular development. For many, examination of phrenic nerve/diaphragm preparations has proved to be a fruitful avenue in this regard for a variety of reasons. In comparison to other endplate systems such as those found in the hind limb muscles, the diaphragm provides a relatively simple preparation of motor nerve and muscle fiber. The muscle fibers of the diaphragm exist as a thin sheet of parallel myotubes, allowing for clear visualization and microscopic histology without sectioning. The primary and

secondary axon branches of the phrenic nerve and subsequent innervation are easily visualized by immunolabeling. Thus, both nerve and muscle can be studied in parallel, with high spatiotemporal resolution (Burden, 1998; Witzemann, 2006). Furthermore, extraction and analysis of diaphragms is possible as early as embryonic day 12 (E12), when the first myofibers begin to form and the motor nerve first approaches the muscle, allowing a study of the initial stages of neuromuscular genesis.

Described in this dissertation, is a genetic approach toward studying specific aspects of presynaptic development. Chapter 2 discusses the possible roles of cholinergic signaling in directing aspects of presynaptic development, while chapter 3 speculates as to the requirement of Schwann cells in the regulation of the timing of presynaptic differentiation.

CHAPTER 2:

Presynaptic specialization in the absence of postsynaptic neurotransmitter receptor

Introduction

Neuromuscular junctions (NMJs) are the synaptic contacts between motor neurons and muscles that use acetylcholine (ACh) as the neurotransmitter for communication. NMJs are one of the best-studied synapses, and have been an excellent model for understanding the mechanisms underlying patterning and formation of chemical synapses. Individual synapses comprise a synaptic vesicle-rich nerve terminal closely apposed by a perisynaptic Schwann cell and a postsynaptic apparatus that contains high density of ACh receptors (AChRs). Aspects of presynaptic and postsynaptic differentiation are controlled by bi-directional interactions between presynaptic and postsynaptic cells in a step-wise, time-dependent fashion.

During development of the mouse NMJ, motor axons are guided to muscle fibers and undergo regulated nerve branching within a narrow, central region of muscle fibers (Lin et al., 2001; Yang et al., 2001; Yang et al., 2000b). Shortly after embryonic day 14.5 (E14.5), AChRs are clustered along the central band of the muscle through a process initiated by a muscle intrinsic mechanism. Thus, neuromuscular synaptogenesis is pre-patterned in muscle (Yang et al., 2000a). This event occurs prior to the onset of presynaptic specialization, which,

as indicated by the accumulation of synaptic vesicles at nerve terminals, is not observed until E15.5-16.5, thus AChR clusters exist initially in an unapposed, aneural state (Lin et al., 2001; Yang et al., 2001). Previous studies have shown that aneural clusters participate in mediating the patterning of synapses by a process in which aneural clusters are dispersed and recruited to new ones (Flanagan-Steet et al., 2005; Panzer et al., 2006). However, in addition, we have previously postulated that the aneural AChR clusters may also attract navigating axons in response to ACh and limit nerve branching and nerve terminal growth, which in turn controls the location of future synapses (Brandon et al., 2003a; Lin et al., 2001). In supporting this hypothesis, the motor nerve is hyper-branched and innervates a broader muscle territory, accompanied by an expansion of the boundary of synapse distribution in mice deficient in choline acetyltransferase (ChAT), the key enzyme for ACh biosynthesis (Brandon et al., 2003b; Misgeld et al., 2002). Thus, postsynaptic AChRs may be required for regulating the process of nerve branching and consequent pre-patterning of neuromuscular synapses.

In addition to the negative regulation of nerve branching, several lines of evidence suggest that ACh and postsynaptic AChR clusters may positively or negatively regulate presynaptic specialization. First, postsynaptic differentiation is required for eliciting a retrograde signal to initiate presynaptic specialization (Glass and Yancopoulos, 1997; Nguyen et al., 2000). Muscle-specific kinase (MuSK), which is absolutely required for initiating and maintaining postsynaptic

differentiation, is essential for presynaptic specialization. Though not essential in initiating postsynaptic differentiation, agrin is required for maintaining MuSK clusters (Glass and Yancopoulos, 1997). Consistent with the requirement of postsynaptic differentiation, specialization of nerve terminals is not observed in MuSK (DeChiara et al., 1996) and agrin mutants (Gautam et al., 1996). *In vitro* studies demonstrate that the AChR is required for agrin signaling through the MuSK complex for some aspects of postsynaptic differentiation (De La Porte et al., 1998; Grow and Gordon, 2000; Marangi et al., 2001). Raising the possibility that postsynaptic AChR clusters promote presynaptic specialization by facilitating MuSK-mediated retrograde signaling *in vivo*. However, analysis of synaptic differentiation in ChAT/agrin mutants suggests that ACh may inhibit presynaptic specialization. ACh inhibits postsynaptic differentiation by dispersing MuSK and AChR clusters in the absence of agrin (Lin et al., 2005; Misgeld et al., 2005). Relieving inhibition by blocking ACh production leads to the restoration of both presynaptic and postsynaptic specializations in agrin mutants (Lin et al., 2005; Misgeld et al., 2005). The restoration of postsynaptic specialization may lead to subsequent presynaptic specialization in ChAT/agrin mutants. Alternatively, ACh may inhibit specialization of nerve terminals via AChRs present on nerve terminals (Minic et al., 2002; Role and Berg, 1996; Wessler, 1992) and/or Schwann cells (Garcia et al., 2005; Rochon et al., 2001). The absence of ACh relieves the inhibition and may thus result in presynaptic specialization in ChAT/agrin mutants (Lin et al., 2005; Misgeld et al., 2005).

In the present study, we address these issues by focusing on the roles of ACh and postsynaptic AChRs in nerve branching and specialization of nerve terminals. Available data show that embryonic muscle expresses two types of AChR complexes that use AChRα1 or AChRα7 as the ligand binding subunit (Fischer et al., 1999; Romano et al., 1997), but no other nicotinic AChR complexes or muscarinic AChR subtypes (Garcia et al., 2005; Minic et al., 2002; Rochon et al., 2001; Wessler, 1992). The AChR complex at the developing NMJ is a pentamer of the composition $\alpha1_2\beta1\gamma\delta$. A wealth of receptor assembly and trafficking studies suggest that the α1 subunit is expressed only in muscle and is necessary for any of the other subunits to be located to the membrane (Green and Millar, 1995). The second AChR complex is an $(\alpha7)_5$ pentamer and is not clustered at the NMJ. The AChRα7 subunit is transiently expressed in embryonic muscle (Fischer et al., 1999; Romano et al., 1997). To determine whether these two AChR complexes play a role in presynaptic development, we analyzed mice deficient in AChRα1 and AChRα7 subunits. The results suggest that ACh, acting through the $\alpha1_2\beta1\gamma\delta$ pentamers, induces retrograde signals to regulate nerve branching and nerve terminal growth, which in turn regulates motor neuron number and distribution of synaptic sites. The results support the hypothesis that the E14.5 aneural AChR clusters play a role in pre-patterning of neuromuscular synapses. Comparison of presynaptic specialization between AChRα1/agrin (AGD), AChRα1/ AChRα7/AGD and ChAT/AGD mutants suggests that ACh

inhibits presynaptic specialization via non-muscle AChRs. The ACh-mediated inhibition is antagonized by an agrin-dependent signal.

Results

AChRα1 Mutants Lack a Functional Muscle AChR complex

To investigate the role of muscle AChR, we first generated mice deficient in AChRα1 subunit. A targeting vector, in which exon 1 and upstream promoter region was deleted and replaced with a neo-cassette as shown in Figure 1, was constructed and electroporated into J1 embryonic stem cells. Chimeric mice with germline contribution of the mutant allele were bred to a C57BL/6 background to produce viable and fertile heterozygote mice. By breeding heterozygous mice, we were able to obtain homozygous mutants in embryonic stage litters at a frequency 1/4 of all embryos collected. However, homozygous mutants die at birth, similarly to ChAT mutants, most likely due to a loss of diaphragm function. AChRα1 mutants are also morphologically similar to ChAT mutants with kyphosis and carpoptosis phenotypes apparent at embryonic stages as early as E15.5.

When stained with Texas Red-conjugated α-bungarotoxin (TR-αBTX), AChR clustering is completely absent in E17.5 AChRα1 mutant muscle (Figure 4d and Figure 3a). Similar results were observed when E14.5, E15.5 and E16.5 muscles were examined (data not shown). To determine whether AChR δ

subunit is expressed in AChRα1 mutants, intercostal muscle sections were *in situ* hybridized with riboprobes for AChRα1 or δ subunits. As shown in Figure 3a, AChRα1 transcripts are absent in the mutants. AChR α1 subunit immunoreactivity is not detectable in the mutants (Figure 3b). Although AChRδ subunit transcripts are detected along the central region of mutant muscle (Figure 3a), AChRδ subunit protein is not clustered on the membrane in the absence of AChRα1 subunit. These results are consistent with the idea that AChRα1 subunit plays an essential role in the assembly of the pentameric AChR complex and, hence, the trafficking of the complex to cell surface for clustering (Green and Millar, 1995). Consistent with the absence of AChR clusters, electrophysiological analysis showed that spontaneous miniature and nerve evoked end-plate potentials are absent in AChRα1 mutant muscle (Figure 2a and Figure 2b). Therefore AChR clusters and activity are absent in AChRα1 mutant muscle.

Loss of Muscle Receptors Results in Increased Nerve Branching and Motor Neuron Number

Because previous results showed that ChAT mutants display increased nerve branching and a concomitant increase in motor neuron number (Brandon et al., 2003b; Misgeld et al., 2002), we then studied innervation patterns and motor neuron number in AChRα1 mutants. E17.5 whole-mount diaphragm muscles were immunostained with anti-neurofilament (NF) antibodies to

visualize the innervating nerve processes. As shown in Figures 2c-f, AChRα1 mutant diaphragms are hyperinnervated, with nerve branches extending throughout a wider region of the muscle in a striking contrast to the centrally restricted pattern in control muscle. Furthermore, as shown in Figure 2g, the number of motor neurons at the lumbar levels is increased by approximately 60% in both AChRα1 and ChAT mutants as compared to that of controls. These results support the idea that initial aneural muscle AChR clusters play a role in limiting the growth and branching of the developing motor nerve, thereby restricting the formation and distribution of future synaptic sites along the central band of muscle. The close similarities in the electrophysiological and hyperinnervation defects and increased motor neuron survival between the AChRα1 and ChAT mutant mice suggest that these defects are due to a lack of ACh signaling in large part, if not solely, through postsynaptic AChRs (see comparison of presynaptic differentiation of AChRα1 mutant with various mutants in Table 2). An absence of ACh activation through postsynaptic receptors may elevate expression or release of muscle-derived trophic factors to increase nerve branching and motor neuron number. Alternatively, or in addition, activation of centrally located AChR clusters in control mice may render other regions of muscle less attractive for innervation. A lack of such muscle activity may permit the nerve to increase branching and to innervate a broader muscle territory.

Muscle Receptors Are Not Required for Presynaptic Differentiation

Much evidence has suggested that postsynaptic differentiation is required for eliciting a retrograde signal to initiate presynaptic specialization (Glass and Yancopoulos, 1997; Nguyen et al., 2000). MuSK is absolutely required for postsynaptic and, hence, presynaptic differentiation (DeChiara et al., 1996; Gautam et al., 1996). As muscle AChR has been shown to play a role in MuSK-dependent clustering of several postsynaptic proteins in muscle cultures (Grow and Gordon, 2000; Marangi et al., 2001), we determine if AChR clusters are required for eliciting a retrograde signal to promote presynaptic differentiation. E17.5 diaphragm muscles were co-stained with TR-αBTX and antibodies against synaptophysin, a synaptic vesicle protein (Figure 4). In control diaphragms, synaptophysin immunoreactivity is highly concentrated at the nerve terminals that are closely apposed by AChR clusters. Surprisingly, synaptophysin immunoreactivity is accumulated as distinct clusters at the nerve terminals despite the absence of AChR clusters in mutant muscles. Synaptophysin staining is also observed to some degree in many of the axon bundles, however as shown by several groups, detection of low levels of synaptic vessels in the axons of embryonic mice is to be expected (Polo-Parada et al., 2001). Despite this fact, nerve terminal clusters are clearly distinct from the relatively low levels of axonal staining. Furthermore, when antibodies against another synaptic vesicle protein SV2 were used, similar results were obtained (Figure 6b). These synaptophysin-rich nerve terminals occupy a broader region of the diaphragm,

similar to what was seen in the ChAT mutant mice (Brandon et al., 2003b; Misgeld et al., 2002). So while it is clear that postsynaptic MuSK-mediated retrograde signaling is necessary for the development of nerve terminal specialization, these results suggest that muscle AChR clustering is not essential in this process.

Ultrastructure at AChRα1-deficient synapses

Despite the lack of AChRs, the immunohistochemical experiments revealed the co-localization of nerve terminals and some post-synaptic proteins, suggesting that neuromuscular synapses appeared to be formed in mutant muscles. We further examined these synapses at the ultrastructure level. EM observation of E17.5 diaphragm muscles revealed that AChR-deficient mutant NMJs showed features typical of the embryonic NMJ as seen in controls (Figure 5). The multiple motor nerve terminals, covered by the processes of perisynaptic Schwann cell, closely apposed to and made synaptic contacts on the postsynaptic membrane of the myotubes. The nerve terminals contained mitochondria and clusters of synaptic vesicles. There was little difference in the abundance of synaptic vesicles between mutants and controls. The basal lamina was seen in the synaptic cleft. The postsynaptic membrane showed more electron-dense material than other muscle plasma membranes, which is one of the characteristics of postsynaptic specialization, in both genotypes. However, morphometric analysis revealed that an average of postsynaptic

membrane length was significantly shorter at mutant NMJs than that at controls, most likely due to the lack of junctional folds in mutants (Table 1). The subsynaptic nucleus of the myotubes was present as usual. In short, all of the synaptic components found at control NMJs were present at mutant NMJs, although the postsynaptic specialization appeared more immature in mutant muscles compared to that of controls. Thus, the absence of AChRs did not prevent formation of presynaptic specialization at the ultrastructure level.

MuSK and AChE are Clustered at NMJs in the Absence of Muscle Receptor

While receptor clustering has long been used as a marker to represent aggregation of the postsynaptic apparatus, this could not be the case in our analysis of the α1 mutant mice. In fact it is likely that other postsynaptic proteins could aggregate in the absence of the receptor, supporting the idea that localized elements of the postsynaptic apparatus may serve to elicit a positive retrograde signal to the nerve. Furthermore, AChR has been shown to be required for agrin-induced acetylcholine esterase (AChE) clustering in myotube cultures (De La Porte et al., 1998). Thus, we examined clustering of AChE, MuSK and rapsyn in AChRα1 mutant mice. AChE is normally localized to the basal lamina of the synaptic cleft, and is a common marker for synaptic sites. Histochemical AChE staining of mutant diaphragms reveals that AChE appears to be clustered along the central band of mutant diaphragms, although the clusters occupy a broader region and seem to be smaller than those in controls

(Figure 6a). To determine whether AChE clusters are apposed with SV-rich nerve terminals, leg muscle sections were immunostained with fasciculin toxin conjugated to a fluorescent dye and anti-synaptophysin or anti-SV2 antibodies to label AChE clusters and nerve terminals, respectively. As shown in Figure 6b, fasciculin-stained AChE clusters are co-localized with synaptophysin-rich nerve terminals in both control and mutant muscles. Similarly, MuSK is aggregated and co-localized with nerve terminals in mutant muscle. In contrast, rapsyn is no longer clustered and co-localized to nerve terminals in mutant muscle. The absence of rapsyn clustering is consistent with previous findings that rapsyn binds AChRβ1 or all other subunits prior to the trafficking to muscle membrane surface (Burden et al., 1983; Maimone and Merlie, 1993) and is required for association of the AChR with other postsynaptic proteins in cultures (Fuhrer et al., 1999). Similarly, AChRs direct rapsyn clusters to the neuromuscular synapses in zebrafish (Ono et al., 2004), and rapsyn is not clustered at synaptic sites in zebrafish lacking AChR (Ono et al., 2001).

Interestingly, MuSK has been recently shown to be required for anchoring AChE at the NMJ in cultures (Cartaud et al., 2004). The presence of MuSK clusters may explain the occurrence of presynaptic specialization (Figure 4) (Table 2) and AChE clustering (Figure 6) in AChRα1 mutants.

Evidence Suggests that ACh Inhibits Presynaptic Specialization

Previous analysis demonstrated that agrin mutant mice lack both pre- and postsynaptic specializations. When ACh is removed from the developing endplates, as was seen in ChAT/ agrin (AGD) double mutants (Lin et al., 2005; Misgeld et al., 2005) (see summary in Figure 7c and Table 2), both presynaptic and postsynaptic specializations are restored. Furthermore, ACh inhibits postsynaptic differentiation by dispersing the postsynaptic apparatus that is not stabilized by agrin signaling (Lin et al., 2005; Misgeld et al., 2005). Thus, ACh and agrin elicit opposing activities in postsynaptic differentiation. The presence of presynaptic specialization in ChAT/AGD double mutants may be due to the restoration of postsynaptic differentiation. Alternatively, ACh may directly inhibit presynaptic specialization. However, as distinct AChR subtypes are present in postsynaptic muscle cells and non-muscle cells (nerve terminals and perisynaptic Schwann cells), it is not clear whether ACh acts through muscle or presynaptic AChRs to exert inhibitory activity on presynaptic specializations. To address this issue, we analyzed the presynaptic specialization of AChRα1 and AChRα1/AGD double mutants (Fig. 7a) and compared with previous results in ChAT and ChAT/agrin mutants (Figure 7c and Table 2). As shown in Figure 7a, in contrast to ChAT/AGD double mutants (Lin et al., 2005; Misgeld et al., 2005) (Figure 7c and Table 2), no presynaptic specialization was observed in AChRα1/AGD double mutant muscle. As previously mentioned, low levels of synaptic vessels can be observed in the embryonic axons of mice, however the

characteristic disc-shaped staining of nerve terminal specializations in ChAT/AGD double mutant muscle is clearly distinct from the axonal staining, while the clear lack of such specialized staining in AChRα1/AGD double mutant muscle is in stark contrast. These results indicate that ACh inhibits presynaptic specialization by a mechanism that does not involve postsynaptic receptor clusters, suggesting that the inhibitory activity is likely mediated through AChRs on nerve terminals or Schwann cells. However, the AChRα7 subunit is expressed in embryonic muscle and forms a homo-pentamer complex that is physically and pharmacologically distinct from the muscle AChR complex containing the AChRα1 subunit (Fischer et al., 1999; Romano et al., 1997). AChRα7 mutants display no overt defects in NMJ development (data not shown). Although AChRα7 pentamers are present at low levels and are not clustered, we considered the possibility that AChRα7 pentamers play a compensatory role to mediate inhibition by ACh in muscle in AChRα1/AGD. To test this possibility, we analyzed AChRα1/AChRα7/AGD triple mutants, where only postsynaptic AChRs have been abolished, leaving ACh signaling through non-muscle AChRs intact. As shown in Figure 8, presynaptic specialization was not observed in AChRα7/AGD double and AChRα1/AChRα7/AGD triple mutants. Lastly, while we have not obtained data to corroborate the light microscopy results for the double and triple mutants by EM, many previous studies have shown that there are little or no discordance between immunostaining with anti-synaptic markers and EM analysis (Misgeld et al.,

2005; Wang et al.) Because $(\alpha7)_5$ and $(\alpha1)_2\beta\delta\gamma$ pentamers are the only AChR complexes present in embryonic muscle (Fischer et al., 1999; Romano et al., 1997), our results support the hypothesis that ACh inhibits presynaptic specialization through a non-postsynaptic mechanism.

These results raise the possibility that this ACh-mediated inhibitory activity is antagonized by an agrin-dependent signal through the MuSK clusters. Consistent with this possibility, MuSK clusters are observed in ChAT/AGD mutants (Misgeld et al., 2005), but not in AChRα1/AGD (Figure 9) (Table 2) and AChRα1/AChRα7/AGD (data not shown). Interestingly, ACh, acting through postsynaptic receptors, disperses AChR and MuSK clusters that are not stabilized by an agrin-dependent activity. Thus, AChR and MuSK clusters are dispersed in AGD mutants, but rescued in ChAT/AGD mutants (Lin et al., 2005; Misgeld et al., 2005) (Figure 7c and Table 2). Furthermore, in contrast to previous results showing that AChE is clustered in ChAT/AGD double mutants (Lin et al., 2005), no AChE clusters were detected in AChRα1/AGD double mutants (Figure 7b). The absence of MuSK clusters may explain a lack of AChE clusters in the AChRα1/AGD double mutants.

Discussion

Previous studies demonstrated that ACh negatively regulates postsynaptic differentiation by dispersing the postsynaptic apparatus that is not apposed by a specialized nerve terminal (Lin et al., 2005; Misgeld et al., 2005).

In the present study, we provide genetic evidence that ACh negatively and differentially regulates two aspects of presynaptic differentiation. Specifically, ACh, acting through postsynaptic AChRs, inhibits nerve branching, whereas ACh inhibits presynaptic specialization via non-postsynaptic AChRs. The ACh-mediated inhibition on presynaptic specialization may be antagonized by agrin-dependent retrograde signals. These conclusions are based on analyzing synaptic differentiation in controls, AChRα1, AGD, AChRα1/AGD, and AChRα1/AChRα7/AGD mutants and on comparing the results with previous analysis in MuSK, ChAT, and ChAT/AGD mutants from our and others' laboratories (Figure 7c and Table 2). The results demonstrate that postsynaptic AChRs mediate the nerve-released ACh signal to negatively regulate nerve branching and motor neuron number in a retrograde manner (Oppenheim et al., 2003). Furthermore, synaptic sites, as indicated by the apposition of synaptic vesicle-rich nerve terminals with the postsynaptic apparatus containing MuSK clusters (Figure 6), are distributed in a broader muscle territory in AChRα1 mutants, reminiscent of ChAT mutants (Brandon et al., 2003b) (Table 2). These findings are separate from and unique to previous studies in zebrafish that showed that aneural clusters play a role in the patterning of synapses via the mechanism of dispersal and recruitment to new sites. These results are not contradictory however, and in concert, strengthen and support the hypothesis that the aneural AChR clusters detected at E14.5 along a narrow central band of muscle are a component of the muscle intrinsic mechanism for pre-patterning of

neuromuscular synapses. They primarily function to restrict nerve branching and nerve terminal growth within a limited, central region of muscle fiber, thereby controlling the boundary for the formation and distribution of mature synapses (Brandon et al., 2003b). We suggest that the ACh-mediated inhibition on presynaptic specialization is to ensure that only those nerve terminals apposing a postsynaptic apparatus undergo specialization.

Based on our results, we propose the following step-wise model for regulation and coordination of pre- and postsynaptic specializations from initiation to maturation stages. During the initiation of neuromuscular synaptogenesis at E14.5, a muscle intrinsic MuSK clusters at the central band, facilitating the accumulation of aneural AChR clusters on the muscle membrane (Lin et al., 2001; Yang et al., 2001; Yang et al., 2000b). At the same time, the nerve is guided to the central band of muscle, a period during which ACh, acting through non-muscle AChRs, inhibits specialization of nerve terminals. Once the nerve reaches muscle, ACh released from the nerve signals through the E14.5 aneural AChRs, which activates a retrograde signal that negatively regulates nerve branching and nerve terminal growth. Nerve-released agrin acts to support and stabilize the clustering of MuSK and the postsynaptic apparatus that are otherwise dispersed by ACh. As discussed previously (Lin et al., 2005), because agrin may be partially bound to extracellular matrix associated with nerve terminals, ACh may exert inhibitory activity over a larger diffusible area than agrin, thus at extrasynaptic sites, ACh serves to disperse clusters, while at

nerve-apposed sites, the high concentration of agrin serves to antagonize the inhibitory activities. Finally, nerve terminals that are apposed to postsynaptic specializations are relieved of inhibition in an agrin-dependent manner, allowing the nerve terminal to begin to differentiate.

How might ACh and agrin play opposing roles in presynaptic specialization? Neural activity has been shown to affect cytoskeletal proteins and expression of synaptic adhesion molecules that play a role in presynaptic specialization (Schaefer and Nonet, 2001), including actin, N-cadherin, β-catenin and neuronal cell adhesion molecule (NCAM). Presynaptic maturation and SV clustering at the NMJ are impaired in NCAM mutant mice (Polo-Parada et al., 2001). ACh may also regulate expression and signaling through other synaptic adhesion molecules, including neurexin-neuroligin (Scheiffele, 2003), SynCAM (Biederer et al., 2002) and β-catenin (Bamji et al., 2003; Murase et al., 2002). Signaling through the MuSK complex may lead to the release of trophic factors to induce presynaptic specialization such as fibroblast growth factors and collagen IV chains (Fox et al., 2007; Umemori et al., 2004). Agrin may act through the MuSK complex to stabilize MuSK clusters and regulate expression or function of trophic factors. Interestingly, recent results showed that low-density lipoprotein (LDL) receptor-related protein 4 (Lrp4) and MuSK mutant mice display similar phenotypes at the NMJ (Weatherbee et al., 2006). As members of the Lrp family play a role in Wnt signaling, the results raise the possibility that the

interplay of agrin and Wnt signaling pathways may be required for a retrograde signal to induce presynaptic specialization.

In conclusion, we have demonstrated that ACh inhibits synaptic vesicle clustering through a non-postsynaptic mechanism, whereas ACh, acting through postsynaptic AChRs, controls nerve branching and nerve terminal number, and, hence, location of the synapses and motor neuron number. Taken together, these results suggest that ACh plays essential roles in controlling the location and multiple aspects of specialization of the synapse it subserves through distinct pathways.

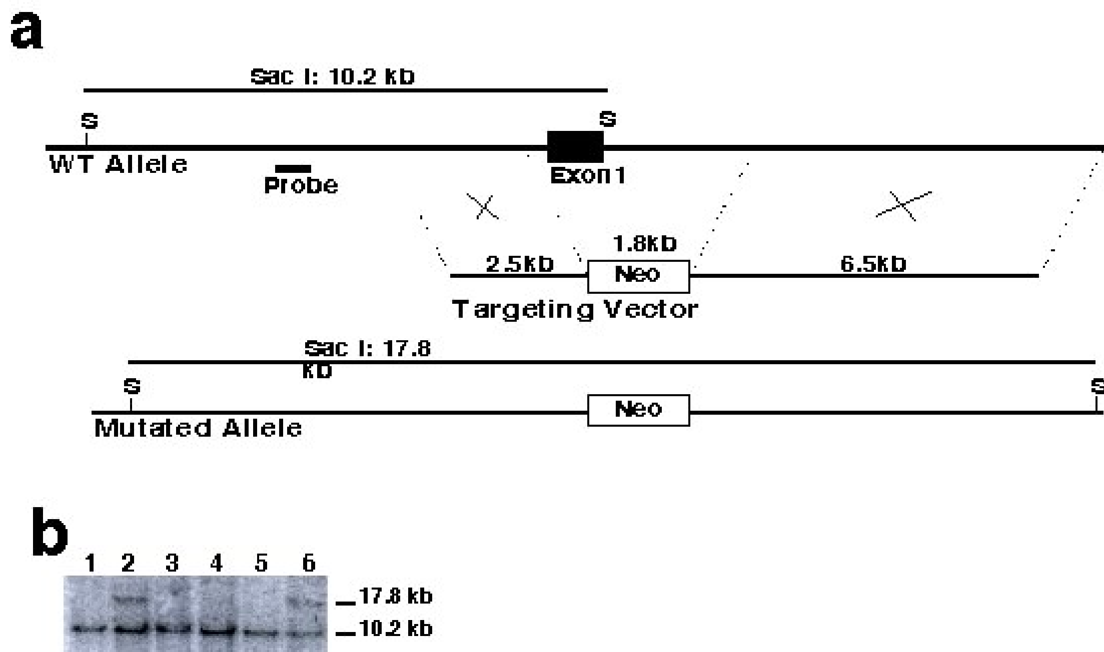

Figure 1. Generation of AChRα1 mutant mice

(a) Diagram showing targeted deletion of AChR α1 genomic regions. Top, Genomic region surrounding Exon 1 of the α1 WT Allele is shown. Middle, The targeting vector, with 2.5kb and 6.5kb homologous arms. A 4 kb region that includes Exon 1 is replaced by a PGK-neo cassette (neo). Bottom, α1 mutated allele that results from homologous recombination. Sac I sites (S), with resulting genomic fragments that will bind the indicated probe by southern analysis is shown. (b) ES clones identified by Southern blot analysis to have undergone homologous recombination.

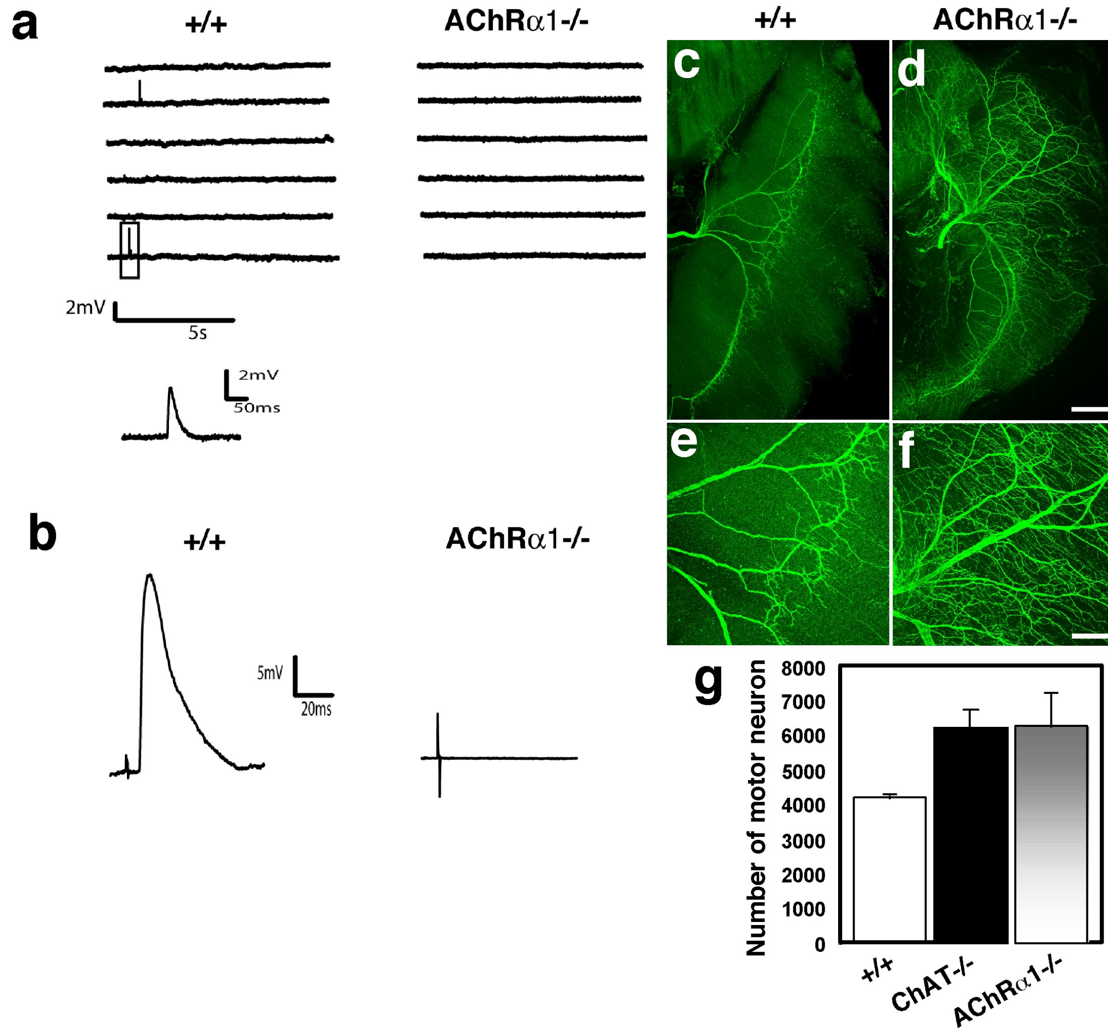

Figure 2. Postsynaptic transmission deficiency, muscle hyperinnervation and increased motor neuron number in AChRα1 mutant mice

a. Spontaneous mepps were observed in control diaphragm but not in AChRα1 mutant diaphragm. One mepp is expanded below. b. Nerve-evoked epps were observed in control but not in mutant muscle. c-f. E17.5 wholemount diaphragm muscles from controls (+/+) and AChRα1 mutants were immunostained with anti-neurofilament (NF) antibodies (green). Both low (c, d) and high (e, f) power magnifications showed that the phrenic nerve is highly branched in mutant muscle (d, f). g. A similar increase in the number of lumbar motor neurons was observed in ChAT and AChRα1 mutants as compared to controls. Scale Bars: c and d, 200 μm; e and f, 100 μm.

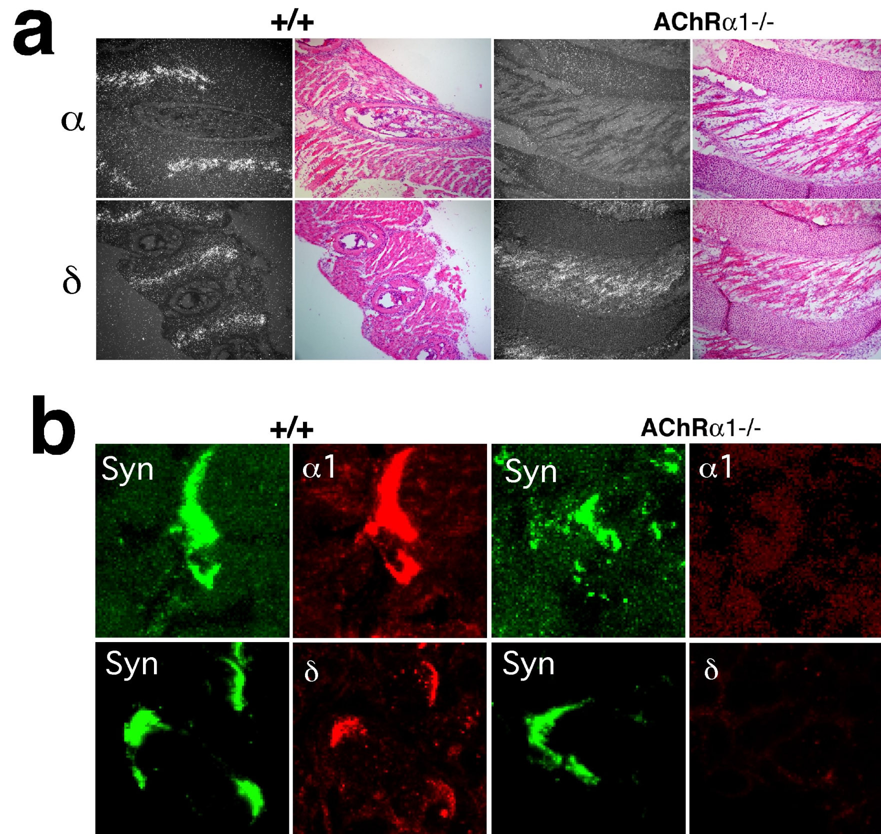

Figure 3. Expression of AChR α and δ subunit mRNA and proteins in AChRα1 mutants

a. E17.5 intercostal muscle sections were subjected to *in situ* hybridization for AChR α1 and δ transcripts. In control (+/+) muscle, α1 and δ mRNAs are concentrated along a central band. In mutant muscle, α1 transcripts are absent, while δ mRNA is distributed across a broader region of the muscle compared to controls. b. E17.5 hind limb muscle sections were immunostained with antibodies against SV2 and AChR α1 or δ subunit. In control muscle, both AChR α and δ were aggregated on the membrane and apposed by SV2-rich nerve terminals. Consistent with the absence of AChRα1 subunit mRNA, no AChRα1 subunit immunoreactivity was detected in mutant muscle. Although AChR δ subunit mRNA was detected in mutant muscle, no AChRδ subunit proteins were aggregated and apposed to SV2-rich nerve terminal.

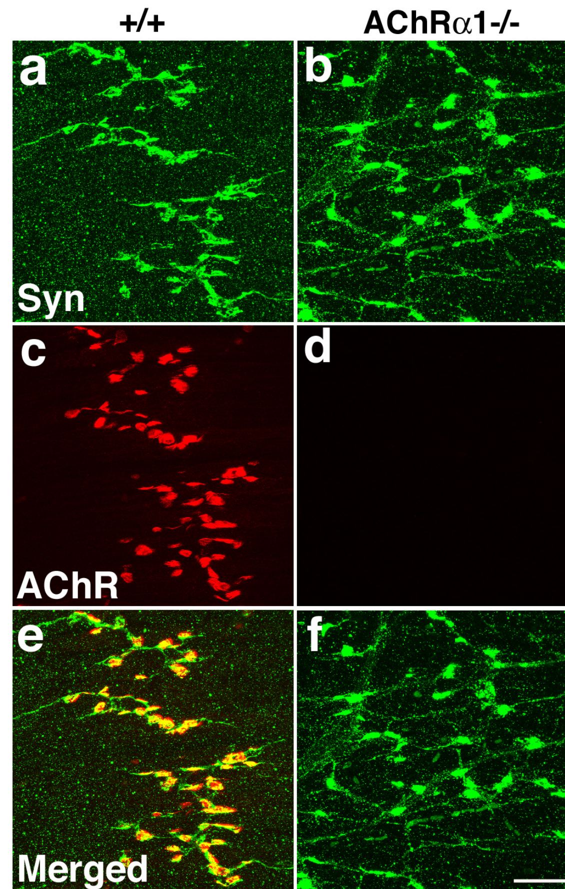

Figure 4. Presynaptic nerve terminals differentiate in AChRα1 mutants

Mutant (-/-) and control (+/+) E17.5 wholemount diaphragm muscles were immunostained with anti-synaptophysin antibodies (green), and co-stained with Texas Red-conjugated α-bungarotoxin (red). Synaptophysin immunoreactivity is accumulated at the nerve terminals and is co-localized with receptor clusters in control diaphragms. Mutant diaphragms lack receptor clusters, but maintain synaptophysin accumulations at the nerve terminals. Scale bar: a-f, 50 μm.

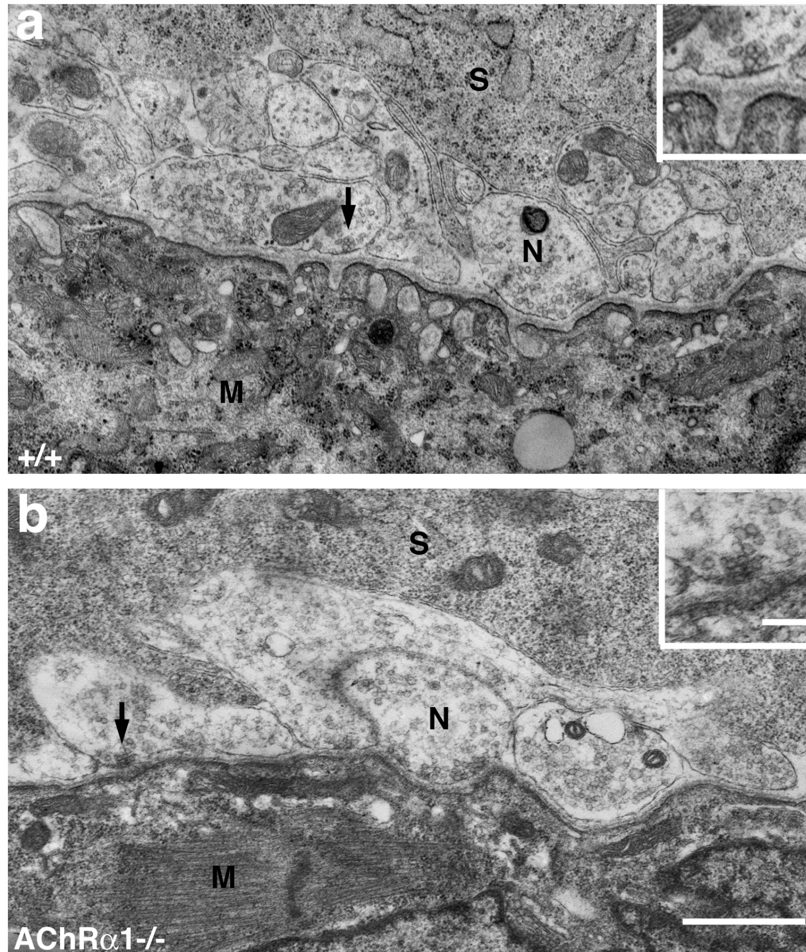

Figure 5. Ultrastructure of the NMJ in the AChRα1 mutant mice

Electron micrographs of NMJs from E17.5 control (a) and AChRα1-deficient mutant (b) diaphragm muscles show similar ultrastructures. A representative micrograph from a control (a) shows features typical of embryonic NMJs. The multiple motor nerve terminals (N), capped by the processes of perisynaptic Schwann cells (S), make synaptic contacts on the postsynaptic membrane of the myotube (M). The basal lamina is seen in the synaptic cleft. The nerve terminals contain mitochondria and clusters of synaptic vesicles (arrow). Similarly, a representative AChRα1 mutant NMJ (b) also shows features typical of the embryonic NMJ, including the multiple nerve terminals (N), the perisynaptic Schwann cell (S), the myotube (M) and the basal lamina. The clusters of synaptic vesicles (arrow) are clearly seen in the nerve terminals. In controls, junctional folds were frequently observed in the postsynaptic membrane. In mutants (b), the postsynaptic membrane has only indentations but lacks junctional folds. Inset in a: a higher magnification of the area indicated by the arrow depicts a cluster of synaptic vesicles over a junctional fold, resembling the active zone in mature NMJs. Inset in b: a higher magnification of the area indicated by the arrow depicts an active zone with a docked synaptic vesicle in the mutant nerve terminal, despite the lack of the junctional fold. Scale bars: a and b, 1 µm; insets, 0.2 µm.

Table 1. Comparison of ultrastructural parameters in neuromuscular synapses from AChRα1 mutants and control embryos

	Mutant	Control
Nerve terminal area (μm^2)	0.55 ± 0.08	0.50 ± 0.05
Nerve terminal perimeter (μm)	2.97 ± 0.23	3.08 ± 0.20
Synaptic contact length (μm)	0.69 ± 0.05*	0.91 ± 0.10
Synaptic vesicle density (per 0.04 μm^2)	5.47 ± 0.34	4.98 ± 0.32
Active zone number/nerve terminal	0.25 ± 0.07	0.44 ± 0.08
Docked SV number/active zone	1.50 ± 0.22	1.21 ± 0.10
Postsynaptic membrane length (μm)/NMJ	4.95 ± 0.85**	10.14 ± 1.75
Synaptic bouton number/NMJ	5.33 ± 1.19	4.36 ± 0.75

Morphometric analysis was performed on EM images of NMJs from E17.5 diaphragm muscles. The electron micrographs were digitized and analyzed using NIH Image software. The following measurements were made from each presynaptic nerve terminal profile: perimeter length, nerve terminal area, synaptic contact length, active zone number, docked synaptic vesicle number and synaptic vesicle density according to the morphological criteria previously described (Brandon et al., 2003b). Briefly, the synaptic contact was measured as the length of the presynaptic plasma membrane that was apposed to the postsynaptic muscle membrane at a distance of 50-80 nm. The active zone was defined as a cluster of synaptic vesicles at the electron-dense presynaptic membrane. A docked synaptic vesicle was defined as a presynaptic plasma membrane-attached vesicle at the active zone. The synaptic vesicle density was determined as the number of synaptic vesicles in a 0.04 μm^2 area surrounding the active zone or immediately adjacent to the presynaptic membrane in the region of synaptic contact for sections not passing through active zones. In addition, the postsynaptic membrane length including junctional folds, if present, was also measured. Data were collected from 48 nerve terminals of 11 NMJs in three mutants and of 12 NMJs in three control embryos and are expressed as means ± SEM. Statistical significance was assessed by two-tailed *t* test; *p<0.05, **p<0.017.

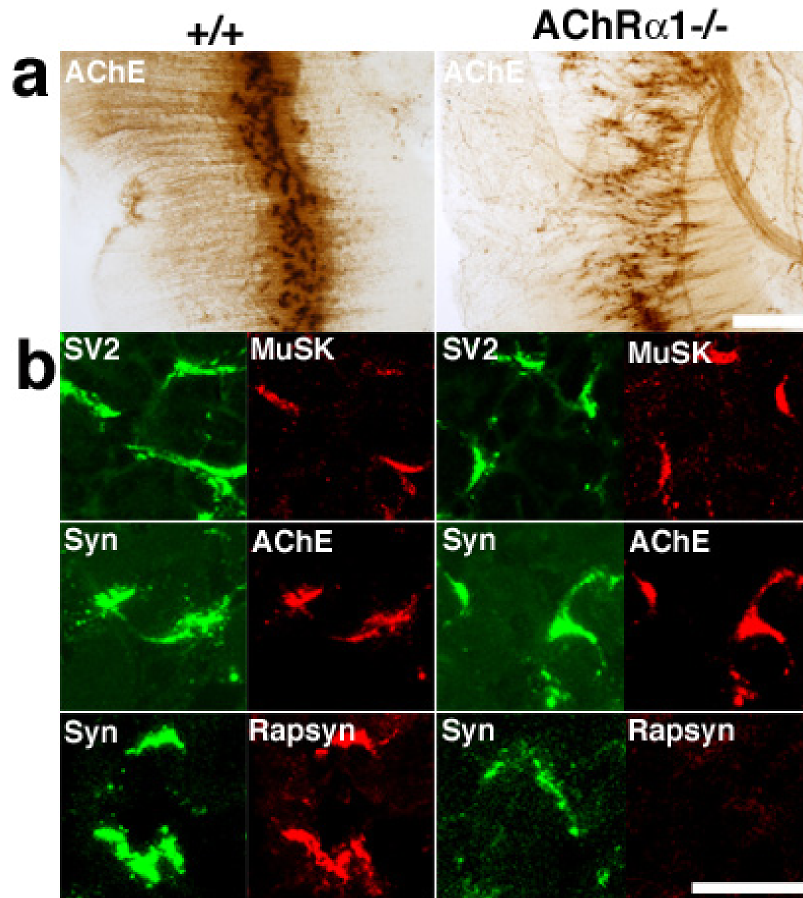

Figure 6. Clustering of AChE and localization of MuSK and rapsyn at the NMJ in AChRα1 mutants

a. E17.5 diaphragms were subjected to AChE histochemistry. AChE was clustered in AChRα1 mutants. b, E17.5 leg muscle sections were immunostained with markers for the presynaptic nerve terminal: anti-SV2 or anti-synaptophysin (syn) in green; and for various postsynaptic proteins: anti-MuSK, anti-rapsyn, or Alexa-fluor 544-conjugated fasciculin (AChE) in red. While MuSK and AChE are accumulated at the synapses in mutant muscle as in controls, rapsyn is not clustered at the synaptic sites in mutants. Scale bars: a, 200 μm; b, 20 μm.

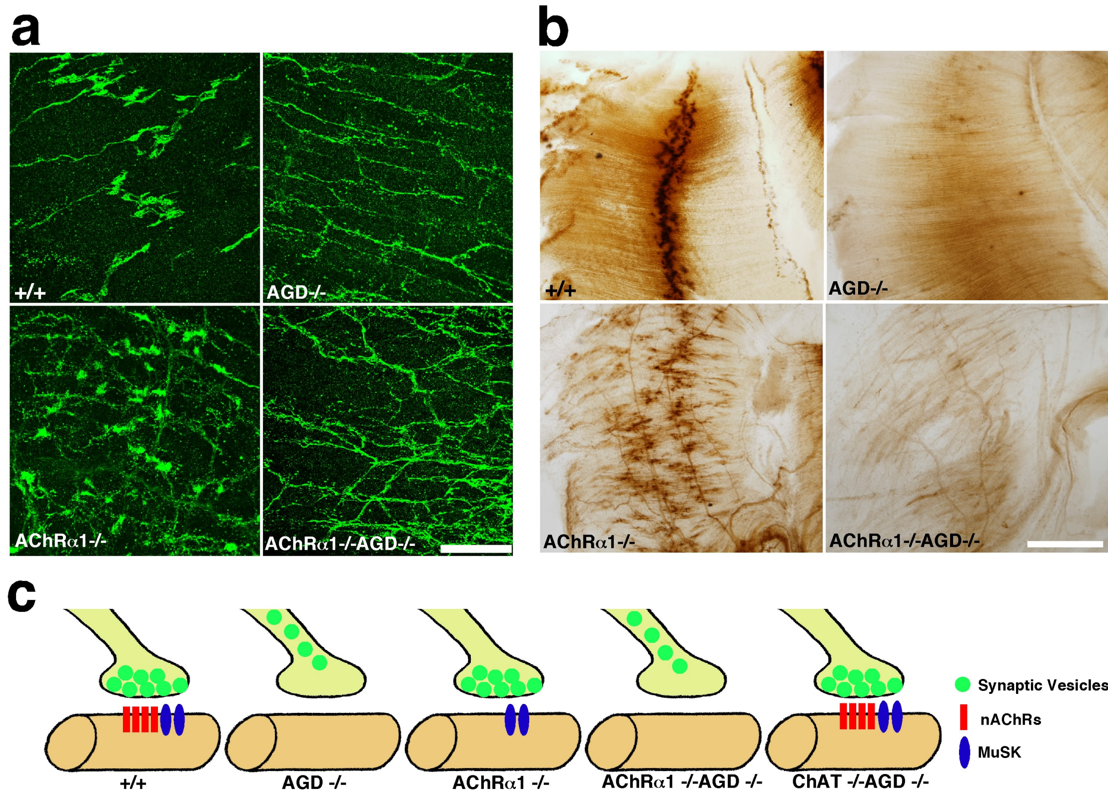

Figure 7. Absence of presynaptic differentiation in AChRα1/agrin double mutants
a. Diaphragm muscles from control, AGD, AChRα1 and AChRα1/AGD mutants were immunostained with antibodies against synaptophysin. Presynaptic specialization is not present in AChRα1/AGD mutants. b. Absence of AChE clusters in AChRα1/AGD mutants. c. Summary of analysis of accumulation of synaptic vesicles in control, agrin (AGD), AChRα1, AChRα1/AGD and ChAT/AGD mutants. Scale bars: a, 50 μm; b, 200 μm.

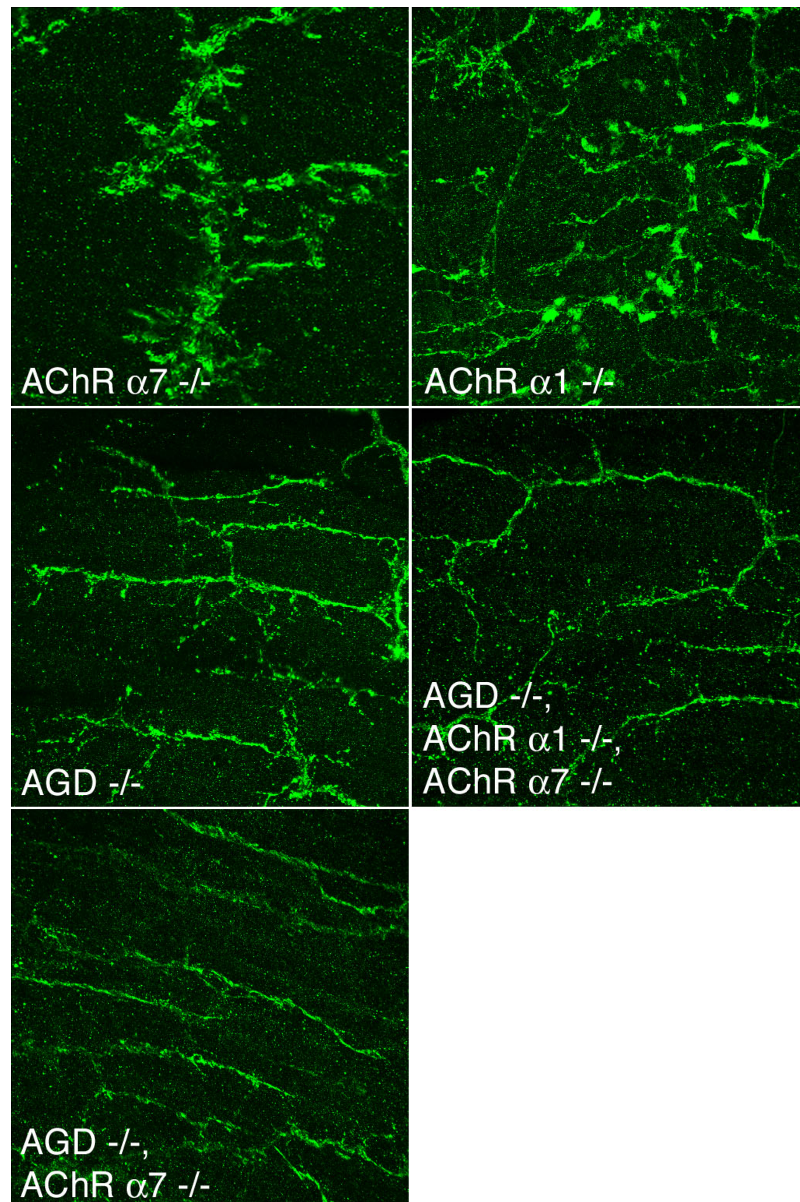

Figure 8. No presynaptic specialization in AGD/AChRα1/AChRα7 triple mutants
Diaphragms from AChRα7 (a), AChRα1 (b), AGD (c), AChRα7/AGD (d) and AChRα1/AChRα7/AGD (e) were immunostained with anti-synaptophysin antibodies. No presynaptic specialization was observed in AChRα7/AGD double and AChRα1/AChRα7/AGD triple mutants.

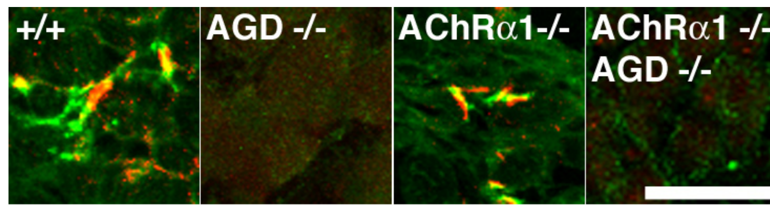

Figure 9. Absence of MuSK clustering in AChRα1/AGD double mutants
Leg muscle sections from control (+/+), AGD, AChRα1 and AChRα1/AGD mutants were double immunostained with anti-synaptophysin and anti-MuSK antibodies. MuSK was not clustered in AGD-/- and AChRα1-/-AGD-/- muscle.

Table 2. Summary of NMJ differentiation in various mutant mice.

Genotype	Nerve-derived signals		Postsynaptic differentiation		Presynaptic differentiation		Synapse distribution
	Agrin	ACh	AChR clustering	MuSK clustering	SV Accumulation	Nerve branching and growth	
+/+	+	+	+	+	+	Limited[a]	Narrow
AGD	-	+	-	-	-	Excess growth[b]	-
MuSK	+	+	-	-	-	Excess growth[b]	-
ChAT	+	-	+	+	+	Highly branched[c]	Broad
AChRα1	+	+	-	+	+	Highly branched[c]	Broad
ChAT/AGD	-	-	+	+	+	Excess growth[b]/Highly branched[c]	Broad
AChRα1/AGD	-	+	-	-	-	Excess growth[b]/Highly branched[c]	-

Synaptic differentiation of MuSK, agrin, ChAT and ChAT/AGD mutants was previously published. SV, synaptic vesicle.

[a]Axons exhibit limited growth and branching within a narrow, central band of muscle fibers.
[b]Axons exhibit excess growth in parallel with muscle fibers.
[c]Axons are highly branched and grow across multiple muscle fibers.

The text in this chapter is a modified version of a manuscript under submission for publication in Neuron (**Mahru C. An**, Weichun Lin, Bertha Dominguez, Daniel Padgett, Yoshie Sugiura, Prafulla Aryal, Thomas W. Gould, Ronald W. Oppenheim, Chien-Ping Ko and Kuo-Fen Lee. Presynaptic specialization in the absence of postsynaptic neurotransmitter receptor.)

CHAPTER 3:

The possible role of Schwann cells in the timing of presynaptic differentiation

Introduction

The function and development of the vertebrate synapse is highly dependent on the coordinated efforts between three entities: the presynaptic cell, postsynaptic cell, and glial cells. During development of the vertebrate neuromuscular junction, formation of synapses occurs through the differentiation of presynaptic and postsynaptic components in a coordinated manner, such that functionally specialized elements are juxtaposed to each other. The independently derived, but convergent nature of nerve-muscle synaptogenesis requires that there exist mechanisms by which both the location and timing of differentiation are directed. It has been shown that the postsynaptic muscle is predisposed to initiate the clustering of AChRs along a central band of the target muscle fiber, providing a prepatterned framework on which neuromuscular synapses are built in a coordinated manner (Lin et al., 2001). This initial process of differentiating the postsynaptic membrane is an event that occurs 1-2 days ahead of presynaptic specialization, as hallmarked by the accumulation of synaptic vesicles. Thus, determining the location of synapses is, in part, a process initiated by a muscle-intrinsic mechanism, and eventually refined in a coordinated process. More importantly, the timing of

presynaptic development and subsequent events is relative to this initial step, and must be carefully controlled. Little is known about how the timing of presynaptic differentiation is controlled, but as with location, it is likely to be a process that is coordinated through the interactions between presynaptic, postsynaptic, and glial components of the NMJ.

Glial cells are important mediators of vertebrate neuronal function and development, coexisting with neurons as an active and fundamental partner (Feng et al., 2005; Jessen and Mirsky, 1997). Schwann cells of the peripheral nervous system, as members of the glial cell family, exist in support of both peripheral nerve and synapse, with roles in the growth, development, and function in both cases. Schwann cells arise primarily from the neural crest as precursor Schwann cells, which give rise to both mature myelinating and non-myelinating Schwann cells (Jessen et al., 1994; Mirsky and Jessen, 1996). Schwann cell precursors are dependent on the mitogenic activity of erbB receptor family signaling to regulate these developmental steps, and thus they depend on association with developing axons, which express NRG. Loss of erbB signaling in Schwann cell precursors results in a failure to correctly regulate Schwann cell development, and a resulting abnormal development of the peripheral nervous system (Lin et al., 2000; Morris et al., 1999). Schwann cells are absent from the NMJs of mice deficient in NRG signaling, and axons are abnormally fasciculated upon initially innervating the muscle. Furthermore, nerve retraction begins to occur by later stages of development, followed by

widespread motoneuron death (Lin et al., 2000; Morris et al., 1999). In this study, which is part of a larger set of experiments directed at determining the relationship between Schwann cells and the timing presynaptic development, we describe that premature onset of presynaptic development occurs during the neuromuscular development of erbB2 deficient mice.

Results

Synaptic elements are prematurely apposed in ErbB2 mutant mice

Previous work in the lab on the role of neuregulin signaling in neuromuscular development led to the study of mice deficient in erbB the receptors. Because both erbB2 and erbB3 mutant mice die at embryonic stage E11 of a cardiac trabeculation defect, transgenic mice expressing the rat erbB2 under the control of an a-myosin heavy chain (α-MHC) promoter were previously crossed with the erbB2 null background to achieve a rescued delay of embryonic death (Morris et al., 1999). ErbB2-/-,α-MHC survive until birth, but the loss of erbB2 in Schwann cells results in a complete absence of Schwann cells and, not surprisingly, a severe loss of both sensory and motor neurons at later stages of neuromuscular development. It was observed, however, that motoneurons survived through the initial stages of development, long enough to innervate muscle fibers and begin early stages of synaptogenesis (Lin et al., 2000). From these experiments, it was noted that the early innervating nerve of embryos deficient in erbB2 formed many

presynaptic nerve terminal accumulations along the central band of muscle at E14.5. Recent findings suggest that AChR clusters are able to be formed in a pre-determined nerve-independent manner along the central band of muscle, which led to some increased interest on our part on the earliest stages of NMJ development, when the nerve first reaches the muscle. Some of our recent observations on the timing of neuromuscular development have suggested that postsynaptic AChR clusters are largely unapposed at E14.5, and do not become apposed until E15.5-16.5. The previous experiments by Lin and colleagues, however, lacked a marker for postsynaptic specialization, and it could not be determined whether the observed presynaptic specializations were apposed to AChR clusters on the muscle fiber.

In order to determine whether synaptic apposition is formed at an earlier stage of neuromuscular development in erbB2 deficient mice, we analyzed the development of the phrenic nerve. Embryonic diaphragm was collected from E14.0 stage erbB2-/-,α–MHC embryos and co-immunostained with anti-synaptophysin (syn) antibodies and Texas Red-conjugated α-bungarotoxin (TR-αBTX) to visualize AChR clusters. Previous results described that at E14.5, the phrenic nerve in erbB2 mutant mice was highly defasciculated, in contrast to the tightly bundled wildtype nerve, and was diffusely projected across the diaphragm upon reaching the muscle surface. In both cases, however, nerve terminal staining was restricted along the central band of muscle. At E14.0, our co-immunostaining analysis was consistent with those

observations, and additionally we observed that in erbB2 mutants, many nerve terminals were apposed by postsynaptic AChR clusters (Figure 10b,d). While in wildtype littermate controls (Figure 10a,c), though the phrenic nerve was tightly branched along the central band of AChR clusters, there were far fewer nerve terminal staining apposed to postsynaptic clusters. Also, the abundance of discrete presynaptic specializations was visually much less in our wildtype E14.0 diaphragms than was observable at E14.5. Consistent with previous observations by several groups that relatively low levels of synaptic vesicles are able to be detected along the embryonic axons of mice (Polo-Parada et al., 2001), we observed synaptophysin staining at low levels along the axons of both mutant and wild type mice. In some cases in wildtype, synaptophysin-stained axon bundles overlapped with AChR clusters on the muscle, and while some vesicle release may exist at these sites, we believe that these do not specifically constitute nerve terminals.

Conditional knockout of erbB2 via Schwann cell-specific cre promoter

ErbB2 is also expressed, together with erbB3 and erbB4 in skeletal muscle, and co-localizes with AChR clusters, playing a role in neuregulin-mediated regulation of synaptic transcription. To eliminate the possibility that loss of erbB2 mediated signaling play a role in regulating the timing of presynaptic development, we crossed conditional erbB2 mutant mice with mice expressing the Schwann-cell specific P0-cre so that we might study NMJ

development in embryos lacking erbB2 specifically in Schwann cells. Mice homozygous for the floxed erbB2 allele and carrying the P0-cre transgene, however, were viable at birth. These erbB2floxed/P0-cre mice were similar in size to littermate controls, but exhibited stunted growth early in their development. By two weeks age, severe neuromuscular defecits were observed in the mutants, marked by a lack of limb strength and shaking, particularly in the hind limbs. Though they were able to use their forelimbs to some degree, general mobility of the mutants was impaired, which probably accounted for the early stunted growth due to an inability feed effectively. Given supportive care, however these mutants were able to eventually grow to normal size, and live to normal age.

Analysis of Schwann cells at NMJs of erbB2floxed/P0-cre mice

In order to determine whether Schwann cells are present at NMJs of P0-cre expressing erbB2 conditional mice, diaphragms were dissected from E18.5 erbB2floxed/P0-cre embryos and immunostained for α-bungarotoxin and Schwann cell marker S100. As shown in figure 11, Schwann cell staining was present at NMJs of mutant mice, but not all receptor clusters were apposed by Schwann cells (figure 11 arrows). Immunustaining with antibodies specific for nerve (neurofilament, SMI-31) revealed that the nerve was present and bundled similar to littermate controls (data not shown). These results show that conditional deletion of erbB2 using the P0-cre does not result in loss of

Schwann cells as it does in the complete knockout of erbB2. The nerve is not defasciculated, and does not degenerate at E15.5, but Schwann cells are absent from some NMJs, which may cause some neuromuscular defects, as observed in the adult mice.

Myelination of P0-erbB2 sciatic nerve

In order to further assay the extent to which Schwann cells and myelination exists in the conditional knockout mice, P0-erbB2 adult sciatic nerve along with littermate controls were dissected and sectioned crosswise for histological analysis. Sciatic nerve diameter was reduced dramatically in mutants by half, as seen by histological staining (figure 12a,b). Sections immunohistochemically stained for Schwann cell (S100) and axonal (SMI-31) markers showed that Schwann cell association with axon fibers was reduced in mutant nerve (figure 12c), compared to control nerve (figure 12d). Subsequent EM analysis of cross-sections confirmed that while axons were myelinated in mutant nerve, myelination thickness was greatly reduced (data not shown). These results, in combination with the presence of Schwann cell staining at mutant NMJs, suggest that in erbB2floxed/P0-cre mice, there are some defects in Schwann cell migration and development, which result in the reduction of myelination of axons, and the reduction of Schwann cells at NMJs. However, in contrast to mice in which erbB2 is completely absent, Schwann a total loss of Schwann cells does not occur. This is likely due to the timing of which

erbB2 deletion occurs in P0-cre conditional mice. P0 is thought to begin being expressed as early as E12 (Sommer et al., 1995). Meanwhile, gliogenesis in the mouse PNS begins at E11, and migration through the nerve root thought to occur by E11.5. Since it is thought that loss of Schwann cells at the NMJs of erbB2 deficient mice is due to the decreased ability of Schwann cell precursors within the DRG to migrate into peripheral nerves, it is possible that the P0-driven cre does not express early enough to cause the full cessation of Schwann cell precursor migration. Some level of Schwann cell migration and development may take place, although to a lesser degree than in normal mice, and therefore, the reduced myelination and Schwann cell presence in erbB2floxed/P0-cre mice, as well as the ability of mutant mice to live and display some level of neuromuscular function.

Discussion

These preliminary studies in which Schwann cells are absent from the developing NMJ raise several interesting questions concerning the manner in which Schwann cells may regulate synaptogenesis. It is clear that Schwann cells are essential for the long-term survival of presynaptic neurons at the NMJ. Early synaptic structures are able to initially form in mice lacking Schwann cells, but are not maintained, with their retraction and the eventual loss of spinal cord motoneurons. What is the cause of this loss of presynaptic integrity? One possibility is that Schwann cells are essential to the maturation

and maintenance of presynaptic axons. PSCs ablation from developing NMJs in vivo by complement-mediated cell lysis results in a reduction of synaptic growth, accompanied by an increase in retraction. Another possibility is that neurons in erbB2 mutant NMJs may not receive proper trophic support due to aberrant projections of axons to incorrect targets. The presynaptic axons are highly defasciculated and were aberrantly projected in erbB2 mutant mice.

Alternatively, there is the issue of timing of synaptogenesis, which may also play a role in the regulation of synaptogenesis. In wildtype animals, postsynaptic specialization precedes the onset of presynaptic specialization by a very short window. Though the length of this window was not specifically quantified (AChR clusters were observed at E14.0, some apposition of nerve terminals were observed as early as E14.5), it is clear that one follows the other, if not by necessity. In erbB2 mutant embryos, onset of presynaptic specialization and apposition to AChR clusters occurs earlier, which may reflect either a premature initiation of synaptogenesis by the muscle, or a self-initiation process by the nerve. Although the extent to which muscle may direct or induce the presynaptic nerve to differentiate is not well understood, there is evidence to suggest that such signals may be a part of the general program of synaptogenesis and maintenance of synaptic integrity (Campagna et al., 1997; Fox and Umemori, 2006; Knight et al., 2003; Nishimune et al., 2004). Thus, early apposition in erbB2 mutants may reflect a premature direction of presynaptic differentiation by the postsynaptic target. Consistent with the

notion that this is a process that is directed by muscle, erbB2 mutant nerve projections are profusely distributed across the surface of the diaphragm, but synaptic terminals are only formed along the central band of muscle where AChR clusters exist. If the premature terminals were non-specific, or self-directed, they would tend to form across the entire surface of the diaphragm to the extent that the nerve projections extend. This supports the idea that a signal from the preexisting AChR clusters may recruit the differentiation of the developing nerve to develop along the central band. In wildtype mice, this recruitment is delayed until E14.5, while in erbB2 mutants, the delay is seemingly reduced, or possibly abolished. In both cases, the extending axons of the nerve exist in close proximity to the preexisting AChR clusters, but wildtype axons are able to avoid recruitment until a designated stage.

Recent data from our lab shows that in mice lacking both erbB2 and ChAT, the phrenic nerve does not degenerate at E16.5 as it does in erbB2 mutants despite a similar lack of Schwann cells (data not shown). Since ChAT mutant mice lack the ability to synthesize acetylcholine, it is likely that degeneration of the nerve in erbB2 mutants is the result of activity induced through AChRs on the muscle membrane. If this were the case, it might suggest that the degeneration of the nerve in erbB2 mice stems from early synaptogenesis, which leads to premature activity-induced signaling, and subsequent effects to the immature nerve. In this scenario, we might attribute the survival of the wildtype nerve to its ability to delay the onset of activity-

induced signaling. In support of the idea that muscle may negatively regulate presynaptic development in response to nerve activity is the hypothesis in chapter 2, that ACh from the incoming nerve signals through postsynaptic receptors to initiate a retrograde signal that negatively regulates nerve growth and branching. We are currently crossing erbB mutant mice with the AchRa1 mutant mice to determine whether the nerve degeneration in erbB2 mutant mice is a result of activity-induced signaling through postsynaptic AChR clusters.

How does the nerve delay premature apposition to postsynaptic clusters? Absence of Schwann cells in erbB2 mutant mice may play a role in the existence of early nerve terminal differentiation. Schwann cells may play a protective role, in preventing early presynaptic differentiation, either by providing negative signals, or possibly by shielding the axon from positive signals. Alternatively, Schwann cells may prevent the axons from becoming defasciculated as they are in erbB2 mutants. Tightly bundled axons may be less susceptible to differentiation than singular axons. Conversely, defasciculated axons may be more amenable to turning toward, or being recruited by, an adjacent AChR cluster.

It is possible that erbB2 signaling in other tissues plays a role in regulation of presynaptic support and differentiation. Because erbB2 is expressed in muscle, it is possible that erbB2 may be required for the target tissue to release cues that are involved with either the fasciculation, proper

guidance, or synaptogenesis. Our study of a Schwann cell conditional erbB2 in an effort to eliminate this alternate possibility was inconclusive due to the possibility that Schwann cell migration to the NMJ is not impaired in P0 conditional erbB2 mice to the extent that it is in the complete knockout. P0-driven expression of cre in Schwann cells may not occur early enough during Schwann cell precursor development to halt the migration of Schwann cells at the nerve root. Thus, Schwann cells are present in a lower capacity at NMJs of the conditional mutant mice. Even in light of this unfortunate finding, it is possible that a mouse with partial deficit of Schwann cells could be useful for study of a Schwann cell specific roles in NMJ development. Further characterization of NMJ development in these mice could reveal defecits in timing or patterning of synapse development. Alternatively, the role of Schwann cells, in synaptic maturation and maintenance during development, or alternatively, the role of erbB2 in Schwann cell signaling during synaptogenesis could be explored. Furthermore, defects in synaptic function and maintenance can be studied in the adult mouse, because the mice do not die at birth as full erbB2 knockouts do.

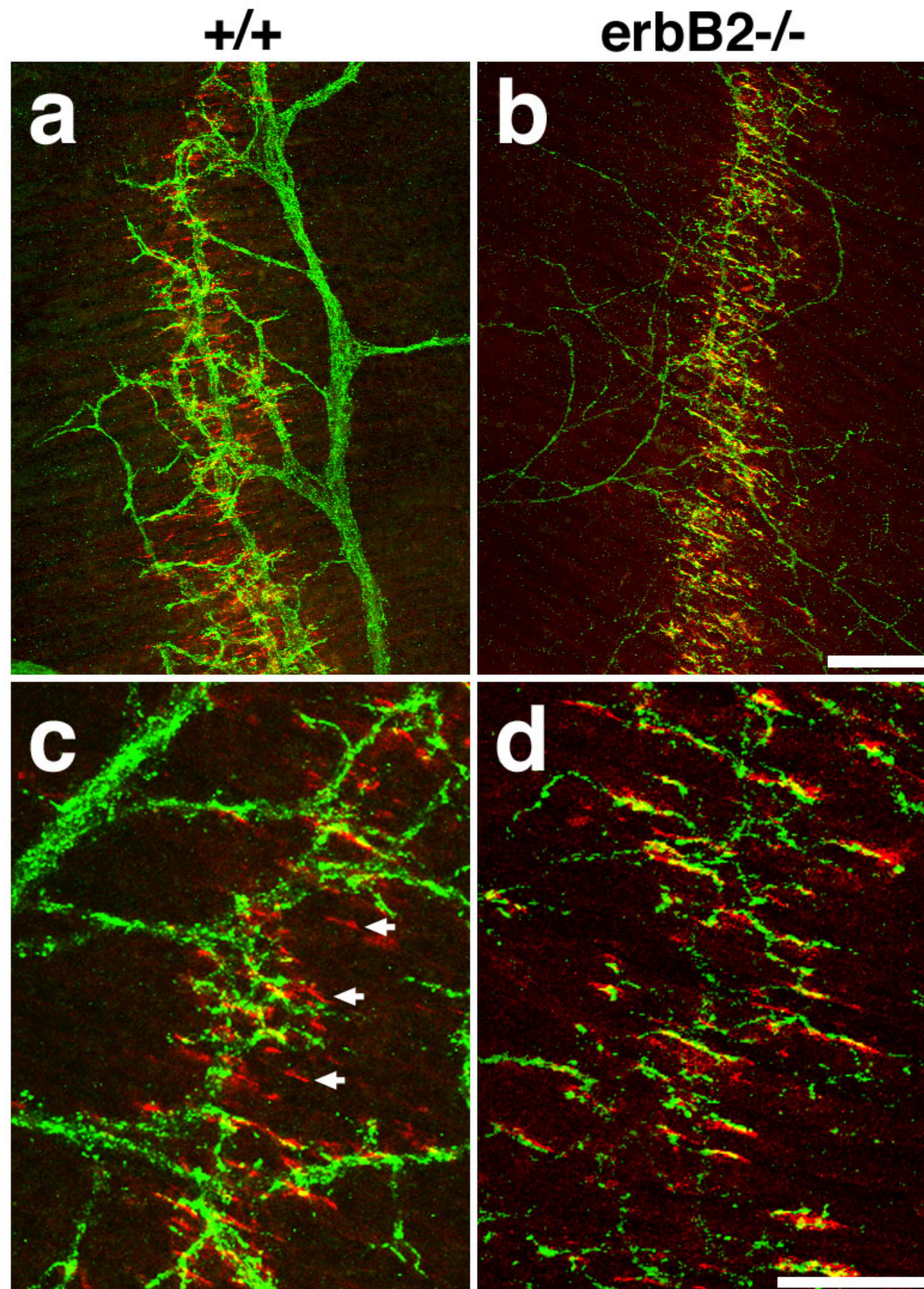

Figure 10: Premature differentiation of nerve terminal differentiation in erbB2 mutant mice. Mutant (erbB2-/-) (b,d) and control (+/+) (a,c) E14.0 wholemount diaphragm muscles were immunostained with anti-synaptophysin antibodies (green), and co-stained with Texas Red-conjugated α-bungarotoxin (red), shown in low (a,b) and high (c,d) magnification. In control animals, many clusters are not apposed by nerve terminals marked by synaptophysin immunoreactivity (c, arrows). In mutant animals, a majority of receptor clusters are apposed by nerve terminals (d). Scale bar: a,b, 100 m; c,d 50 mm.

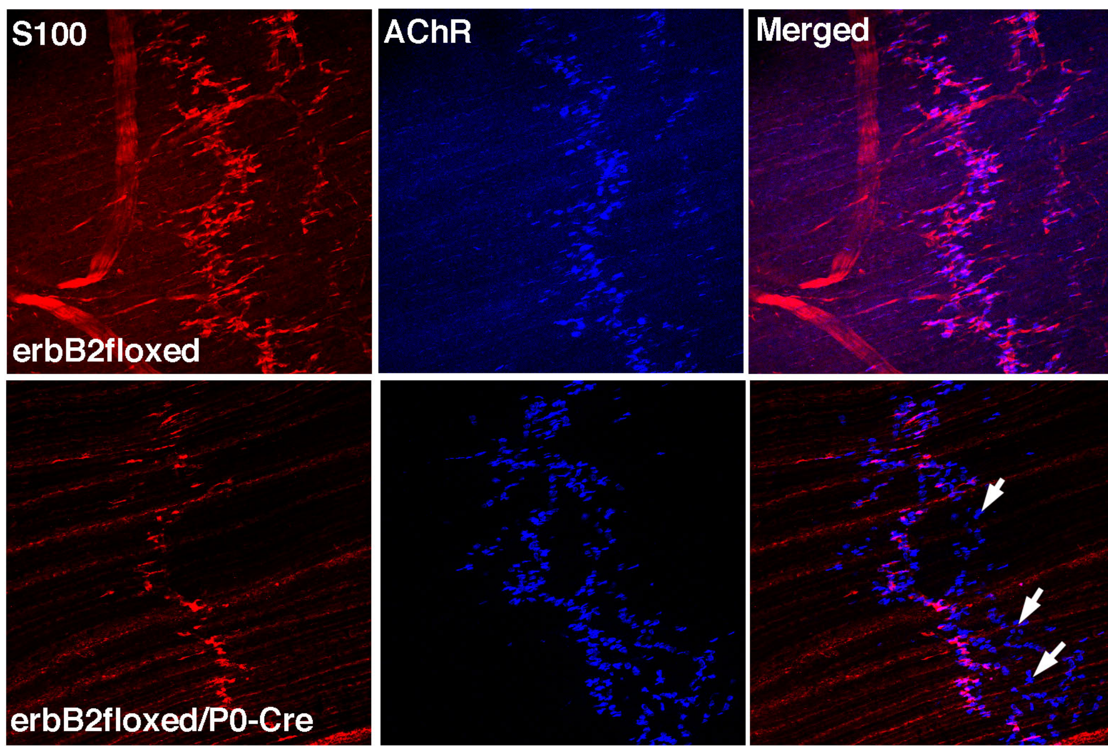

Figure 11: Presence of Schwann cells at NMJ of erbB2floxed/P0-cre mice. Mutant (erbB2floxed/P0-Cre) and control (erbB2floxed) E18.5 wholemount diaphragm were immunostained with anti-S100 antibodies (red), and co-stained with Texas Red-conjugated α-bungarotoxin (blue). Schwann cells as immunolabeled by S100 are present in both mutant and control, but in mutant mice Schwann cells are fewer, and many receptor clusters are not apposed (arrows). In control animals, a majority of receptor clusters are apposed by Schwann cell staining.

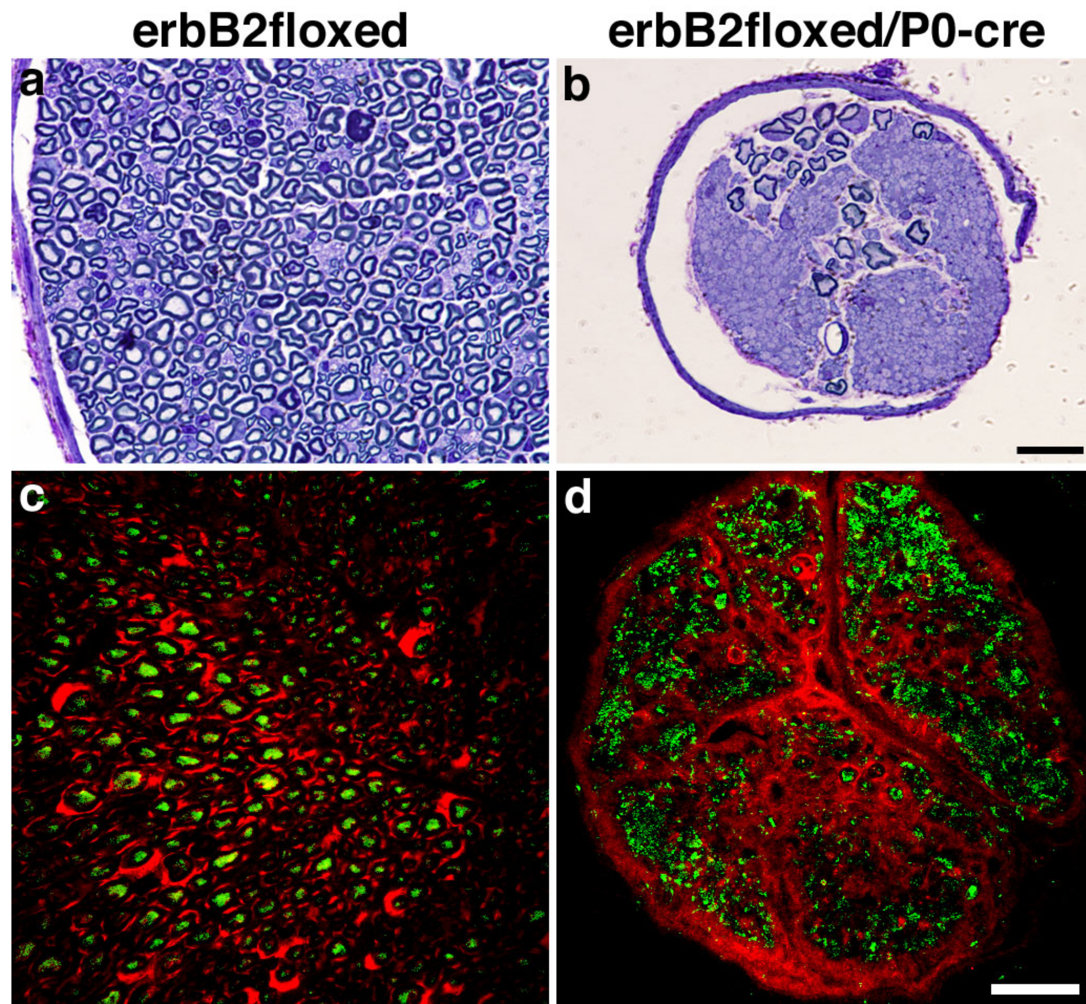

Figure 12: Defects in the sciatic nerve of P0-cre conditional erbB2 mice. Mutant (erbB2floxed/P0-Cre) and control (erbB2floxed) sciatic nerve were dissected and sectioned crosswise, and then subjected to toluidine blue staining (a,b), and immunostained with anti-S100 antibodies (red), and anti-SMI-31 antibodies (green) (c,d). Mutant sciatic nerve is markedly smaller in diameter, and shows a reduced density of Schwann cell staining in association with nerve axon staining as marked by SMI-31 immunoreactivity. Scale bars: a,b 50 mm; c,d 30 mm.

Chapter 4:

GENERAL DISCUSSION

Synaptogenesis is a process that involves the coordinated presentation of positive and negative signals between presynaptic, postsynaptic, and glial partners. It is a highly evolved conversation that directs everything from survival to location, with the goal of maximizing efficiency at both the synaptic and system-wide level. The intricate process defines not only the ability of the developing organism to form its nervous system, but is also essential to the ability of complex neural networks to be dynamic entities, allowing us to possess traits such as learning, memory, and consciousness.

In this dissertation I explore the process of synaptogenesis, through a discussion of experiments that were performed during my graduate studies in an effort to contribute to the growing knowledge of synaptogenesis in our field today. Presented, are genetic evidence for two distinct mechanisms by which ACh regulates presynaptic development. ACh acts through postsynaptic receptors to inhibit nerve branching in a retrograde manner, while ACh also acts to inhibit specialization of nerve terminals via non-postsynaptic AChRs. Our results further suggest that this ACh-mediated inhibition on presynaptic specialization may be antagonized by agrin-dependent retrograde signals. In addition, I suggest that the loss of erbB2 receptors in mice leads to premature presynaptic differentiation during the initial stages of NMJ development. In

general, the approach chosen involved the use of mouse genetics as a tool to study the contribution of various key molecules towards the stepwise assembly of the neuromuscular junction, and while it is a powerful method for studying mammalian synapse development in vivo, there are still limitations to this approach that lead to further discussion regarding both the specific nature and the greater context of these processes.

Increased motor axon branching has been observed in many knockout mice in which clustering of AChRs has been affected (DeChiara et al., 1996; Fu et al., 2005; Gautam et al., 1996; Okada et al., 2006; Weatherbee et al., 2006). The increased branching due to loss or decrease of synaptic function caused by muscle specific deficiencies supports a non-autonomous mechanism for regulation of nerve branching, which is consistent with our conclusions. Similar observations have been made in zebrafish mutants in which muscle-specific defects in receptor clustering led to increased nerve branching (Saint-Amant et al., 2007). Furthermore, increased axonal branching have also been observed when acetylcholine metabolism is disrupted in both c. elegans and drosophila, though a direct or indirect effect of ACh on axons is not determined in these studies (Yang and Kunes, 2004; Zhao and Nonet, 2000). ACh-dependent motor neuron survival has been studied extensively in chick motor neuron development. It has been observed that neuromuscular activity blockers, but not neuronal nicotinic AChR blockers alone increase motor neuron survival (Oppenheim et al., 2000), which is

consistent with a post-synaptic receptor mediated mechanism for regulating nerve growth and survival.

Increased motor neuron survival in both AChRα1 and ChAT mutant mice could be attributed to several possibilities. In both mutant mice, there is increased muscle innervation, which may lead to increased access of motor neurons to neurotrophic factors for survival. This is supported by evidence from other mutant mice that exhibit increased muscle innervation, such as MuSK, which also have increased motor neuron numbers. Conversely, there is the possibility that absence of synaptic transmission in AChRα1 and ChAT mutant mice may lead to an increase in the production of neurotrophic factors, although it has previously been shown that muscle denervation does not result in increased neurotrophic factor levels for chick motor neuron survival (Houenou et al., 1990).

It is unclear through our current genetic analysis, how ACh acts as a negative regulator. Inhibition of presynaptic differentiation may be mediated through Schwann cells, by binding to muscarinic AChRs. Rat and chick Schwann cells have been shown to express muscarinic receptors (Bernardini et al., 1998; Loreti et al., 2006). Alternatively, ACh could bind to neuronal AChRs to directly affect nerve terminal differentiation. It has been suggested that neurotransmitters are capable of acting as an anterograde signal to regulate synapse formation. At central synapses, activation of AMPA or kainite-type glutamate receptors on hippocampal neurons has been shown to

decrease filopodial motility, which may contribute to stability and promote maturation of synaptic contacts (Chang and De Camilli, 2001; Goda and Davis, 2003; Tashiro et al., 2003). In further support of a direct effect of ACh, neural activity has also been shown to affect the expression of a variety of synaptic adhesion molecules which may be involved in presynaptic specialization, including actin, N-cadherin, β-catenin, and NCAM (Schaefer and Nonet, 2001). The self-inhibitory effect of ACh may reflect a nerve-intrinsic mechanism to prevent specialization until the nerve is appropriately apposing a postsynaptic apparatus. An agrin-dependent signal is likely involved in antagonizing this inhibition. This signal could be mediated through the MuSK complex, which may lead to the release of trophic factors such as fibroblast growth factors and collagen IV chains (Fox et al., 2007; Umemori et al., 2004). Alternatively, Wnt-family signaling pathways may play a role. Wnt-3 secreted by spinal cord motor neuron dendrites has been shown to promote terminal arborization of sensory neuron axons in the spinal cord (Krylova et al., 2002). In the cerebellum, Wnt7a may act retrogradely on Frizzled receptors to support growth cone remodeling and accumulation of synaptic vesicle proteins (Hall et al., 2000).

Finally, while studies in ErbB receptor mice have led us to determine that loss of ErbB2 receptors leads to loss of Schwann cells and premature presynaptic differentiation, recent data from ChAT/ErbB2 mice show that loss of Schwann cells does not by itself necessitate nerve regeneration, suggesting

that it is the combination of the loss of Schwann cells, and the presence of ACh signaling, that necessitate degeneration of the nerve. Based on the involvement of ACh in this neuronal degradation, and the hypothesis from chapter 2 that suggests that ACh acts through postsynaptic receptors to negatively regulate the growth and branching of the nerve, this could lead us to speculate that perhaps the premature formation of synaptic connections contributes to degeneration of the nerve via ACh mediated inhibition. Loss of Schwann cells may lead to premature presynaptic differentiation, which in turn leads to neuronal retraction and degeneration. There is increasing support for a role of glial cells as an important mediator of synapse formation, however, while there is much evidence of glial cells acting to support synaptogenesis, there is little to no data to suggest a glial repression or delay of synaptogenesis. Nonetheless, it has been speculated that Schwann cells could act to switch motor neurons from a growth state to a synaptogenic state (Peng et al., 2003). In developing NMJs treated with neuregulin glial growth factor II (GGF2), which causes the Schwann cells to extend processes and migrate away from the synapse, it was observed that motor axons were unable to make synapses until the GGF2 was removed (Trachtenberg and Thompson, 1997). It is possible, therefore, that Schwann cells in a growth or differentiation state may repress or inhibit the differentiation and synaptogenesis of the accompanying nerve.

In conclusion, we have demonstrated that ACh regulates the development of nerve in two distinct ways. ACh inhibits presynaptic branching and nerve number through postsynaptic receptors, while ACh acts through non-muscle receptors to inhibit synaptic vesicle clustering. In addition, we have brought forward preliminary evidence suggesting a possible role for Schwann cells in the timing of synapse formation. These results have further demonstrated the complexity of the mechanisms involved in the regulation and direction of synapse formation at the neuromuscular junction.

Chapter 5:

MATERIALS AND METHODS

Mice

AChR α1 mutant mice were generated by standard procedures. AChRα7 (Orr-Urtreger et al., 1997) and agrin (AGD) mutant mice (Lin et al., 2001) were described previously. The use of animals is in compliance with the guidelines of the Animal Care and Use Committee of the Salk Institute.

Electrophysiology

Intracellular sharp-electrode recording was performed blind to genotype on phrenic nerve/diaphragm preparations from E17.5 embryos. Tissue was dissected in oxygenated normal mouse Ringer's (NMR) solution: 135 mM NaCl, 5 KCl, 15 mM $NaHCO_3$, 1 mM Na_2HPO_4, 1 mM $MgSO_4$, 2.5 mM Ca gluconate, and 11 mM glucose, pH7.4, pinned to Sylgard-coated dishes, and continuously perfused with oxygenated NMR. Glass microelectrodes filled with 3 M KCl were used to record spontaneous miniature end plate potentials (mepps) at 22–24℃ for 5 min. End plate potentials (epps) were evoked by suprathreshold stimulation of the phrenic nerve via suction electrode and were recorded in NMR containing 10 mM Ca gluconate and 5–12 mM dTC (to prevent muscle contractions). Data were collected and analyzed using pClamp (Axon Instruments) and Minianalysis (Synaptosoft).

Motor neuron count

Spinal cords were dissected free from the spinal column and fresh-frozen in OCT compound (Tissue-Tek). The lumbar spinal cord begins near T11 and ends at L4 at this age, based on the position of emerging lumbar ventral roots and the presence of laterally situated MNs in cross sections. Sixteen μm-thick cross sections of the lumbar spinal cord were processed for *in situ* hybridization with a riboprobe made against the mouse vesicular acetylcholine transporter (VAChT). Every fifth section was cut for counting. Sections were stained with 1μg/ml Hoechst 33342 to identify mRNA-positive cells whose nuclei were completely within the plane of section and which contained at least two nucleoli, and counts were made blindly and multiplied by 5 (Gould et al., 2006).

Immunohistochemistry

Diaphragm muscles were fixed in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.3) overnight at 4°C, rinsed briefly with phosphate-buffered saline (PBS, pH 7.3), incubated in 0.1 M glycine in PBS for 1 h, rinsed briefly with PBS and then washed with 0.5% Triton X-100 in PBS. The muscles were blocked in dilution buffer (500 mM NaCl, 0.01 M phosphate buffer, 3% BSA, 5% goat serum and 0.01% thimerosal) overnight at 4°C, and then incubated with primary rabbit antibodies against neurofilament-150 (Chemicon) or synaptophysin (DAKO) in dilution buffer overnight at 4°C. After

being washed three times for 1 h each in 0.5% Triton X-100 in PBS, the muscles were incubated with fluorescein-conjugated goat anti-rabbit IgG (1:600; Cappel) and Texas Red conjugated α-BTX (Molecular Probes) overnight at 4°C. The muscles were then washed three times for 1 hr each with 0.5% Triton X-100 in PBS and once with PBS and flat-mounted in Vectorshield solution (Vector). Muscle sections were double stained with Alexa-544-conjugated fasciculin and antibodies against, synaptophysin, SV2 (Developmental Hybridoma), AChRα, AChRδ, MuSK, or rapsyn in dilution buffer followed by the appropriate secondary antibodies.

Electron microscopy

E17.5 embryos were placed in a solution of 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. The diaphragm muscles were dissected out and fixed in the same solution overnight at 4°C. The tissue was then rinsed with buffer and post-fixed in 2% osmium tetroxide in buffer for 1 hr on ice. The tissue was then dehydrated in a graded series of ethanol, infiltrated, and polymerized in Epon 812 (Polysciences). Ultrathin sections were stained with uranyl acetate and lead citrate, and electron micrographs were recorded using a JEOL 100CXII electron microscope operated at 80 kV.

AChE histochemistry

Muscles were dissected from fixed embryos with 4% PFA, rinsed in TBS several times, and incubated with solution containing ethopropazine (0.2 mM), acetylthiocholine iodine (4 mM), glycine (10 mM), cupric sulfate (2 mM), and sodium acetate (65 mM, pH5.5) for 2-4 hr at 37^0C. AChE reaction was developed by incubating the muscle for 1.5 min in sodium sulfide solution (1.25%, pH6.0). Tissues were then washed extensively with water, cleared in PBS containing 50% glycerol, and mounted for photography.

***In situ* hybridization**

For whole-mount *in situ* hybridization, we fixed intercostals muscles in 2% PFA in 0.1 M phospate buffer at 4℃ overnight. Digoxygenin-labeled AChRα AChRδ riboprobes were transcribed *in vitro*. We carried out hybridization at 70℃ overnight in hybridization buffer (50% formamide, 1.3x SSC, 5 mM EDTA, 50 μg ml^{-1} yeast transfer RNA, 0.2% Tween-20, 0.5% CHAPS and 100 μg ml^{-1} heparin). After hybridization, the samples were washed three times for 1 h with TBS containing 1% Tween-20, blocked with 5% goat serum in dilution buffer, and incubated with alkaline phosphatase conjugated anti-digoxygenin (1:1,000; Boeringer Manneheim) overnight at 4℃. Detection was carried out in 100 mM Tris (pH 9.5) containing nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP). For *in situ* hybridization with ^{33}P-labelled AChRα and AChRδ riboprobes.

Slides were dipped in NTB2 emulsion, exposed for 5 days, photographically processed and counterstained with eosin/haematoxylin.

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