L-TYPE CALCIUM CHANNELS MEDIATE NICOTINIC ACETYLCOLINE RECEPTOR AGGREGATION ON CULTURED MYOTUBES

by

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ABSTRACT

In this dissertation, I have presented new information on several aspects of the signaling pathway responsible for the clustering of AChRs on muscle cells. First, I have shown that activation of L-CaChs is both necessary for agrin induced clustering of AChRs and sufficient to stimulate AChR clustering even in the absence of agrin. Additionally, I have shown that activation of AChRs causes their own clustering by influencing the activity of L-CaChs. I have also shown that neither AChRs nor L-CaChs play a role in MuSK activation or AChR β subunit phosphorylation suggesting that the role of AChR and L-CaCh is downstream of MuSK activation and phosphorylation of the AChR β subunit in the signaling cascade that leads to the aggregation of AChRs. Finally, I have shown that calcium induced clustering and phosphorylation of AChRs require L-CaCh activation. These data suggested that although L-CaCh activation is insufficient to cause AChR β subunit phosphorylation L-CaCh may modulate an intermediate step between MuSK activation and AChR phosphorylation. These data therefore support the hypothesis that L-CaCh activation delivers extracellular calcium to the intracellular machinery that regulates AChR clustering. Furthermore, these data establish the position of L-CaChs in the signaling hierarchy responsible for AChR clustering as being downstream of or parallel to both MuSK activation and AChR phosphorylation in the signaling cascade behind AChR clustering.
The data presented in this paper begin to provide an integrated view of NMJ formation in which neuromuscular transmission, calcium signaling, and signaling cascades mediated by neurotrophic factors act in concert to regulate the localization of synaptic molecules to junctional regions of the muscle fiber. Many questions remain, however, regarding the events downstream of MuSK and L-CaCh activation.
CHAPTER 1: INTRODUCTION

1.1 Nervous System Plasticity: A Brief Synopsis

The nervous system provides the control center for an animal's entire body from involuntary functions such as breathing, heart beat, and hormone release to voluntary activities such as coordinated muscle movement, learning, and memory. For these processes to occur properly, the nervous system must remain plastic not only during development of the organism but into adulthood as well.

Plasticity within the nervous system involves processes that occur within multiple physiological tiers including systems, cellular, and molecular levels. For example, one form of synaptic plasticity is synaptogenesis. Synaptogenesis is the formation of new synapses and occurs via a complex set of processes that can be regulated by a gradual strengthening of synapses in response to activity in the presynaptic neuron, release of neural factors from the presynaptic neuron, or chemical mediators displayed on the extracellular membranes of other cells (Nicoll, 1992; Bernard and Weal, 1995).

At the molecular level, one extremely important part of synaptogenesis is the localization of ligand-gated ion channel receptors to synaptic areas of the postsynaptic membrane (Sanes and Lichtman, 1999). This localization of receptors to synaptic areas is essential for fast, efficient signal transduction between cells (Hoch, 1999). Localization of receptors is regulated by synaptic activity and receptor mediated signaling cascades that act to appropriately
localize postsynaptic proteins and to regulate the configuration of the cytoskeleton for proper maintenance of protein aggregates (Nicoll, 1992; Hoch, 1999; Sanes and Lichtman, 1999; Burden, 2002). Although an essential component of synapse formation, many of the molecular mediators in the signaling pathways leading to localization of synaptic components remain unknown. Thus, for my dissertation work, I have chosen to study some of the molecular events leading to the localization of postsynaptic molecules using the Neuromuscular Junction (NMJ) as a model system.

1.2 Development of the Neuromuscular Junction: A General Overview

The experimental accessibility of the NMJ and repeating sarcomeric structure of the multinucleate myofibrils have rendered the NMJ an ideal model system with which to study synaptogenesis (Hoch, 1999; Sanes and Lichtman, 1999). Indeed, one of the earliest demonstrations of synaptic transmission was shown using the vertebrate NMJ (Dale, 1936).

Further studies of the NMJ over the next several decades gave birth to the modern understanding of synaptic function including concepts such as chemical synaptic transmission in the form of neurotransmitters, quantal neurotransmitter release due to storage in packets or vesicles, neurotransmitter receptors as transmitters of signals from the outside to the inside of the postsynaptic cell, and the requirement for receptor aggregation for efficient synaptic transmission (Katz, 1966; Kidokoro and Yeh, 1982; Duclert and Changeux, 1995). These concepts
have now been generalized to virtually all other synapses in both the CNS and the Peripheral Nervous System. Over the years, the NMJ has remained an ideal model system with which to study synaptogenesis and as such is the focus of my dissertation work.

1.2.a Early Development of the Neuromuscular Junction:

During development of the skeletomuscular system, motor neurons arise from multipotent progenitor cells in the ventral horn of the spinal cord and send axonal projections out from the spinal cord via the ventral roots to make contact with muscles in the periphery (Leber et al., 1990; Mirsky and Jessen, 1996). The muscle cells themselves arise from the mesoderm and migrate to the sites of muscle formation as myoblasts (single cells). Once they have reached the sites of muscle formation, the myoblasts differentiate into multinucleate cells called myofibrils (Brand-Saberi et al., 1996).

1.2.b Initial Contact:

Initial contact between the motor neuron terminal and muscle occurs while the myoblasts are still fusing, resulting much later in the NMJ being localized to the centers of the muscle. Spontaneous, quantal release of the neurotransmitter acetylcholine (ACh) from the nerve terminal results in stimulation of the nicotinic acetylcholine receptor (AChR) expressed in the muscle membrane (Kidokoro and Yeh, 1982; Chow and Poo, 1985; Xie and Poo, 1986; Evers et al., 1989). AChR is a pentameric ligand gated cation channel composed of two α, one β, one δ, and one ε or γ subunit (Figure 1.1) (Witzemann et al., 1987). Each subunit
AChR is a pantamic ligand-gated ion channel. It is composed of two α subunits, one β, one δ, and one γ or one ε subunit. Each subunit contains 4 transmembrane domains with the N and C termini both extracellular. When folded properly, the five subunits form a cationic channel. The channel pore is lined by the M2 transmembrane segments of each subunit. The intracellular side of the pore is lined with negative charges which act to select for positive charges. The receptor’s ligand Acetylcholine (ACh), binds the alpha subunits. Two molecules of ACh are required for the channel pore to open. Once open, the AChR channel’s primary conductances are the influx of sodium and the efflux of potassium ions although some influx of calcium has been observed.
contains 4 transmembrane domains. The quaternary structure of the protein forms an ion channel pore lined with negatively charged residues (Sperelakis, 2001). These negatively charged residues render the AChR channel cation selective and the primary conductances through the channel pore are sodium influx and potassium efflux (Conti-Tronconi and Raftery, 1982; Ragozzino et al., 1998). Thus, activation of AChR by its ligand ACh results in depolarization of the muscle fiber membrane.

Myoblasts express very low levels of AChRs. However, upon fusion, AChR expression increases dramatically to a uniform distribution of approximately 1,000 molecules/μm. Prior to innervation, spontaneous aggregates of these AChRs begin to form. Upon innervation, the motor neuron induces localization of these aggregates to junctional areas. Thus, in the mature muscle, junctional areas possess a high density of AChRs (10,000 molecules/μm) whereas extrajunctional areas possess a very low density of receptors (10 molecules/μm) (Salpeter and Loring, 1985; Salpeter et al., 1988). This ability of motor neurons to regulate the behavior of AChRs in the muscle membrane results from the release of neural factors from the nerve terminal which control both AChR expression patterns and aggregation.

1.2. c Junctional Maturation:

1.2.c.i Presynaptic Differentiation:

Shortly after initial contact between motor neuron and muscle fiber, the number of synaptic vesicles in the presynaptic terminal increases and the release
of neurotransmitter becomes more frequent (Dennis, 1981; Buchanan et al., 1989). Additionally, the vesicles begin to become clustered in particular areas of the nerve terminal called active zones (Kelly and Zacks, 1969; Kidokoro and Yeh, 1982; Lupa and Hall, 1989). These changes occur in parallel with increases in the area and volume of the synaptic terminal (Dennis, 1981).

Additionally, early in development, each muscle fiber is innervated by multiple motor neurons whose terminals share the same postsynaptic plaque or motor endplate (Balice-Gordon and Lichtman, 1993). As development proceeds, synapses are eliminated such that each muscle fiber is innervated by a single motor neuron (Nystrom, 1968; Balice-Gordon and Lichtman, 1993).

1.2.c.ii Synapse Elimination:

Synapse elimination occurs through competition between nerve terminals innervating the same postsynaptic plaque (Slater, 1982; Colman et al., 1997). The molecular basis for this competition is unclear, however one of several hypotheses suggests that the muscle fiber releases a "maintenance factor" in limited supply in an activity dependent manner. The maintenance factor is then taken up by a nerve terminal in an activity dependent manner and promotes the survival and terminal sprouting of that neuron. Thus, the most active nerve terminals "starve" the other terminals of the "maintenance factor," resulting in their elimination (Snider and Lichtman, 1996).

Synapse elimination is a long process which begins even as postsynaptic changes are still occurring and continues past birth in most vertebrate species.
(Redfern, 1970). In most adult mammals, synapse elimination results in a system in which each muscle fiber is innervated by a single motor neuron. In contrast each motor neuron innervates multiple muscle fibers (Anderson et al., 1977). Thus, a single motor neuron and the muscle fibers it innervates constitutes a motor unit (Redfern, 1970; Rosenthal and Taraskevich, 1977).

1.2.c.iii Postsynaptic Differentiation:

As the motor neuron terminal becomes larger and the process of synapse elimination is occurring, the distribution of AChRs in the postsynaptic plaque changes from a uniform distribution to a perforated "pretzel" shape due to gradual elimination of synaptic terminals (Steinbach, 1981; Balice-Gordon and Lichtman, 1993). Within weeks, postsynaptic folds begin to form in the postsynaptic membrane directly opposite the active zones of the presynaptic terminal (Antony et al., 1995; Jasmin et al., 1995). In the mature NMJ, voltage gated potassium channels, AChRs and other synaptic molecules reside at the crests of these synaptic folds, with voltage gated sodium and calcium channels residing deeper in the folds (Flucher and Daniels, 1989; Robitaille et al., 1993; Sugiura et al., 1995).

Shortly after birth, an AChR class switch occurs in which γ subunit expression switches to ε subunit expression (Mishina et al., 1986; Witzemann et al., 1987; Gu and Hall, 1988; Martinou and Merlie, 1991). The functional consequences of this switch include a change in AChR conductance properties from a long open time and a small current to a short open time and large current
(Fischbach and Schuetze, 1980; Siegelbaum et al., 1984; Mishina et al., 1986). Additionally, the half-life of AChR increases dramatically contributing to the overall stability of the mature NMJ (Michler and Sakmann, 1980; Salpeter and Loring, 1985).

Additionally, the basal lamina grows to ensheath the muscle fiber and extends into the junctional folds and the pre and postsynaptic membrane are “zippered” together (Wood and Slater, 1997). The end result is a mature junction capable of fast, efficient signaling whose adhesive properties render it stable for the lifetime of the organism (Wood and Slater, 1997).

1.3 Molecular Modulators of NMJ Formation

1.3.a Synapse Specific Expression:

1.3.a.i Induction of Junctional Expression:

Although first shown to increase expression of AChRs at Neuromuscular synapses, release of the Acetylcholine Receptor Inducing Activity (ARIA or neuregulin-1 (Nrg-1) from motor neuron terminals also controls the expression of many other post synaptic proteins, such as Muscle Specific Kinase (MuSK), utrophin, rapsyn, N-CAM, sodium channels, and acetylcholine esterase (Corfas et al., 1993; Carraway and Burden, 1995; Chu et al., 1995; Fromm and Burden, 1998). Nrg-1 is a member of a family of molecules called neuregulins, which are ligands of a family of receptor tyrosine kinases, the ErbB receptors (Altik et al., 1995; Carraway and Burden, 1995).
Nrg-1 is concentrated at NMJs, expressed in motor neurons, and released from motor neuron terminals (Jo et al., 1995; Sandrock et al., 1995). Additionally, some of the Nrg-1 localized at NMJs is synthesized by myofibrils and a such could potentially act as an autocrine mediator (Moscoso et al., 1995; Rimer et al., 1998; Burden, 2002). Modulation of Nrg-1 activity is mediated at least in part by the myofiber itself via the Agrin-MuSK signaling pathway (below). Neural agrin or a constitutively active receptor tyrosine kinase, MuSK, can induce the clustering of Erb receptors and expression of muscle derived Nrg-1 and result in an increase in the expression of AChRs (Meier et al., 1998a; Loeb et al., 2002). These data suggest that the expression of muscle NRG-1 and the Erb receptor are mediated by the agrin-MuSK pathway (Burden, 2002). Thus, since NRG-1 regulates MuSK expression, the agrin-MuSK and NRG-1-Erb Receptor pathways appear to be positively reinforcing and act together to regulate synapse specific expression of synaptic components and localization of those components to the junctional areas of the muscle membrane.

1.3.a.ii Inhibition of Postjunctonal Expression:

Inhibition of expression of AChRs in extrajunctional regions is achieved in a very different manner. Denervation of a muscle fiber or chronic blockade of the AChR in a muscle fiber results in a phenomenon called denervation supersensitivity (Lomo and Westgaard, 1975; Fambrough, 1979). Denervation supersensitivity refers to a condition in which there is an increased sensitivity to ACh following removal of presynaptic signal. It results from an increase in the
expression of AChRs. This phenomenon suggests that normal synaptic activity in skeletal muscle acts to inhibit the expression of AChRs in extrajunctional areas (Klarsfeld and Changeux, 1985; Goldman et al., 1988). The release of NRG-1 from the motor neuron terminal acts to "override" this inhibition and therefore to allow localized (junctional) expression of AChRs, as stated above.

1.3.b Regulation of the Aggregation of AChRs:

1.3.b.i Agrin:
Release of neural agrin from the motor neuron terminal regulates the localization of AChRs expressed in the muscle fiber membrane (Magill-Solc and McMahan, 1988; Fallon and Gelfman, 1989; Magill-Soic and McMahan, 1990b; Magill-Solc and McMahan, 1990a; Reist et al., 1992). Agrin is a 200 kd protein that is a heparin sulfate proteoglycan and is a natural component of the basal lamina that ensheaths the muscle fiber (Figure 1.2). Agrin contains a laminin binding domain, 9 folistatin repeats, a laminin type III domain, 3 laminin-G domains, and 4 EGF-like signaling domains (Rupp et al., 1991; Rupp et al., 1992) (Rupp et al., 1991; Burden, 2002)

Agrin’s role in NMJ formation has been dramatically illustrated through the use of agrin knockout mice. Agrin knockout mice produce AChR clusters concentrated at sites of nerve-muscle contact early in the development of the neuromuscular system (E14), but fail to form mature neuromuscular junctions during later stages of development (Gautam et al., 1996; Gautam et al., 1999). Additionally, agrin knockout mice have deficiencies in sympathetic synapse
The agrin protein consists of a laminin binding domain (LN binding), 9 folistatin-like repeats, a laminin B1 domain (same as laminin type III), an EGF-like domain, and 3 laminin G domains. Neural isoforms possess a 8, 11, or 19 amino acid insertion at the Z-site (Burden, 2002).
formation (Gingras et al., 2002). The role of agrin was confirmed by experiments that showed that extrajunctional ectopic expression of agrin is sufficient to induce postsynaptic differentiation (Meier et al., 1997).

The agrin gene is expressed in many tissues, and post-transcriptional modification results in the production of multiple isoforms (Fallon and Gelfman, 1989; Magill-Solc and McMahan, 1990a; Ruegg et al., 1992; Biroc et al., 1993). Indeed, both myofibers and neurons express agrin (Magill-Solc and McMahan, 1990a; Lieth and Fallon, 1993). Due to an insertion of 8, 11, or 19 amino acids at the Z-site of neural forms of agrin however, only neural agrin is effective in stimulating NMJ formation (Gesemann et al., 1995).

Treatment of muscle cells in culture with agrin results in the activation of the kinase domain of MuSK and results in AChR aggregation (Nitkin et al., 1987; Smith et al., 1987; Godfrey et al., 1988; Glass et al., 1996). Although the C-terminal laminin-G domains along with the EGF-like domains have been shown to be sufficient for MuSK activation, biochemical studies have not shown a direct interaction between agrin and MuSK (Hoch et al., 1994).

Although there does not appear to be a direct interaction between agrin and MuSK, agrin does bind to some factor in the postsynaptic membrane, and that binding is saturable (Ma et al., 1993). Additionally, following treatment with agrin for at least 2 hr., those "agrin binding sites" co-cluster with AChRs (Nastuk et al., 1991). These and other data have lead researchers to hypothesize the existence of an Agrin Receptor Complex, which includes the theoretical
members, Muscle Associated Specificity Component (MASC) and Rapsyn Associated Transmembrane Linker (RATL), the α subunit of dystroglycan, and MuSK (Ma et al., 1993; Bowe et al., 1994; Hoch, 1999).

1.3.b.ii The α-dystroglycan, Syntrophin, Sarcoglycan Complex:

α-dystroglycan has been shown to bind agrin and is the extracellular portion of a 2-protein complex that also includes β-dystroglycan (Sugiyama et al., 1994; Yamada et al., 1996; Brancaccio et al., 1997; Gesemann et al., 1998). β-dystroglycan is a transmembrane protein and interacts with the actin cytoskeleton as a member of a complex of proteins, including dystrophin or utrophin (synaptic form), syntrophin, and a sarcoglycan complex (Ervasti and Campbell, 1993; Deyst et al., 1995; Chan et al., 1998). β-dystroglycan has also been shown to associate with rapsyn (Apel et al., 1995; Marangi et al., 2002). Dystrophin is thought to be important for the structural integrity of skeletal muscle and is the protein mutated in the human disease, Duchene's muscular dystrophy (Campbell, 1995; Moll et al., 2001).

1.3.b.iii Rapsyn:

Rapsyn co-localizes in a one to one ratio with AChRs from the earliest times in development at which AChR clusters can be observed (Sealock et al., 1984; Peng and Froehner, 1985). Co-expression experiments in quail fibroblasts showed that AChRs were distributed diffusely in the absence of rapsyn, but when co-expressed with rapsyn, both rapsyn and AChRs formed large surface clusters,
suggesting that rapsyn can cause and is required for AChR clustering (Phillips et al., 1991; Brennan et al., 1992). Additionally, when co-transfected with MuSK, rapsyn induces clustering of MuSK and stimulates tyrosine phosphorylation of MuSK, suggesting a role for rapsyn in MuSK signaling (Gillespie et al., 1996).

In support of the idea that rapsyn plays a role in the agrin-MuSK signaling pathway, rapsyn deficient myotubes fail to show a clustering response, an increase in the co-association between AChRs and MuSK, or downstream tyrosine phosphorylation of AChRs in response to agrin (Apel et al., 1997; Gautam et al., 1999). MuSK, however, remains localized at synaptic sites in rapsyn deficient mice, and rapsyn deficient myotubes show normal MuSK activation in response to agrin (Gillespie et al., 1996; Apel et al., 1997). These data suggest that rapsyn acts as a linker between MuSK and its effector molecules.

Additionally, rapsyn is required for the association of AChRs, and the dystroglycan, syntrophin, sarcoglycan complex; this may provide an indirect link between AChRs and the actin cytoskeleton, allowing for specific localization of AChRs to the tops of postjunctional folds during development of the NMJ (Apel et al., 1995; Fuhrer et al., 1999). In support of this idea, AChRs and utrophin are co-localized at the crests of postjunctional folds, and utrophin deficient mice exhibit a decrease in the density of AChRs at NMJs (Pons et al., 1993; Sanes et al., 1998; Adams et al., 2000). One might envision a system in which Rapsyn, could act as a scaffold by which a postsynaptic complex of synaptic proteins can
be formed. Within this complex, MuSK would mediate the clustering of AChRs, and the dystroglycan, syntrophin, sarcoglycan complex would acting to stabilize this complex in the proper region of a muscle fiber (Figure 1.3).

1.3.b.iv Muscle Specific Kinase:

MuSK is a 120 kd protein that functions as a receptor tyrosine kinase; its expression is restricted to skeletal muscle in mammalian systems (Figure 1.4) (DeChiara et al., 1996). The extracellular domains of the MuSK protein include 4 immunoglobulin-like domains, a cysteine-rich domain, and a kringle domain. Additionally, there is one transmembrane domain and a 50 amino acid juxtamembrane domain near the intracellular leaflet of the cellular membrane. The intracellular portions of the MuSK protein contain a kinase domain and a short 8 amino acid carboxy tail (Jennings et al., 1993; Valenzuela et al., 1995; Fu et al., 1999; Burden, 2002).

The ectodomain near the IgG domains of MuSK is required for agrin activation of MuSK, and the MuSK kinase domain, specifically a tyrosine residue near the juxtamembrane domain, is required for agrin activation of the MuSK kinase domain and for induction of AChR clustering (Zhou et al., 1999; Herbst and Burden, 2000; Watty et al., 2000). Activation of MuSK by agrin occurs by dimerization of MuSK monomers and results in rapid auto-phosphorylation of tyrosine residues in the MuSK kinase domains (Hopf and Hoch, 1998).

Similar to agrin knockout mice, MuSK knockout mice fail to produce neuromuscular junctions. More specifically, MuSK knockout mouse muscle
Figure 1.3. **Model of Rapsyn as a linker protein between MuSK signaling and the cytoskeleton.** Agrin activates MuSK and binds alpha dystroglycan (αDG). αDG associates with beta dystroglycan (βDG), a transmembrane protein which in turn associates with the sarcoglycan complex (SG) and with utrophin (Utr). Utrphin associates with syntrophin and the actin cytoskeleton. Intracellularly, rapsyn associates with AChR, beta dystroglycan and potentially with MuSK.
Figure 1.4. **MuSK Structure.** The ectodomain of the MuSK protein consists of 4 IGg domains and a cysteine rich kringle domain. There is one transmembrane domain and an intracellular juxtamembrane region. The intracellular juxtamembrane region is required for agrin activation of the MuSK kinase domain. The intracellular portions consist of a kinase domain (Burden, 2002).
development proceeds normally, and there is expression of molecules such as AChRs, acetylcholine esterase, and Nrg-1 usually concentrated in synaptic areas, but the pattern of localization of these molecules is diffuse rather than concentrated to areas of nerve muscle contact. Dissimilar to the agrin knockout mice, however, MuSK knockout mice do not produce AChR clusters at any point in their development (Gautam et al., 1996). These data indicate that MuSK is required for initiation of AChR clustering whereas agrin is necessary to maintain AChR clusters (DeChiara et al., 1996).

1.3.b.v AChR Tyrosine Phosphorylation:

MuSK co-localizes with AChRs, and AChRs and MuSK can be co-immuno-precipitated in an agrin dependent manner (Fuhrer et al., 1997; Hopf and Hoch, 1998). Following activation of MuSK there is rapid tyrosine phosphorylation of AChR β and δ subunits (Wallace et al., 1991; Qu and Huganir, 1994; Mohamed and Swope, 1999; Mittaud et al., 2001). The kinase domain of MuSK is sufficient for phosphorylation of AChRs but not their clustering indicating a role for the MuSK ectodomain (Glass et al., 1997). The functional consequences of this tyrosine phosphorylation on AChRs are not known.

One possibility is that phosphorylation of AChR subunits is required for or causes clustering of the AChRs. However, mutant AChR β subunits in which tyrosine residues have been mutated to phenylalanine residues followed by expression in myotubes do become incorporated into AChR clusters, albeit less
efficiently than wildtype AChRs (Meyer and Wallace, 1998). These results suggest that AChR β subunit phosphorylation is not required for AChR clustering. This conclusion is questionable, however, since the myotubes tested still expressed endogenous AChR β and δ subunits.

A clue to the role of AChR tyrosine phosphorylation came from biochemical data which showed that mutant (tyrosine mutated to phenylalanine) β subunits were extracted from cell lysates with detergent more readily than were wildtype β subunits (Borges and Ferns, 2001). Additionally, tyrosine phosphorylated AChR δ subunits (Y393) have been shown to be a docking site for the adaptor protein Grb2 (Colledge and Froehner, 1997). Grb2 has been shown in other systems to act as a linker between cellular proteins including cytoskeleton (Chardin et al., 1995). These results suggest that phosphorylation of AChRs could promote association of AChRs with cytoskeletal components.

Equally unclear is the identity of the kinase that phosphorylates AChRs. Tyrosine kinase inhibitors inhibit AChR phosphorylation but not MuSK activation by agrin, suggesting a requirement for the activity of an intervening kinase between MuSK and AChRs (Ferns et al., 1996; Fuhrer et al., 1997). Src kinases were originally thought to be likely candidates for agrin induced AChR phosphorylation.

Src kinases are a family of cytosolic tyrosine kinases of approximately 60 kd which include Src, Fyn, Fyk, and Yes kinases. Src kinase has been shown to associate with and phosphorylate AChR β subunits whereas Fyn binds to
phosphorylated AChR β subunits (Fuhrer and Hall, 1996; Mohamed and Swope, 1999; Mohamed et al., 2001). Additionally, agrin treatment of myotube cultures induces tyrosine phosphorylation of Src (Mohamed et al., 2001).

Evidence contrary to the idea that src kinases phosphorylate AChRs downstream of MuSK comes from mice deficient in Src and Fyn or Src and Yes. Src and Fyn and Src and Yes knockout mice breathe and move poorly and die shortly after birth, but have normal motor neuron path-finding, postsynaptic molecule aggregation, synapse specific expression of molecules and muscle development (Smith et al., 2001). Additionally, agrin induces tyrosine phosphorylation of AChR β subunits and AChR clustering in muscle cell lines deficient in Src and Fyn or Src and Yes. Upon removal of agrin, AChR clusters do dissolve more quickly in these mutant cell lines than in normal myotube cultures (Smith et al., 2001). Taken together, these data suggest that Src family kinases are dispensable during the early stages of NMJ formation, but may come into play later in the maturation process.

1.3.b.vi Nitric Oxide Synthase:

One other possible pathway toward the phosphorylation of AChRs is via Nitric Oxide Synthase (NOS). NOS is an enzyme which acts to produce nitric oxide by converting L-arginine to citriline and nitric oxide (NO). NO is a signaling mediator in systems throughout the body (Bredt, 1999; Blottner and Luck, 2001). Treatment of muscle cell cultures with NOS inhibitors inhibits agrin induced AChR clustering and also prevents agrin induced phosphorylation of the AChR β
subunit, whereas treatment with NO donors stimulates both AChR clustering and phosphorylation of the AChR β subunit in the absence of agrin (Jones and Werle, 2000).

The most abundant form of NOS in skeletal muscle is the calcium dependent form, called neuronal NOS (nNOS or NOS-1) (Kone, 2000). NOS-1 has been shown to co-cluster with synaptic molecules such as AChRs, MuSK, Rapsyn, and the dystroglycan, syntrophin/utrophin, sarcoglycan complex in skeletal muscle cells in culture (Luck et al., 2000; Ebert et al., 2003). These results strongly indicate a role for NOS downstream of MuSK and upstream of AChR β subunit phosphorylation in the Agrin-MuSK signaling cascade.

1.3.b.vii Rho Family GTPases:

In addition to AChRs and NOS, Rho GTPases are also downstream targets of MuSK signaling. Rho GTPases are a family of monomeric GTPases which include Rac, CDC42, and Rho. Rho GTPases are involved in a myriad of signaling pathways in cells and have been implicated in multiple cellular processes such as growth, motility, and membrane trafficking, and are often activated by receptor tyrosine kinases such as growth factor receptors.

In their inactive state, Rho GTPases are bound to GDP. An upstream signal stimulates the exchange of GDP for GTP and results in the activation of the enzyme. GTP bound Rho GTPase can then interact with a host of intracellular proteins and influence their activity. After a time, the GTPase's
intrinsic enzymatic activity hydrolyzes the GTP to GDP, inactivating the enzyme (Sahai and Marshall, 2002).

Agrin treatment of muscle cells in culture results in the activation of the small GTPases Rac, Rho, and CDC42. Furthermore, dominant negative Rac or CDC42 both abolish agrin induced AChR clustering. Alternatively, expression of constitutively active Rac and Cdc42 result in an increase in the frequency of AChR clustering in the absence of agrin (Weston et al., 2000). More specifically, (Weston et al., 2003) showed that agrin induced AChR clustering is mediated by the sequential and coordinated activities of Rac and Rho. Activation of Rac was transient, but resulted in the activation of Rho. Furthermore, the activity of both enzymes was required for the growth of agrin induced microclusters into mature AChR clusters.

One useful area of future research would be to determine whether or not there is a requirement for Rho family GTPase activity on AChR β and δ subunit tyrosine phosphorylation. Additionally, given the data suggesting both NOS and small GTPases are downstream of MuSK in the Agrin-MuSK signaling pathway, and furthermore, that NOS is involved in the signaling cascade which results in AChR β subunit tyrosine phosphorylation it seems likely that NOS and the small GTPases, Rho, Rac, and Cdc42 are part of the same signaling pathway. Indeed, iNOS activity (inducible NOS) has been shown to be regulated by Rho family GTPases (Schonhoff et al., 2001).
1.3. Calcium Signaling and NMJ Formation

Aside from neural factors such as agrin and ARIA released from the motor neuron terminal and activity in the postsynaptic muscle fiber, calcium ions have also been shown to influence the signaling behind synaptogenesis at the neuromuscular junction.

1.3.c.1 Extracellular Calcium:

Extracellular calcium is required for agrin binding to the agrin receptor complex, for both neuron- and agrin-induced AChR clustering, and for the stability of agrin induced clusters (Bloch, 1983; Henderson et al., 1984; Peng, 1984; Wallace, 1988; Nastuk et al., 1991). Furthermore, removal of extracellular calcium prevents agrin-induced MuSK activation (Borges et al., 2002). Increasing the extracellular calcium concentration in the medium of cultured myotubes also results in an increased frequency of AChR clustering, activation of MuSK, and downstream tyrosine phosphorylation of AChR β subunit (Zhu and Peng, 1988; Grow et al., 1999). These data taken together firmly establish a role for extracellular calcium in AChR clustering.

1.3.c.2 Intracellular Calcium:

In addition to extracellular calcium, intracellular calcium has also been shown to play a role in the agrin-MuSK signaling cascade. Chelation of intracellular calcium with BAPTA blocks agrin induced AChR clustering and causes the dispersal of spontaneous AChR clusters in cultured myotubes (Megeath and Fallon, 1998). Additionally, intracellular calcium is required for phosphorylation of
the AChR β subunit but not activation of MuSK (Borges et al., 2002). These data suggest that intracellular calcium mediates both initiation and maintenance of protein aggregates at the NMJ.

1.3.c.iii Calcium Flux

Shortly after birth, the half-life of AChRs in junctional regions increases whereas the half-life of extrajunctional AChRs decreases (Michler and Sakmann, 1980; Salpeter and Loring, 1985). Data has shown that calcium flux through dihydropyridine sensitive voltage gated calcium channels (L-CaCh) but not release from intracellular stores results in an increase in the half-life of junctional AChRs in skeletal muscle (Caroni et al., 1993). Thus, calcium flux induced by muscle activity regulates the stability of AChRs at mature NMJs.

1.3.d Summary of Signaling Behind the Clustering of AChRs in Skeletal Muscle.

The regulation of AChR clustering requires in two major steps: AChRs aggregation and anchoring of AChRs to the cytoskeleton. The mechanism behind this phenomenon is complex but involves the agrin-MuSK signaling cascade and calcium. Both extracellular calcium and agrin regulate MuSK activation. MuSK in turn mediates both the tyrosine phosphorylation and aggregation of AChRs. Downstream of MuSK, NOS-1 mediates MuSK tyrosine phosphorylation of AChRs. Additionally, intracellular calcium may regulate NOS-1 activity and thereby act to mediate signaling downstream of MuSK.
Rapsyn clusters AChRs and MuSK aggregation and may act as a link between MuSK signaling, AChRs, and the cytoskeleton. Additionally, Src kinases and Rho GTPases appear to play a role in linking AChRs to cytoskeleton and/or maintenance of AChR clusters. A summary model is shown in Figure 1.5.

1.4 Theoretical Justification

Even though the requirement for both intracellular and extracellular calcium in the signaling cascade behind both AChR clustering and NMJ formation is apparent, the mechanism by which calcium exerts these effects is largely unknown. One question central to understanding the mechanism of calcium's effects is the mechanism by which calcium is delivered to its effector targets. There are several possible routes/mechanisms by which delivery of calcium to its effectors might occur.

1.4.a Extracellular Calcium:

Calcium could act outside the cell to influence the stability of interactions between synaptic molecules and ECM or between agrin and the MuSK receptor complex. Indeed, as stated above, both agrin binding to the MuSK receptor complex and agrin activation of MuSK require the presence of extracellular calcium (Nastuk et al., 1991; Borges et al., 2002). Chelation of intracellular calcium does not, however, inhibit agrin induced MuSK activation (Borges et al., 2002). These data taken together suggest that extracellular calcium may promote binding of agrin to the Agrin Receptor Complex.
Figure 1.5. **Model of Postsynaptic protein complex at the NMJ.** Agrin, released from the nerve terminal, activates MuSK and binds α-DG. Activation of MuSK results in the rapid phosphorylation of and increases the aggregation of AChR. NOS-1, Src, and Rho GTPases are thought to be signaling mediators that act downstream of MuSK. Extracellular calcium is required for agrin binding and for agrin induced activation of MuSK. Intracellular calcium is required for agrin induced AChR clustering.
1.4.b Intracellular Calcium:

The requirement for intracellular calcium in agrin induced AChR clustering suggests that an increase in intracellular calcium acts downstream of MuSK (Megeath and Fallon, 1998). One mechanism for a rise in intracellular calcium could be influx of calcium via an ion channel. There are two possibilities as to the identity of this ion channel: the AChR itself, and voltage gated calcium channels.

1.4.b.i AChR:

Although both neuronal and skeletal muscle forms of AChRs have been shown to conduct calcium ions, in addition to sodium and potassium ions, the neuronal form conducts a significantly greater amount (7 times greater than the skeletal muscle form) (Vernino et al., 1992). Furthermore, the fetal form of the skeletal muscle AChR (containing the γ subunit) conducts 2 fold less calcium than the adult form of the AChR (containing the ε subunit) (Cens et al., 1997). Additionally, the class of AChR expressed in cultured skeletal muscle systems including C2C12 cells, a skeletal muscle cell line and embryonic day 18 primary cultured myotubes from mice have been shown to be the γ subunit (Mishina et al., 1986; Witzemann et al., 1987; Martinou and Merlie, 1991; Mileo et al., 1995; Meier et al., 1998b). Thus as all of the experiments illustrated within this paper were performed on myotubes in culture, C2C12 cells and primary mouse myotube cultures, it can be assumed that the conductance properties of the of
the AChRs in my experimental system are of the embryonic skeletal muscle form.

Measurement of calcium transients in C2C12 myotubes in culture has shown that current through the AChRs accounts for only 12% of the total Acetylcholine induced increase in intracellular calcium concentration whereas 69% of the total increase can be accounted for by voltage gated calcium currents (Grassi et al., 1994). These data taken together suggest that AChR is a potential candidate for delivery of calcium to the clustering machinery behind AChR aggregation however, the small magnitude of calcium conductance through the AChR channel renders it unlikely to be the sole mediator.

1.4.b.ii Voltage Gated Calcium Channels:

Voltage gated calcium channels are a family of ion channels which open in response to membrane depolarization and allow the flow of calcium ions across cell membrane with a high degree of selectivity. Members of the family of voltage gated calcium channels are composed of an α₁ subunit, an α₂ subunit, a δ subunit, a β subunit, and a γ subunit (Figure 1.6) (Perez-Reyes and Schneider, 1995; Jones, 1998; Keef, 2003).

The α₁ subunit is made up of 4 internally homologous domains, each containing 6 transmembrane domains. The tertiary structure of the α₁ subunit forms the pore of the ion channel and contains the voltage sensor of the channel (Tanabe et al., 1988; Chaudhari et al., 1989; McCleskey et al., 1993). The β subunit resides on the intracellular side of the cell membrane and facilitates
Figure 1.6. **Structure of Voltage Gated Calcium Channels.** The α subunit is composed of 4 homologous domains containing 6 transmembrane domains each. The tertiary structure of the α₁ subunit forms the pore of the ion channel. The S4 region of each homologous domain forms the voltage sensor. The β subunit exists near the intracellular membrane leaflet and is thought to regulate channel activation kinetics. The α₂-δ subunit complex has one transmembrane domain and extracellular glycosylated domains. The α₂-δ subunit complex and the β subunit are thought to regulate membrane trafficking of the α₁ subunit. There is also a putative gamma subunit associated with voltage gated calcium channels whose function is not well understood (not shown in this illustration).
activation and modulates membrane trafficking of the pore-forming $\alpha_1$ subunit (Gregg et al., 1996). The $\alpha_2$ subunit has one transmembrane domain and is bound to the $\delta$ subunit peptide via a disulfide bond. Both the $\alpha_2$ subunit and the $\delta$ subunit are heavily glycosylated and their function is to modulate membrane trafficking of the $\alpha_1$ subunit (De Jongh et al., 1990; Jay et al., 1991). The function of the $\gamma$ subunit is largely unknown, but there have been some data to suggest that it regulates the inactivation kinetics of the channel (Letts et al., 1998; Arikkath et al., 2003).

The different family members of voltage gated calcium channels differ in their threshold for activation, inactivation kinetics, conductance, expression patterns, and physiological function. These differences result in the ability of the voltage gated calcium channel family members to be involved in a myriad of physiological processes such as neurotransmitter release, hormone release, excitation-contraction (E-C) coupling, and action potential generation. A summary of the characteristics of voltage gated calcium channel family members is shown in Table 1.1 (De Waard et al., 1996; Jones, 1998; Database, 2003).

1.4.b.iii Voltage Gated Calcium Channels in Skeletal Muscle:

Both T-type (low voltage activated, fast inactivation) and L-type (high voltage activated and slowly inactivating) voltage gated calcium channels have been shown to be expressed in skeletal muscle (Beam and Knudson, 1988a; Jones, 1998). T-type calcium channels (T-CaCh) are the major channel expressed in embryonic and neonatal skeletal muscle. T-CaCh activation is
<table>
<thead>
<tr>
<th>Type</th>
<th>Sub-unit</th>
<th>Threshold</th>
<th>Inactivation</th>
<th>Conduc tance</th>
<th>Expression/ Function</th>
</tr>
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<tbody>
<tr>
<td>L</td>
<td>α1-c</td>
<td>High Voltage (above 30 mV)</td>
<td>Slow (&gt;500ms) inactivation at &lt;60 mV)</td>
<td>25 pS</td>
<td>Muscle, endocrine, E-C coupling</td>
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<tr>
<td></td>
<td>α1-d</td>
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<td>α1-f</td>
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<td>α1-s</td>
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<tr>
<td>N</td>
<td>α1-b</td>
<td>High Voltage (above -20 mV)</td>
<td>Partial, fast inactivation,</td>
<td>13 pS</td>
<td>Neurons, neurotransmitter release</td>
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<tr>
<td>P/Q</td>
<td>α1-e</td>
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<td>R</td>
<td></td>
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</tr>
<tr>
<td>T</td>
<td>α1-g</td>
<td>Low Voltage (above -70 mV)</td>
<td>Complete, fast inactivation, inactive between -60mV and -10 mV</td>
<td>8pS</td>
<td>Heart, Neuron, pacemaker, muscle</td>
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<td></td>
<td>α1-h</td>
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Table 1.1. Characteristics of members of the voltage gated calcium channel family. Members of the voltage gated calcium channel family possess diversity in channel activation kinetics, inactivation kinetics, conductance, tissue specific expression, and physiological roles. This incredible diversity of channel properties allows these ion channels to play a role in a myriad of physiological functions such as: E-C coupling, hormone release, neurotransmitter release, and action potential generation (Mouse Synapse Database, L-CaCh table, http://www.biochem.unierlangen.de/MouseDB/db/multprot/CaCH.html)
thought to contribute a large fraction of the intracellular calcium required for muscle development and is thought to play a role in processes such as gene expression and enzymatic reactions (Beam and Knudson, 1988b). T-CaCh expression gradually decreases and eventually disappears in the first postnatal weeks.

In contrast, L-type calcium channels (L-CaCh), otherwise known as the dihydropyridine receptor, are the major voltage gated calcium channel expressed in adult skeletal muscle. L-CaCh is expressed in embryonic muscle but its expression increases postnatally (Beam and Knudson, 1988b). L-CaChs have been most thoroughly studied for their role in E-C coupling in both skeletal and cardiac muscle. Briefly, acetylcholine released from the motor neuron terminal activates AChRs at the crests of the synaptic folds resulting in depolarization of the muscle membrane. This depolarization activates voltage gated sodium and potassium channels resulting in the generation of an action potential. The action potential travels down into the T-tubules, activating L-CaChs. A conformational change in the L-CaCh results in the opening of ryanodine receptors in the membrane of the sarcoplasmic reticulum, resulting in the release of calcium from intracellular stores. The calcium then stimulates the contractile machinery to produce muscle contraction (Moffett, 1993).

In addition to their role in E-C coupling, although less thoroughly studied, L-CaChs mediate the clustering of glycine receptors in the spinal cord (Kirsch and Betz, 1998). Glycine is the major inhibitory neurotransmitter in the spinal
cord and binds and activates the glycine receptor (GlyR), a ligand gated anion channel that belongs to the same superfamily of receptors as AChRs (Kneussel and Betz, 2000; Meier et al., 2000). GlyR clustering requires the anchoring protein gephyrin, GlyR function, and calcium influx mediated by L-CaChs (Kirsch et al., 1993; Kirsch and Betz, 1998).

1.4. c Hypothesis:

Given the large amount of calcium current due to T-CaCh in developing tissues, T-CaCh could potentially play a role in AChR clustering however, no data to date has shown that these channels to play a role in the aggregation of any type of neurotransmitter receptor. In contrast, data showing that L-CaChs mediate the clustering of GlyRs in the spinal cord suggests the possibility that L-CaChs play a role in the clustering of AChRs in the skeletal muscle. These data together with the data showing that intracellular calcium is required for agrin induced AChR clustering lead me to hypothesize that L-CaChs deliver extracellular calcium to the intracellular AChR clustering machinery to mediate the clustering of AChRs downstream of both MuSK and AChR activity.

I tested this hypothesis by employing pharmacological, biochemical, immuno-histochemical, and mutant mouse techniques. This dissertation reports the results of these experiments.
CHAPTER 2: MATERIALS AND METHODS

2.1 Pharmacological Agents

Recombinant rat neural agrin was from R & D Systems (Minneapolis, MN); nifedipine, from Calbiochem (San Diego, CA), S(-)BAYK8644, nicotine, β-tubocurarine (curare), and verapamil HCl from Sigma-Aldrich (St. Louis, MO); and calcium chloride, from Fisher Scientific (Los Angeles, CA).

2.2 Cell Culture

All cells were cultured at 37°C with 8% CO₂. Cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA).

2.2.a C2C12 Cell Culture

In order to study molecular mechanisms for the clustering of AChRs in skeletal muscle I used the mouse skeletal muscle cell line, C2C12, originally generated in 1977 (Yaffe and Saxel, 1977). In addition to the normal advantages of cell line use, such as homogeneous cell type and the ability to passage the cells multiple times, in culture these cells exhibit remarkably similar behavior to primary cultured skeletal muscle. For example, C2C12 myoblasts fuse into myotubes, express many synaptic molecules expressed at in vivo NMJs, and twitch after approximately 72 hr. of differentiation (Grow et al., 1999).

Additionally, agrin treatment of differentiated C2C12 cells results in activation of MuSK, phosphorylation of AChR, and an increase in AChR clustering (Ferns et al., 1996; Fuhrer et al., 1997; Grow and Gordon, 2000). Given these properties, the C2C12 cell line provided an excellent culture system with which to begin to examine the potential role of L-CaCh in the clustering of AChRs.
C2C12 myoblasts were cultured according to the protocol outlined by (Grow et al., 1999). C2C12 myoblasts were cultured on 22 mm X 22 mm glass coverslips (VWR, Westchester, PA) or culture treated plastic dishes (BD Biosciences, Bedford, MA, or Sarstedt, Princeton, NJ) at an initial plating density of 2500 to 3000 cells/cm².

The cells were cultured in 20% fetal bovine serum (FBS), 0.5% chicken embryo extract, 100 U/ml penicillin, and 100 μg/ml streptomycin in Dulbecco’s Modified Eagle’s Medium (DMEM, low glucose, with L-glutamine) for 48 hr. Once the cells had reached approximately 70% confluence, the medium was exchanged for 2% horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in DMEM, and the cells were allowed to differentiate for 48 hr. prior to treatment.

2.2.a.i Determination of the Optimal Plating Density for C2C12 Cells

The optimal initial plating density was determined by examining cells plated at initial densities of 3,000 and 3,600 cells/cm². Cells were then allowed to grow for 48 hr., followed by differentiation for 48 hr. The differentiated myotubes were then treated with pharmacological agents, and a clustering assay was performed (see section 2.5 below).

The initial plating density dramatically affected the number of clusters of AChR observed in both untreated and treated groups (Figure 2.1). Cells plated at 3,600 cells/cm² had more clusters under control and agrin treated conditions.
Figure 2.1. The initial plating density of myoblasts affects both the baseline number of AChR clusters and the response to treatment. Cells were plated and allowed to grow for 48 hours, followed by differentiation for 48 hours. Samples were then treated with pharmacological agents for 18 hr., and AChRs were labeled with ALEXA594 conjugated α-bungarotoxin.
than cells plated at 3,000 cells/cm$^2$. The fold increase in the frequency of AChR clustering in response to agrin, however, was greater with the lower initial plating density (4 vs. 2.6 fold greater than control). These data suggested that the assay for AChR clustering was less sensitive to changes in the frequency of AChR clustering under conditions in which cells were densely plated.

This idea was further supported by data showing that the inhibition of agrin induced AChR clustering by nifedipine was much diminished in the higher density cultures (Figure 2.1). Chapter 3 contains a more detailed explanation of the mechanism of nifedipine's blockade of AChR clustering. Based on these data, in all experiments using C2C12, cells were plated at an initial density of 3,000 cells/cm$^2$ or less.

2.2.b Mouse Myotube Primary Culture

Mouse myotube primary cultures were generated according to a published protocol (Beam and Knudson, 1988a). Hindlimbs and forelimbs were dissected from E18 embryonic mice. Skin and bone fragments were removed, and the remaining tissues were minced. The homogenate was then digested in 0.25% trypsin from Invitrogen Corporation (Carlsbad, CA), 1 mM EDTA-4 Na for 10 minutes at 37° C, followed by triturating with 2 ml and then 1 ml pipettes. The samples were then incubated for an additional 20 minutes in 0.25% Trypsin, 1 mM EDTA-4Na at 37° C with mixing every 5 minutes. Following digestion, cells were dissociated from large pieces of tissue using a syringe top cell strainer (VWR, Westchester, PA). To select for satellite cells, the cell preparations were
then pre-plated in uncoated cell-culture treated T25 flasks for 2 hours at 37°C, 8% CO₂. Cells not attached to the dish were then replated on glass coverslips at a density of 60,000 cells/cm². Cells were cultured in 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, in DMEM for 48 to 72 hr. Once the cells had reached approximately 70% confluence, the medium was exchanged for 10% Horse Serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in DMEM, to stimulate myotube formation. Cultures were then allowed to differentiate for 48 hr. prior to treatment.

**2.3 Muscular Dysgenesis Mouse Colony**

**2.3.a Muscular Dysgenesis Mice**

**2.3.a.i Genotype and Phenotype:**

The muscular dysgenesis mutant mouse (MDG mouse) was originally characterized in 1963 and has since been shown to possess a naturally occurring mutation in the gene for an L-CaCh. The mutant gene is transmitted as an autosomal recessive allele (Gluecksohn-Waelsch, 1963; Pai, 1965a; Pai, 1965b). The abnormality in the gene is a single nucleotide deletion in the α1S subunit of L-CaCh (skeletal muscle alpha subunit) which results in a shift in the reading frame for the gene (Knudson et al., 1989; Chaudhari, 1992). As a result, although mRNA for the dysgenic α1S subunit is transcribed, no functional protein is translated, and therefore no functional L-CaCh are produced (Chaudhari, 1992).
Due to the absence of L-CaCh, homozygous dysgenic mice fail to produce effective Excitation-Contraction Coupling (E-C) coupling or skeletal muscle contraction and as a result die due to asphyxiation shortly after birth. Cardiac and smooth muscle function remain intact. Morphologically, dysgenic mice are born with a fixed posture at multiple joints, a small lower jaw, often with a cleft palette, a lack of muscle tendon connections, and overall immature skeletal muscle structure, presumed to reflect the lack of muscle contraction during development (Banker, 1977)(Figure 2.2).

In terms of normal fine muscle structure (Figure 2.3) (Lodish and Darnell, 1995; Gleeson, 1999), in dysgenic muscle, Z disks are disorganized and longer than normal. Additionally, the sarcoplasmic reticulum is swollen, there is an absence of normal junctional triads, there are nuclear inclusions, the myofibrils are disorganized, and there are regions of vacuolization near NMJs (Banker, 1977; Essien et al., 1977; Friedman and Powell, 1981).

The vacuolization near NMJs suggests that at least some muscle contraction does occur during the development of dysgenic embryos. Indeed, dysgenic myotubes in culture are capable of producing action potentials and to a very limited degree muscle contractions. The muscle contractions that are produced are localized to small areas of the muscle near NMJs (Bournaud and Mallart, 1987). These contractions can be induced either by nerve stimulation or
Figure 2.2 Examples of dysgenic and normal mouse embryos (E18). Images shown in A. and B. are examples of homozygous mdg/mdg mice and image C. is an example of a wildtype mouse.
A. **Skeletal Muscle Contractile Apparatus.** Whole muscle is organized into bundles of circular contractile fibers called muscle fibers. Each muscle fiber is further packaged into myofibrils. Myofibrils consist of multinucleate cells descended from fused myoblasts. Each myofibril consists of repeating contractile units called sarcomeres. One sarcomere encompasses the regions between Z-disks. The actin thin filaments attach to the Z-disk at one end and are the I band. The myosin thick filaments overlap with the actin thin filaments forming the A band. The H band is the region between thin filaments. With contraction, interaction between thick and thin filaments acts to shorten the sarcomere by pulling the Z-disks closer together (Gleeson, 1999, [http://www.medicdirectsport.com/exercisetheory/default.asp?step=4&pid=54](http://www.medicdirectsport.com/exercisetheory/default.asp?step=4&pid=54)).

B. The sarcoplasmic reticulum (SR) surrounds the myofibrils and together with invaginations from the surface of the myofiber called t-tubules propagate the action potential throughout the muscle fiber. The SR, t-tubule network connects to intracellular calcium stores via the L-CaCh-ryanodine receptor complex forming junctional triads (Lodish and Darnell, 1995).
treatment with acetylcholine and can be mediated by calcium flux through the AChR ion channel (Powell et al., 1984a; Bournaud and Mailart, 1987; Melliti et al., 1996).

In addition to AChR, two other potential sources of calcium influx are expressed in dysgenic myotubes. A transient calcium conductance has been attributed to T-type voltage gated calcium channels, whereas a slow calcium conductance has been attributed to a novel voltage gated calcium channel expressed in dysgenic muscle that is termed I\textsubscript{dys} (Adams and Beam, 1989; Bournaud et al., 1989; Shimahara and Bournaud, 1991).

I\textsubscript{dys} is expressed at extremely low levels in dysgenic muscle and as such can be difficult to detect. The I\textsubscript{dys} channel is dihydropyridine sensitive but activates at more negative potentials, and activates more rapidly than wildtype α1S containing L-CaCh. The genetic identity of I\textsubscript{dys} is not known. The conductance properties are very similar to those of the cardiac form of L-CaCh, but I\textsubscript{dys} does not display calcium dependent inactivation (Adams and Beam, 1989). Interestingly, the cardiac form of L-CaCh (α1C) expressed in dysgenic myotubes does not display calcium dependent inactivation either. Thus, the composition of the channels responsible for I\textsubscript{dys} could be an α subunit of L-CaCh normally expressed in cardiac muscle (Tanabe et al., 1990). Data showing that proper localization of the α2 subunit in skeletal muscle requires the α1S subunit suggests that differences in the inactivation kinetics between I\textsubscript{dys}/α1C expressed in dysgenic muscle and the cardiac channel expressed in cardiac muscle could
be due to improper expression or localization of accessory subunits in dysgenic muscle (Flucher et al., 1991).

As indicated above, NMJ do form in dysgenic muscle. However, there are several abnormalities in synaptogenesis. There is nerve overgrowth, extensive axon terminal sprouting of motor neurons, and residual poly-innervation of single muscle fibers (Pincon-Raymond and Rieger, 1982; Powell et al., 1984b). Additionally, along with the increase in the overall number of the nerve terminals there is an increase in the number of AChR clusters in the muscle fiber, in the variability in the shape of AChR clusters, and in extrajunctional expression of AChRs (Powell et al., 1984b).

2.3.a.ii Rescue of the Phenotype:

The dysgenic phenotype can be rescued in several different ways. Once dissociated from whole muscle, dysgenic myoblasts display an enhanced fusion ability and can fuse with fibroblasts, exogenous myoblasts, and Schwann cells. As a result, E-C coupling can be restored in dysgenic skeletal muscle cultures by co-culture with myoblasts, fibroblasts, or Schwann cells from wildtype mice (Powell et al., 1984a; Courbin et al., 1989a; Courbin et al., 1989b).

This rescue of the phenotype of cells in culture does not, however, translate to the whole animal. Mice in which chimeric embryos containing both Mdg/Mdg and wildtype cells were generated at the eight cell stage did not survive (Rieger et al., 1984). These data indicate that fusion with wildtype cells did not
occur in such embryos or that it was insufficient to rescue the dysgenic phenotype in the whole animal.

In addition to fusion with other cell types, the phenotype of dysgenic myotubes in culture can be rescued through transfection with the α1 subunit of L-CaCh. Transfection of dysgenic cultures with a gene for the wild-type α1S subunit restores calcium independent, skeletal muscle type E-C coupling, whereas transfection with α1C subunit results in the production of calcium dependent, cardiac type E-C coupling (Perez-Reyes et al., 1989; Tanabe et al., 1990). In addition to E-C coupling, transfection with the α1S subunit restores junctional triad formation and fine structural organization of the myotubes, such as the reappearance of sarcomeres (Seigneurin-Venin et al., 1994).

The data suggesting that NMJ formation is abnormal in dysgenic mice together with my hypothesis that L-CaCh mediates agrin induced AChR clustering in skeletal muscle rendered these mice an ideal model system in which to study of the role of L-CaCh in AChR aggregation. Therefore, I generated a mouse colony of heterozygous dysgenic mice from founders obtained as a generous gift from Dr. Kurt Beam. I then used primary cultured skeletal muscle from embryos of these mice for experiments on AChR clustering.

2.3.3.iii Genotyping:

Mouse tail samples were digested with protease K in lysis buffer, and the DNA was extracted using the DNeasy Tissue Kit from Qiagen (Valencia, CA). The tail DNA samples were then subjected to PCR analysis using the Taq PCR
Master Mix Kit from Qiagen. The PCR primer sequences and cycling protocol were a generous gift provided by Dr. Kurt Beam (unpublished data, protocol sent to me in a personal correspondence). The composition of the PCR reaction mixture, the primer sequences, and the PCR cycling protocol are shown in Tables 2.1, 2.2, and 2.3.

In order to distinguish the single nucleotide deletion mutation in the dysgenic vs. wildtype sequence using PCR, primers were designed such that the 3' most base corresponded to the position of the mutation. If the last base of a primer is not complimentary to the sequence of DNA present, it often will fail to prime (Qiagen, 1999).

Thus, when the proper set of primers are used, the presence or absence of a 100 bp product distinguishes between the wildtype and mutant alleles of the L-CaCh gene (Figure 2.4).

2.4. Immunocytochemistry

Myotubes cultured on glass coverslips were labeled for AChR by incubation with 0.3 μM Alexa594 conjugated α-bungarotoxin from Molecular Probes (Eugene, OR) for 30 minutes at 37° C. The only exception to this protocol were those samples treated with curare (see below). In order to avoid potential interference with α-bungarotoxin binding by curare, curare-treated samples were washed 4 times with cold DMEM containing 20 mmol HEPES from Sigma-Aldrich (St. Louis, MO) for five minutes each at 4° C, followed by incubation with Alexa594-α-bungarotoxin for 1 hr. at 4° C.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Wildtype Reaction</th>
<th>Dysgenic Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR master mix</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2.0 mmol</td>
<td>2.5 mmol</td>
</tr>
<tr>
<td>Primer</td>
<td>INT200, WT36</td>
<td>INT200, MDG35</td>
</tr>
<tr>
<td>Sterile Water</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1 Table of PCR reaction mixture for Wildtype and Dysgenic Reactions.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDG35</td>
<td>CGT GCT GCT GCT CTT A</td>
</tr>
<tr>
<td>WT 36</td>
<td>GTC CTG CTG CTC TTC</td>
</tr>
<tr>
<td>INT200</td>
<td>TCC AGT GTC AAA CCT CG</td>
</tr>
</tbody>
</table>

Table 2.2 Sequences of Primers used in PCR reactions for wildtype and dysgenic L-CaCh genes. Sequences are reported in the 5' to 3' direction.
<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (minutes)</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hot start</td>
<td>94</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>2. Denature</td>
<td>94</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3. Anneal</td>
<td>53.2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>4. Amplify</td>
<td>72</td>
<td>1</td>
<td>40 (steps 2-4)</td>
</tr>
<tr>
<td>5.</td>
<td>72</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>6. cool</td>
<td>4</td>
<td>terminal</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.3 Cycling protocol for PCR reactions.
Figure 2.4 Representative PCR reaction products. A. Results from a wildtype reaction. The presence of a 100 bp product in samples 4-12 indicates the presence of the wildtype allele of L-CaCh. The absence of product in samples 1-3 indicates the absence of the wildtype allele. B. Results from a dysgenic reaction. The presence of a 100 bp product in samples 1-6 and 8-12 indicates the presence or the dysgenic allele of L-CaCh. The absence of product in sample 7 indicates the absence of the dysgenic allele. Thus, the genotypes for the samples are as follows: samples 1-3 are homozygous for the dysgenic allele, samples 4-6 and 8-12 are heterozygous, and sample 7 is homozygous for the wildtype allele.
Following incubation in α-bungarotoxin and washing, samples were fixed in 2% paraformaldehyde (Ted Pella, Redding, CA) at 4°C for 20 minutes, and then washed. Samples to be double labeled for L-CaCh were then solublized in 0.1% saponin with 5% bovine serum albumin (BSA) from Sigma-Aldrich (St. Louis, MO), calcium magnesium free phosphate buffered saline (CMF-PBS) from Invitrogen Corporation (Carlsbad, CA), pH 7.4 for 10 minutes, followed by blocking of non-specific sites by incubation in 5% BSA, PBS pH 7.4 for 1 hr. at room temperature. Samples were then labeled with a primary antibody to the L-CaCh α1S subunit (MA3-920, diluted 1:500 in 5% BSA, PBS, from Chemicon, Timecula, CA) for 1 hr., followed by incubation with Alexa488 conjugated secondary antibody (rat anti-mouse conjugated to ALEXA 488, diluted 1:1000 in 5% BSA, PBS, from Molecular Probes, Eugene, OR) for 45 minutes at room temperature. Finally, samples were fixed in ice-cold methanol from (Merck) Darmstadt, Germany), and then the coverslips were mounted on glass slides using Prolong antifade reagent from Molecular Probes (Eugene, OR).

2.5 Assay for AChR Clustering

Myotubes cultured on coverslips were treated with pharmacological agents for 18 hr. followed by labeling of AChR with Alexa594 α-bungarotoxin as described above. Samples were examined using a Zeiss Axiovert Microscope using the 40X oil immersion objective with a 1.6X optivar. Clusters of labeled AChRs were identified as bright spots of greater than 5μM in diameter (Figure 2.5). The average number of clusters/mm² was calculated by dividing the
Figure 2.5 Identification of AChR clusters. AChRs on C2C12 myotubes were labeled with ALEXA594 conjugated α-bungarotoxin. A cluster is identified as a bright spot 5 μM in diameter or greater. Scale bar = 25 μM.
field size by the number of clusters per field in 25 random fields. For primary cultures, percent of control was calculated by comparison to untreated cultures using each embryo as a within-subjects control.

Data were analyzed using 2 tailed t-test comparison and 2X2 or 2X3 way Analysis of Variance (ANOVA). Images were obtained using a digital camera (Fugifilm S2 Pro, Elmsford, NY, or Photometrix Sensys, Tucson, AZ).

2.6 Assay for Tyrosine Phosphorylation of MuSK and AChR

2.6.a Precipitation and Analysis by SDS-page

Myotubes were treated with pharmacological agents for 1 hr. (calcium, agrin, nicotine, BAYK8644) or 1.5 hr (nifedipine or curare). Dishes were then washed three times with cold 1X CMF-PBS from Invitrogen Corporation (Carlsbad, CA) containing 1 mM Na orthovanadate and 50 mM NaF from Sigma-Aldrich (St. Louis, MO). Samples were then solubilized in 25 mmol Tris-HCl, pH 7.4, 25 mmol glycine, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 mM N-ethylmaliamide, 0.4 mM PMSF, 1 mM tetrathionate, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 20 μg/ml aprotinin, 1 mM Naorthovanadate, 50 mM NaF, 1% Triton-X100 from Sigma-Aldrich (St. Louis, MO) for 20 minutes at 4°C. Nuclei and other cell components were then removed by centrifugation for 4 minutes, at 17,000 g, 4°C. The supernatants were then collected and incubated with MuSK specific rabbit antiserum for 1 hour (for characterization of the antibody, see section 2.6.b), or α-bungarotoxin conjugated aminolink agarose
beads from Pierce (Rockford, IL) for 2 hours, at 4 °C. The antibody-MuSK complex was then precipitated by incubating samples with Immunopure Protein G conjugated sepharose beads from Pierce (Rockford, IL) for 1 hour, 4 °C. Samples were then washed 3 times with solublizing buffer (from above) and eluted from the beads by boiling for 10 minutes. Proteins were resolved via SDS-PAGE on a 6% (MuSK) or 8% (AChR) polyacrylamide gel and transferred to a nitrocellulose membrane from Bio-rad (Hercules, CA). Non-specific binding on the membranes was blocked for 1hr. with 5% BSA, 1X Tris-Buffered Saline, 0.1% Tween 20. The membranes were incubated with anti-phosphotyrosine antibody (PY20, diluted 1:10,000 in 5% BSA, 1X Tris-Buffered Saline, 0.1% Tween 20, from Transduction Laboratories/BD Biosciences, Lexington, KY), followed by incubation with anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (diluted 1:10,000 in 5% BSA, 1X Tris-Buffered Saline, 0.1% Tween 20) from Amersham Pharmacia (Arlington Heights, IL). The chemiluminescence signal was visualized using enhanced chemiluminescence from Amersham Pharmacia (Arlington Heights, IL). The identity of phosphorylated MuSK or AChR was confirmed by stripping each blot with Re-blot from Chemicon, (Temecula, CA) followed by reanalysis via western blotting with rabbit anti-MuSK specific primary antibody (GW002; a generous gift from Dr. Sheridan Swope), or rat anti-AChR β subunit primary antibody (MAB111, Sigma-Aldrich, St. Louis, MO).
2.6.b Generation and Characterization of an Anti-MuSK Antibody:

Due to the limited availability of antibodies specific to MuSK protein, I opted to have an antibody generated by Research Genetics (Huntsville, AL). Research Genetics synthesized a peptide with the sequence, CSIHRILQRMCREAGTGVG, which represented the C-terminal 23 amino acids of the MuSK protein. The peptide was then injected into rabbits and after several weeks, serum was collected and shipped to our laboratory, where I characterized it.

In order to characterize the anti-MuSK antibody, C2C12 cells were treated with agrin, and then cells were homogenized, solubilized, immuno-precipitated using the anti-peptide rabbit serum, and subjected to western blot as described above. The antiserum precipitated a band of a protein of the size of MuSK, 120 kd (Figure 2.6). This band contained phosphotyrosine at low levels in samples from control cells, and at much higher levels in agrin-treated cells (Figure 2.6). These data strongly indicated that the identity of the protein precipitated by the peptide antiserum was MuSK.

In addition to the 120 kd band presumed to be MuSK, a protein slightly larger than 120 kd was also labeled by the anti-phosphotyrosine probe. The amount of phosphotyrosine labeling in this second band was not altered by agrin treatment, and it was hypothesized to be precipitated non-specifically, either by the protein G beads or by the anti-peptide antibody.
Figure 2.6. **Peptide inhibition of labeling by putative anti-MuSK peptide antibody.** Lanes 1-8 samples immunoprecipitate with anti-peptide serum in the presence of 0 to 5 µg peptide. Lane 9: cell lysates precipitated with protein G beads in the absence of anti-peptide serum. Incubation with competing peptide to anti-MuSK antibody inhibited agrin induced tyrosine phosphorylation of a protein of approximately 120 kd in size presumed to be MuSK but not the protein responsible for the slightly larger band. Additionally, a band slightly larger than 120 kd binds to the protein G agarose beads in the absence of cell lysate.
In order to test which of these hypotheses was correct, two experiments were performed: 1) samples were pre-incubated with several concentrations of the original peptide, followed by immuno-precipitation with the anti-peptide serum, 2) protein G beads were incubated with cell lysates in the absence of anti-peptide serum. The signal from the agrin responsive protein was eliminated in a dose dependent manner by pre-incubation with peptide, whereas the slightly larger band was not. Additionally, a signal the same size as the agrin non-responsive band was observed following the incubation of protein G beads with cell lysates in the absence of anti-peptide serum (pre-clear).

These data strongly supported the conclusion that the anti-peptide antibodies present in the rabbit serum were specific for MuSK protein, and were capable of immuno-precipitating MuSK. Additionally, the signal from the protein slightly larger than MuSK was confirmed as a non-specific signal which had precipitated on the protein G beads. The identity of MuSK as the agrin responsive band has subsequently been confirmed by re-probing numerous anti-MuSK phosphotyrosine assay membranes with an independently raised anti-MuSK antibody (GW002) (Mohamed et al., 2001); a generous gift from Dr. Sheridan Swope); several examples are illustrated throughout the following chapters.
CHAPTER 3: L-TYPE CALCIUM CHANNELS MEDIATE AGRIN-MUSK SIGNAL TRANSDUCTION

3.1 Introduction

The focal nature of synapses in the nervous system facilitates high efficiency signaling with minimal crosstalk. On the postsynaptic membrane neurotransmitter receptors are highly concentrated beneath presynaptic terminals. Both activity and neurotrophic factors have been implicated in different systems as causing the focal aggregation of postsynaptic molecules (Nicoll, 1992; Bernard and Wheal, 1995). Much attention has focused on the development of the neuromuscular synapse and the role of agrin in inducing the aggregation of AChR. Agrin induces tyrosine phosphorylation of both Muscle Specific Kinase (MuSK) and AChR (Qu and Huganir, 1994; Glass et al., 1996a; Mittaud et al., 2001). Phosphorylation of MuSK results in the activation of the MuSK kinase domain and in the aggregation of AChR and other postsynaptic molecules in a calcium dependent manner (Nitkin et al., 1987; Nastuk et al., 1991; Hopf and Hoch, 1998a; Hopf and Hoch, 1998b; Borges et al., 2002). Activity has also been implicated in the stabilization of presynaptic contacts on muscle at various stages of development (Lichtman et al., 1985). At glycinergic synapses of the central nervous system, activity and calcium flux through L-type calcium channels have been shown to be required for the aggregation of GlyR at synapses (Kirsch and Betz, 1998).

It was hypothesized that L-type calcium channels might also mediate signaling that underlies the aggregation of AChR and other postsynaptic
molecules on muscle. To address this hypothesis, the C2C12 mouse muscle cell line which clusters AChR spontaneously and which shows a strong response to agrin was used (Yaffe and Saxel, 1977; Ferns et al., 1996). In addition, the muscular dysgenesis mouse which possesses a natural mutation in the α1S subunit of L-CaCh was used (Pai, 1965a; Pai, 1965b; Banker, 1977; Chaudhari, 1992).

Here it was shown that an L-type calcium channel (L-CaCh) acts downstream of MuSK. Blockade of L-CaCh in differentiated C2 muscle cultures prevented agrin-induced AChR clustering without interfering with the induction of MuSK tyrosine phosphorylation. Conversely, stimulation of L-CaCh induced clustering of AChR without inducing MuSK tyrosine phosphorylation. Agrin responsiveness was dramatically reduced in primary muscle cultures from the muscular dysgenesis mouse which lack L-CaCh α1S subunit as well as from normal mouse muscle cultures in which L-CaCh was blocked pharmacologically. The findings suggest that L-CaCh is an important downstream mediator of Agrin-MuSK signal transduction but leave open the possibility of a parallel signaling pathway.

3.2 Results
Figures presented in this chapter illustrate representative assays for responses to treatment conditions. Each experiment was repeated 3 or more times with the same results.
3.2.a L-CaCh activity is necessary and sufficient for Agrin-induced AChR clustering:

To test whether L-CaCh are necessary for the clustering of AChR, C2C12 myotube cultures were treated with agrin in the presence or absence of the L-CaCh blocker nifedipine and a clustering assay was performed. The following comparisons and their p values were determined via two sided t-test analysis: untreated versus 60 pM agrin (p < 0.0001), untreated versus 60 pM agrin + 10 μM nifedipine (p < 0.0001), untreated versus 60 pM agrin + 50 μM nifedipine (p = 0.76), untreated versus 10 μM nifedipine (p = 0.46), untreated versus 50 μM nifedipine (p = 0.10), 60 pM agrin versus 60 pM agrin + 10 μM nifedipine (p = 0.0009), 60 pM agrin versus 60 pM agrin + 50 μM nifedipine (p < 0.0001), 60 pM agrin + 10 μM nifedipine versus 10 μM nifedipine (p < 0.0001), 60 pM agrin + 50 μM nifedipine versus 50 μM nifedipine (p = 0.1). 2-way ANOVA analysis revealed an interaction between agrin and nifedipine treated groups (p < 0.0001) indicating that nifedipine significantly inhibited agrin-induced AChR clustering in a dose-dependent manner (Figure 3.1). Verapamil, another L-CaCh blocker, similarly inhibited agrin-induced clustering (data not shown).
Figure 3.1. **Blockade of L-type calcium channels inhibited agrin-induced AChR clustering.** (A) C2C12 myotubes were treated with 60 pM agrin and/or 10 or 50 μM Nifedipine for 18 hr. AChRs were then labeled with ALEXA594 conjugated α-Bungarotoxin. Images were obtained using a digital camera. Scale Bar = 25 μm. (B) The average number of AChR clusters/mm² was calculated based on 25 fields of view sampled for each experimental condition. * indicates samples significantly different from untreated samples (p < 0.01), ** indicates samples significantly different from agrin treated samples (p < 0.01).
These data suggested that activation of L-CaCh was required for agrin-induced clustering of AChR and that stimulation of L-CaCh might promote clustering.

To test whether L-CaCh activation is sufficient for clustering of AChRs, C2C12 myotube cultures were treated with the L-CaCh activator S(-)BAYK8644 and a clustering assay was performed. The following comparisons and their p values were determined via two sided t-test analysis: untreated versus 60 pM agrin (p < 0.0001), untreated versus 10 μM S(-)BAYK8644 (p < 0.0001), untreated versus 10 μM S(-) BAYK8644 + 50 μM nifedipine (p = 0.53), 10 μM S(-) BAYK8644 versus 10 μM S(-) BAYK8644 + 50 μM nifedipine (p < 0.0001). S(-)BAYK8644 significantly increased the frequency of AChR clustering in the absence of agrin although to lesser degree than agrin (Figure 3.2). 2-way ANOVA analysis revealed an interaction between S(-) BAYK8644 treated and nifedipine treated groups (p < 0.0001) indicating that nifedipine significantly inhibited S(-) BAYK8644 induced AChR clustering (Figure 3.2). These data demonstrated that the effect of S(-) BAYK8644 is specific to L-CaCh. Together, these data show that L-CaCh activation is necessary for agrin induced AChR clustering and sufficient to induce AChR clustering.

3.2.b L-CaCh activation acts downstream of MuSK in the clustering pathway behind AChR clustering:

Agrin stimulation of MuSK results in tyrosine phosphorylation and activation of the MuSK kinase domain. Thus, tyrosine phosphorylation of MuSK has been
Figure 3.2. **Stimulation of L-CaCh increased the frequency of AChR clustering.** (A) Myotubes were treated with 60 pM agrin, 10 μM S(-)BAYK8644 and/or 50 μM nifedipine for 18 hr. AChRs were then labeled with ALEXA594 conjugated α-Bungarotoxin. Images were obtained using a digital camera. Scale Bar = 25μm (B) The average number of AChR clusters/mm² was calculated based on 25 fields of view sampled for each experimental condition. * indicates samples significantly different from untreated samples (p < 0.01), ** indicates samples significantly different from agrin treated samples (p < 0.01).
used as a tool with which to detect MuSK activation and as an indicator of the signaling behind AChR clustering (Glass et al., 1996b; Hopf and Hoch, 1998a; Hopf and Hoch, 1998b). Therefore, to determine the hierarchical position of L-CaCh in the agrin/MuSK signaling pathway C2C12 cells were treated with Agrin, alone or in the presence of Nifedipine followed by an assay for MuSK tyrosine phosphorylation. Blockade of L-CaCh with nifedipine failed to inhibit agrin-induced MuSK phosphorylation indicating that L-CaCh acts downstream of MuSK activation to regulate AChR clustering (Figure 3.3). These data suggested that activation of L-CaCh would be insufficient to induce phosphorylation of MuSK.

Thus, to confirm the role of L-CaCh downstream of MuSK, C2C12 myotubes were treated with S(-) BAYK8644 in the presence or absence of nifedipine, followed by immuno-precipitation of MuSK, and analysis as above. S(-) BAYK8644 did not induce MuSK phosphorylation (Figure 3.4). Together, these results substantiate a role for L-CaCh activation downstream of MuSK in the AChR clustering pathway.

3.2. c Dysgenic Mice Exhibited a Markedly Reduced Response to Agrin

Dysgenic mice of the mdg strain carry a frame-shift mutation in the gene for the α1S subunit of L-CaCh, which results in the absence of functional protein (Chaudhari, 1992). Additionally, individual dysgenic myotubes have been shown to contain multiple, smaller than normal NMJs (Powell et al., 1984; Rieger et al., 1984). These data indicated that dysgenic mice might have a defect in the
Figure 3.3. **L-CaCh activity was not required for agrin induced MuSK phosphorylation.** C2C12 myotubes were treated with 60 pM agrin for 1 hr. and/or 10 or 50 μM Nifedipine for 1.5 hr. Myotubes were solublized and immuno-precipitated using an anti-MuSK primary antibody followed by incubation with protein G agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel) or reanalyzed using an anti-MuSK antibody (GW002, bottom panel).
Figure 3.4. **L-CaCh activity was not sufficient to induce MuSK phosphorylation.** C2C12 myotubes were treated with 60 pM agrin for 1 hr. and/or 10 or 50 μM nifedipine for 1.5 hr. Myotubes were solubilized and immuno-precipitated using an anti-MuSK primary antibody followed by incubation with protein G agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel) or reanalyzed using an anti-MuSK antibody (GW002, bottom panel).
development of the NMJ. We tested whether myotubes from dysgenic mice responded to agrin in the absence of functional L-CaCh.

Cultured myotubes from wildtype (+/+), heterozygous (+/-), and dysgenic (mdg/mdg) myotubes were co-labeled for AChR and L-CaCh followed by a clustering assay. Wild-type and heterozygous samples showed no differences in response to agrin (data not shown), so the data from these two groups were combined and are referred to as "normal." As expected, normal myotubes expressed L-CaCh whereas L-CaCh expression was never observed in dysgenic myotubes (Figure 3.6 A). Additionally, AChR clusters and L-CaCh did not co-localize in normal primary cultured myotubes expressing L-CaCh (Figure 3.6 A).

To confirm the results of the clustering assays performed using C2C12 cells, normal primary cultured myotubes were treated with agrin, alone or in the presence of 10 or 50 μM nifedipine and a clustering assay was performed. The following comparisons and their p values were determined via two sided t-test analysis using clusters/mm² from untreated cultures from each embryo as a within subjects control: untreated versus 60 pM agrin (p < 0.0001), untreated versus 60 pM agrin + 10 μM nifedipine (p < 0.0001), untreated versus 60 pM agrin + 50 μM nifedipine (p < 0.0001), untreated versus 10 μM nifedipine (p = 0.21), untreated versus 50 μM nifedipine (p = 0.16). The following comparisons and their p values were determined via two sided t-test using clusters/mm² data combined from multiple embryos in a between subjects analysis: 60 pM agrin versus 60 pM agrin + 10 μM nifedipine (p < 0.0001), 60 pM agrin versus 60 pM agrin + 50 μM nifedipine (p < 0.0001), untreated versus treated (p < 0.0001).
agrin + 50 μM nifedipine (p < 0.0001), 60 pM + 10 μM nifedipine versus 10 μM nifedipine (p < 0.0001), 60 pM + 50 μM nifedipine agrin versus 50 μM nifedipine (p < 0.0001). 2-way ANOVA analysis revealed an interaction between agrin treated and nifedipine treated groups (p = 0.05) indicating that nifedipine significantly inhibited agrin induced AChR clustering in a dose dependent manner. Thus, the analysis showed that as in C2C12 cells, blockade of L-CaCh with nifedipine markedly inhibited agrin-induced clustering in normal primary myotubes, but unlike C2C12 cells, normal primary myotubes retained a small response to agrin even at the highest dose of nifedipine (Figure 3.5). These results indicated that L-CaCh are central to clustering of AChR in primary myotubes, but may not be uniquely necessary.

Normal and Dysgenic (mdg/mdg) primary cultured myotubes were treated with agrin, alone or in the presence of nifedipine followed by a clustering assay. The following comparisons and their p values were determined via two sided t-test analysis using clusters/mm² data from untreated cultures from each homozygous mdg/mdg embryo as a within subjects control: untreated versus 60 pM agrin (p < 0.0001). The following comparisons and their p values were determined via two sided t-test analysis using clusters/mm² data combined from multiple embryos in a between subjects analysis: untreated normal (+/+, mdg/+) versus untreated dysgenic (mdg/mdg) (p < 0.0001), 60 pM agrin normal (+/+, mdg/+) versus 60 pM agrin dysgenic (mdg/mdg) (p < 0.0001), 60 pM agrin dysgenic (mdg/mdg) versus 60 pM agrin + 50 μM nifedipine normal (+/+, mdg/+).
Figure 3.5. **Blockade of L-type calcium channels inhibited agrin induced AChR clustering in primary cultured myotubes.** (A) Wild type and heterozygous MDG cultured primary myotubes were treated with 60 pM agrin and/or 10 or 50 μM nifedipine for 18 hr. AChRs were labeled with ALEXA 594 conjugated α-bungarotoxin. Scale Bar = 25 μm. (B) Percent of control clusters/mm² from the experiment illustrated in (A). Percent of control was calculated using untreated cultures from each embryo as a within subjects control. * indicates samples significantly different from untreated samples (p < 0.01), ** indicates samples significantly different from agrin treated samples (p < 0.01).
Figure 3.6. **Dysgenic mice exhibited a dramatically reduced response to agrin** (A) Normal and dysgenic primary myotubes in culture were treated with 60 pM agrin alone or with 50 μM Nifedipine for 18 hr. Samples were double labeled with ALEXA 594 conjugated α-bungarotoxin and anti-L-CaCh antibody (MA3-920) and ALEXA 488 conjugated secondary antibody. Images were collected using a digital camera. Scale bar = 25 μm. (B) Average number of AChR clusters/mm² from the experiment shown in (A). (C) Percent of control clusters/mm² from the experiment shown in (A). Percent of control was calculated using untreated cultures from each embryo as a within subjects control. * indicates samples significantly different from normal myotubes (p < 0.01).
(p = 0.028). 2-way ANOVA analysis revealed an interaction (p < 0.0001) between genotype and agrin treated groups indicating that genotype significantly affected the response to agrin. Dysgenic myotubes exhibited a higher baseline frequency of AChR clustering than normal myotubes as well as a much smaller response to agrin (Figure 3.6 B). The response to agrin in dysgenic myotubes was reduced both in the overall frequency of agrin-induced AChR clusters and in the fold response relative to baseline levels (Figure 3.6 B and C). Nifedipine had no effect on the agrin response in dysgenic myotubes, indicating that the effect of nifedipine on normal myotubes is specific to L-CaCh (Figure 3.6 C). Moreover, although significantly different, the average frequency of clustering (clusters/mm²) in dysgenic myotubes treated with agrin was very similar to that in normal samples treated with agrin and nifedipine, confirming that nifedipine effectively mimicked the effect of the mdg mutation. These results suggest that L-CaCh activity is an essential component of the signaling pathway that controls the clustering of AChR in skeletal muscle.

3.3 Discussion

3.3.1 Summary

In this chapter, I have presented evidence that L-CaCh mediates agrin-induced AChR aggregation on C2C12 and primary mouse myotubes. Agrin can cause a four to five-fold increase in the frequency of AChR aggregates, but in the presence of verapamil or nifedipine, 2 blockers of L-CaChs, agrin's ability to cause clustering is abrogated. Additionally, L-CaCh activation was not required
for agrin induced MuSK activation. Stimulation of L-CaCh with S(-)BAYK8644, induced AChR clustering but not MuSK activation. The data indicate that L-CaCh regulates AChR clustering downstream of MuSK activation in the agrin-MuSK signaling cascade.

3.3.b Is the mediation of agrin induced AChR clustering observed specific for L-CaCh?

A previous attempt to inhibit agrin-induced clustering with inhibitors of L-CaCh had no effect on agrin induced AChR clustering (Wallace, 1988). The earlier work, however used chick primary cultures. It is possible that redundant mechanisms exist in chick myotubes that are absent from mouse myotubes but that are capable of compensating for a loss of L-CaCh function in this species.

Partial blockade of the AChR has been reported for verapamil and nifedipine even at the doses used in this paper (Edeson et al., 1988). I have shown, however that nifedipine had no effect on agrin induced clustering in myotubes from dysgenic mice. The specificity of the response observed was further confirmed by the ability of the specific activator of L-CaCh, S(-)BAYK8644 to induce AChR clustering albeit with less magnitude than that induced by agrin. One potential explanation for the submaximal response, is that S(-)BAYK8644 was unstable over the assay period. This seems unlikely however, since as I discuss in chapter 4 in detail, AChR activation induced AChR clustering not only requires activation of L-CaCh, but also produces a clustering response similar in magnitude to that of S(-)BAYK8644. Another more likely possibility is that L-
CaCh is responsible for only a portion of the agrin-MuSK signaling response. Indeed, the residual response to agrin in myotubes from muscular dysgenesis mice and in normal primary mouse myotubes treated with agrin and nifedipine suggests the possibility of parallel or alternate signaling pathways downstream of MuSK independent of L-CaCh. Finally, taken together the data in this chapter clearly suggest a role for L-CaChs downstream of MuSK but leave open the possibility for alternate signaling pathways in the regulation of AChR aggregation.

3.3.3 What are potential downstream mediators of the residual agrin response in dysgenic myotubes?

The presence of the residual response to agrin in dysgenic myotubes implies the existence of a signaling pathway downstream of MuSK that does not depend on L-CaCh. The source of the residual response to agrin in dysgenic myotubes remains unknown, however there are several potential candidates. AChR is a non-specific cation channel and although the primary conductances are Na+ and K+, AChRs have been shown to conduct small amounts of calcium as well (Nicoll, 1992; Vernino et al., 1992; Moffett, 1993). Indeed, calcium flux via AChRs has been shown to be sufficient to induce contraction in dysgenic myotubes (Melliti et al., 1996). Thus, the source of the residual response to agrin in dysgenic myotubes could potentially be due to flux of calcium via AChR.

Additionally, NOS has also been implicated as a mediator of agrin/MuSK signaling (Jones and Werle, 2000; Luck et al., 2000; Ebert et al., 2003). NOS-1, the primary isoform of NOS present in skeletal muscle is allosterically modulated
by calmodulin released from CAM kinase by calcium (Bredt, 1999; Kone, 2000; Blottner and Luck, 2001). Thus, being modulated by both MuSK and Calcium, NOS-1 might provide a mechanism for crosstalk between calcium and MuSK signaling cascades and an explanation for the residual response to agrin observed dysgenic myotubes.

3.3.d What is the nature of the modulation of L-CaCh by MuSK?

The role of L-CaCh downstream of MuSK in agrin induced AChR clustering suggests that MuSK might modulate the function of L-CaCh through phosphorylation of the α-subunit of L-CaChs. Indeed, phosphorylation of the α-subunit of L-CaChs in other systems by PKA (cytosolic serine-threonine kinase), PKC (cytosolic serine-threonine kinase), or Src kinase (cytosolic tyrosine kinase) potentiates L-CaCh activation. These data suggest that phosphorylation of L-CaCh induced by MuSK signaling might promote L-CaCh activation and therefore AChR aggregation (Sculptoreanu et al., 1993; Gutierrez et al., 1994; Wijetunge and Hughes, 1995; Fratacci et al., 1996; Wijetunge and Hughes, 1996; Hu et al., 1998).

3.3.e Significance to NMJ formation

Data presented in this chapter suggest the model shown in Figure 3.7, in which agrin activation of MuSK causes a modification of L-CaCh that results in a net increase in calcium flux. In the model, this flux of calcium via L-CaCh thus
provides an important source of the intracellular calcium that is required for agrin-
induced AChR aggregation (Megeath and Fallon, 1998; Borges et al., 2002).

The model shown in Figure 3.7 also suggests that the L-CaCh acts in close
proximity to clustering machinery. Indeed, this idea is supported by data showing
that the rapid acting chelator BAPTA blocks agrin-induced clustering but the
slowly acting chelator EGTA does not (Megeath and Fallon, 1998). Such
proximity has been demonstrated elsewhere between L-CaCh and calmodulin
(Wu et al., 2001). Such localization would also help to explain how L-CaCh could
play roles in both AChR aggregation and in extrajunctional down-regulation of the
transcription of postsynaptic mRNAs (Walke et al., 1994).

My data provides experimental evidence of an integrating role for synaptic
activity and agrin signal transduction in the formation of the neuromuscular
synapse (Moffett, 1993). This may be relevant to later stages of neuromuscular
synaptogenesis during the period of synapse elimination. In particular, Lichtman
has shown a requirement for activity through AChRs in determining successful
synaptic input to the muscle (Lichtman et al., 1985). Illustrated in the model
shown in Figure 3.7, there is also a positive feedback component in which
clustered AChRs cause a larger local membrane depolarization that in turn
results in greater local calcium influx through nearby L-CaCh and hence more
aggregation of AChR. Such a mechanism could explain the role of AChR activity
in synapse elimination.
Figure 3.7. **Model of the role of L-CaChs in Agrin/MuSK signaling.** The model places calcium flux via L-CaChs downstream of MuSK in the agrin-MuSK signaling cascade. The data shown in this chapter support placement of BAPTA inhibition of agrin-induced AChR aggregation without inhibition of tyrosine phosphorylation on MuSK downstream of L-CaCh (Borges, et al., 2002). The model also suggests that MuSK signaling may influence the conductance properties of the L-CaCh channel, promoting further influx of calcium via a positive feedback loop.
The model in Figure 3.7 together with data presented here suggests that synaptogenesis at the NMJ might be controlled by parallel signaling by both activity and kinase mediated signaling cascades. Similarly, data from the post synaptic density at glutamatergic synapses in the hippocampus shows that both protein-protein interactions within the PSD and synaptic activity regulate PSD composition and therefore specific functional capacity (Kennedy, 1997). Together, the data from brain and the data shown here suggest that integration of activity with kinase mediated signaling cascades may be central to synaptogenesis not only at NMJ but throughout the nervous system.
CHAPTER 4: THE ROLE OF ACHR ACTIVITY IN ACHR CLUSTERING AND PHOSPHORYLATION IS DOWNSTREAM OF THE ACTIVATION OF L-CACH

4.1 Introduction

The aggregation of AChRs to junctional regions of the NMJ is an extremely important event in NMJ formation. The neural factor agrin, released from motor neuron terminals in vivo, activates the receptor tyrosine kinase, MuSK (Magill-Solc and McMahan, 1988; Magill-Solc and McMahan, 1990; Glass et al., 1996). Activation of MuSK causes tyrosine phosphorylation of MuSK and rapid downstream tyrosine phosphorylation of the β and δ subunits of AChR, and promotes AChR clustering (Nitkin et al., 1987; Qu and Huganir, 1994; Hopf and Hoch, 1998a; Hopf and Hoch, 1998b; Mohamed and Swope, 1999; Zhou et al., 1999; Godfrey et al., 2000; Watty et al., 2000).

While it is clear that activation of MuSK causes AChR clustering, the functional consequences of AChR phosphorylation have been more difficult to ascertain. Recent data have shown that mutant AChRs that lack tyrosine residues as potential phosphorylation substrates solublize more easily in detergent and incorporate less efficiently into AChR clusters than do wildtype receptors (Meyer and Wallace, 1998; Borges and Ferns, 2001). These results have been used to suggest that tyrosine phosphorylation of AChRs regulates anchoring of AChR protein complexes to the cytoskeleton. Whatever the functional significance of the result, phosphorylation of AChR β subunits can
serve as an indicator of signaling downstream of MuSK and will be used as such throughout this chapter.

In chapter 3, my data showed that L-CaChs mediate agrin induced AChR clustering downstream of MuSK, but left questions regarding the hierarchical position of L-CaChs with respect to downstream signaling events such as AChR β subunit phosphorylation. Additionally, MuSK may modulate the activity of L-CaChs. However, the voltage sensitivity of L-CaChs (Jones, 1998) suggests that neuromuscular signal transmission could also modulate the clustering of AChRs in skeletal muscle.

*In vivo,* muscle activity is stimulated by the release of ACh from the nerve terminal. ACh released from the motor neuron activates AChRs resulting in depolarization of the muscle membrane. This depolarization normally generates an action potential, which travels into the T-tubules and activates L-CaChs in the T-tubule membrane. Activation of L-CaChs stimulates release of calcium from intracellular stores and induces contraction (Moffett, 1993).

Muscle activity is known to regulate AChRs in a variety of ways. Denervation of a muscle or chronic blockade of AChRs results in an increase in sensitivity to ACh over the surface of the muscle fiber due to an increase in the expression of extrajunctional AChRs. The expression of extrajunctional AChRs in denervated fibers can be blocked by direct electrical stimulation of the muscle (Lomo and Rosenthal, 1972; Klarsfeld and Changeux, 1985; Goldman et al., 1988). Normally, Neuregulin (Nrg-1) released from the motor neuron acts to
override this activity induced inhibition of AChR expression in junctional regions (Corfas et al., 1993; Moscoso et al., 1995; Si et al., 1998). Muscle activity, via L-CaCh, also regulates the increase in the stability of junctional AChRs which occurs during normal maturation of the NMJ (Caroni et al., 1993).

Finally, activity may regulate agrin induced clustering of postsynaptic molecules including AChR. In skeletal muscle cultures which lack AChRs, Agrin does not induce the clustering of synaptic molecules such as MuSK and β dystroglycan in spite of MuSK activation (Grow and Gordon, 2000; Marangi et al., 2001). These data suggest that the presence of AChR protein is required for agrin induced clustering of synaptic molecules but do not illustrate the mechanism by which AChRs modulate agrin induced clustering of synaptic molecules. Perhaps AChR protein provides a nucleus by which protein-protein interactions form a junctional "scaffold" from which NMJ formation begins. Another possibility is that AChR activity regulates agrin induced AChR clustering. Both mechanisms potentially play a role in the formation of the NMJ. In this chapter I have chosen to examine the role of AChR activity in agrin induced AChR clustering.

To study the role of AChR activity in AChR aggregation I used the skeletal muscle cell line C2C12 as a model system. Here I show that AChR activity is required for agrin induced AChR clustering and that activation of AChRs is sufficient to induce AChR clustering. Furthermore, this AChR induced AChR clustering required L-CaCh activity. Activation of AChRs did not induce either
MuSK activation or AChR β subunit phosphorylation. L-CaCh activity was not sufficient to induce AChR β subunit phosphorylation. Additionally, L-CaCh activity was not required for agrin induced AChR β subunit phosphorylation. These data establish a role for AChR activation in the regulation of its own aggregation. These data also establish that AChR mediates AChR clustering by influencing the activation of L-CaChs and provide a potential mechanism by which neuromuscular signal transmission might act in concert with the agrin-MuSK signaling cascade to regulate NMJ formation. Furthermore, these data suggest that AChR β subunit phosphorylation requires MuSK activation even though the clustering of AChRs does not require MuSK activation.

4.2 Results

Figures presented in this chapter illustrate representative assays for responses to treatment conditions. Each experiment was repeated 3 or more times with the same results.

4.2.a AChR activity is necessary and sufficient for AChR clustering:

In order to test whether AChR activation is necessary for AChR clustering, cells were treated with agrin, alone or in the presence of the AChR blocker curare, followed by a clustering assay. The following comparisons and their p values were determined via two sided t-test analysis: untreated versus 60 pM agrin (p = 0.001), untreated versus 10 μM curare (p = 0.16), 60 pM agrin + 10 μM curare versus 10 μM curare (p = 0.91), 60 pM agrin versus 60 pM agrin + 10 μM curare
curare \((p < 0.0001)\). 2-way ANOVA analysis revealed an interaction between agrin treated and curare treated groups \((p < 0.0001)\) indicating that curare significantly inhibited agrin induced AChR clustering (Figure 4.1). These data indicated that AChR activation is required for agrin induced AChR clustering.

In order to determine whether AChR activity is sufficient to induce AChR clustering, cells were treated with the AChR agonist nicotine, followed by a clustering assay. The following comparisons and their \(p\) values were determined via two sided t-test analysis: untreated versus 60 pM agrin \((p < 0.0001)\), untreated versus 3 \(\mu\)M nicotine \((p < 0.0001)\) (Figure 4.2). Nicotine significantly increased the frequency of AChR clustering in the absence of agrin indicating that AChR activity is sufficient for AChR clustering.

### 4.2.b AChR activity is not required for AChR or MuSK phosphorylation:

I was also interested in determining the hierarchical position of AChR activity in the signaling pathway underlying AChR clustering. An assay commonly used to test for the signaling behind AChR clustering is the tyrosine phosphorylation assay (see Chapter 2). Tyrosine phosphorylation of MuSK reflects the activation of the MuSK kinase domain and, hence, activation of MuSK. Tyrosine phosphorylation of AChR \(\beta\) subunit provides additional evidence of MuSK signaling downstream of MuSK activation.

Therefore, in order to test whether AChR activity is required for MuSK signaling, cells were treated with agrin, either alone or in the presence of curare,
Figure 4.1. **Blockade of AChR inhibited agrin induced AChR clustering.**

(A) C2C12 myotubes were treated with 60 pM agrin and/or 10 μM d-tubocurarine (curare) for 18 hr. Cells were then labeled with ALEXA594-conjugated α-bungarotoxin. Images were obtained using a digital camera. Scale Bar = 25μm (B) Graph of the results of the experiment shown in (A). The average number of AChR clusters/mm² was calculated based on 25 fields of view sampled for each experimental condition. * indicates samples significantly different from untreated samples (p < 0.01), ** indicates samples significantly different from agrin treated samples (p < 0.01).
Figure 4.2. **Blockade of L-CaCh inhibited nicotine induced AChR clustering.** (A) C2C12 myotubes were treated with 3 μM nicotine and/or 50 μM nifedipine, or 60 pM Agrin for 18 hr. Cells were then labeled with ALEXA594-conjugated α-bungarotoxin. Images were obtained using a digital camera. Scale Bar = 25μm. (B) Graph of the results of the experiment shown in (A). The average number of AChR clusters/mm² was calculated based on 25 fields of view sampled for each experimental condition. * indicates samples significantly different from untreated samples (p < 0.01). ** indicates samples significantly different from nicotine treated samples (p < 0.01).
followed by a tyrosine phosphorylation assay for MuSK and AChR. Curare did not inhibit agrin induced MuSK activation or AChR β subunit phosphorylation (Figure 4.3 and 4.4). These data showed that AChR activation is not necessary for agrin induced MuSK activation or AChR phosphorylation and suggest that AChR activation acts downstream of or in parallel to MuSK in the signaling pathway that regulated AChR aggregation.

To confirm that AChR activation does not regulate MuSK activation or AChR β subunit phosphorylation, cells were treated with nicotine, and phosphorylation assays were performed for both MuSK activation and AChR β subunit phosphorylation. Nicotine did not induce tyrosine phosphorylation of either protein (Figure 4.5 and 4.6). These data showed that AChR activation was not sufficient to activate MuSK or to induce AChR phosphorylation and reaffirmed that the role of AChR activation in agrin-MuSK signaling is downstream of MuSK activation and AChR β subunit phosphorylation in the signaling cascade that mediates AChR aggregation.

4.2.3 L-CaCh activity is necessary for AChR activity induced AChR clustering:

To determine whether AChRs modulate their own clustering by influencing the activation of L-CaChs, C2C12 cells were treated with nicotine in the presence of the L-CaCh blocker nifedipine and a clustering assay was performed. The following comparisons and their p values were determined via two sided t-test analysis: untreated versus 50 μM nifedipine (p = 0.13), untreated versus 3 μM
Figure 4.3. **AChR activity does not mediate agrin induced AChR β subunit phosphorylation.** C2C12 myotubes were treated with 60 pM agrin for 1 hr. and/or 10 μM d-tubocurarine for 1.5 hr. C2C12 myotubes were solubilized and precipitated using α-Bungarotoxin conjugated agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel) or reanalyzed using an anti-AChR β subunit antibody (MAB111, bottom panel).
Figure 4.4. **AChR activity does not mediate agrin induced MuSK phosphorylation.** C2C12 myotubes were treated with 60 pM agrin for 1 hr. and/or 10 μM d-tubocurarine (curare) for 1.5 hr. Myotubes were solublized and precipitated using an anti-MuSK primary antibody followed by precipitation by protein G agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel) or reanalyzed using an anti-MuSK antibody (GW002).
Stimulation of AChR did not induce AChR \( \beta \) subunit phosphorylation. C2C12 myotubes were treated with 60 pM agrin or 3 \( \mu \)M nicotine for 1 hr. and/or 50 \( \mu \)M nifedipine for 1.5 hr. Myotubes were solublized and precipitated using \( \alpha \)-Bungarotoxin conjugated agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel) or reanalyzed using an anti-AChR \( \beta \) subunit antibody (MAB111, bottom panel).
Figure 4.6. **Stimulation of AChR did not induce MuSK phosphorylation.** C2C12 myotubes were treated with 60 pM agrin or 3 μM nicotine for 1 hr. and/or 50 μM nifedipine for 1.5 hr. Myotubes were solubilized and precipitated using an anti-MuSK primary antibody followed by precipitation by protein G agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel) or reanalyzed using an anti-MuSK antibody (GW002, bottom panel).
nicotine + 50 μM nifedipine ($p = 0.003$), 3 μM nicotine versus 3 μM nicotine + 50 μM nifedipine ($p < 0.0001$), 3 μM nicotine + 50 μM nifedipine versus 50 μM nifedipine ($p = 0.16$), 3 μM nicotine versus 60 pM agrin ($p = 0.003$). 2-way ANOVA analysis revealed an interaction between nicotine treated and nifedipine treated groups ($p < 0.0001$) showing that nifedipine significantly inhibited nicotine induced AChR clustering (Figure 4.2). These data indicated that L-CaCh activation is required for AChR induced clustering of AChRs. Therefore, AChRs regulate their own clustering by influencing the activity of L-CaChs.

4.2.d L-CaCh activity is not required for AChRs β subunit phosphorylation:

In Chapter 3, I showed that L-CaChs act downstream of MuSK activation in the pathway behind AChR clustering. The experiments in Chapter 3 did not, however, reveal whether L-CaCh activity was required for signaling events downstream of MuSK activation, such as AChR β subunit phosphorylation. Thus, to test whether L-CaCh activation was required for AChR β subunit phosphorylation, C2C12 cells were treated with agrin, alone or in the presence of nifedipine, followed by a tyrosine phosphorylation assay for AChR β subunit. Nifedipine did not inhibit agrin induced AChR phosphorylation (Figure 4.7). These data indicate that L-CaCh does not regulate AChR β subunit phosphorylation in the agrin-MuSK signaling cascade.

If L-CaCh is not involved in AChR β subunit phosphorylation, one would not expect stimulation of L-CaCh to induce AChR phosphorylation. In order to test
Figure 4.7. **L-type calcium channels do not mediate agrin-induced AChR β subunit phosphorylation.** C2C12 myotubes were treated with 60 pM agrin for 1 hr. and/or 10 or 50 μM nifedipine for 1.5 hr. Myotubes were solubilized and precipitated using α-Bungarotoxin conjugated agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel) or reanalyzed using an anti-AChR β subunit antibody (MAB111, sigma).
this prediction, cells were treated with the L-CaCh activator S(-)BAYK8644, and assays were performed for AChR β subunit phosphorylation. S(-)BAYK8644 failed to induce AChR phosphorylation, even though it did induce AChR clustering, as shown in Chapter 3. This result suggested that L-CaCh activation does not regulate AChR phosphorylation and suggested that the role of L-CaChs is downstream or in parallel to both MuSK activation and AChR phosphorylation in the signaling pathway behind AChR clustering (Figure 4.8).

4.2.e AChR clustering does not induce AChR β subunit phosphorylation

In Chapter 3, I showed that S(-)BAYK8644 promoted AChR clustering although to a lesser degree than 60 pM Agrin (repeated in figure 4.9 A). Additionally, in Chapter 3 and in the current chapter I have shown that L-CaCh activation is not sufficient to induce MuSK activation or AChR β subunit phosphorylation. These results suggest that AChR clustering does not induce activation of MuSK or AChR β subunit phosphorylation.

It could be argued, however, that S(-)BAYK8644 causes only a partial activation of the cluster-signaling pathway, not sufficient to induce a detectable level of AChR β subunit phosphorylation. Therefore, to test whether AChR β subunit phosphorylation could be detected with a sub-maximal clustering response, a dose of agrin (10 pM) was determined to produce a clustering response similar in magnitude to S(-)BAYK8644 using a clustering assay. The results of that clustering assay are shown in Figure 4.9 A. The following comparisons and their p values were determined via two sided t-test analysis: untreated versus 60 pM
Figure 4.8. **Stimulation of L-type calcium channels did not induce AChR β subunit phosphorylation.** C2C12 myotubes were treated with 60 pM agrin or 10 μM S(-)BAYK8644 for 1 hr. and/or 50 μM nifedipine for 1.5 hr. Myotubes were solublized and precipitated using α-Bungarotoxin conjugated agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel) or reanalyzed using an anti-AChR β subunit antibody (MAB111, bottom panel).
Figure 4.9. 10 pM agrin induced a submaximal AChR clustering response and AChR phosphorylation whereas stimulation of L-CaCh induced AChR clustering without inducing AChR \( \beta \) subunit phosphorylation. C2C12 myotubes were treated with 60 pM agrin, 10 pM agrin or 10 \( \mu \)M S(-)BAYK8644. A. Cells were treated for 18 hr. and labeled with ALEXA594-conjugated alpha-bungarotoxin. The average number of clusters/mm\(^2\) was calculated based on 25 fields of view sampled for each experimental condition. * indicates samples significantly different from untreated samples \( (p < 0.01) \), ** indicates samples significantly different from 60 pM agrin treated samples \( (p < 0.05) \), # indicates samples significantly different from S(-)BAYK8644. B. C2C12 myotubes were treated for 1 hr. Myotubes were solublized and precipitated using \( \alpha \)-Bungarotoxin conjugated agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel) or reanalyzed using an anti-AChR \( \beta \) subunit antibody (MAB111, bottom panel).
agrin ($p < 0.0001$), untreated versus 10 pM agrin ($p < 0.0001$), untreated versus 10 μM S(-)BAYK8644 ($p < 0.0001$), 10 μM S(-)BAYK8644 versus 10 pM agrin ($p = 0.30$), 10 μM S(-)BAYK8644 versus 60 pM agrin ($p = 0.016$), 60 pM agrin vs. 10 pM agrin ($p = 0.015$). This result, coupled with the finding that L-CaCh activation does not induce activation of MuSK or AChR β subunit phosphorylation, suggests that AChR β subunit phosphorylation is not induced by clustering.

Once a dose of agrin (10 pM) that induced a clustering response similar in magnitude to that of S(-)BAYK8644 was determined, C2C12 myotubes in culture were treated with 10 and 60 pM agrin and 10 μM S(-)BAYK8644 and then assayed for AChR β subunit phosphorylation. Although the reduced dose of agrin (10 pM) induced a clustering response similar in magnitude to S(-)BAYK8644, 10 pM agrin also induced detectable phosphorylation of AChR β subunit, albeit less than the 60 pM dose of agrin. In contrast, however, S(-)BAYK8644 did not induce detectable phosphorylation of AChR β subunit (Figure 4.9 B). These data support the conclusion that AChR β subunit phosphorylation occurs independent of AChR clustering and is not required for AChR clustering.

4.3 Discussion

4.3.a Summary

In this chapter, I have shown that AChR activation is required for agrin induced AChR clustering and that stimulation of AChRs by nicotine is sufficient to induce AChR clustering. Furthermore, I showed that nifedipine inhibited nicotine
induced AChR clustering. AChR function was neither necessary for agrin induced MuSK activation or AChR β subunit phosphorylation nor sufficient to induce MuSK activation or AChR β subunit phosphorylation. L-CaCh function was not required for agrin induced AChR β subunit phosphorylation. These data showed that signaling due to AChR function converges downstream of MuSK and acts upstream of L-CaChs in the signaling pathway that mediates agrin induced AChR clustering.

4.3.b The Role of AChR activity in AChR localization

Previous research has shown that muscle activity regulates the behavior of AChRs in skeletal muscle. Much of this research has focused on the role of activity in controlling the expression and number of AChRs in muscle membrane. Normally, muscle activity inhibits extrajunctional AChR expression (Klarsfeld and Changeux, 1985; Goldman et al., 1988). Nrg-1, released from the motor neuron terminal, overrides the activity induced inhibition in the region of the NMJ and stimulates junctional expression of AChRs (Falls et al., 1993). Treatment with antagonist increases the expression of AChRs, and treatment with the AChR agonists, nicotine or carbachol, decreases the number of AChRs in myotube membranes (Goldman et al., 1988; St John and Gordon, 2001).

Here I show data that extends the role of muscle activity from AChR expression to the aggregation of AChRs. These data are consistent with previous studies which showed a requirement for AChR protein in agrin induced clustering of synaptic proteins such as MuSK and β-dystroglycan, and extend the
scope of those data in two ways (Grow and Gordon, 2000; Marangi et al., 2001). First, these data show that AChR function is required for agrin induced AChR clustering second, that this AChR induced AChR aggregation requires L-CaCh activation.

I established the hierarchical position of AChRs with respect to L-CaChs and MuSK in the signaling pathway behind agrin induced AChR clustering. These experiments showed that 1) AChR activation does not cause MuSK activation 2) AChR activation is not required for agrin induced AChR β subunit phosphorylation and 3) L-CaCh activation is not involved in agrin induced AChR β subunit phosphorylation. Data presented in this chapter therefore extend the model shown in Chapter 3 to include AChR function as a mechanism by which L-CaChs can be activated and shows signaling from AChRs and L-CaChs converging downstream of MuSK in the signaling cascade leading to AChR clustering (Figure 4.10).

4.3.c The Role of AChR Phosphorylation in Clustering

Data presented in this chapter and throughout Chapter 3 shows that, although activation of L-CaChs or AChRs induces clustering of AChRs, neither protein plays a role in MuSK activation or AChR β subunit phosphorylation suggesting that the role of AChR and L-CaCh activation in the regulation of AChR aggregation acts downstream of early events in the agrin-MuSK signaling pathway. In addition these data demonstrate that AChR phosphorylation is not required for AChR clustering.
Figure 4.10. **Model of the role of AChR activity in the formation of the NMJ.** Release of ACh from the motor neuron terminal results in depolarization of the myotube membrane due to AChR activity. AChR activity inhibits expression of AChRs. Nrg-1 released from the motor neuron terminal overrides the inhibition and increases expression of junctional AChRs. The depolarization also activates L-CaCh inducing an increase in the aggregation and stability of AChRs. Release of agrin from the nerve terminal results in activation of MuSK which promotes aggregation of AChRs to junctional regions and results in phosphorylation of AChR. Phosphorylation may then increase the efficiency with which AChRs are incorporated into the newly formed junctional region and promote anchoring of AChRs to the cytoskeleton. Muscle activity and the Agrin-MuSK signaling cascade thus act in concert to inhibit expression of extrajunctional receptors while promoting aggregation and anchoring of junctional receptors.
It is possible, nonetheless, that AChR β subunits phosphorylation modulates the efficiency with which AChRs are incorporated into clusters. AChR β subunit phosphorylation has been uncoupled from the clustering response in experiments using cultures of myotubes expressing mutant AChR β subunits lacking tyrosine residues (Meyer and Wallace, 1998). The fold response to agrin treatment was much less in magnitude in the mutant cultures than in wildtype cultures suggesting that in the absence of AChR β subunit phosphorylation AChRs are incorporated less efficiently into aggregates.

Although experiments presented in this chapter used myotubes expressing wildtype receptors, AChR aggregation was uncoupled from AChR β subunit phosphorylation following treatment with Nicotine or S(-)BAYK8644. Both Nicotine and S(-)BAYK8644 induced a significant increase in the frequency of AChR clustering in the absence of AChR β subunit phosphorylation. The magnitude of the clustering response however, was significantly less than the clustering response to 60 pM agrin (Nicotine, p = 0.003, S(-)BAYK8644 , p = 0.016). Thus, the experimental conditions used in this chapter mimicked the conditions in which mutant AChRs are present and support the conclusion that AChR phosphorylation may play a role in the efficiency with which AChRs are incorporated into clusters.

4.3.d Implications for NMJ Development

The data in this chapter begin to establish a model in which both neuromuscular transmission and signaling cascades act in concert to regulate
the formation of the NMJ. In this model, during the development of the NMJ, ACh released from the motor neuron terminal opens AChR channels, resulting in depolarization of the muscle membrane and inhibition of the expression of AChRs. Nrg-1, also released from the motor neuron terminal, acts to override this inhibition of AChR expression in junctional regions. This increase in the expression of junctional AChRs allows additional ACh released from the motor neuron to induce depolarization of the muscle membrane. This depolarization results in downstream activation of L-CaCh, allowing a focal influx of calcium via L-CaChs and promotes both increased stability of AChRs and AChR aggregation. Parallel release of agrin from the nerve terminal promotes AChR localization by activating MuSK and possibly beginning a positive feedback loop in which L-CaCh activity is enhanced by MuSK signaling (Figure 4.10).
CHAPTER 5: THE ROLE OF L-TYPE CALCIUM CHANNELS IN CALCIUM INDUCED ACHR AND MUSK PHOSPHORYLATION AND IN ACHR CLUSTERING

5.1 Introduction

The formation of the NMJ involves a complex set of coordinated changes in both pre and postsynaptic cells that is mediated by muscle activity, calcium flux, and signaling cascades. The aggregation of AChRs to junctional regions of the muscle fiber membrane enables fast, efficient transmission of signals from the nerve terminal to the muscle fiber (Wood and Slater, 1997). The aggregation of AChRs is mediated by agrin released from the nerve terminal (Fallon and Gelfman, 1989; Magill-Solc and McMahan, 1990b; Magill-Solc and McMahan, 1990a). Agrin activation of MuSK results in the rapid phosphorylation of both MuSK and AChR. Activation of MuSK promotes the aggregation of AChRs, whereas the functional consequences of AChR phosphorylation remain unclear (Swope et al., 1995; Glass et al., 1996; Hopf and Hoch, 1998a; Hopf and Hoch, 1998b; Mohamed and Swope, 1999).

Extracellular calcium is required for both agrin binding to the agrin-binding complex and for agrin activation of MuSK (Bloch, 1983; Peng, 1984; Nastuk et al., 1991; Borges et al., 2002). Additionally, raising the concentration of extracellular calcium induces MuSK activation, AChR phosphorylation, and AChR aggregation in the absence of agrin (Zhu and Peng, 1988; Grow et al.,
1999). These data have shown that extracellular calcium is both necessary and sufficient for agrin induced MuSK activation and AChR clustering.

Experiments which showed that chelation of intracellular calcium with BAPTA abolishes agrin induced AChR phosphorylation and clustering, but not MuSK activation, indicated that intracellular calcium is required for agrin induced AChR clustering (Megeath and Fallon, 1998). This uncoupling of AChR phosphorylation from MuSK activation was also observed in experiments in which inhibitors of tyrosine kinase activity allowed agrin-induced activation of MuSK, but blocked AChR phosphorylation (Borges and Ferns, 2001). Together, these data suggest that there is an intermediary signaling step or steps between MuSK activation and AChR phosphorylation that is regulated by intracellular calcium.

Thus, both extracellular and intracellular calcium are important mediators of the signaling behind AChR clustering. These data have suggested conclusion that extracellular calcium both modulates MuSK activation and flows into the cell via an ion channel conduit to produce increases in intracellular calcium which then modulate the intracellular signaling responsible for AChR clustering (Megeath and Fallon, 1998; Borges et al., 2002).

In chapter 3, I showed that L-CaChs act downstream of MuSK signaling to mediate agrin-induced AChR clustering. Here I hypothesize that L-CaCh functions in calcium-induced AChR clustering, as well. Further, since intracellular calcium is required for AChR phosphorylation but not MuSK
activation, I hypothesize that L-CaChs act downstream of MuSK activation but upstream of AChR phosphorylation in the pathway that leads to AChR phosphorylation following a rise in extracellular calcium (Borges et al., 2002).

I chose to use the skeletal muscle cell line C2C12 as a model system with which to study the role of L-CaChs in calcium induced AChR clustering. Here I show that blockade of L-CaCh with nifedipine inhibits calcium induced AChR clustering. Additionally, blockade of L-CaCh with verapamil inhibited calcium induced AChR phosphorylation but not MuSK activation.

5.2 Results

Figures presented in this chapter illustrate representative assays for responses to treatment conditions. Each experiment was repeated 3 or more times with the same results.

5.2.a L-CaCh Activity is Necessary for Calcium Induced AChR clustering:

C2C12 cells require 1.8 mM calcium in the medium for proper growth and differentiation. Therefore all "control" samples throughout this chapter contain 1.8 mM calcium in the medium. Treatment of cells with 4.8 mM calcium referred to a final concentration in the medium of 4.8 mM resulting in a rise in extracellular calcium of 3 mM.

In order to determine whether L-CaChs are required for calcium induced AChR clustering, cells were treated with calcium, alone or in the presence of the L-CaCh blocker nifedipine, and a clustering assay was performed. The following
comparisons and their \( p \) values were determined via two sided t-test analysis: control versus 60 pM agrin (\( p < 0.0001 \)), control versus 4.8 mM calcium (\( p < 0.0001 \)), control versus 4.8 mM calcium + 50 \( \mu \)M nifedipine (\( p < 0.096 \)), control versus 50 \( \mu \)M nifedipine (\( p = 0.89 \)), 4.8 mM calcium versus 4.8 mM calcium + 50 \( \mu \)M nifedipine (\( p < 0.0001 \)), 4.8 mM calcium versus 60 pM agrin (\( p < 0.0001 \)), 4.8 mM calcium + 50 \( \mu \)M nifedipine versus 50 \( \mu \)M nifedipine. 2-way ANOVA analysis revealed an interaction between calcium treated and nifedipine treated groups (\( p < 0.0001 \)) indicating that nifedipine significantly inhibited calcium induced AChR clustering (Figure 5.1). These data support the hypothesis that L-CaCh activity is required for calcium induced AChR clustering.

5.2.b L-CaCh Activity is Not Required for Calcium Induced MuSK Activation:

To test whether L-CaCh activity is required for calcium induced MuSK activation, cells were treated with calcium, alone or in the presence of the L-CaCh blocker, verapamil, followed by an assay for MuSK tyrosine phosphorylation. Although calcium did induce MuSK phosphorylation, the magnitude of this response was much less than the response seen to agrin treatment. In any case, verapamil did not inhibit calcium induced MuSK activation at any dose. These data indicated that the L-CaCh is not involved in calcium induced MuSK activation and suggest that L-CaChs role is downstream of MuSK activation in the signaling cascade responsible for calcium induced AChR clustering (Figure 5.2).
Figure 5.1 **Blockade of L-CaChs inhibited calcium induced AChR clustering.** (A) C2C12 myotubes were treated with 4.8 mmol calcium chloride and/or 50 μM Nifedipine, or 60 pM agrin for 18 hr. Cells were then labeled with ALEXA594-conjugated α-bungarotoxin. Images were obtained using a digital camera. Scale Bar = 25μm (B) Graph of the results of the experiment shown in (A). The average number of AChR clusters/mm² was calculated based on 25 fields of view sampled for each experimental condition. * indicates samples significantly different from untreated samples (p < 0.01), ** indicates samples significantly different from calcium treated samples (p < 0.01).
Figure 5.2. **L-CaChs do not mediate calcium induced MuSK phosphorylation.** C2C12 myotubes were treated with 60 pM Agrin or 4.8 mmol calcium chloride for 1 hr. and/or 3 or 30 μM Verapamil for 1.5 hr. Myotubes were solubilized and precipitated using an anti-MuSK primary antibody followed by precipitation by protein G agarose beads and analyzed via SDS-PAGE followed by western blot using an anti-phosphotyrosine antibody (PY20). Reanalysis of this blot was not possible because at the time of the experiment the antibody for reprobing was not available.
5.2. c L-CaCh Activity Modulates Calcium Induced AChR Phosphorylation:

In order to determine the role, if any of L-CaCh activity in calcium induced AChR phosphorylation, cells were treated with calcium, alone or in the presence of verapamil, followed by an assay for tyrosine phosphorylation of AChR β subunits. Calcium induced phosphorylation of AChRs but, similar to calcium induced MuSK phosphorylation, calcium induced less phosphorylation of AChR β subunits than did agrin. Additionally, verapamil inhibited calcium induced AChR phosphorylation in a dose dependent manner. These data indicate that L-CaCh function is necessary for calcium induced AChR β subunit phosphorylation and suggest that L-CaChs role in calcium induced AChR clustering is downstream of MuSK activation or acts via a calcium regulated pathway in parallel to MuSK signaling.

5.3 Discussion

5.3.a Summary

In this chapter, I have shown that L-CaCh activity is required for calcium induced AChR clustering. Additionally, I have shown that L-CaCh acts downstream of MuSK and upstream of AChR phosphorylation in the signaling pathway that leads to calcium induced AChR clustering.

5.3.b The Role of Calcium In AChR Aggregation:

In this chapter I repeated previous experiments, and presented data that showed that a rise in extracellular calcium induced AChR clustering (Bloch, 1983;
Zhu and Peng, 1988; Grow et al., 1999). However, the magnitude of the clustering response was less than that observed following treatment with agrin (Figure 5.1). This result is reminiscent of my earlier findings that activation of L-CaCh, either directly with S(-)BAYK8644 or indirectly by upstream activation of AChR with nicotine, also produced an AChR clustering response similar in magnitude to calcium (Figure 3.3 and Figure 4.1).

Additionally, blockade of L-CaChs inhibited calcium induced AChR clustering (Figure 5.1). These data together suggest that the mechanisms for calcium and S(-)BAYK8644 mediated induction of AChR clustering are similar. Both depend on calcium delivery to the intracellular clustering machinery by the flux of calcium ions through the L-CaChs. While the S(-)BAYK8644 depends on pharmacological activation of L-CaCh to allow calcium flux, increasing extracellular calcium likely stimulates increased calcium flux based on the increased calcium driving force.

5.3.c The Role of Calcium in MuSK Activation:

Here I confirmed the finding that increasing extracellular calcium induced MuSK activation (Grow et al., 1999) (Figure 5.2). Similar to the calcium induced clustering response, the calcium induced MuSK phosphorylation was again much less than that observed with agrin. These data, coupled with the data showing that extracellular calcium is required for agrin induced MuSK activation, suggest that extracellular calcium promotes MuSK activity, perhaps through the
promotion of agrin binding or dimerization of MuSK monomers (Nastuk et al., 1991; Borges et al., 2002).

In chapter 3, I showed that blockade of L-CaCh does not inhibit agrin induced MuSK activation. In this chapter I have shown that blockade of L-CaCh does not inhibit calcium induced MuSK activation (Figure 5.2). These data indicate that regardless of the mechanism by which MuSK is stimulated, the role of L-CaCh in AChR clustering lies downstream of MuSK activation.

5.3.d The Role of Calcium in AChR β Subunit Phosphorylation:

In this chapter, I also repeated experiments which showed that increasing extracellular calcium increases AChR phosphorylation (Grow et al., 1999; Figure 5.3). Again, the increase in AChR phosphorylation was less than that observed in response to agrin. It is likely that this AChR phosphorylation is initiated by calcium induced MuSK activation, and as such it would be expected to be in proportion to the sub-maximal MuSK activation induced by calcium.

Additionally, I showed that blockade of L-CaCh inhibited calcium induced AChR phosphorylation in a dose dependent manner (Figure 5.3). At first these data seem contradictory to the data shown in chapter 3 in which activation of L-CaCh with S(-)BAYK8644 failed to induce MuSK activation or AChR phosphorylation. I now suggest, therefore, that calcium flux through L-CaChs modulates some intermediary effector activated by MuSK, which in turn mediates AChR phosphorylation. In the absence of MuSK activation, such as in the case
Figure 5.3. L-CaCh activity mediates calcium induced AChR phosphorylation. C2C12 myotubes were treated with 60 pM agrin or 4.8 mmol calcium chloride for 1 hr. and/or 3 or 30 μM Verapamil for 1.5 hr. Myotubes were solublized and precipitated using α–bungarotoxin conjugated agarose beads and analyzed via SDS-Page followed by western blot using an anti-phosphotyrosine antibody (PY20, top panel). The blot was then reanalyzed using an anti-AChR β subunit antibody (MAB111).
of direct stimulation of L-CaChs with S(-)BAYK8644, that intermediary is not activated and therefore is not available for regulation by L-CaCh or for inducing phosphorylation of AChRs. When MuSK is activated, such as in the case of agrin stimulation or increased extracellular calcium, this intermediary is available for regulation by L-CaCh activity (Glass et al., 1996; Grow et al., 1999).

What, then, is the reason that agrin induced AChR phosphorylation is not inhibited by blockade of L-CaChs, whereas calcium induced AChR phosphorylation is inhibited by blockade of L-CaChs? The use of electrochemiluminescence (ECL) to visualize protein does not allow quantification of protein present because the signal produced is not linear. In other words, as the amount of protein increases, the ECL signal increases to a maximal level. Once maximal signal level is reached, the magnitude of the signal plateaus so that regardless of further increases in protein levels, the signal magnitude remains the same (Laskey). Thus, in presence of a large magnitude signal, changes in that signal become increasingly difficult to visualize. I propose that a maximal dose of agrin (60pM) produces such a large activation response of MuSK and subsequent downstream phosphorylation of AChR, as to render changes in phosphorylation of AChR in response to Nifedipine treatment too small to visualize. Therefore, in the presence of sub-maximal activation of MuSK, such as in the presence of a rise in extracellular calcium, the signal from AChR phosphorylation remains in the linear range for ECL detection and
modulation of AChR phosphorylation by the hypothesized intermediary is visualizable.

5.3.e The Role of Calcium in the modulation of the Intermediary Between MuSK Activation and AChR Phosphorylation:

Nitric Oxide Synthase (NOS) has been shown to act downstream of MuSK to regulate both AChR clustering and β subunit phosphorylation (Jones and Werle, 2000). Interestingly, the predominant form of NOS in skeletal muscle is NOS-1, which can be regulated by intracellular calcium through calmodulin (Kone, 2000). Thus, NOS-1 could provide an ideal molecule to act as the intermediary between MuSK activation and AChR phosphorylation hypothesized in this chapter.

5.3.f Calcium and NMJ formation:

The data presented in this chapter further extend the model proposed in chapter 3 and expanded in chapter 4 to include specific roles for both extra- and intracellular calcium in NMJ development, confirm the role of L-CaChs in this process, and suggest the model shown in Figure 5.4. In the model, release of ACh from the motor neuron terminal activates AChRs and induces depolarization of the muscle fiber membrane. Depolarization activates L-CaCh, which results in a rise in intracellular calcium. In parallel, extracellular calcium and agrin, released from the motor neuron terminal, together activate MuSK, which in turn stimulates activation of NOS. Perhaps the rise in intracellular calcium then promotes or increases this NOS activity. NOS then stimulates a signaling cascade which results in phosphorylation of AChR β subunits, and this in turn
Figure 5.4. Model of the role of calcium in the aggregation of AChRs at the NMJ. Release of ACh from the motor neuron terminal results in depolarization of the myotube membrane due to AChR activity. AChR activity inhibits expression of extrajunctional AChRs. The depolarization also activates L-CaCh inducing an increase in the aggregation of AChRs. Release of Agrin from the nerve terminal results in activation of MuSK which promotes aggregation of AChR's to junctional regions and activates NOS-1. This activity of NOS-1 is increased or prolonged by the calcium influx through the L-CaCh and results in downstream phosphorylation of AChR. This phosphorylation may then increase the efficiency with which AChRs are incorporated into the newly formed junctional region and promotes anchoring of AChR to the cytoskeleton.
promotes anchoring of synaptic components to protein complexes or cytoskeletal components.
CHAPTER 6: SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

6.1 Summary

In this dissertation, I have presented new information on several aspects of the signaling pathway responsible for the clustering of AChRs on muscle cells. First, I have shown that activation of L-CaChs is both necessary for agrin induced clustering of AChRs and sufficient to stimulate AChR clustering even in the absence of agrin. Additionally, I have shown that activation of AChRs causes their own clustering by influencing the activity of L-CaCh. I have also shown that neither AChRs nor L-CaChs play a role in MuSK activation or AChR β subunit phosphorylation suggesting that the role of AChR and L-CaCh is downstream of MuSK activation and phosphorylation of the AChR β subunit in the signaling cascade that leads to the aggregation of AChRs. Finally, I have shown that calcium induced clustering and phosphorylation of AChRs require L-CaCh activation. These data suggested that although L-CaCh activation is insufficient to cause AChR β subunit phosphorylation L-CaCh may modulate an intermediate step between MuSK activation and AChR phosphorylation. These data therefore support the hypothesis that L-CaCh activation delivers extracellular calcium to the intracellular machinery that regulates AChR clustering. Furthermore, these data establish the position of L-CaChs in the signaling hierarchy responsible for AChR clustering as being downstream of or parallel to both MuSK activation and AChR phosphorylation in the signaling cascade behind AChR clustering. For a summary of the responses to pharmacological agents see Table 6.1.
<table>
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<td>- (partial)</td>
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Table 6.1 Summary Table of Results
The data presented in this paper begin to provide an integrated view of NMJ formation in which neuromuscular transmission, calcium signaling, and signaling cascades mediated by neurotrophic factors act in concert to regulate the localization of synaptic molecules to junctional regions of the muscle fiber. Many questions remain, however, regarding the events downstream of MuSK and L-CaCh activation.

6.2 At what point to the calcium and MuSK signaling cascades converge?

Although I have shown that L-CaChs deliver extracellular calcium to intracellular clustering machinery, data presented in this paper do not identify the point at which the role of L-CaChs and calcium and agrin-MuSK signaling pathways converge to influence the aggregation of AChRs. If the two pathways do converge within the first few steps in the agrin-MuSK signaling cascade, there might be a direct relationship between MuSK activation and L-CaChs role in AChR clustering (Figure 6.1). For example, MuSK might influence the phosphorylation state of L-CaChs promoting L-CaCh activation and calcium influx resulting an increase in the frequency of AChR aggregation. Alternatively, if the two pathways do converge many steps downstream of MuSK activation and AChR β subunit phosphorylation, the two pathways would act in parallel to promote AChR aggregation during early signaling events (Figure 6.1). For example, MuSK signaling might promote initiation of clustering, and calcium flux through L-CaChs might promote later stages of clustering such as anchoring of
Figure 6.1. **Model of the role of L-CaChs in the signaling cascade behind AChR aggregation.** Although I have shown that calcium flux via L-CaChs delivers calcium to intracellular clustering machinery to influence the aggregation of AChRs, data presented in this paper do not establish the point of convergence between between the role of L-CaChs, calcium signaling and MuSK signaling cascades. There are several possibilities including: very early convergence (1), parallel pathways in which convergence occurs very late in the signaling cascade (2), and multiple points of convergence (early and late) along the signaling cascade leading to the aggregation of AChRs (3).
molecules to the cytoskeleton. Finally, convergence between the two pathways might occur at multiple levels along the signaling hierarchies behind AChR clustering (Figure 6.1). For example, MuSK might influence the phosphorylation state of L-CaChs promoting its activation while stimulating NOS-1 activation. Influx of calcium through L-CaChs might then promote further NOS-1 activity and activate CAM kinase through CAM. CAM kinase might then phosphorylate L-CaChs inducing further influx of calcium resulting in a positive feedback loop for calcium influx. NOS-1 and CAM kinase might then act further downstream to promote anchoring of molecules to the cytoskeletal membrane. Future research should focus on determining the specifics of the hierarchical positions of L-CaChs in the signaling cascade which results in the aggregation of postsynaptic molecules.

6.3 How might MuSK signaling regulate L-CaCh function?

The ability of L-CaCh activity to promote the clustering of AChRs downstream of MuSK activation suggests that in the presence of agrin, MuSK activation may regulate L-CaCh activity in some way. The classification of MuSK as a receptor tyrosine kinase suggests that the most likely mechanism by which it might regulate L-CaCh function would be via phosphorylation of the channel (Hopf and Hoch, 1998). This might occur through direct phosphorylation of L-CaCh by MuSK. Although the identities of molecules that directly interact with MuSK remain unknown, downstream events such as AChR phosphorylation, Src phosphorylation, and NOS activation imply their existence (Jones and Werle,
One of those molecules which interacts with MuSK could therefore be L-CaCh.

Alternatively, L-CaCh could be phosphorylated by cytosolic tyrosine or serine/threonine kinases downstream of MuSK. In skeletal muscle, two serine/threonine kinases, protein kinase A (PKA) and C (PKC), can regulate L-CaCh function through phosphorylation (Gutierrez et al., 1994; Johnson et al., 1997). For example, phosphorylation of the α1S subunit of L-CaChs by PKC promotes activation of the L-CaCh (Gutierrez et al., 1994).

Interestingly, PKC also plays a role in agrin induced AChR clustering. nPKC is the predominant isoform of PKC in skeletal muscle and is localized to the NMJ in vivo (Keef et al., 2001; Miles and Wagner, 2003). Overexpression of nPKC in myotubes inhibits agrin induced AChR clustering, indicating that nPKC activity inhibits MuSK signaling (Miles and Wagner, 2003). Conversely, these data also suggest that MuSK activation inhibits nPKC. Since L-CaCh activity is normally potentiated PKC and my data have shown that L-CaCh activation is required for agrin induced AChR clustering, it appears unlikely that PKC plays a role in the regulation of L-CaCh function by MuSK.

In contrast to PKC, PKA may be a likely downstream candidate for MuSK regulation of L-CaChs. PKA regulates L-CaCh function in multiple systems and even co-purifies with L-CaChs under certain conditions (Gray et al., 1997). Phosphorylation of serines in the α1S subunit of L-CaCh by PKA results in the potentiation of L-CaCh current (Chang et al., 1991; Mitterdorfer et al., 1996).
(Figure 6.2). As yet, it is not known whether PKA is involved in either MuSK signaling or AChR aggregation. However, preliminary data from our laboratory have shown that forskolin, which activates PKA by stimulating adenylyl cyclase, induces clustering of AChRs (data not shown), suggesting that PKA may indeed regulate AChR clustering. These data together with the major role that PKA plays in L-CaCh regulation suggest that future experiments should aim to determine whether or not MuSK regulation of L-CaChs might act via PKA.

In addition to serine/threonine kinases, protein tyrosine kinases have also been shown to regulate the activity of L-CaCh. Inhibition of tyrosine phosphatases or activation of Src increases L-type calcium current in vascular cells (Hu et al., 1998). Additionally, src becomes phosphorylated following agrin stimulation of MuSK, and plays a role in the maintenance of AChR clusters (Mohamed et al., 2001; Smith et al., 2001). Therefore, Src seems to be a reasonable candidate for mediating MuSK regulation of L-CaChs.

6.3.a Future Experiments:

As a first step in identifying a kinase or kinases that mediate MuSK regulation of L-CaCh, one could stimulate myotubes with agrin, and then assay for phosphorylation of serine, threonine, and tyrosine residues on the α subunit of L-CaCh. If one type of phosphorylation predominated, this would narrow the search to that class of kinases.

Once the class of kinase was determined, one could begin to narrow the identification of that kinase by using different selective pharmacological agents to
Figure 6.2. Regulation of L-CaCh by phosphorylation. Phosphorylation of L-CaCh by PKA, PKC, or Src promotes L-CaCh activation. In addition to the possibility of direct phosphorylation by MuSK, there is potential for MuSK to act to regulate L-CaCh activity via any of these three kinases.
inhibit that kinase. If inhibition of agrin induced phosphorylation of L-CaCh was observed, the identification of the phosphorylating kinase would be further narrowed.

6.3b How might MuSK regulation of L-CaCh be manifested?

If MuSK does regulate the function L-CaCh, the most likely manifestation of this regulation might be changes in the conductance properties of L-CaChs such as, open time probability, duration of activation, magnitude of current, etc. One way to test whether MuSK activation by agrin alters the functional properties of L-CaChs one might record electrophysiologically from myotubes and look for changes in the overall calcium current in the myotube. Since phosphorylation of L-CaChs often leads to potentiation of current, and since activation of L-CaChs promotes clustering of AChRs, if MuSK activation does influence L-CaCh function, it seems likely that agrin treatment might be expected to induce a calcium current whose characteristic shape (inward current that is slowly activating and inactivating) would identify it as that of an L-type calcium current. Indeed, preliminary experiments suggest this to be the case (A. Yool and H. Gordon, personal communication) (Gutierrez et al., 1994; Johnson et al., 1997).

6.4 Does L-CaCh mediation of agrin induced clustering require the influx of calcium?

In skeletal muscle, E-C coupling requires L-CaCh activation, but not the flow of calcium through L-CaChs. The conformational change in L-CaCh is sufficient to induce the release of calcium from intracellular stores (Moffett, 1993). Thus, in
skeletal muscle E-C coupling, L-CaCh acts as a voltage sensor rather than an ion channel. My data do not show whether calcium flux is required for AChR clustering, only that activation of L-CaCh is required.

6.4.a Future Experiments:

To test whether calcium flux is required for L-CaCh mediation of AChR clustering, cells could be treated with the open channel blocker of calcium channels, cadmium chloride, in the presence of agrin. In this case, extracellular calcium would be present, so agrin binding and activation of MuSK could be expected to occur normally. L-CaCh activation would also remain possible, but calcium flux would be prevented. In that case, inhibition of agrin induced clustering would suggest that L-CaCh activation but not calcium flux is required for agrin induced AChR clustering.

6.5 What is the source of the residual response to agrin in dysgenic myotubes?

As I mentioned in chapter 3, although the clustering response to agrin is dramatically reduced in myotubes from dysgenic mice, there is some residual response. The source of this residual response is unknown, but one possible source could be AChR activation. Indeed, calcium flux through AChRs is sufficient to induce contraction in dysgenic myotubes, and, as I have shown, AChR activity is sufficient to induce clustering of AChRs in C2C12 myotubes (Melliti et al., 1996). If the source of the residual response to agrin in dysgenic
myotubes were AChR activity, this might indicate that calcium flux through AChR is involved in AChR clustering.

Another possible source for the residual clustering response to agrin in dysgenic myotubes might be the activity of T-type calcium channels (T-CaCh, low voltage activated, fast inactivating). Indeed, in normal muscle, the T-type calcium channel (T-CaCh) is the major voltage gated calcium channel expressed in skeletal muscle prenatally and as such contributes a significant percent of the calcium flux required for muscle development in utero (Beam and Knudson, 1988a). T-CaCh expression begins to decrease postnatally and is virtually undetectable in adult skeletal muscle (Beam and Knudson, 1988b). Additionally, T-CaChs are also expressed and functional in dysgenic myotubes (Shimahara and Bournaud, 1991; Garcia and Beam, 1994). Thus, T-CaCh could provide sufficient calcium influx to partially compensate for the lack of L-CaChs in dysgenic myotubes and therefore be responsible for the residual agrin response observed in dysgenic samples.

6.5.a Future Experiments:

To test whether the residual response to agrin is due to AChR or T-CaCh activity, future experiments should therefore include treating wildtype and dysgenic myotubes with: 1) nicotine or with agrin and curare to test for the role of T-CaCh blocker amiloride in the presence of agrin to test for the role of T-CaCh (Tang et al., 1988). Based on data from the C2C12 cells, one would expect that nicotine would induce AChR clustering and curare would block agrin...
induced clustering in wildtype myotubes. If that were found to be the case, a lack of response to nicotine or a lack of inhibition of agrin induced clustering by curare in dysgenic myotubes would eliminate AChRs as the source of the residual response to agrin and would support the data from chapter 4 that suggested that AChRs influence their own clustering by influencing L-CaChs.

If inhibition of agrin induced clustering were observed in response to amiloride in both normal and dysgenic myotubes this would indicate that T-CaCh was involved in the agrin-MuSK signaling cascade and was responsible for the residual response to agrin in dysgenic myotubes. As compared to L-CaCh, the functional properties of T-CaCh are very different, thus, if T-CaCh were involved this would provide yet another level of activity related regulation of AChR clustering.

If both AChRs and T-CaChs were eliminated as the source of the residual response to agrin, this would support the existence of additional signaling pathways downstream of MuSK which regulates AChR clustering independent of or in parallel with calcium signaling. This separate pathway could act through any of the potential downstream mediators of MuSK signaling discussed below.

6.6 What are the identity and function of the downstream signaling mediators of MuSK?

Previous experiments, including my own, have focused on the identification of targets downstream of MuSK either very early or very late in the signaling cascade behind AChR clustering. For example, following agrin
treatment, activation of MuSK and tyrosine phosphorylation of AChRs occurs very rapidly, within minutes of treatment (Fuhrer et al., 1997). I have shown that the activation of neither AChRs nor L-CaCh causes AChR phosphorylation, suggesting that AChRs and L-CaChs are not involved in or act downstream of MuSK in the agrin-MuSK signaling cascade.

Since, AChR phosphorylation occurs within minutes of agrin treatment, it is a very early indicator of the signaling cascade downstream of MuSK and may influence anchoring of AChRs; perhaps AChR phosphorylation even initiates creation of a "nucleus" around which additional synaptic components might aggregate (Fuhrer et al., 1997; Borges and Ferns, 2001). Given that AChR phosphorylation occurs very early in the agrin-MuSK signaling cascade, the presence of AChR phosphorylation does not shed much light on later steps in the cascade which actually result in AChR aggregates.

Other research has focused on the very late endpoint of changes in the frequency of AChR clustering. For example, agrin induces AChR clustering (Nitkin and Rothschild, 1990). I have shown that activation of both AChRs and L-CaChs are required for agrin induced clustering. These data show that the endpoint, AChR clustering, requires the activity of AChRs and L-CaChs at some point downstream of MuSK in the processes behind the formation, growth, and maintenance of AChR clusters, but gives very little information regarding the point in those processes at which AChRs and L-CaChs might be important.
There are a few clues concerning downstream mediators between MuSK activation and AChR aggregation. For example, the Rho small GTPases become activated in the presence of agrin, and their activity can induce AChR clustering (Weston et al., 2000; Weston et al., 2003). Small GTPases can activate NOS in PC12 cells, and NOS acts downstream of MuSK to regulate both AChR phosphorylation and clustering (Jones and Werle, 2000; Luck et al., 2000; Schonhoff et al., 2001). Thus, Rho small GTPases could act downstream of MuSK and upstream of NOS to influence the clustering of AChRs.

Additionally, the cytosolic tyrosine kinase Src becomes phosphorylated following MuSK activation (Mohamed et al., 2001). Src can phosphorylate AChRs and has been shown to be involved in the maintenance of AChR clusters (Mohamed and Swope, 1999; Smith et al., 2001). Thus, Src family tyrosine kinases seem to act at a late stage in AChR clustering (Smith et al., 2001).

6.6.a Future Experiments:

Although a few downstream mediators of MuSK signaling have been identified, there is still much to be learned. Future experiments should aim to discover the shape of missing pieces and put the pieces of the puzzle together. In other words, the goal of future work should be to identify downstream signaling mediators of MuSK and to establish the order in which those mediators act in the pathway behind the clustering of AChRs in skeletal muscle. Identifying downstream mediators of MuSK activation should aid in the integration of our
understanding of the roles for calcium signaling, muscle activity, and anchoring of synaptic molecules in the formation of the NMJ.

Future experiments might begin the hunt by using proteomic methods to identify the proteins associated with activated MuSK. Proteomics include techniques in which a protein of interest is immuno-precipitated. Co-immunoprecipitated proteins are digested into peptides which are analyzed by mass spectrometry to determine their masses. These values are then compared with a database of protein sequences to deduce the amino acid sequences of the peptides. A comparison of the peptide complement of untreated and agrin treated cells might yield proteins complexed with MuSK specifically when activated by agrin. Once some members of a MuSK signaling complex are identified, the potential next steps will become more narrow. At that point, functional studies using pharmacological agents, biochemistry, and transfection systems can be undertaken to more firmly establish the roles for the identified proteins.

6.7 What is the role of phosphorylation of AChR in AChR clustering?

Activation of MuSK by agrin results in rapid phosphorylation of AChR β and δ subunits (Qu and Huganir, 1994; Glass et al., 1996; Glass et al., 1997; Mittaud et al., 2001; Mohamed et al., 2001). This phosphorylation may be involved in the regulation of anchoring of AChRs to protein complexes or cytoskeleton, and has been suggested by myself and others to regulate the efficiency with which AChRs are incorporated into AChR clusters (Meyer and Wallace, 1998; Borges
and Ferns, 2001). As yet, the identity of the phosphorylating enzyme remains unclear.

NOS can regulate AChR phosphorylation downstream of MuSK and seems to act at early stages in the normal MuSK signaling cascade (Jones and Werle, 2000; Luck et al., 2000; Ebert et al., 2003). Additionally, Src has been shown both to become phosphorylated in response to MuSK activation and to phosphorylate AChRs (Mohamed et al., 2001). The role of Src, however, seems to be in later stages in the signaling cascade behind AChR clustering, such as maintenance of clusters (Smith et al., 2001).

These data suggested to me that there may be both early and late stage phosphorylation of AChRs and, furthermore, that early and late stage phosphorylation of AChRs may be mediated by different enzymes and serve different purposes. For example, perhaps early phosphorylation of AChRs by one enzyme initiates clustering by promoting the formation of a nucleus for a protein complex. Perhaps later phosphorylation on AChRs by a second enzyme stabilizes a connection between that protein complex and cytoskeletal components.

Indeed, such a sequence of events may underlie the long time course for AChR phosphorylation observed in myotubes in culture. Peak phosphorylation of AChRs occurs 2 hr after MuSK activation, and is still observable at to 18 hr (Fuhrer et al., 1997). Perhaps the reason for the long time course is that multiple phosphorylation events are actually occurring simultaneously, but the “hand-off”
is seamless and as such is unobservable using simple biochemistry that has been used up to now (Figure 6.3). Furthermore, differential phosphorylation of the β and δ subunits of AChRs, if it could be achieved, could offer extremely fine control over whatever events are triggered by AChR phosphorylation, whether they be protein complex formation or anchoring of AChRs (Mittaud et al., 2001).

6.7.a Future Experiments:

Experiments on wildtype cells transfected with mutant β subunits of AChRs that lacked sites for tyrosine phosphorylation showed that AChR anchoring to protein complexes and the efficiency with which AChR was incorporated into clusters was decreased. The presence of endogenous AChR β subunits in those cells limits the conclusions that can be drawn from these experiments, however (Meyer and Wallace, 1998). Additionally, similarly mutated δ subunits have not yet been tested, and therefore the specific function of phosphorylation of AChR δ subunits is unknown.

In order to understand the functional significance of AChR phosphorylation in the signaling cascade behind AChR clustering, future experiments should express mutant (tyrosines mutated to phenylalanines) β, δ or both AChR subunits in skeletal muscle cell lines not expressing wildtype AChRs. Cultures of these transfected cells could then be analyzed for their clustering response to agrin treatment. If tyrosine phosphorylation on the β or δ subunit of AChR is necessary for AChR clustering, one or more of the mutant AChRs should not cluster in response to agrin.
Figure 6.3 Time course for AChR phosphorylation. Onset of AChR phosphorylation following agrin treatment occurs within minutes and peaks at 4-6 hr. and is still present at 18 hr. Perhaps this phosphorylation occurs in stages, early and late such that the peaks of these stages coincide and as such appear as one long time course for AChR phosphorylation.
In order to test the hypothesis that AChR phosphorylation occurs in stages, experiments should be aimed at separating the stages. Since NOS is likely to induce phosphorylation of AChRs early (within minutes), cells could be treated with inhibitors of NOS at different times after exposure to agrin, followed by a phosphorylation assay for AChRs. If inhibition of NOS changed the time to onset of AChR phosphorylation following agrin treatment from minutes to hours, this might indicate that NOS activation was required for an early stage of AChR phosphorylation and another kinase was involved in a later stage.

To examine the possibility that Src family kinases perform later stages of AChR phosphorylation, further experiments might make use of primary cultured myotubes from Src family knockout mice, including Src- and Yes- or Src- and FYK- knockout mice (Smith et al., 2001). Cultured cells could be treated with agrin for different amounts of time, followed by assays for phosphorylation of AChRs. If the time course for AChR phosphorylation was shortened, perhaps from several hours to a few minutes, this would suggest that the Src family kinases do indeed normally mediate late stages of AChR phosphorylation. A combination of the experiments with NOS and with Src family kinases would thus separate a two stage phosphorylation time course of AChRs downstream of MuSK activation.
6.8 What are the downstream signaling mediators of intracellular calcium?

Both extracellular and intracellular calcium are required for agrin induced clustering of AChRs. Extracellular calcium is required for agrin activation of MuSK (Nastuk et al., 1991; Borges et al., 2002). I have shown that L-CaChs act as a conduit for a rise in intracellular calcium due to a flux of calcium from outside the myotube membrane. None of these data, however, identify the downstream mediators of calcium’s promotion of AChR clustering.

Given that calcium is a major signaling mediator in skeletal muscle, there are several potential mediators for calcium’s influences on AChR clustering. One obvious potential target for calcium signaling is the calcium calmodulin/calmodulin dependent kinase (CAM-kinase) cascade. Calcium stimulates the release of the regulatory protein calmodulin from the cytosolic serine/threonine kinase CAM kinase, activating the enzyme. CAM kinase then autophosphorylates other molecules of CAM kinase to produce calcium independent CAM kinase. CAM kinase is eventually inactivated through the activity of a phosphatase. CAM kinase works through a variety of effectors and influences a wide range of cellular functions (Abraham et al., 1997; Lisman et al., 2002). Indeed, CAM kinase phosphorylation of L-CaCh results in facilitation of L-CaCh activation, with a prolonged activation (Dzhura et al., 2000; Wu et al., 2001). Given that L-CaCh activity is required for agrin induced AChR clustering, MuSK activity may promote activation of L-CaCh. This promotion of L-CaCh
activation could result in a rise in intracellular calcium and subsequent activation of CAM kinase. CAM kinase phosphorylation of L-CaCh might in turn promote further L-CaCh activation. Thus, activation of CAM kinase might provide a positive feedback loop whereby calcium influx could act in concert with MuSK signaling to promote the clustering of AChRs.

Another potential target for intracellular calcium signaling is NOS-1. NOS-1, the primary isoform of NOS expressed in skeletal muscle, is calcium dependent (Kone, 2000). As mentioned above, NOS-1 is required for both clustering of AChRs and phosphorylation of the AChR β subunit (Jones and Werle, 2000; Luck et al., 2000; Ebert et al., 2003). Thus, MuSK might promote L-CaCh and NOS-1 activation in parallel. Calcium influx via L-CaChs might then prolong the activation of NOS-1, thereby promoting the clustering of AChRs. Indeed, I have shown that L-CaCh activity is required for calcium induced AChR phosphorylation but not MuSK activation, suggesting that L-CaCh activity regulates a downstream target of MuSK that is upstream of AChR. I hypothesize that that effector is NOS-1.

6.8.a Future Experiments:

To determine the role, if any, of CAM kinase downstream of influx of calcium via L-CaChs, cells might be treated with S(-)BAYK8644 and then tested for activation of CAM kinase using a serine-threonine phosphorylation assay. If an increase in phosphorylation of CAM kinase was observed in response to S(-)BAYK8644, this would indicate that calcium flux through L-CaCh was sufficient to
activate CAM kinase but would not illustrate a role for CAM kinase in AChR clustering.

Transfection of cells with a kinase-dead form of CAM kinase could be used to determine if CAM kinase is required in AChR clustering. Given that the activation of CAM kinase involves both calcium and autophosphorylation, a kinase-dead CAM kinase would be expected to act as a dominant negative to inhibit all CAM kinase activity in transfected cells. The cells might then be treated with S(-)BAYK8644, and a clustering assay performed. A lack of clustering response to S(-)BAYK8644 would indicate that CAM kinase is required for L-CaCh activity induced AChR clustering, possibly as a downstream mediator of the calcium signaling cascade which results in the clustering of AChRs.

I have shown that L-CaCh activity regulates calcium induced AChR β subunit phosphorylation but not calcium induced MuSK activation. However, since L-CaCh activation was not sufficient to cause AChR phosphorylation (see chapter 4), in chapter 5 I suggested that the signaling mediator responsible for AChR β subunit phosphorylation must be first activated by another signaling event, perhaps by MuSK to be modulated by L-CaCh activity. I hypothesize that the mystery effector is NOS-1.

To determine whether calcium flux via L-CaCh is necessary for NOS-1 mediated clustering of AChRs, cells might be treated with the NOS substrate SNAP in the presence of the L-CaCh blocker nifedipine, followed by an assay for AChR clustering. Inhibition of NOS induced AChR clustering by nifedipine would
indicate that L-CaCh activation was required for NOS induced AChR clustering, and would suggest that calcium flux via L-CaCh acts to modulate NOS-1 downstream of MuSK.

To determine whether L-CaCh activity was necessary for NOS-1 induced AChR phosphorylation, cells might again be treated with the NOS substrate in the presence of nifedipine, followed by an assay for AChR phosphorylation. Inhibition by nifedipine would indicate that L-CaCh activity is required for NOS-1 induced AChR phosphorylation and would confirm that calcium flux via L-CaCh acts downstream of MuSK to regulate NOS-1 activity.

6.9 Conclusions

Figure 6.4 shows a model of potential signaling events that regulate the aggregation of AChRs. The model includes both known mediators of the Agrin-MuSK signaling cascade and hypothesized downstream mediators of calcium signaling discussed in this chapter.

Previous research has lead to the current understanding that release of neurotransmitters and neurotrophic factors from the motor neuron act in parallel to regulate synaptogenesis at the NMJ. Data presented in this dissertation expand that understanding to suggest that the integration of signaling due to neuromuscular transmission, calcium signaling, and kinase cascades are required for the proper formation of the NMJ. Given the similarities in the development of the NMJ with neuronal synapses in the brain, data presented
Figure 6.4 Summary model of known and hypothesized signaling cascades that regulate AChR clustering.
herein combined with the proposed experiments could also have a more general value to understanding synaptogenesis throughout the nervous system. Thus, the data presented in this dissertation could provide a significant contribution to our understanding of neurobiology. The End.
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