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Characterization of the Molecular Mechanisms Regulating the Agrin Signaling Pathway: a Dissertation

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A Dissertation Presented

by

LAURA JALSO MEGEATH

Submitted to the Faculty of the
University of Massachusetts Graduate School for Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY

October 4, 1999

Cell Biology
CHARACTERIZATION OF THE MOLECULAR MECHANISMS
REGULATING THE AGRIN SIGNALING PATHWAY

A Dissertation Presented

By

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October 4, 1999
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ABSTRACT

The nervous system requires rapid, efficient, and accurate transmission between cells for proper functioning. Synapses are the predominant structures through which such vital communication occurs. How synapses are formed, maintained, and eliminated are questions of fundamental importance.

At the nerve-muscle synapse, formation of the postsynaptic apparatus is directed by agrin. The hallmark activity of agrin is the aggregation of acetylcholine receptors (AChRs) into dense clusters opposite the presynaptic nerve terminal. Early events in the agrin signal transduction cascade include activation of the receptor tyrosine kinase MuSK and tyrosine phosphorylation of AChRs, but how these events lead to AChR cluster formation is unknown. Using the calcium buffer BAPTA, we demonstrate that intracellular calcium fluxes are necessary for agrin-induced formation of AChR clusters. However, clamping calcium fluxes before agrin stimulation does not alter agrin-induced phosphorylation of either MuSK or AChRs, indicating that this calcium-dependent step occurs downstream of both MuSK and AChR phosphorylation. These results identify a new step in the agrin signaling pathway required for the formation of AChR clusters.

We show that intracellular calcium fluxes also play an important role in stabilizing AChR clusters. Clamping intracellular calcium fluxes results in rapid dispersal of AChR clusters and dephosphorylation of both MuSK and AChRs, even if agrin is continually present. Furthermore, the protein tyrosine phosphatase inhibitor pervanadate inhibits both the dispersal and dephosphorylation, indicating a role for a
tyrosine phosphatase in AChR cluster dispersal. Our data indicate that AChR clusters are maintained by agrin/MuSK-induced intracellular calcium fluxes that tonically inhibit a tyrosine phosphatase localized to AChR clusters. Our findings also show that distinct molecular mechanisms mediate the formation and the dispersal of agrin-induced AChR clusters.

The work presented here expands our understanding of synaptic differentiation in several ways. First, I characterized a new, calcium-dependent step required for the formation of agrin-induced AChR clusters. Next, I showed that postsynaptic specializations must be actively maintained, and describe a molecular mechanism that stabilizes AChR clusters. Finally, dispersal and formation of AChR clusters occurs by distinct pathways. Our understanding of the mechanisms regulating the formation and modulation of synapses will help us to better understand how the nervous system develops and responds to the world around us.
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CHAPTER I
INTRODUCTION

From the perception of light to the development of abstract ideas, the nervous system is responsible for an enormous range of functions. Vital for all of these functions is an intricate network of communication between approximately $10^{11}$ neurons in the brain (Kandel et al., 1991) and a multitude of additional neurons in the peripheral nervous system (PNS). Auxiliary sensory and effector cells, such as the eye’s rods and the muscle’s myofibers, are wired into this network. Communication between these cells must be rapid, efficient, and precise. Extensive and specific neural circuitry is established before birth, and throughout life this circuitry is continually developed and refined.

Each connection, or synapse, is a specialized structure through which information is transmitted from one cell to another. Both presynaptic and postsynaptic cells contain numerous molecules dedicated to the sending and receiving of impulses. The substantial body of research dedicated to the study of synapse formation, maintenance, modulation, and elimination is indicative of their fundamental importance to our understanding of the nervous system.

The arrangement of synaptic connections is not a hard-wired circuit and is subject to many modifications. Synapse formation begins early in development and continues into postnatal life. There is also a period after birth in which numerous synapses are disassembled (Nguyen and Lichtman, 1996). Several factors, particularly synaptic
activity, determine whether an individual synapse is sustained or eliminated. Connectivity within the nervous system is therefore the product of the formation and elimination of synapses. Those synapses that are maintained are not rigid structures; rather electrical activity continually sculpts synaptic architecture. The term synaptic plasticity refers to this feature of neuronal connections whereby long term changes in transmission can be made. To learn about these processes, the vertebrate skeletal neuromuscular junction (NMJ) has proved to be an informative model synapse.

**Structure of the NMJ**

Every NMJ involves a motor neuron, a Schwann cell, and a muscle cell (Fig. 1-1). Each of these three cells contains specialized molecules and organelles localized to the synaptic site and will be described in turn. Surrounding these cells and interposed between them is the extracellular matrix or basal lamina, which is also specialized at synapses.

Motor neurons receive impulses from descending tracts of the spinal cord and transmit these signals to muscle fibers. At the final tip of the motor neuron is a presynaptic terminal or bouton, filled with characteristic active zones and synaptic vesicle arrays. The numerous synaptic vesicles can rapidly release acetylcholine (ACh) into the synaptic cleft. Molecules necessary for synaptic transmission are enriched in presynaptic terminals, including calcium (Robitaille et al., 1990) and potassium (Robitaille et al., 1993b) channels, choline acetyltransferase (for synthesis of ACh) (Kan and Chao, 1981), and synaptic vesicle associated proteins (Calakos and Scheller, 1996). In mature animals,
one motor neuron can innervate multiple muscle fibers though each muscle fiber is innervated by just one motor neuron. A neuron and those muscle fibers it innervates together constitute a motor unit.

Another important component of NMJs is the Schwann cells. Schwann cells myelinate neuronal processes and surround both the presynaptic terminal and the accompanying postsynaptic membrane. These cells modulate synaptic efficacy (Georgiou et al., 1999) and are also believed to play a vital role in regeneration at the NMJ of the adult (Fu and Gordon, 1997; O’Malley et al., 1999).

Schwann cells are derived from crest cells and commitment to a glial fate appears to be influenced by neuronally synthesized neuregulins (Marchionni et al., 1993; Shah et al., 1994), though some crest cells express the Schwann cell-specific P0 mRNA and protein before encountering neurons (Bhattacharyya et al., 1991; Zhang et al., 1995b). Neurons supply mitogenic signals to Schwann cells and also regulate programmed cell death in vitro (Dong et al., 1995; Morrissey et al., 1995). After neurons provide trophic support for Schwann cells during the early stages of development, Schwann cells in turn provide support for neurons. During the period of motor neuron cell death, Schwann cells express high levels of glial derived neuron survival factor (GDNF) (Henderson et al., 1994), as yet the most potent neuronal survival factor known.

Ultimately three or four Schwann cells surround the motor neuron terminal and muscle fiber end plate at mature NMJs (Hirata et al., 1997). Terminal or perisynaptic Schwann cells are specialized and do not form the myelin sheaths. Instead, these
perisynaptic cells express other molecules such as N-CAM and agrin (a non-clustering isoform, see below) (Martini et al., 1994; Astrow et al., 1998; Werle et al., 1999).

Extracellular matrix (ECM) is a network of secreted extracellular macromolecules that surrounds cells in most tissues (Alberts et al., 1994). The ECM aids processes such as cell migration, cell-cell interactions and adhesion. Regions of specialized ECM called basal lamina are associated with muscle fibers, epithelial, fat, and Schwann cells. Basal lamina is found in the synaptic cleft between motor neurons and muscle fibers and reaches into postjunctional folds of the myotube. Specific isoforms of collagen, laminin and entactin are associated preferentially with synaptic basal lamina (Sanes and Lichtman, 1999). Acetylcholinesterase (McMahan et al., 1978), synapse-specific carbohydrate bearing molecules (Scott et al., 1988), neuregulin, and agrin are also associated with the basal lamina specifically at NMJs. The association of neuregulin and agrin with basal lamina may assist their functions in formation, maintenance, and regeneration of the NMJ.

Muscle cells are derived from cells in the somites that become committed to a muscle precursor phenotype (Zigmond et al., 1999). These cells migrate and proliferate into mononucleated cells called myoblasts that synthesize muscle-specific proteins. Myoblasts then fuse together and become multi-nucleated myotubes, and after this step innervation occurs. Maturation of myotubes involves movement of nuclei from the center to the periphery of the cells, after which the cells are referred to as muscle fibers or myofibers (Sanes and Lichtman, 1999). Within each muscle fiber are numerous myofibrils that appear as long parallel threads. These myofibrils are further subdivided into sarcomeres,
longitudinal arrays of thick myosin and thin actin fibers that ratchet into more compact arrangements during contraction of the muscle.

The specialized area of the muscle fiber directly beneath the nerve terminal is referred to as the motor end plate (Fig. 1-2). A cardinal feature of this region is the high density of AChRs: as many as 1000 fold more than are found in extrasynaptic regions (Fertuck and Salpeter, 1976). These AChRs are ligand-gated ion channels that bind ACh released from the nerve terminal. ACh binding induces opening of the channel and rapidly initiates contraction of the myofiber. Ultrastructural analysis of AChR distribution revealed that these receptors are positioned on the crests of junctional folds, which are ~1 micron deep (Matthews-Bellinger and Salpeter, 1978). The bottom portions of these folds are enriched in sodium channels (Flucher and Daniels, 1989) and neural cell adhesion molecule (N-CAM) (Covault and Sanes, 1986). The cytoskeleton is also specialized at the NMJ. Rapsyn (Sealock et al., 1984), α-dystrobrevin-1, and utrophin (Peters et al., 1998) are found beneath the folds’ crests and ankryin (Flucher and Daniels, 1989), β-spectrin (Wood and Slater, 1998), α-dystrobrevin-2 and dystrophin (Peters et al., 1998) are below the troughs.

Though the NMJ is similar to other synapses, there are unique characteristics that make it the most accessible synapse for study. Only one neurotransmitter, ACh, and one neurotransmitter receptor, the AChR, is found at the NMJ. Unlike the multiply innervated neurons in the CNS, each mature myofiber is innervated by just one motor neuron. During development numerous NMJs are formed and eliminated, and these processes can be reproduced in adult animals by denervation of muscles. A valuable
reagent for studying NMJs is α-bungarotoxin (BTx), a toxin derived from venom of the snake *Bungarus coeruleus*. Since BTx binds virtually irreversibly to AChRs, it has made detailed studies of AChRs possible.

Contact between a motor neuron and myotube in the early stages of development initiates synaptic differentiation. Biochemical and morphological changes occur in both pre- and postsynaptic cells. In the myotube, transcription is altered, synaptic molecules aggregate, nuclei accumulate below the synaptic area, and postjunctional folds form. Though AChRs are expressed on the myotube surface prior to innervation, and some receptors do form spontaneous clusters or “hot spots” (Frank and Fischbach, 1979), the pattern of expression changes dramatically following neuronal contact. Local transcription of AChRs is increased, extra-synaptic transcription is depressed, and previously inserted AChRs are translocated to the NMJ site. Directing these changes are the molecules agrin and ARIA. The signaling pathways through which agrin and ARIA affect synaptic differentiation have been partially characterized.

**Synaptic Differentiation**

**the Agrin Signaling Pathway**

Agrin is the factor responsible for the aggregation of AChRs and other synaptic molecules at the NMJ. It was first identified and purified by McMahan and colleagues from Torpedo electric organ, a tissue rich in synaptic components (Godfrey et al., 1984; Fallon et al., 1985; Nitkin et al., 1987). Secreted from motor neuron terminals, agrin becomes stably associated with synaptic basal lamina (Reist et al., 1987) and can be
detected at the earliest NMJs (Fallon and Gelfman, 1989). This pattern of expression and its AChR clustering activity in culture assays lead to the formulation of the “agrin hypothesis”: neuronally secreted agrin directs postsynaptic differentiation (McMahan, 1990). The sequence of events initiated by agrin binding to myotubes is referred to as the agrin signaling pathway (Fig. 1-3).

Structural analysis revealed that agrin is a >400 kD heparan sulfate proteoglycan with a protein core of ~200-250 kD (Tsen et al., 1995). The amino terminal half of the molecule is necessary for secretion and laminin binding, and contains three sites for the addition of heparin sulfate side chains (Fig. 1-4) (Denzer et al., 1995). The carboxy terminal half of agrin contains both the α-dystroglycan binding domain and AChR clustering activity.

Agrin is spliced at sites that are referred to as the γ and ζ sites in mammals and A and B in chick. Agrin isoforms differ in their biological activity and in the cell type that synthesize them. A four amino acid insert at the ‘γ’ site (agrin4,4) is necessary for agrin to interact with heparin (Gesemann et al., 1996; O’Toole et al., 1996). The ‘ζ’ splice site can include inserts of eight, eleven, or nineteen amino acids. Isoforms with these inserts are expressed exclusively in neurons, hence the term “neuronal agrin”, and have the highest AChR-clustering activity. Myotubes and Schwann cells synthesize agrin4,0 (muscle agrin) and agrin0,0, both of which are considerably less effective at clustering AChRs than agrin4,8 (Burgess et al., 1999; Werle et al., 1999). Agrin4,8 was used in the work presented in Chapters II and III.
Agrin-deficient mice provided strong support for agrin's role in NMJ formation (Gautam et al., 1996). These animals are unable to synthesize any neuronal agrin and express very low levels of all other isoforms. Agrin-deficient mice die before birth and display four major defects. (1) Postsynaptic differentiation is disrupted. AChRs do aggregate, though the clusters are rare and fail to achieve normal size or density. The presence of such rudimentary AChR clusters indicates that another molecule – perhaps muscle agrin or laminin – is capable of compensating and triggering AChR cluster formation. (2) Synapse-specific molecules, including AChRs, cluster at extrasynaptic sites. Aneural AChR aggregates are frequently observed in culture but rarely in vivo unless synaptic activity is blocked. Insufficient synaptic activity in these embryos may therefore account for this observation. (3) Presynaptic differentiation is abnormal. In these mice, motor neurons extend unusually long processes parallel to muscle fibers, fewer neuronal branches are observed, and no arbors are observed at their tips. Some presynaptic specializations were observed, including the accumulation of synaptic vesicles at sites of nerve-muscle contacts, but such specializations were quite rare in comparison to littermate controls. (4) Less specialization is observed in the subsynaptic nuclei of agrin-deficient mice. In normal mice, transcription in the subsynaptic nuclei is different from that in non-synaptic nuclei. Since neuregulins have been shown to regulate synaptic transcription, it has been proposed that agrin anchors neuregulins in the ECM (Sanes, 1997). In support of this model, ectopically expressed agrin has been observed to cluster neuregulins extrasynaptically (Meier et al., 1998).
In addition to triggering synaptic differentiation at the NMJ, agrin may have additional functions in the peripheral nervous system. In coculture experiments, agrin is an adhesive substrate for motor neurons and inhibits neurite outgrowth (Campagna et al., 1995; Chang et al., 1997; Halfter et al., 1997). Presynaptic differentiation is induced by both neuronal and muscle agrin in culture as determined by accumulation of the synaptic vesicle marker synaptogamin (Campagna et al., 1995; Campagna et al., 1997). Additional work is needed to clarify the role agrin isoforms play in neuronal outgrowth and differentiation.

Although agrin is also expressed in the central nervous system (CNS) (Ma et al., 1995; Escher et al., 1996; Cohen et al., 1997), its function there is unclear. Agrin expression is regulated during development and increased expression does correspond to periods of synaptogenesis (Li et al., 1997). However, agrin does not appear to be necessary for synaptogenesis in cultured CNS neurons (Li et al., 1999; Serpinskaya et al., 1999). Other studies hint at a more complex role for agrin within the CNS in normal and pathological states. Lesion-induced seizures altered agrin mRNA levels without corresponding changes in agrin protein levels, suggesting that transcription of agrin is regulated by activity (O'Connor et al., 1995). In brain tissue from patients with Alzheimer's Disease, agrin is concentrated in neuritic plaques and neurofibrillary tangles (Donahue et al., 1999). The association of agrin with β-amyloid suggests that agrin may be involved in the formation of amyloid plaques characteristic of Alzheimer's Disease. Recently, neuronal agrin was found to specifically induce CREB phosphorylation in
hippocampal neurons (Ji et al., 1998), so further investigation may reveal a role for agrin in the regulation of CNS gene expression.

The signaling component of the agrin receptor complex is MuSK (Muscle Specific Kinase) (Valenzuela et al., 1995; Glass et al., 1996). MuSK, a receptor tyrosine kinase, is expressed specifically in muscle and is localized to the motor endplate by birth (Valenzuela et al., 1995). From the earliest observable AChR clusters and throughout development, MuSK colocalizes with AChRs (Valenzuela et al., 1995; Bowen et al., 1998). In normal muscle, MuSK mRNA is preferentially expressed in subsynaptic nuclei similarly to the expression of AChRs. Upon denervation, physical immobilization, or block of electrical activity (via application of tetrodotoxin), extrajunctional expression of MuSK is upregulated until normal synaptic activity resumes (Bowen et al., 1998).

Several lines of evidence indicate that MuSK activation is both necessary and an early event in the agrin signaling pathway. Agrin rapidly activates MuSK both in culture and in denervated skeletal muscle (DiStefano et al., 1996; Glass et al., 1996). Agrin causes MuSK to co-aggregate with AChRs (Bowen et al., 1998), and the ability of different agrin isoforms to induce MuSK phosphorylation closely parallel the isoforms' AChR clustering activities (Hopf and Hoch, 1998b). Antibody-induced dimerization of MuSK results in activation of MuSK and both clustering and tyrosine phosphorylation of AChRs (Xie et al., 1997; Hopf and Hoch, 1998a). Constitutively active MuSK constructs induce clustering of MuSK with other elements of the postsynaptic apparatus, including AChRs, laminin, sodium channels, β-dystroglycan, neuregulins, and erbB3 (a neuregulin receptor) (Jones et al., 1999). Interestingly, studies with MuSK-TrkC chimeras
demonstrated that different domains of MuSK are responsible for activating different branches of the agrin signaling pathway. The intracellular kinase domain is necessary for agrin-induced tyrosine phosphorylation of AChRs, and the extracellular domain is both necessary and sufficient for the interaction of MuSK with rapsyn, an intracellular molecule necessary for agrin-induced AChR clustering (Gautam et al., 1995; Apel et al., 1997; Glass et al., 1997). Discovery of a new MuSK transcript expressed in *Xenopus* CNS has lead to speculation that MuSK may also play a synaptogenic role in the CNS (Fu et al., 1999).

Investigation of MuSK deficient mice has provided further insights into its physiological role (DeChiara et al., 1996). Though the gross morphology of the skeletal muscles appears normal, these mice asphyxiate rapidly after birth because they lack NMJs. Expression of agrin is normal (Ruegg and Bixby, 1998) but no AChR clusters are observed (DeChiara et al., 1996). Other molecules normally clustered in the postsynaptic apparatus -AChE, ErbB4, utrophin, and rapsyn- are distributed throughout the myofibers. In addition, transcription specific to subsynaptic nuclei is disrupted and AChR genes are uniformly expressed in MuSK-/− myofibers. Presynaptic defects are also observed; motor neuron terminals are unusually long and fail to form arbors, as if the neurons had been searching for a missing stop signal. Finally, myotubes cultured from MuSK mutant mice do not phosphorylate or cluster AChRs in response to agrin (Glass et al., 1997).

Together these data indicate that MuSK is an early and necessary component of the agrin signaling pathway. However, agrin does not bind directly to MuSK. To account for this, a coreceptor has been proposed that forms a complex with MuSK and
that is necessary for agrin binding (Glass et al., 1996). This coreceptor is not dystroglycan since agrin can induce MuSK phosphorylation in α-dystroglycan-deficient myotubes (Jacobson et al., 1998). Furthermore, because agrin will activate MuSK in myotubes but not in myoblasts, this coreceptor is deduced to be developmentally regulated and present only in myotubes. The coreceptor MASC, or the Myotube Associated Specificity Component, remains to be identified (Glass et al., 1996).

MuSK may be the first molecule of the postsynaptic apparatus to be clustered and thus serve as a primary scaffold for the subsequent addition of other molecules. The evidence for this is derived from three observations. (1) In myotubes, all spontaneous and agrin-induce AChR clusters are associated with MuSK (Bowen et al., 1998). (2) Not all MuSK clusters have associated AChRs. Approximately 20% of all MuSK clusters are observed without corresponding AChR aggregates. (3) Mice that lack rapsyn, an intracellular molecule necessary for AChR aggregation, have no AChR clusters but do display MuSK clusters on their myofibers (Apel et al., 1997). MASC is likely to be part of this primary scaffold as well since agrin-induced MuSK activation occurs within 2 minutes, too rapidly to suggest MASC recruitment to a pre-existing MuSK-only scaffold.

Following MuSK activation, the next known step in the agrin signaling pathway is the phosphorylation of AChRs. First described by Wallace (Wallace et al., 1991), subunits γ and δ are phosphorylated on serine residues and the β subunit is phosphorylated on tyrosine residues. Since blocking phosphorylation of γ and δ subunits with the serine kinase inhibitor H-7 did not alter agrin-induced clustering, these serine phosphorylation events are not necessary for AChR clustering. Inhibiting both serine and
tyrosine kinases with staurosporine did prevent AChR clustering (Wallace, 1994). This result spurred further work to determine whether tyrosine phosphorylation of AChRs was necessary for AChR cluster formation.

Several early lines of evidence indicated that tyrosine phosphorylation of AChRs was necessary for cluster formation. Both agrin-induced AChR phosphorylation and clustering are dose-dependent, staurosporine (a serine and tyrosine kinase antagonist) inhibits both phosphorylation and clustering, and agrin withdrawal causes both receptor phosphorylation and the number of clusters to decrease (Wallace, 1992; Wallace, 1994). In addition, incubating cells with agrin slowed AChR solubilization by detergent extraction, and the degree of receptor extractability correlated with levels of AChR phosphorylation. Herbimycin A, a Src kinase inhibitor, inhibits both phosphorylation and extraction of AChRs (Swope et al., 1999). In other experiments, the mobility of AChRs in the plasma membrane was measured by fluorescence recovery after photobleaching (Meier et al., 1995). The tyrosine phosphatase inhibitor pervanadate decreased the mobility of unclustered AChRs by ~40% while increasing phosphorylation of the receptors. The model to emerge from these data suggested that tyrosine phosphorylation of AChRs served to anchor the receptors to the cytoskeleton.

However, recent evidence suggests that tyrosine phosphorylation of AChRs is neither necessary nor sufficient for agrin-induced AChR clustering. The most compelling evidence is from experiments in which phosphorylation of AChRs was prevented by substituting phenylalanine for each tyrosine residue in the cytoplasmic domain of the AChR β subunit (Meyer and Wallace, 1998). When these mutant receptors were
expressed in myotubes, agrin stimulation still resulted in the formation of aggregates that included these mutant receptors (though endogenous receptors were still present). AChR clustering without phosphorylation occurs in other contexts as well. In developing Torpedo electrocytes, tyrosine phosphorylation of the AChR β and δ subunits occurs at a late stage of embryonic development, well after the accumulation of AChRs and rapsyn in the membrane and the onset of innervation (Camus et al., 1999). In addition, agrin-induced AChR clustering, but not phosphorylation, is inhibited by heparin (Wallace, 1990; Hopf and Hoch, 1998a). Finally, laminin treatment (in the absence of agrin) induces receptor clustering without concomitant phosphorylation (Sugiyama et al., 1997).

It is also possible to induce the phosphorylation of AChRs without receptor aggregation. A chimera was constructed of the extracellular domain of Trk C and the intracellular domain of MuSK. Activation of this chimera with the Trk C ligand neurotrophin 3 resulted in AChR phosphorylation but not aggregation (Glass et al., 1997). We also observed AChR phosphorylation without aggregation. By clamping intracellular calcium fluxes, agrin-induced AChR cluster formation was inhibited but MuSK and AChR phosphorylation were unaffected (Chapters II and III). These results indicate that neither MuSK activation nor AChR phosphorylation is sufficient for AChR aggregation. Current models place tyrosine phosphorylation of AChRs as either a nonessential event in the pathway leading to cluster formation or as a different branch of the agrin signaling pathway.

Although the function of agrin-induced AChR β subunit tyrosine phosphorylation remains to be determined, roles have been discovered for agrin-independent
phosphorylation of the other subunits. Phosphorylation of gamma and delta subunits increases the rate of receptor desensitization (Huganir et al., 1986; Hoffman et al., 1994). Gamma subunit phosphorylation is linked to receptor assembly (Green et al., 1991) and mediates binding to the adapter protein Grb2 (Colledge and Froehner, 1997).

Additional efforts to understand the role of AChR phosphorylation in the agrin signaling pathway have focused upon the kinase(s) that might be activated by agrin. Though agrin-activated MuSK has tyrosine kinase activity, MuSK is unlikely to phosphorylate AChRs directly. MuSK can be activated without AChRs becoming phosphorylated (Fuhrer et al., 1999). The one group to successfully copurify MuSK with AChRs estimates that only 2% of total cellular MuSK is associated with AChRs (Fuhrer et al., 1997). This group also finds that MuSK and AChR phosphorylation can be differentially regulated with staurosporine. Other candidate tyrosine kinases have been tested in this system. Fyn and fyk are tyrosine kinases purified from Torpedo electric organ that coimmunoprecipitate with AChRs and phosphorylate AChRs in vitro (Swope and Huganir, 1993). Src also co-purifies with AChRs (Fuhrer and Hall, 1996). Src was shown to phosphorylate glutathione S-transferase fusion proteins containing the N-terminal half of the cytoplasmic loop of the AChR β subunit. Though the AChR may be a substrate of fyn, fyk, and src, there is as yet no evidence of agrin regulating the activity of these kinases.

One essential element of the agrin signaling pathway is rapsyn, also known as the 43 kD protein. Rapsyn is colocalized with AChRs at the earliest clusters observed in vivo (Noakes et al., 1993). Rapsyn associates with AChRs in an agrin-independent fashion
(Fuhrer et al., 1999) and is necessary for the formation of AChR clusters in several circumstances. When expressed in heterologous cells such as fibroblasts, QT-6 cells, or *Xenopus* oocytes, rapsyn and AChRs will cluster spontaneously with or without MuSK present (Froehner et al., 1990; Phillips et al., 1991; Dai et al., 1996; Apel et al., 1997). Expression of AChRs with rapsyn in heterologous cells also leads to the tyrosine phosphorylation of AChRs, though this phosphorylation is not required for AChR-rapsyn interactions (Qu et al., 1996). Studies with the plant lectin VVA-B4 have demonstrated that rapsyn’s role is not limited to the agrin signaling pathway. Stimulating myotubes with VVA-B4 instead of agrin will cluster AChRs (Martin and Sanes, 1995). VVA-B4 can induce AChR clusters in MuSK- or agrin-deficient myotubes, but not in cells lacking rapsyn (Gautam et al., 1999). Together these studies show that rapsyn participates in the AChRs clustering in multiple cell types and contexts.

Studies of transgenic mice lacking rapsyn provided further compelling evidence that rapsyn is necessary for the clustering of AChRs (Gautam et al., 1995). These mice have normal muscles and myotubes, but like the MuSK mutant mice, die soon after birth because of abnormal NMJs. In contrast to the MuSK deficient mice, which had virtually no molecular components of NMJs assembled, the rapsyn mice have incomplete nerve-muscle synapses. Immunostaining revealed localization of MuSK, laminin, and the synaptic vesicle protein SV2 at their NMJs (Gautam et al., 1995; Apel et al., 1997). Missing from these synapses were AChRs, utrophin, acetylcholinesterase (AChE), syntrophin, α- and β-dystroglycan, and erb B3 and 4 (Gautam et al., 1995; Moscoso et al., 1995). Interestingly, the total number of AChRs in rapsyn-deficient myotubes was
increased approximately 60% compared to controls. Since synaptic activity normally suppresses synthesis of AChRs (Hall and Sanes, 1993) and rapsyn deficient mice have difficulty moving, Gautam et al. (1995) suggest that reduced fetal movements caused this over-abundance of AChRs.

Studies of myotubes cultured from rapsyn deficient mice contributed further insights into the agrin signaling pathway. These myotubes did not form AChR clusters either spontaneously or in response to agrin, confirming in culture that rapsyn is necessary for AChR cluster formation (Gautam et al., 1995). When rapsyn deficient cells were stimulated with agrin, phosphorylation of MuSK but not AChRs was observed (Apel et al., 1997).

Presynaptic differentiation is also disrupted in rapsyn knock-out mice (Gautam et al., 1995). Motor neurons do form simplified terminals but lack terminal arbors. The branching pattern is more extensive than observed in neurons of control mice. As noted previously, neuronal branching was absent in muscles of MuSK-deficient mice, whose motor neurons tended to extend longer distances with fewer branches, indicating that both MuSK and rapsyn influence presynaptic differentiation though perhaps by different mechanisms.

Rapsyn has other functions in addition to its role in AChR aggregation. It is necessary for associations between AChRs, dystroglycan, and utrophin (Fuhrer et al., 1999). Rapsyn binds β-dystroglycan (Cartaud et al., 1998) and interacts with MuSK indirectly (Apel et al., 1997). Rapsyn-MuSK interactions were investigated using MuSK chimeras, and surprisingly an extracellular domain of MuSK was found to be necessary
and sufficient for the two molecules to cocluster (Apel et al., 1997; Zhou et al., 1999). Since rapsyn is entirely intracellular, this finding led to the proposal that a connecting molecule links the extracellular domain of MuSK to rapsyn. This connecting molecule was dubbed RATL for rapsyn associated transmembrane linker. It has not been unidentified.

Agrin can bind to a number of other molecules not directly implicated in the agrin signaling pathway. These molecules include heparin-binding growth-associated molecular (HB-GAM) (Daggett et al., 1996), midkine (Zhou et al., 1997), NCAM (Burg et al., 1995; Storms et al., 1996), FGF-2, thrombospondin, merosin, laminin, and tenascin (Cotman et al., 1999). The functions of most of these interactions remain to be determined, but none are essential for NMJ formation in vivo. FGF, tenascin, and NCAM knockout mice have relatively normal NMJs, indicating that the interaction of agrin with these molecules is not necessary for NMJ development (Moscoso et al., 1998). Some of these molecules, such as HB-GAM, midkine, and laminin, appear to supplement or change the pattern of agrin induced AChR clusters (Daggett et al., 1996; Sugiyama et al., 1997; Zhou et al., 1997). Laminin binding has also been suggested to anchor agrin to the basal lamina (Denzer et al., 1997). Though not necessary for the formation of agrin-induced AChR clusters, these interactions may modulate agrin signaling.

Several studies indicate that integrins are involved in AChR clustering. Integrins bind the G domain of laminin and a similar domain in agrin suggests an interaction may be possible (Bowe and Fallon, 1995). The integrins α7A, α7B, and α1 are enriched at NMJs (Martin et al., 1996), but how they affect AChR distribution is unclear. Antibodies
to the β1 integrin subunit were found to completely block agrin-induced AChR clustering (Martin and Sanes, 1997), yet in another study α7 β1 integrin preferentially colocalized with laminin-induced rather than with agrin-induced AChR clusters (Burkin et al., 1998). α7 integrin deficient mice display abnormal myotendinous junctions that result in a progressive muscular dystrophy (Mayer et al., 1997). Future work may reveal whether integrins function in postsynaptic specializations via the agrin signaling pathway or by some other mechanism.

Before MuSK was discovered, the search for the agrin receptor identified dystroglycan as a likely candidate (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994). Derived from a single mRNA, dystroglycan is posttranslationally cleaved into two proteins, α- and β-dystroglycan. α-Dystroglycan is a peripheral membrane protein associated with the extracellular matrix. β-Dystroglycan is a transmembrane protein that binds the cytoskeletal molecule dystrophin (Fig. 1-3). However, dystroglycan does not appear to be the primary agrin receptor. Experiments with different forms of agrin demonstrate that though α-dystroglycan can bind all agrin isoforms (including those with no AChR clustering activity), some agrin fragments can cluster AChRs without detectable binding to dystroglycan (Gesemann et al., 1996; Jacobson et al., 1998). Further investigations of the functions of dystroglycan have been unfortunately limited by the early embryonic lethality of dystroglycan mutant mice (Sanes, 1997).

A variety of observations suggest that dystroglycan is likely to play a downstream role in the agrin signaling pathway. For example, cells with low levels of α-dystroglycan
bear fewer AChR clusters (Jacobson et al., 1998). Dystroglycan becomes concentrated at AChR clusters in both agrin-stimulated cultures and nerve-muscle co-cultures (Cohen et al., 1995; Montanaro et al., 1998). Some studies showed that antibodies that recognize dystroglycan (mAb IIH6) inhibit agrin induced AChR clustering (Campanelli et al., 1994; Gee et al., 1994), however others have been unable to duplicate this inhibition (Sugiyama et al., 1994). β-Dystroglycan can bind two molecules concentrated at the NMJ, rapsyn (Cartaud et al., 1998) and utrophin (Ohlendieck et al., 1991; Bewick et al., 1992). Interestingly, utrophin-deficient mice have 20-40% fewer postsynaptic folds and a similar decrease in clustered AChRs (Deconinck et al., 1997; Grady et al., 1997).

the ARIA signaling pathway

AChR gene transcription is selectively elevated at the synapse. This transcription is thought to be controlled by ARIA, the acetylcholine receptor-inducing factor. Discovered by Fischbach and colleagues (Falls et al., 1993), ARIA is one of several alternatively spliced products from a single gene that are collectively referred to as the neuregulins. The family of neuregulins includes neu differentiation factor, heregulin, and glial growth factor (Lemke, 1996). ARIA is secreted from motor neurons and becomes associated with synaptic basal lamina (Goodearl et al., 1995). Activation of erbB kinases by ARIA (Altiok et al., 1995) initiates a local intracellular signaling pathway that includes PI3-Kinase, MAP kinase, ras, raf, and erk2 (Si et al., 1996; Tansey et al., 1996; Altiok et al., 1997). This pathway ultimately stimulates local synthesis of AChRs, and the promoter for the AChR epsilon subunit has been clearly shown to be responsive to
ARIA (Chu et al., 1995; Jo et al., 1995). It also induces a switch from the embryonic delta subunit to the epsilon AChR subunit observed in mature muscle (Meier et al., 1998). ARIA may also be responsible for upregulation of the utrophin transcript (Gramolini et al., 1999). Mice lacking neuregulins die early in embryogenesis (Meyer and Birchmeier, 1995). However, heterozygotic animals have only half as much neuregulin mRNA as control animals and the density of postsynaptic AChRs is also decreased by half (Sanes, 1997).

These results support a model for neuronally-secreted neuregulin enhancing AChR transcription specifically at the NMJ. However, there are data indicating that the role of neuregulins may be more complex. (1) Neurons synthesize a small proportion of the neuregulins found at the NMJ. Schwann cells secrete neuregulins and express erbB kinase (Rosenbaum et al., 1997). Neuregulins are also synthesized by muscle cells and are found in extrasynaptic regions of myofibers (Moscoso et al., 1995). (2) Of the four isoforms of erbB kinases, only erbB3 is localized to the postsynaptic region perinatally (Moscoso et al., 1995). Furthermore, the concentration of erbB3 at the NMJ is disrupted in mice lacking rapsyn. (3) Localization of ARIA signaling may be influenced by agrin. Ectopically expressed agrin induces clustering of neuregulins extrasynaptically (Meier et al., 1998). Also, synapse specific transcription is disrupted in agrin- and MuSK-deficient mice (DeChiara et al., 1996; Gautam et al., 1996), yet in myotubes cultured from these knock-out mice, neuregulin can activate AChR gene expression (Gautam et al., 1999). Together these observations indicate that neuregulins require agrin signaling for synapse
specific transcription and that neuregulins may have as yet unrecognized functions at the NMJ.

Localized transcription induced by ARIA leads to a local accumulation of AChRs at the NMJ. A complementary phenomenon is the extrasynaptic inhibition of AChR transcription observed after the process of cluster formation begins. Electrical activity inhibits transcription of AChR subunits (Klarsfeld and Changeux, 1985; Goldman et al., 1988) and only denervation or loss of synaptic activity will induce extrasynaptic nuclei to resume transcription of AChRs (Fambrough, 1979; Merlie et al., 1984; Tsay and Schmidt, 1989). The mechanism by which extrasynaptic transcription is inhibited begins with ACh binding to its receptor. The resulting action potentials release calcium from the sarcoplasmic reticulum (Adams and Goldman, 1998) which activates protein kinase C (PKC) (Klarsfeld et al., 1989; Huang et al., 1992). PKC in turn phosphorylates myogenic factors, including myoD, myf5, MRF4, and myogenin (Rudnicki and Jaenisch, 1995). These factors recognize regulatory sites in AChRs genes including sequences known as E boxes (Bessereau et al., 1998). PKC-dependent phosphorylation of myogenic factors results in their inactivation and eventual down-regulation (Eftimie et al., 1991; Huang et al., 1994b), ultimately diminishing transcription of AChR subunits.

An interesting aspect of this mechanism is the regulation of AChR expression through a pathway that includes the activation of AChRs. Furthermore, transcriptional downregulation is not observed locally even though synaptic activity activates AChRs in a highly localized domain. Instead, local activation of AChRs leads to downregulation of
AChRs specifically in extrasynaptic regions of the myotube where AChR transcription is not rescued by ARIA.

Establishing a synapse between a motor neuron and a muscle cell requires the combined efforts of agrin and ARIA to accumulate all of the necessary molecular machinery in one discrete location. The structure of this machinery is continually manipulated, particularly by synaptic activity. In the next section, the most extreme modification—the elimination of a synapse—is described.

**Synapse Elimination**

In mammalian muscle, embryonic synaptic formation is followed by selective postnatal synaptic disassembly (Fig. 1-5) (Nguyen and Lichtman, 1996). The necessity of removing synapses is a consequence of poly-innervation of muscle fibers during embryogenesis. In rat pups, for example, from two to six motor axons innervate each myofiber at birth (Redfern, 1970; Brown et al., 1976). Subsequent pruning reduces the number of contacts to one input per myofiber. This process of exuberant innervation followed by synapse elimination ensures that each muscle cell has one and only one input.

Initially, the terminal arbors of the innervating axons are intertwined. Gradually these branches atrophy, detach, and withdraw until each axonal terminal occupies its own non-overlapping domain (Gan and Lichtman, 1998). Though initial synaptic efficacies may be equal, one synapse will gradually become stronger by increasing the amount of neurotransmitter released (Colman et al., 1997). Quantal content and efficacy from all
other synapses diminish (partially due to decreased AChR density) (Dunia and Herrera, 1993; Colman et al., 1997), and ultimately these axons form retraction bulbs and withdraw from the myofiber (Balice-Gordon and Lichtman, 1993).

What determines which synapse will be maintained and which will be eliminated? Studies of synapse elimination in vivo revealed that the first morphologic change in a synapse is the dispersal of postsynaptic elements, including AChR clusters, rapsyn, and utrophin (Rich and Lichtman, 1989; Balice-Gordon and Lichtman, 1993; Culican et al., 1998). Thus it is the postsynaptic cell that may mediate the competition between multiple synapses. Moreover, since all axons converge on one region of the myotube surface (Sanes and Lichtman, 1999), the postsynaptic signals for maintenance and elimination must be very highly localized. Synaptic activity greatly influences the decision of which synapse will be maintained (reviewed in Nguyen and Lichtman, 1996). For example, blocking synaptic activity slows synapse elimination (Thompson, 1985) while inhibiting AChE (thereby increasing postsynaptic stimulation) hastens elimination (Duxson and Vrbova, 1985). Paralyzing a muscle may even prevent synapse elimination (Srihari and Vrbova, 1978; Thompson et al., 1979; Miyata and Yoshioka, 1980).

Therefore the myofiber directs the elimination of synapses in a manner that is activity dependant, competitive, and localized. Three principal mechanisms have been proposed to explain how such signaling might occur (Sanes and Lichtman, 1999): synaptotrophins, synaptotoxins, and synaptomedins.

The model of synaptotrophins suggests that the postsynaptic cell synthesizes limited quantities of a trophic factor that axons require to sustain their synapses. Axons
may take up such synaptotrophins in an activity-dependant manner. Though quite plausible, there is as yet little data that supports this model.

A complementary model is the synaptotoxins. Postsynaptic cells may release compounds that promote the dissolution of synapses. Their level of electrical activity determines the degree to which axons are susceptible to synaptotoxins. Thrombin (Chang and Balice, 1997) and calcium-activated neutral protease (Connold et al., 1986; Swanson and Vrbova, 1987; Tyc and Vrbova, 1995) are suggested to act as synaptotoxins. Electrical activity might trigger myotubes to release proteases that would cleave linkages between axons, myotubes, Schwann cells, and the ECM. Protective protease inhibitors might be released by electrically active synapses. In support of this model, muscle cells synthesize prothrombin in vivo (Zoubine et al., 1996) and release proteases when stimulated with ACh in vitro (O'Brien et al., 1978). The protease nexin is specifically localized to the NMJ (Festoff et al., 1991) and pharmacologically inhibiting thrombin reduces or delays synapse elimination in culture and in vivo (Connold et al., 1986; Liu et al., 1994; Zoubine et al., 1996). Interestingly agrin does inhibit the activity of some proteases though not thrombin (Biroc et al., 1993). An attractive aspect of the synaptotoxin model is that the connection between synaptic activity and elimination can be readily explained. However, this model predicts that total synaptic activity, and not relative levels of activity, determine the maintenance or elimination of a synapse.

Synaptomedins can either weaken or strengthen a synaptic connection depending upon local, activity-dependant differences between synapses. Synaptomedins are proposed to be signals targeted to all of a myofiber's inputs. Indeed, local postsynaptic
differences are observed prior to synaptic elimination (Rich and Lichtman, 1989; Balice-Gordon and Lichtman, 1993) (Culican et al., 1998). In vivo, denervated cells will maintain inactive sites in the absence of any electrical activity (Frank et al., 1976; Moss and Schuetze, 1987) but inactive sites are removed after reinnervation (Stanco and Werle, 1997). These data demonstrate that active synapses signal the dissolution of inactive synapses without being eliminated themselves. Two candidates have been suggested for such dispersive signals: tyrosine phosphatases (Dai and Peng, 1998) and intracellular calcium fluxes (Sanes and Lichtman, 1999). The findings presented in Chapter III support a role for these candidates in the dispersal of postsynaptic specializations.

Potential regulators of synaptic structure

Intracellular calcium signaling

Calcium is the most common element used for intracellular signaling, being used in cells as different as bacteria and neurons. Calcium signaling is central to fertilization, secretion, cell division, cell motility, gene transcription and synaptic plasticity. At the NMJ, calcium plays specific roles in neurotransmitter release and contraction. Our studies revealed that intracellular calcium signals participate in the formation, maintenance, and dispersal of agrin-induced postsynaptic specializations as well (Chapters II and III). We might better understand the ways in which calcium participates in these processes by considering how calcium is regulated in other pathways.

Though calcium participates in numerous signaling cascades, it conveys information specific to individual pathways. This selectivity is made possible by
regulating the amplitude, frequency, and localization of calcium transients. Cells have
developed several means of modulating cytosolic calcium and some of these mechanisms
have been characterized (Fig. 1-6).

Cells draw from two sources of calcium, the extracellular pool and intracellular
stores. The high concentration of extracellular calcium, approximately 2 mM, serves as a
virtually limitless reservoir of calcium (Clapham, 1995). In contrast, the concentration of
intracellular calcium is maintained at approximately 100 nM. This concentration
gradient, plus polarization of the plasma membrane, results in a rapid calcium influx at
any open channel or pore. More than $10^6$ ions are estimated to pass through a 5 angstrom
pore open for 1 second (Allbritton et al., 1992). Once inside, calcium-binding molecules
and endogenous buffers find these ions so rapidly that the estimated effective range for
free calcium is 0.1 micron. For comparison, the range of the signaling molecule IP$_3$ is
estimated to be 24 microns.

Intracellular calcium levels are kept low by the activity of two pumps (Pozzan et
al., 1994). Named according to their location, the plasma membrane calcium ATPase
(PMCA) and smooth endoplasmic reticulum calcium ATPase (SERCA) actively transport
cytosolic calcium out of the cell or into the endoplasmic reticulum (ER), respectively.

Calcium from the extracellular space can enter a cell through a variety of channels
that are generally classified according to their gating mechanisms. Agonists, antagonists,
and gating kinetics further subdivide this large family of molecules. The three principle
types of channels are the voltage operated calcium channels (VOCs; also known as the
voltage-sensitive calcium channels), the receptor operated calcium channels (ROCs), and the store operated calcium channels (SOCs).

VOCs are calcium selective and are closed at resting membrane potentials. They are opened by changes in membrane potential, then either close or become inactivated. VOCs play a major role in triggering release of neurotransmitter from presynaptic terminals. Interestingly, the presynaptic distribution of N-type VOCs closely corresponds with the postsynaptic localization of AChRs at the frog NMJ (Robitaille et al., 1993a).

ROCs transmit signals from the outside of a cell to the inside. Ligand binding induces conformational changes in these molecules that open up a channel for ions to pass through. AChRs are one type of ROC and are permeable to sodium and potassium in addition to calcium. Dissociation of the ligand-receptor complex allows the channel to close.

SOCs are a very different type of channel, opening in response to signals from within the cells. When intracellular stores of calcium are depleted, a signal triggers SOCs to open and a slow calcium current begins, thereby replenishing the cells’ supply of calcium. This calcium current is referred to variously as SOCE (store-operated calcium entry), I_{CRAC} (calcium release-activated current), or I_{DAC} (depletion-activated calcium current). Though no SOC has yet been purified, the means by which emptying of intracellular stores is coupled to calcium entry through SOCs appears to involve a coupling mechanism akin to secretion (Patterson et al., 1999; Yao et al., 1999).

Cells have multiple sources of intracellular calcium. Many organelles such as mitochondria, Golgi, and endosomes, sequester calcium (Pozzan et al., 1994). However,
these calcium pools may serve local functions since they do not appear to respond with rapid calcium release in response to intracellular messengers. In contrast, the sarcoplasmic reticulum (SR), an organelle found in muscle cells, and the ubiquitous ER are critical stores for calcium signaling.

The SR contains 1-2 mM calcium (Brini et al., 1997) for use in contraction of muscle fibers. The SR surrounds myofibrils and is also closely associated with invaginations of the plasma membrane called transverse (T) tubules. When a motor neuron releases ACh and AChRs are activated, an action potential is generated in the plasma membrane of the myotube. T tubules transmit this action potential towards the cell interior. The change in voltage is detected by dihydropyridine-sensitive receptors (DHPRs) in the T tubule membrane that are directly coupled to ryanodine receptors (RyRs). The calcium that is then released from the SR further amplifies the signal by calcium-induced calcium release (CICR). Calcium also binds to troponin, causing the release of troponin from the myosin binding site on actin filaments. Myosin is then able to bind the filaments and contraction of the myotube ensues.

The best characterized intracellular calcium store is the ER. This membrane-enclosed compartment appears to be a continuous membrane throughout the cell (Berridge, 1998), and in some places comes in close association with both the nuclear and plasma membranes. This compartment contains high capacity, low affinity calcium buffers such as calsequestrin and calreticulin, which bind calcium without any further signaling functions (Pozzan et al., 1994).
Embedded in the membrane of the ER are two types of channels, inositol triphosphate receptors (IP3Rs) and RyRs. The IP3R is a nonselective cation pore comprised of four subunits (Mikoshiba, 1993), each of which can bind one molecule of IP3. The IP3R is regulated by IP3 and by calcium itself. IP3 opens the channel, but IP3 can also cause desensitization (Hajnoczky and Thomas, 1994). The IP3R shows a biphasic sensitivity to calcium concentrations: at <300 nM calcium the IP3-mediated release is enhanced but >300 nM calcium depresses calcium release (Iino, 1990). Furthermore, the IP3R cytoplasmic domain binds calcium (Mignery and Sudhof, 1990) and cytosolic calcium concentrations modulate receptor inactivation by IP3 (Hajnoczky and Thomas, 1994).

Like IP3Rs, RyRs are sensitive to intracellular calcium concentrations. Interestingly, the ranges of concentrations at which these two receptors operate are quite different. In lipid bilayers, the maximum probability of opening for RyRs is estimated to lie between 1 and 100 uM calcium compared to 0.2 uM for IP3Rs (Bezprozvanny et al., 1991). In cells, >10 uM calcium inhibits RyRs whereas >0.3 uM depresses IP3Rs (Iino, 1990; Ghosh and Greenberg, 1995). These differences in gating characteristics suggest differences in the functions of these receptors. Furthermore, a study of the distribution of these channels in the CNS indicates that the two receptors have complementary distributions in different brain regions and at the ultrastructural level (Sharp et al., 1993). Together these two types of channel enable cells to utilize a variety of calcium signals.

A significant property of both IP3Rs and RyRs is their sensitivity to intracellular calcium. Local increases in calcium concentrations from one channel lead to opening of
neighboring channels. This phenomenon is known as calcium-induced calcium release (CICR) (Berridge, 1997b). This mechanism ensures that a sufficient calcium flux is generated for a particular function. Receptor inactivation prevents this feedback loop from driving calcium concentrations to potentially toxic levels. An interesting aspect of CICR is its potential to generate a wave of calcium away from an initial calcium signal.

Not every calcium efflux from the ER results in a large cascade however. Calcium signals may be repetitive oscillations, brief or longer-lasting transients. Activation of individual or groups of ER receptors may account for the different responses observed (Berridge, 1997a). Increasing temporal and spatial resolution in calcium imaging techniques permit closer examination of calcium fluxes. The elementary units of release from the ER have been named blips and quarks when released from individual IP3Rs and RyRs, respectively (Cheng et al., 1993; Yao et al., 1995; Parker and Yao, 1996). Small groups of receptors are organized to open simultaneously, causing larger releases of calcium called puffs and sparks.

By adjusting characteristics of a calcium signal, cells can regulate the downstream consequences. For example, naïve B lymphocytes presented with antigen respond with a large rise in intracellular calcium whereas B cells tolerant to the antigen respond with a smaller, sustained calcium flux (Dolmetsch et al., 1997). These two signals selectively activate different transcriptional regulators. These results illustrate how a stimulus can elicit separate effects via intracellular calcium by varying the amplitude and duration of the transient. The importance of the frequency of calcium signals was demonstrated in studies with calcium/calmodulin-dependent protein kinase II (CaM kinase II), a kinase
that responds to elevated calcium with increased enzymatic activity. Experimentally varying the frequency of calcium spikes resulted in distinct amounts of kinase activity (De Koninck and Schulman, 1998). The amplitude and duration of the calcium fluxes also modulated activity.

A further aspect of calcium signaling is localization of the signals. Calcium can be elevated within different subcellular compartments and induce different outcomes. In one example, increases in nuclear calcium concentrations were shown to stimulate transcription mediated by the cyclic-AMP-response element whereas cytoplasmic calcium signals triggered transcription through the serum-response element (Hardingham et al., 1997). However, many calcium signals are more highly localized. Calculations of calcium dynamics indicate that this ion is bound within ~50 microseconds of entering the cytoplasm and less than 0.5 microns from an open channel (Clapham, 1995). Free calcium therefore falls exponentially with increasing distance from its source (Stern, 1992). This high concentration of calcium surrounding the mouth of an open channel is referred to as a microdomain.

Calcium is bound either by trigger proteins that modulate effector molecules or by endogenous buffer proteins, which differ in their kinetics and mobility. Most trigger proteins fall into one of three categories: annexins, EF hand domain proteins, and PKC C₂ domain proteins (Kasai, 1993). The annexins bind to phospholipids and membranes in a calcium-dependent manner and function in membrane trafficking (Waisman, 1995). EF hand proteins are largely cytosolic and include molecules such as calmodulin, α-actinin, and calpain. Proteins with domains homologous to the C₂ domain of PKC are
translocated and activated upon calcium binding. This last category includes synaptogamin and phospholipase C.

**Protein tyrosine phosphatases**

The activities of numerous molecules are modulated by tyrosine phosphorylation. Protein tyrosine kinases (PTKs) catalyze this reaction and are themselves highly regulated. Phosphorylation often increases the enzymatic activity of the molecule that receives the phosphate group; hence the term activation is often synonymous with tyrosine phosphorylation. Removal of this phosphate group by protein tyrosine phosphatases (PTPs) is necessary to deactivate many molecules and to silence signaling pathways. Once thought to be constitutively active, recent work indicates that PTPs are as highly regulated as are PTKs. Little is known about PTP ligands, mechanisms of activation and inactivation, and their substrates. As these features of PTPs are revealed, it is now clear that dephosphorylation may have activating as well as deactivating affects depending upon the pathway. Our studies indicate that a PTP activity is involved in the mechanism of AChR cluster dispersal (Chapter III).

Studies of phosphotyrosine regulation lead to the first purification and characterization of a PTP (PTP1B) in 1988 (Tonks et al., 1988b; Tonks et al., 1988a). By 1998, over 100 PTPs had been identified (Burke and Zhang, 1998) with as many as 500 PTPs predicted to lie within the human genome (Tonks and Neel, 1996). PTPs are commonly divided into three categories based upon molecular structure and substrate specificity. (1) The classical PTPs remove phosphate groups from tyrosine residues
exclusively and are further subdivided into transmembrane or non-transmembrane families (Fig. 1-7). Transmembrane PTPs are also known as receptor PTPs (R-PTPs). (2) The dual-specificity PTPs are capable of dephosphorylating threonine and serine residues in addition to tyrosines. As yet, all members of this class are cytosolic molecules. (3) The low molecular weight PTPs are short polypeptides which primarily target tyrosine residues.

All PTPs are single polypeptides with a shared catalytic domain of approximately 250 amino acids (Burke and Zhang, 1998). Within this PTP signature motif lies the active site, $(H/V)C(X)_2R(S/T)$ (amino acid single-letter code; $X$ represents any amino acid). With the exception of this domain, PTPs have minimal sequence similarity within the family and no sequence similarity with the family of serine/threonine phosphatases. The superfamily of PTPs displays much more similarity at the level of tertiary structure than at the level of amino acid sequence. Significantly, all PTPs appear to use the same catalytic mechanism, an observation that has fostered mutational analysis of many PTPs. PTP “substrate traps” have been generated that continue to bind phosphorylated substrates but are catalytically inactive (Neel and Tonks, 1997). Substrates for some PTPs were discovered using this approach (Garton et al., 1996).

Unlike dual-specificity and low molecular weight PTPs, most receptor PTPs have two catalytic domains. Of the two domains, the more amino-terminal, membrane proximal domain appears to catalyze the majority of reactions. The function(s) of the carboxy-terminal domain, including its possible catalytic activity, is in dispute (Streuli et al., 1989; Streuli et al., 1990; Wang and Pallen, 1991; Lim et al., 1997; Wu et al., 1997).
PTP expression is developmentally and spatially regulated. In the nervous system, PTP expression is restricted by region and cell type, and some PTPs are further localized to particular subcellular domains. For example, SHP-2 is expressed exclusively in neurons where it is diffusely distributed in the cytoplasm (Reeves et al., 1996) and PTPα is found in glia but not in neurons (Ledig et al., 1999). Localized expression suggests distinct functions for PTPs in the nervous system.

The regulatory mechanisms for PTPs discovered thus far rely on two general approaches. First, access to the catalytically active domain can be restricted by ligand-induced dimerization, binding of other PTPs, or autoinhibitory domains. Alternatively, catalytic activity may be regulated by modifying the PTP itself, either by cleavage or phosphorylation.

One proposed model of PTP regulation has garnered considerable attention for its striking parallels to the activation mechanism for receptor tyrosine kinases (RTKs) (Stoker and Dutta, 1998; Weiss and Schlessinger, 1998). Ligand binding induces RTK dimerization. Within these dimers, each RTK phosphorylates its partner, resulting in increased kinase activity of each RTK. Similarly, it has been proposed that ligand binding may induce R-PTPs to dimerize. However, rather than activating R-PTP activity, dimerization is proposed to decrease phosphatase activity. Ligand induced inactivation provides a mechanism by which PTP activity can be directly controlled.

Evidence for this dimerization model of PTP regulation comes from structural analysis. First, the extracellular domains of R-PTPs are highly variable, suggesting that these domains are receptors capable of selectively binding ligands in a manner analogous
to RTKs. Second, PTPα, a R-PTP, was crystallized and found to form dimers (Bilwes et al., 1996). Within each dimer, a hinge domain on each molecule was inserted into the catalytic site of its partner's amino-terminal phosphatase domain. With hinge domains in this position, it is unlikely that the R-PTP would be capable of binding substrates.

Further support for this model comes from studies of lymphocytes. The R-PTP CD45 is necessary for normal lymphocyte development as well as for the signal transduction pathway triggered by activation of T cell antigen receptors (Kishihara et al., 1993). In CD45-deficient cells, a chimera made of the extracellular domain of the epidermal growth factor receptor (EGFR) and the CD45 intracellular domain can restore cell responsiveness (Desai et al., 1993). The recovery of signaling with this chimera indicated that the CD45 intracellular domain was necessary and sufficient for activation. Importantly, epidermal growth factor induced the chimeras to dimerize, and subsequently antigen receptor signaling was diminished. This loss of function suggests that dimerization results in PTP inactivation. Of the two phosphatase domains in CD45, only the amino-terminal domain was found to be catalytically active. So to further test the dimerization model, mutations were introduced into the EGFR-CD45 chimera hinge domain that was proposed to associate with the amino-terminal phosphatase domain. Addition of epidermal growth factor continued to induce dimerization of the mutant chimera, but antigen receptor signaling was not inhibited (Majeti et al., 1998). Thus the hinge domain appears to functionally block the catalytic site during dimerization-induced PTP inactivation.
Though many aspects of this model are appealing, this mechanism may not be widely used. Structural analysis of the PTPμD1, which is 46% identical to PTPα, revealed no such steric hindrance of the catalytic site (Hoffmann et al., 1997). Other PTPs appear to use different domains to control access to catalytic sites without homodimerization. For example, the carboxy-terminal phosphatase domain of PTPδ binds to the amino-terminal phosphatase domain of PTPσ, and this binding decreases the catalytic activity of PTPσ in vitro (Wallace et al., 1998). In the molecules Src-homology 2-domain phosphatase-1 and -2 (SHP-1 and SHP-2), the amino-terminal SH-2 domain (a protein-protein binding domain) acts as a conformational switch to regulate the active site (Pei et al., 1996; Hof et al., 1998). Without a phosphotyrosine binding partner, this SH-2 domain binds to the catalytic site of the PTP and suppresses most catalysis. When the amino-terminal SH-2 domain binds to another molecule, the catalytic site is exposed and phosphatase activity increases.

Another mechanism of regulating PTPs is proteolytic processing which can alter both the localization of a PTP and its activity. Some of the PTPs that are known to be subject to proteolysis include PTP1B, PTPζ/β, PTPMEG, and LAR (Streuli et al., 1992) (Cool et al., 1990) (Gu and Majerus, 1996). Cleavage of SHP-2 increases catalytic activity by 27-fold (Sugimoto et al., 1994). Increased PTP activity following proteolysis suggests that PTPs may have autoinhibitory domains with which to regulate their activities. Interestingly, proteolysis of both PTPMEG and LAR has been linked to influx of intracellular calcium. In the case of PTPMEG, either calcium ionophore or thrombin treatment of platelets induced both cleavage by calpain and activation (Gu and Majerus,
1996). Similarly, treatment with a calcium ionophore resulted in proteolytical processing of LAR and PTPσ (Aicher et al., 1997). After proteolysis, both of these PTPs shed their extracellular domains and the cytoplasmic, catalytically active domains were redistributed within the cells.

PTPs may also be phosphorylated themselves. In some cases phosphorylation alters catalytic activity (Vogel et al., 1993; Garton and Tonks, 1994), in other cases it does not (Flint et al., 1993; Zhang et al., 1995a). Phosphorylation of PTPs can regulate their interactions with other molecules (Conboy, 1993; Li et al., 1994). Within some PTPs are domains that suggest protein-protein interactions, such as SH-2 domains. A pair of SH-2 domains is found in both SHP-1 and SHP-2, and each domain can bind tyrosine phosphorylated molecules (Pei et al., 1996; Hof et al., 1998). These domains are thought to recruit substrates and to participate in determining subcellular localization, but do not appear to determine ligand specificity (Tenev et al., 1997).

Our understanding of PTP functioning in the nervous system has profited greatly from genetic manipulation of PTPs. By mutating or deleting individual genes, important roles for PTPs have been discovered in determining cell fates, neuronal outgrowth, and axonal guidance. Each of these processes will be discussed in turn.

The commitment of precursors to neuronal cell fates is an important step in the development the nervous system. An informative model for examining cell fate induction has been the compound eye of Drosophila (Van Vactor, 1998). Each eye is an array of functional units named ommatidia and each ommatidium has eight specialized photoreceptors surrounded by twelve supporting cells. Differentiation of the
photoreceptors begins with the centermost cell, R8, and spreads in a stereotyped fashion. The final photoreceptor to develop is R7, the one cell that provides the animal with sensitivity to ultraviolet light. Mutant flies unable to respond to ultraviolet light lacked only R7 (Quinn et al., 1974). A search for the defective gene identified sevenless, a gene that encodes a receptor tyrosine kinase (Banerjee et al., 1987; Hafen et al., 1987). For proper signaling, Sevenless was found to require the dephosphorylation of DOS (daughter of sevenless) by Corkscrew, a non-receptor PTP (Allard et al., 1996; Herbst et al., 1996; Raabe et al., 1996). Corkscrew also acts downstream of other Drosophila RTKs, including the epidermal growth factor receptor (DER) and the fibroblast growth factor receptor (Breathless), and possibly other RTKs as well (Perkins et al., 1996). In an interesting demonstration of conservation, SHP-2, the mammalian homologue, was able to functionally substitute for Corkscrew. In mice, lack of SHP-2 function results in severe developmental defects and embryonic lethality (Saxton et al., 1997).

PTPs are important mediators of neuronal adhesion, axonal guidance, and target recognition. Evidence for these functions is largely derived from phenotypic analysis of animals with absent or mutant PTPs. Within the developing nervous system in Drosophila, four receptor PTPs have been identified so far: DPTP10D, DPTP69D, DPTP99A, and DLAR (Van Vactor, 1998). Mutating DLAR or DPTP69D causes select neurons to fall short of or bypass their targets (Desai et al., 1996; Krueger et al., 1996; Garrity et al., 1999). Flies that lack both DPTP69D and DPTP99A have a more severe phenotype than the single DPTP69D mutants, even though mutating DPTP99A causes no discernable phenotype. In some pathways, either DPTP69, DPTP99, or DLAR is
sufficient for normal targeting, whereas in other pathways specific PTPs are required (Desai et al., 1997). These observations indicate that PTPs may have partially redundant roles and can substitute for each other depending upon the cellular context. Further experiments revealed that targeting mistakes in DLAR mutants were suppressed in DLAR-99 double mutants, suggesting competition between PTPs in other pathways.

LAR also plays a role in axonal guidance in leeches (Gershon et al., 1998). Antibodies that recognized the extracellular domain of LAR injected into leech embryos caused the internalization of some LAR. Interestingly, neurons in these embryos left their normal pathways and traveled shorter distances. LAR may have a different and more complex function in mammals. Notably, mice with decreased LAR expression have smaller cholinergic neurons and less cholinergic innervation of the dentate gyrus (Yeo et al., 1997).

PTPs expressed by glia have also been implicated in neuronal outgrowth and adhesion. PTPζ/β is synthesized by glia and may interact with axonal receptors such as N-CAM, Nr-CAM, TAG1/Axonin 1, Ng-CAM/L1, and contactin (Grumet et al., 1996; Margolis et al., 1996; Milev et al., 1996; Van Vactor, 1998). When PTPζ/β binds contactin and Nr-CAM in vitro, both neuronal differentiation and axonal outgrowth are enhanced (Sakurai et al., 1997). However, the extracellular domains of PTPζ/β which bind to these cell adhesion molecules are commonly cleaved by proteolysis (Van Vactor, 1998).

Disruption of PTPs has revealed additional roles in the mammalian nervous system. Mice with mutant PTPdelta have retarded growth, motor deficits, and shorter life
spans (Van Vactor, 1998). Targeted disruption of PTPσ caused numerous problems in mice: premature death, stunted growth, developmental delays, spastic movements, tremor, ataxic gait, abnormal limb flexion and defective proprioception (Wallace et al., 1999). Fewer neurons were found in the posterior pituitary and forebrain, and in the peripheral nerves slower conduction velocities and reduced myelination was observed. The wide range of defects derived from the loss of one molecule indicates the fundamental importance of individual PTPs in numerous contexts.

The roles of PTPs within the nervous system have lead to the suggestion that these molecules may prove to be promising targets for nerve repair (Stoker and Dutta, 1998). Outside of the nervous system, PTPs have wide ranging functions comparable to those identified for PTKs. As aberrant PTK signaling has been implicated in human diseases, so to might abnormal PTP functions lead to pathological conditions (Neel and Tonks, 1997).
Figure 1-1. Elements of the mature neuromuscular junction. Every myofiber is innervated by a single motor neuron, and basal lamina lies between the myofiber and nerve terminal. Surrounding the area are Schwann cells. Synaptic specialization is orchestrated by agrin and ARIA, which become stably associated with synaptic basal lamina.
Synaptic vesicles containing ACh

BASAL LAMINA

MYOTUBE

POSTJUNCTIONAL FOLDS

NERVE TERMINAL

calcium and potassium channels

AChR, dystroglycan, MuSK, erbB

rapsyn, utrophin, α-dystrobrevin-1

sodium channels

dystrophin, ankryin, β-spectrin, α-dystrobrevin-2

Figure 1-2. Molecular architecture of the NMJ. Synapse-specific molecules accumulate in the ECM and plasma membrane of the neuron and myotube. Cytoplasmic molecules, such as rapsyn, become localized to the synaptic region. Adapted from Sanes & Lichtman (1999).
Figure 1-3. Molecules associated with the agrin signaling pathway. Agrin interacts with a MuSK/MASC complex, α-dystroglycan (α-DG), and the extracellular matrix. Rapsyn binds both AChRs and β-dystroglycan (β-DG). Our results indicate that a protein tyrosine phosphatase is colocalized with MuSK and AChRs. MASC and RATL have not yet been identified.
Figure 1-4. The structural domains and isoforms of agrin. Three sites of alternative exon splicing are indicated by arrow heads. AChR-clustering efficacy is determined by inserts at the 'y' and 'z' sites (denoted 'A' and 'B' in chick agrin). The horizontal arrow indicates the N-terminus of the truncated, soluble form of agrin. Adapted from Ruegg et al. (1992) and Ferns et al. (1993).
Figure 1-5. Schematic of synapse elimination. Myotubes are initially innervated by multiple neurons with equally efficacious synapses (top panel) and may use asynchronous activity to determine which synapses will be disassembled (middle) and ultimately eliminated (bottom). Increased synaptic activity at one terminal may therefore destabilize less active synapses. Adapted from Nguyen and Lichtman (1996).
Figure 1-6. Calcium signaling. The entry of calcium from the extracellular pool and from the endoplasmic reticulum, an intracellular store, involves several highly regulated channels. See Appendix I for explanation of abbreviations. Adapted from Berridge (1998) & Clapham (1995).
Receptor PTPs

Non-transmembrane PTPs

Figure 1-7. The family of classical protein tyrosine phosphatases. Names above and below the line refer to PTPs found in vertebrates and invertebrates, respectively. Adapted from Van Vactor (1998) and Neel & Tonks (1997).

BETD=band 4.1/ezrin/talin-homologous domain; CA=carbonic anhydrase domain; Ig=immunoglobulin domain; MAM=meprin/A5/mu domain; PS=proline, glutamate, serine and threonine-rich sequence; ■=fibronectin type III repeats.
CHAPTER II

INTRACELLULAR CALCIUM REGULATES AGRIN-INDUCED ACETYLCHOLINE RECEPTOR CLUSTERING

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Abstract

Agrin is an extracellular matrix protein that directs neuromuscular junction formation. Early signal transduction events in agrin-mediated postsynaptic differentiation include activation of a receptor tyrosine kinase and phosphorylation of AChRs, but later steps in this pathway are unknown. Here, we have investigated the role of intracellular calcium in agrin-induced AChR clustering on cultured myotubes. Clamping intracellular calcium levels by loading with the fast chelator BAPTA inhibited agrin-induced AChR aggregation. In addition, preexisting AChR aggregates dispersed under these conditions, indicating that the maintenance of AChR clusters is similarly dependent on intracellular calcium fluxes. The decrease in AChR clusters in BAPTA-loaded cells was dose-dependent and reversible, and no change in the number or mobility of AChRs was observed. Clamping intracellular calcium did not block agrin-induced tyrosine phosphorylation of the AChR β-subunit, indicating that intracellular calcium fluxes are likely to act downstream from or parallel to AChR phosphorylation. Finally, the targets
of the intracellular calcium are likely to be close to the calcium source, since agrin-induced AChR clustering was unaffected in cells loaded with EGTA, a slower-binding calcium chelator. These findings distinguish a novel step in the signal transduction mechanism of agrin and raise the possibility that the pathways mediating agrin- and activity-driven changes in synaptic architecture could intersect at the level of intracellular calcium fluxes.

Key words: agrin; intracellular calcium; AChR phosphorylation; neuromuscular junction; synaptogenesis; postsynaptic differentiation

Introduction

Synapses throughout the nervous system are characterized by high concentrations of neurotransmitter receptors in the postsynaptic apparatus (Fertuck and Salpeter, 1976; Triller et al., 1985; Jacob et al., 1986; Nusser et al., 1994). Such dense accumulations of receptors are necessary for efficient synaptic transmission. Regulation of receptor number in the postsynaptic membrane is a hallmark of synaptic development, and is likely to be an important element underlying synaptic plasticity during learning and memory (Bailey and Kandel, 1993; Weiler et al., 1995).

Agrin plays a pivotal role in synaptic differentiation at the neuromuscular junction (Hall and Sanes, 1993; Bowe and Fallon, 1995). Here, agrin secreted by motor neurons activates a receptor tyrosine kinase, MuSK, to trigger synapse formation. Mutant mice lacking either agrin or MuSK display three major abnormalities: grossly defective
presynaptic and postsynaptic differentiation, and a failure in synapse selective transcription (DeChiara et al., 1996; Gautam et al., 1996). The signaling pathways between MuSK activation and these three endpoints must diverge, but in ways that currently are not understood (Gautam et al., 1995; Wells and Fallon, 1996; Apel et al., 1997).

The best-characterized branch of the agrin signaling pathway leads to the differentiation of the postsynaptic apparatus. Agrin secreted from the nerve terminal induces the aggregation of acetylcholine receptors (AChRs) and a host of other postsynaptic molecules on the muscle cell surface, including the dystrophin/utrophin-associated protein complex (Campanelli et al., 1994). The binding of agrin to a MuSK-containing complex is the first known step, with activation of the kinase occurring within minutes of agrin addition (Glass et al., 1996). Increased tyrosine phosphorylation of the AChR β-subunit is detected ~30 min later. The benchmark biological activity of agrin, the clustering of AChRs, manifests ~2 hr after agrin addition (Wallace, 1988; Nastuk et al., 1991). The maximal number of AChR clusters and level of AChR phosphorylation are achieved ~4 hr later (Wallace et al., 1991; Nastuk and Fallon, 1993).

In addition to its role in initiating postsynaptic apparatus formation, agrin also seems likely to be important for synaptic maturation, maintenance, and plasticity. Two lines of evidence point to a longer-term action in the agrin signaling pathway. First, agrin-induced AChR clusters continue to mature for at least 1 d after agrin addition in vitro. The AChR clusters become larger and more stable, and cytoskeletal and basal lamina elements, including agrin synthesized by muscle, accumulate with them (Wallace,
1988; Nitkin and Rothschild, 1990; Lieth and Fallon, 1993). These events require the ongoing action of agrin as well as new protein synthesis. Second, this continued action is also likely to require sustained MuSK activation (Glass et al., 1996). This long-term activation distinguishes MuSK from many other receptor tyrosine kinases, which are activated only transiently (Ullrich and Schlessinger, 1990).

Despite these advances in the understanding of the mechanisms of agrin's activity, many questions remain. For example, there is no direct evidence that tyrosine phosphorylation of AChRs is sufficient, or even necessary, for their clustering by agrin. Moreover, additional intracellular signal transduction events are likely to play a role in agrin-induced postsynaptic differentiation, but their nature is unknown. Of particular interest are elements that could be influenced by synaptic activity. Although it is well established that activity can shape synaptic architecture (Balice-Gordon and Lichtman, 1993; Kasai, 1993; Kirkwood and Bear, 1995; Koch, 1997), the interface between activity and the biochemical machinery that organizes synaptic structure is poorly understood.

In the present study we asked whether intracellular calcium fluxes participate in the agrin signaling pathway. We provide evidence that rapid calcium fluxes are required for agrin-induced AChR aggregation. Moreover, these fluxes act downstream from or parallel to AChR phosphorylation, which we demonstrate is not sufficient for AChR clustering. These findings reveal a novel step in the agrin signal transduction pathway. Intracellular calcium fluxes thus emerge as a potential locus for the integration of agrin- and activity-mediated changes in synaptic architecture.
Materials and Methods

Myotube culture. Embryonic chick myotube cultures were prepared as previously described (Nastuk et al., 1991). Briefly, myoblasts from embryonic day 11 chick embryos were dissociated and plated in medium containing MEM (Alpha Medium, Life Technologies, Gaithersburg, MD) supplemented with 2% chick embryo extract, 10% horse serum, 100 U/ml penicillin G, and 2 mM L-glutamine. For ligand-binding and AChR phosphorylation assays, cells were grown on plastic coated with 100 μg/ml gelatin (Sigma, St. Louis, MO). For AChR clustering assays, cells were grown on glass coverslips coated with 20 μg/ml poly-D-lysine (MW > 300,000; Sigma) and gelatin. Myotubes were used 3-7 d after plating.

Drug treatment. Stock solutions of the aminomethoxy ester of BAPTA (BAPTA-AM; Molecular Probes, Eugene OR) were prepared in DMSO (vehicle). The 10 mM BAPTA-AM stocks were stored at -20°C. Aliquots were thawed immediately before experiments and were not refrozen. BAPTA-AM or vehicle was diluted in serum-free medium (SFM) consisting of MEM (Alpha Medium, Life Technologies), 2 mM L-glutamine (Life Technologies), 0.5% bovine serum albumin (BSA), 100 U/ml penicillin G, and 5 μg/l each insulin, transferrin, and selenium (all from Sigma). To load cells with BAPTA, we incubated cells in BAPTA-AM for 1 hr 37°C (final DMSO concentration 0.5%) and then rinsed them with SFM. EGTA-AM (Molecular Probes) was prepared and used in a similar manner.
AChR clustering assays. Recombinant rat agrin, containing inserts of 12, 4, and 8 amino acids at the x, y, and z splice sites, respectively, was produced in COS cells as described previously (O'Toole et al., 1996). In some experiments, agrin purified from Torpedo electric organ (Cibacron Pool; Nitkin et al., 1987) was used with similar results. Native or recombinant agrin was used at a concentration of 10 U/ml in SFM. One unit is defined as the concentration of agrin at which half-maximal AChR clustering activity is observed (Godfrey et al., 1984).

Cells grown on coverslips were incubated with agrin for 4 hr at 37°C. Agrin was added immediately after BAPTA-AM treatment or 24 hr later (see Washout, Fig. 2-3). To detect AChRs, we included 1 μg/ml rhodamine-α-BTx in the final 45 min of the incubation. In some experiments myotubes were incubated simultaneously with agrin and KN-62 or K-252a (Calbiochem, La Jolla, CA) in DMSO. The 10 mM KN-62 and 1 mM K-252a stocks were stored at 4°C in the dark. The final DMSO concentration was ≤1%. Coverslips were rinsed in HEPES-buffered MEM (MEM-H, Life Technologies), fixed in methanol at -20°C for 5 min, mounted in Citifluor (Pella, Redding, CA), and viewed on a Zeiss Axioplan (Oberkochen, Germany) or a Nikon Eclipse (Tokyo, Japan) microscope. For quantitation of AChR clustering, 20-30 myotube segments (200 μm in length) were chosen randomly from two to three coverslips. AChR clusters (defined as AChR aggregates ≥4 μm in diameter) were scored under rhodamine optics (Nastuk et al., 1991).
Antibody-induced AChR microclustering was performed as described by Nastuk et al. (1991). Cells were loaded with BAPTA-AM or vehicle for 1 hr, rinsed, and then incubated with monoclonal antibody (mAb) 35 (Tzartos et al., 1983) for 30 min at 37°C, followed by goat anti-rat IgG (Sigma) and rhodamine-coupled α-bungarotoxin (α-BTx; Molecular Probes) for 30 min at 37°C. The distribution of AChRs was assessed visually; three coverslips were surveyed for each condition.

Ligand-binding assays. Binding of α-BTx and agrin to myotubes was quantitated as previously described (Bowe et al., 1994). Myotubes grown on gelatin-coated removable 96-well strips (Immulon 4, Dynatech, Chantilly, VA) were blocked for 1 hr in MEM-H with 1% BSA and 10% horse serum and incubated for 30 min with 10 nM 125I-α-BTx (10-20 µCi/µg, DuPont NEN, Boston, MA). For agrin binding, wells were incubated with agrin for 2 hr, followed by 1 µg/ml iodinated anti-agrin mAb 131 for 30 min. The mAb 131 (Hoch et al., 1994) was iodinated by using IODO-GEN (Pierce, Rockford, IL) per the manufacturer’s instructions; the range of specific activities was 5-8 µCi/µg. Wells were washed in MEM-H, immersed twice in HBSS with 1% BSA and 1 mM calcium, dried, and counted. Nonspecific binding was determined by including 1 mM EGTA (in agrin-binding experiments) or 100-fold excess competing unlabeled α-BTx. In each experiment six individual wells were counted for each condition, and then the results of multiple experiments were pooled.
Determination of AChR phosphorylation. AChRs from cultured myotubes were purified according to the method of Wallace et al. (1991) with minor modifications. Biotinylated α-BTx (Molecular Probes) was purified on an ImmunoPure Immobilized Monomeric Avidin column (Pierce). Myotube cultures were loaded with BAPTA or vehicle, incubated for 4 hr in agrin and 0.5 μg/ml biotinylated α-BTx, washed twice in cold PBS, harvested, and centrifuged; the cell pellet was resuspended in extraction buffer containing (in mM) 5 EDTA, 5 EGTA, 20 Tris, pH 7.5, 20 glycine, 150 NaCl, 40 Na-pyrophosphate, 50 NaF, 10 Na-molybdate, 1 Na-orthovanadate, 5 benzamidine, 10 N-ethylmaleimide, and 1 phenylmethylsulfonyl fluoride, with 1% Triton X-100, 1 mg/ml bacitracin, and 50 μg/ml each chymostatin, pepstatin, aprotinin, leupeptin, and antipain. Samples were sonicated for 10 sec with a Branson 450 Sonifier at 70% power, incubated 15 min at 4°C, and then spun for 20 min at 3000 × g. Solubilized AChR-biotinylated-α-BTx complexes were incubated with streptavidin-Sepharose beads (Sigma) for 2 hr with constant mixing at 4°C. Beads were washed four times in extraction buffer containing 1 M NaCl, twice in extraction buffer lacking NaCl and Triton X-100, and eluted in SDS-PAGE sample buffer.

Isolated AChRs were electrophoresed on 5-15% gradient SDS-polyacrylamide gels and transferred to nitrocellulose. Then the blots were blocked in PBS supplemented with 1% BSA. To detect AChR α- and β- subunits, we probed blots with mAb 61 and mAb 111, respectively (Wallace et al., 1991) (generously provided by J. Lindstrom, University of Pennsylvania). In some experiments AChR δ-subunit was detected with mAb 88b (Froehner et al., 1983; Qu and Huganir, 1994) (generously provided by S.
Froehner, University of North Carolina). Tyrosine-phosphorylated polypeptides were detected with anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY). After incubation with primary antibody, blots were washed in PBS and incubated for 1 hr with rabbit anti-mouse IgG (Sigma), followed by 100,000 cpm/ml $^{125}$I-protein A (2-10 μCi/μg, DuPont NEN). Bound radioactivity was quantitated in each lane in regions of equal area with a Molecular Dynamics PhosphorImager and software. The amount of tyrosine phosphorylation that was detected was expressed relative to the amount of AChR loaded, as determined by quantitation of mAb 61 binding to the AChR α-subunit. In some experiments blots originally probed with $^{125}$I-protein A were stripped and reprobed with anti-AChR antibodies. Then bound antibodies were detected by biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, CA) and an alkaline phosphatase-based ABC kit (Vectastain ABC; Vector Laboratories).

**Results**

A rapid intracellular calcium chelator inhibits spontaneous and agrin-induced AChR clustering

Both the AChR clustering activity of agrin and its binding to the cell surface require extracellular calcium (Wallace, 1988; Nastuk et al., 1991). We used BAPTA to manipulate intracellular calcium without depleting calcium outside the cells. Myotubes were loaded with BAPTA, using its membrane-permeable non-calcium-binding AM ester (BAPTA-AM) (Tsien, 1981). Upon traversing the plasma membrane, BAPTA-AM is converted to BAPTA, which is membrane-impermeable, by intracellular esterases.
BAPTA binds calcium rapidly, selectively, and with high affinity ($K_D \sim 100$-180 nM), thus serving to "clamp" intracellular calcium fluxes (Stern, 1992; Roberts, 1993; Deisseroth et al., 1996).

Clamping intracellular calcium inhibited AChR clustering. The number of agrin-induced AChR clusters was reduced >60% in cells loaded with 50 μM BAPTA-AM, the highest concentration tested (Fig. 2-1). Significant inhibition of agrin-induced AChR clustering was observed in all experiments ($n = 7$) and was dependent on the BAPTA-AM concentration used for loading (Fig. 2-2). The 50 μM BAPTA-AM concentration was chosen for all subsequent experiments. The number of spontaneous AChR clusters (also known as "hot spots"; Frank and Fischbach, 1979) was reduced by 40% in BAPTA-loaded cells (Fig. 2-1). The inhibition of spontaneous clusters was more variable than that seen for agrin-induced clusters. Although the number of spontaneous clusters decreased in BAPTA-loaded cells in all experiments ($n = 7$), the inhibition was significant in only five of them. Treatment with vehicle alone had no effect on either spontaneous or agrin-induced clusters. AChR clusters looked similar in control and BAPTA-loaded myotubes, indicating that BAPTA prevented the formation of clusters rather than causing them to form more diffusely. These results indicate that intracellular calcium fluxes are necessary for both the maintenance and the formation of AChR clusters.

We were unable to detect any deleterious effects of BAPTA loading on these cells. Myotubes loaded with BAPTA were morphologically indistinguishable from controls, as judged by phase-contrast microscopy. Moreover, the effects of drug
treatment were reversible. After wash-out, the numbers of agrin-induced and spontaneous AChR clusters returned to control levels (Fig. 2-3).

The results presented above suggest that clamping intracellular calcium may interfere directly with the signal transduction pathway of agrin. However, it is also possible that the inhibition is attributable to indirect effects, such as altering the level of agrin-binding sites or AChRs on the cell surface. Therefore, to test these possibilities, we measured the levels of AChRs and of agrin binding. There was no statistical difference in the number of surface AChRs in BAPTA-loaded cells (102% ± 10 of control, n = 5; p = 0.62, paired Student's t test). Similarly, no differences in agrin binding were observed (90% ± 11 of control, n = 4; p = 0.25, paired Student's t test).

The formation of antibody-induced AChR microclusters is unaffected by clamping intracellular calcium

It is possible that clamping intracellular calcium could inhibit AChR clustering by immobilizing AChRs in the myotube membrane. Such immobilization of AChRs, with concomitant inhibition of agrin-induced receptor clustering, has been reported in myotubes treated with tyrosine phosphatase inhibitors (Meier et al., 1995). To assess AChR mobility, we tested the ability of anti-AChR antibodies to drive the formation of AChR microclusters (Nastuk et al., 1991). This manipulation is distinct from agrin-induced clustering because it relies on the direct antibody-mediated cross-linking of AChRs. Antibody-driven AChR microclustering was equally robust in both untreated and BAPTA-loaded myotubes (Fig. 2-4). Together with the binding data presented
above, these observations support the hypothesis that intracellular calcium fluxes play a
direct role in the agrin signaling pathway.

**A slower-binding calcium buffer does not inhibit agrin-induced AChR clustering**

To better characterize the mode of action of calcium in the agrin signaling
pathway, we tested another calcium chelator, EGTA. This chelator binds calcium with a
similar affinity to BAPTA, but with a 400-fold slower on rate. As a result, calcium
issuing into the cytosol can be buffered to within ~0.1 μm of the membrane in a
BAPTA-loaded cell but only to ~1 μm in the presence of EGTA (Stern, 1992; Roberts,
1993; Deisseroth et al., 1996). In contrast to the results observed in BAPTA-loaded
cells, the numbers of spontaneous and agrin-induced AChR clusters were unaffected in
myotubes loaded with EGTA (Fig. 2-5). Different AM ester compounds may load into
cells at different rates (Deisseroth et al., 1996). Therefore, we tested a wider range of
EGTA-AM concentrations. We observed no inhibition of either agrin-induced or
spontaneous AChR clusters when we used an EGTA-AM concentration ranging from 25
to 100 μM (the highest dose tested; data not shown). These results indicate that global
buffering of calcium is unlikely to account for the BAPTA-mediated inhibition of AChR
cluster formation. Moreover, these results demonstrate that the inhibition of AChR
clustering is not the result of nonspecific side effects resulting from the use of AM ester
compounds. Finally, these findings indicate that AChR clustering is likely to rely on
calcium-sensitive effectors localized <1 μm from the calcium source.
One candidate effector is calcium/calmodulin-kinase II (CaM-KII). To test whether this enzyme plays a role in AChR cluster formation, we incubated myotubes with agrin in the presence of CaM-KII inhibitors (either 10-100 μM KN-62 or 0.01-10 μM K-252a). No effects on AChR clusters were observed (data not shown), suggesting that CaM-KII does not participate in the agrin signaling pathway.

**Clamping intracellular calcium does not perturb agrin-induced AChR phosphorylation**

We next wished to position intracellular calcium fluxes relative to known events in the agrin signaling pathway. As discussed above, early steps in this pathway include the agrin-induced activation of the receptor tyrosine kinase MuSK, followed by the tyrosine phosphorylation of AChR β-subunits (Wallace et al., 1991; Glass et al., 1996). We therefore assessed AChR phosphorylation in BAPTA-loaded cells. Phosphorylation levels of AChRs were assayed 4 hr after agrin addition, the time at which AChR clustering was assessed in the above experiments and at which maximal agrin-induced tyrosine phosphorylation is achieved (Wallace et al., 1991). As shown in Figure 2-6A, agrin induced the tyrosine phosphorylation of AChRs in both BAPTA- and vehicle-loaded cells. Quantitation of phosphotyrosine levels indicated that BAPTA loading did not significantly change agrin-induced AChR β-subunit phosphorylation (Fig. 2-6B). Further, BAPTA treatment did not alter the basal level of AChR phosphorylation in these myotubes. These results demonstrate that tyrosine phosphorylation of AChR β-subunits
is not sufficient to induce their clustering and indicate that intracellular calcium fluxes act downstream or parallel to AChR phosphorylation.

**Discussion**

The goal of this study was to test the role of intracellular calcium in the agrin signaling pathway. Our findings indicate that locally acting intracellular calcium fluxes are necessary for agrin-induced AChR clustering. The calcium-sensitive step or steps are downstream of, or parallel to, agrin-induced tyrosine phosphorylation of AChRs. We also show that agrin-induced AChR phosphorylation is not sufficient for receptor aggregation.

We used BAPTA to buffer intracellular calcium. This compound was designed by Tsien (1981) to bind calcium with high selectivity, affinity, and speed. The efficacy and specificity of this drug have been documented in numerous studies (Stern, 1992; Roberts, 1993). For example, BAPTA has been used to probe rapid calcium-signaling events mediating exocytosis (Tsien, 1981; Penner and Neher, 1988; Adler et al., 1991). Loaded via its AM-ester form, this compound has been used to manipulate calcium-activated potassium channels (Robitaille et al., 1993b), to distinguish among classes of evoked EPSPs (Cummings et al., 1996), and to probe calcium transients regulating different aspects of neuronal differentiation (Gu and Spitzer, 1995).

Several lines of evidence indicate that the clamping of intracellular calcium by BAPTA perturbs a step in the signaling pathway of agrin, rather than working via indirect mechanisms. We found no evidence that BAPTA-AM or BAPTA caused
toxicity in these studies. The treated myotubes were indistinguishable from controls, as judged by phase-contrast microscopy, and the effects of BAPTA were reversible (see Fig. 2-3). The levels of surface agrin-binding sites and AChRs were unchanged in the treated cells. It should be noted that although we used the highly active, MuSK-activating isoform of agrin in these assays (agrin 4, 8; Glass et al., 1996), it is likely that a substantial portion of the observed binding was attributable to interaction with dystroglycan on the cell surface (Bowe et al., 1994; O'Toole et al., 1996). The normal levels of both basal and agrin-induced AChR tyrosine phosphorylation observed in BAPTA-treated myotubes also indicate that basic cell functions were uncompromised and further suggest that the level of MuSK on the cell surface is not altered substantially under these conditions. AChRs in the membrane remained mobile, as judged by the robust AChR microclustering driven by anti-AChR antibodies (see Fig. 2-4). However, the possibility remains that BAPTA causes relatively small changes in receptor mobility that could be beyond the sensitivity of this assay. Finally, nonspecific side effects stemming from the use of AM esters are unlikely, because loading cells with EGTA-AM had no effect on spontaneous or agrin-induced AChR clusters (see Fig. 2-5).

The results presented here provide new insights about the roles of MuSK activation and AChR tyrosine phosphorylation in agrin-induced AChR clustering, and their places in the signaling pathway. Activation of the MuSK receptor complex by agrin is essential for synaptic differentiation (DeChiara et al., 1996). Subsequent to this step, and dependent on MuSK activation (Apel et al., 1997), is the tyrosine phosphorylation of AChR β-subunits. Because AChR tyrosine phosphorylation
proceeds normally in BAPTA-loaded cells (see Fig. 2-6), neither MuSK activation nor the phosphorylation of AChR β-subunit is sufficient for agrin-induced AChR aggregation. Therefore, additional components of the agrin signaling pathway, at least some of which rely on intracellular calcium fluxes, must come into play to achieve and to maintain postsynaptic differentiation.

Our studies show clearly that agrin-induced tyrosine phosphorylation of the AChR β-subunit is not sufficient for clustering AChRs. However, it is not known if this phosphorylation is necessary for agrin-induced AChR clustering. Accordingly, we present two possible models for the agrin signaling pathway (Fig. 2-7). In one model, the calcium-dependent step occurs downstream of agrin-induced AChR phosphorylation. In the second, agrin-induced AChR phosphorylation is a step in a parallel pathway that does not play a direct role in clustering.

Calcium is likely to play a role at both extracellular and intracellular loci during agrin-induced postsynaptic differentiation. Extracellular calcium is necessary for nerve- and agrin-induced AChR clustering (Henderson et al., 1984; Wallace, 1988). Removal of extracellular calcium destabilizes AChR clusters (Connolly, 1984; Wallace, 1988; Caroni et al., 1993; Dmytrenko and Bloch, 1993), and raising extracellular calcium can promote AChR clustering (Mook-Jung and Gordon, 1995). In addition, a large fraction of agrin binding to the cell surface and to the major agrin-binding protein on the cell, dystroglycan, is dependent on extracellular calcium (Nastuk et al., 1991; Bowe et al., 1994).
The source of the intracellular calcium transients that are necessary for AChR aggregation is not known. The requirements for extracellular calcium noted above are consistent with the possibility that the extracellular pool could be one source. Both the AChR itself and voltage-dependent calcium channels are potential conduits for such extracellular calcium influx. However, they are unlikely to be the sole sources, because neither α-BTx nor tetrodotoxin inhibits AChR clustering induced by neurons or agrin (Anderson et al., 1977; Godfrey et al., 1984). However, calcium entering from these sources could modulate postsynaptic differentiation (see below). Alternatively, the calcium fluxes may arise from intracellular stores, such as IP3-mediated release from the endoplasmic reticulum (Verma et al., 1990).

The experiments comparing the effects of EGTA and BAPTA provide information about the location of the calcium targets relative to the sources. As a consequence of its slower-binding kinetics, EGTA, even when present at saturating concentrations, can buffer a calcium transient only to within 1-2 μm of its source. On the other hand, BAPTA is estimated to buffer calcium ions within 0.1 μm from a source (Stern, 1992; Roberts, 1993; Schweizer et al., 1995; Deisseroth et al., 1996). For example, neurotransmitter release mediated by voltage-gated calcium channels is blocked by BAPTA, but not by EGTA (Adler et al., 1991; Augustine et al., 1992). Further, BAPTA, but not EGTA, blocks activity-mediated cAMP response element binding protein (CREB) phosphorylation in hippocampal neurons (Deisseroth et al., 1996). Deisseroth and colleagues also showed that equivalent levels of intracellular EGTA and BAPTA were achieved when the loading concentration of EGTA-AM was
threefold greater than that of BAPTA-AM. In the present study we observed significant inhibition of agrin-induced AChR clustering in cell loaded with 25 μM BAPTA-AM, but we observed no effects when the cells were loaded with 100 μM EGTA-AM (see Figs. 2, 5). The inability of EGTA to inhibit agrin-induced or spontaneous AChR clustering thus indicates that the sources of the calcium fluxes are likely to be close to their targets.

A possible connection between the intracellular calcium requirements observed here and the pathogenesis of muscular dystrophies deserves comment. Many varieties of muscular dystrophy, including the most prevalent forms, Duchenne and Becker, are the result of deficiencies in the dystrophin-associated protein complex. Along with clustering AChRs, agrin also induces the aggregation of many members of this complex. Moreover, altered intracellular calcium levels and calcium channel properties in dystrophic muscle have been reported (for review, see Gillis, 1996). It is also of interest that limb-girdle Muscular Dystrophy type 2A is due to a defect in calpain, a calcium-activated protease (Richard et al., 1995; van Ommen, 1995). We speculate that the organization of the dystrophin-associated protein complex involves a calcium-dependent step. Together, these observations raise the possibility that there may be links between agrin's mechanism of action and the molecular pathophysiology of muscular dystrophies.

Finally, the requirement for intracellular calcium fluxes in the AChR clustering activity of agrin presents an attractive locus for activity-mediated regulation of synaptic structure. Activity has far-reaching effects on synaptic structure and function (Balice-Gordon and Lichtman, 1993; Kasai, 1993; Kirkwood and Bear, 1995; Koch, 1997). Many of these events have been linked to changes in intracellular calcium. For example,
neurotransmitter receptor synthesis in muscle is inhibited by electrical activity in a pathway initiated by calcium influx through voltage-dependent calcium channels (Huang et al., 1994a). Calcium also plays a central role in the mechanisms underlying LTP and LTD at neuronal synapses (Bear and Malenka, 1994). At the neuromuscular junction such calcium fluxes could feed into the cellular and molecular machinery of the agrin signaling pathway to shape the synapse in an activity-dependent manner. It is tempting to speculate that similar mechanisms may underlie the structural plasticity of synapses in the CNS.

Footnotes

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Figure 2-1A.
Figure 2-1B.
Figure 2-1. The number of spontaneous and agrin-induced AChR clusters is decreased in BAPTA-loaded cells. A, Myotubes were incubated with 50 μM BAPTA-AM (*bottom panels*) or vehicle only (*top panels*), washed, and incubated with or without agrin for 4 hr, as indicated. Cultures were then incubated in rhodamine-α-BTtx and examined by fluorescence microscopy to reveal the distribution of AChRs. B, Quantitation of AChR clusters revealed that significantly fewer spontaneous and agrin-induced clusters are observed in BAPTA-loaded cells. AChR clusters were quantitated as described in Materials and Methods. Values are mean ± SEM averaged from seven separate experiments. *p < 0.05, paired Student's t test. Scale bar, 20 μm.
Figure 2-2. Quantitation of agrin-induced and spontaneous AChR clusters in myotubes loaded with varying concentrations of BAPTA-AM. Myotubes were incubated with the indicated concentrations of BAPTA-AM for 1 hr and then incubated in media with or without agrin for 4 hr. Data shown are from one representative experiment and are expressed as mean ± SEM. Similar results were seen in three additional experiments.
Figure 2-3. Inhibition of AChR clustering in BAPTA-loaded cells is reversible. Cells were loaded with 50 μM BAPTA-AM or vehicle for 1 hr, washed, and then incubated with agrin either immediately or 24 hr later (Washout). After the wash-out period, the numbers of spontaneous and agrin-induced AChR clusters returned to levels similar to vehicle-treated cells. Values are expressed as mean ± SEM from one experiment. Similar results were observed in four other experiments.
Figure 2-4. Antibody-driven AChR microclustering is unaffected in BAPTA-loaded cells. Myotubes were incubated with vehicle only (A, B) or 50 μM BAPTA-AM (C) and then directly incubated in buffer alone (A) or anti-AChR antibody mAb 35 and anti-Rat IgG for 1 hr at 37°C (B, C). The distribution of AChRs was then determined by labeling with rhodamine-α-BTx. In the absence of anti-AChR antibody incubation, AChRs were distributed diffusely on the myotube surface (A). Incubation with anti-AChR antibodies caused extensive AChR microclustering in both vehicle (B) and BAPTA-loaded (C) cells. Scale bar, 20 μm.
Figure 2-5. Spontaneous and agrin-induced AChR clusters in EGTA-loaded cells.

Myotubes were loaded with EGTA by incubating them with 50 μM EGTA-AM for 1 hr at 37°C and then incubating them with or without agrin, as in Figure 2-1. The numbers of neither spontaneous nor agrin-induced AChR clusters were significantly different in EGTA-loaded, as compared with vehicle-loaded cells. Mean ± SEM from one representative experiment; $p = 0.392$ and 0.360 for spontaneous and agrin-induced clusters, respectively, Student's $t$ test. Similar results were obtained by using cells loaded with 25 or 100 μM EGTA-AM.
Figure 2-6A.
Figure 2-6B.
Figure 2-6. Agrin-induced tyrosine phosphorylation of AChR β-subunit in BAPTA-loaded cells. Myotubes were loaded with BAPTA or vehicle and then incubated with or without agrin for 4 hr, as indicated. Surface AChRs were affinity-purified with biotinylated α-BTx and separated by SDS-PAGE. A, Immunoblots were probed with mAb 4G10 to visualize tyrosine phosphorylated proteins (p-tyr) or mAb 61 to visualize the AChR α-subunit (anti-α). In parallel blots, mAb 124 was used to identify the AChR β-subunit (data not shown). Agrin induced tyrosine phosphorylation of AChR β-subunit in both the presence and absence of BAPTA. A second polypeptide of slightly slower mobility was phosphorylated also. This polypeptide was identified tentatively as AChR δ-subunit, on the basis of immunoreactivity with mAb 88b (data not shown) and previous reports showing that agrin also induces the phosphorylation of this subunit (Qu and Huganir, 1994). B, Phosphotyrosine levels of the AChR β-subunit were measured and expressed relative to total AChR levels, as described in Materials and Methods. Results were derived from three separate experiments, each normalized to untreated controls. Agrin-induced AChR β-subunit tyrosine phosphorylation was equivalent in vehicle, as compared with BAPTA-loaded cells. The basal level of AChR β-subunit tyrosine phosphorylation was also not significantly different in BAPTA-loaded cells (p > 0.05; Newman-Keuls multiple comparison test, after repeated measures ANOVA of three separate experiments; data not normalized).
Figure 2-7. Two models of the signaling pathway of agrin. The first step in the signaling pathway is the activation of the MuSK receptor tyrosine kinase by agrin. The subsequent tyrosine phosphorylation of the AChR is dependent on MuSK activation. The results presented here are consistent with two models. (1) The intracellular calcium-regulated step occurs downstream of AChR phosphorylation. (2) The AChR phosphorylation is on a pathway parallel to (but not necessarily required for) agrin-induced AChR clustering. See Discussion for details.
CHAPTER III
AGRIN-INDUCED POSTSYNAPTIC SPECIALIZATIONS ARE MAINTAINED BY A CALCIUM-DEPENDENT SUPPRESSION OF TYROSINE PHOSPHATASE ACTIVITY

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Abstract

Although much progress has been made in understanding synapse formation, little is known about the mechanisms underlying synaptic maintenance and loss. The formation of agrin-induced AChR clusters on cultured myotubes requires both activation of the receptor tyrosine kinase MuSK and intracellular calcium fluxes. Here, we provide evidence that such AChR clusters are maintained by agrin/MuSK-induced intracellular calcium fluxes that tonically inhibit a localized tyrosine phosphatase. Clamping intracellular calcium fluxes after AChR clusters have formed leads to rapid MuSK and AChR tyrosine dephosphorylation and cluster dispersal, even in the continued presence of agrin. Both the dephosphorylation and the dispersal are inhibited by the tyrosine phosphatase inhibitor pervanadate. In contrast, clamping intracellular calcium at the time of initial agrin stimulation has no effect on agrin-induced MuSK or AChR phosphorylation, but blocks AChR cluster formation. These findings indicate that the mechanisms mediating AChR cluster formation and maintenance are distinct, and further suggest an avenue by which postsynaptic stability can be regulated by modification of intracellular signaling pathways.

Introduction

Information processing in the nervous system depends upon appropriate patterning of synaptic connectivity. Synaptic organization is the result of competing, often experience-dependent factors that drive the formation, persistence, and loss of synapses between neurons and their targets. These factors are critical for the precise
development of the nervous system. For example, nerve-muscle connectivity is shaped by the activity-dependent elimination of synapses on multiply innervated myofibers (Sanes and Lichtman, 1999). Dynamic patterning of synaptic connectivity persists throughout life in at least some areas of the nervous system and may be a basis for learning and memory (Bailey and Kandel, 1993).

Understanding how proper synaptic organization is achieved requires knowledge of the cellular and molecular mechanisms mediating synapse differentiation and elimination. The best understood aspect of synapse formation is the marshalling of neurotransmitter receptors to the postsynaptic membrane. High density arrays of receptors are characteristic of fast synapses throughout the nervous system (Triller et al., 1985; Jacob et al., 1986; Nusser et al., 1994; Nusser et al., 1995). This clustering of neurotransmitter receptors is mediated by the coordinated action of distinct sets of cytoskeletal, transmembrane, and extracellular matrix molecules (Reist et al., 1987; Apel et al., 1997; Brakeman et al., 1997; O'Brien et al., 1999; Wang et al., 1999a).

Knowledge of the detailed mechanisms underlying postsynaptic differentiation is most advanced at the neuromuscular junction (NMJ). Formation of this synapse is directed by agrin, an extracellular matrix molecule secreted by the motor nerve terminal (McMahan and Wallace, 1989). Postsynaptic differentiation can be modeled in culture where the addition of agrin induces the formation of acetylcholine receptor (AChR) clusters on the myotube surface. These agrin-induced AChR aggregates display many hallmarks of the postsynaptic apparatus including the selective concentration of MuSK, rapsyn, and a variety of other cytoskeletal, transmembrane, and extracellular matrix
components (Wallace, 1988; Nitkin and Rothschild, 1990; Lieth and Fallon, 1993; Gautam et al., 1996; Ruegg and Bixby, 1998). An intracellular signaling cascade is triggered when agrin binds to a receptor complex consisting of the receptor tyrosine kinase MuSK and a putative co-receptor MASC (muscle associated specificity component) (Glass et al., 1996). Downstream of MuSK activation, the pathway leading to AChR cluster formation requires the cytosolic protein rapsyn (Gautam et al., 1995). Activated MuSK also leads to the tyrosine phosphorylation of AChRs, but the significance of this modification is not understood (Gillespie et al., 1996; Meyer and Wallace, 1998). Agrin is also necessary, probably through a less direct route, for presynaptic differentiation and synapse-selective transcription (DeChiara et al., 1996; Gautam et al., 1996; Burgess et al., 1999).

In a recent study we showed that intracellular calcium transients play an integral role in the agrin signaling pathway (Megeath and Fallon, 1998). Clamping intracellular calcium concentrations at the time of agrin addition inhibits AChR cluster formation. Notably, these calcium fluxes are likely to act downstream of the early events in the agrin signaling cascade, since they are not required for the agrin-induced tyrosine phosphorylation of AChRs (or of MuSK, see below). These studies also raised the possibility that agrin-induced calcium transients may serve as a control point for postsynaptic differentiation.

Loss of synapses is essential for normal development of the nervous system, but the underlying mechanisms are poorly understood. About half of the nerve-muscle synapses initially formed in the embryo are eliminated in the first weeks after birth.
Synaptic pruning is a tightly regulated process involving activity-dependent competition between neighboring synapses on the same muscle cell. Interestingly, the loss of synaptic constituents is asynchronous, with the postsynaptic apparatus dispersing before obvious signs of presynaptic withdrawal (Balice-Gordon and Lichtman, 1993; Culican et al., 1998). Proposed molecular mediators of this dispersal include proteases (Liu et al., 1994; Harding et al., 1996) and protein tyrosine phosphatases (PTPs) (Dai and Peng, 1998). In addition, AChR cluster formation is inhibited by trkB activation, suggesting a role for neurotrophin signaling in regulating synaptic architecture (Wells et al., 1999).

In the present study, we investigated the mechanisms mediating the maintenance of agrin-induced postsynaptic specializations. We find that agrin-dependent intracellular calcium fluxes are necessary for AChR cluster stability on cultured myotubes. Moreover, clamping intracellular calcium after AChR clusters have formed causes rapid tyrosine dephosphorylation of MuSK and AChR. Our results indicate that agrin-induced calcium transients suppress the activity of a PTP that is likely to be localized at AChR clusters. These findings indicate that the mechanisms mediating the formation and the maintenance of agrin-induced AChR clusters are distinct, and further suggest an avenue by which postsynaptic stability can be regulated by signaling pathways involving MuSK, intracellular calcium transients, and selectively localized protein tyrosine phosphatases.
Results

Clamping intracellular calcium fluxes accelerates AChR cluster dispersal

Both agrin binding to cells and agrin-induced AChR clustering require extracellular calcium (Wallace, 1988; Nastuk et al., 1991). Therefore, to selectively manipulate intracellular calcium fluxes we used BAPTA, a rapid, high affinity calcium buffer ($K_D \sim 100-180$ nM) (Stern, 1992; Roberts, 1993; Deisseroth et al., 1996; Megeath and Fallon, 1998). Myotubes were loaded with BAPTA via its AM ester form (see Methods). To directly assess the effects of BAPTA treatment we measured intracellular calcium levels using Fura-2 ratiometric imaging. Resting intracellular calcium concentrations in BAPTA-loaded cells were indistinguishable from control (vehicle only) myotubes ($105\% \pm 3.1$ of control; unpaired T test, $p > 0.05; N=4$). To test the efficacy of BAPTA treatment, we used acetylcholine pulses to evoke intracellular calcium fluxes. Acetylcholine-induced intracellular calcium fluxes were dramatically reduced in BAPTA-treated cells (Fig. 3-1). These results show that BAPTA clamps intracellular calcium concentrations in cultured myotubes without disrupting resting levels of this ion.

To test the role of intracellular calcium fluxes in the maintenance of AChR clusters, myotubes were first stimulated with agrin for 4 hr to induce cluster formation. Agrin was then washed out and the number of AChR clusters per myotube was counted at successive time points. Clusters dispersed gradually in control cultures (Fig. 3-2). Twenty percent fewer clusters were observed after 1 hr and half the clusters ($t_{1/2}$) dissipated in $\sim 3$ hr. In contrast, clamping intracellular calcium fluxes with BAPTA
markedly accelerated AChR cluster dispersal. The $t_{1/2}$ was $<1$ hr, and virtually all the clusters dispersed within 2 hr of agrin washout. Loading cells with increasing
concentrations of BAPTA-AM resulted in increasing rates of AChR cluster dispersal (Fig. 3-2f). As in our previous studies (Megeath and Fallon, 1998), no effects of BAPTA loading on cell morphology or viability were observed at any of the tested concentrations.

The rapid loss of AChR clusters following clamping of intracellular calcium fluxes could be the result of either AChR internalization or diffusion of AChRs away from the cluster. To distinguish between these possibilities, we used $^{125}$I-α-bungarotoxin binding to determine the levels of surface AChRs in cells stimulated with agrin and then treated with BAPTA or vehicle for 1 hr. Surface AChR levels were not significantly altered in BAPTA- or vehicle-treated (93% ±9 and 81% ±12, respectively, where 100% is the level of AChRs measured immediately after 4 hr of agrin treatment; $p > 0.05$, two-way ANOVA; N=3). In addition, neither BAPTA treatment nor vehicle altered surface AChR levels in cells that had not been stimulated with agrin (91%±13 and 91%±16, respectively; N=3). Thus, the redistribution of surface AChR is the most likely explanation for the accelerated dispersal of AChR clusters induced by clamping intracellular calcium.

Agrin-induced AChR clusters mature upon prolonged agrin exposure. Aggregates present after >12 hr of agrin treatment are larger, more stable, and contain cytoskeletal and basal lamina elements that are not observed in newly formed clusters (Wallace, 1988; Nitkin and Rothschild, 1990; Shadiack and Nitkin, 1991; Lieth and Fallon, 1993). To test whether mature receptor clusters also require intracellular calcium fluxes for their maintenance, we stimulated cells with agrin for 14 hr and then measured AChR cluster dispersal. Mature clusters dispersed somewhat more slowly than their younger
counterparts ($t_{1/2} \sim 3.5$ hr vs. $t_{1/2} \sim 3$ hr for 14 hr and 4 hr agrin stimulated cells, respectively). Nonetheless, BAPTA treatment accelerated the dispersal of these older clusters ($t_{1/2} \sim 1.5$ hr; not shown). Thus the maintenance of mature AChR clusters is also dependent upon intracellular calcium fluxes.

We next asked whether the accelerated AChR cluster destabilization occurred in the face of persistent agrin stimulation. In previous studies we showed that BAPTA treatment did not alter the level of agrin binding. Myotubes were stimulated with agrin for 4 hr and then the cells were treated with BAPTA in the continued presence of agrin. AChR clusters rapidly disperse under these conditions, with a $t_{1/2}$ of $\sim 1$ hr (not shown). These findings suggest that the accelerated destabilization results from the modulation of intracellular signaling pathways.

The role of intracellular calcium fluxes in MuSK and AChR phosphorylation and dephosphorylation

Agrin-induced MuSK phosphorylation is an early and essential step in the AChR clustering pathway. Since previous studies employed rodent myotubes, we developed an assay for MuSK tyrosine phosphorylation in chick muscle cells. We tested two antisera directed against the intracellular and the extracellular domains of rat MuSK (pAb Ecto-MuSK and pAb Cyt-MuSK, respectively (Hopf and Hoch, 1998a; Hopf and Hoch, 1998b)). Both antisera specifically immunoprecipitated a tightly spaced, tyrosine phosphorylated doublet centered at $\sim 110$ kD (Fig. 3-3). This mobility is similar to that observed for mammalian MuSK (Glass et al., 1996; Fuhrer et al., 1997; Jacobson et al.,
1998). Although others have reported a relative mobility of ~140 kD for avian MuSK expressed in C2C12 cells (Glass et al., 1996), we did not detect any candidate MuSK polypeptides with this mobility. The differences between the reported mobility of avian MuSK and our results could be due to the gel systems used or to posttranslational modifications unique to C2 cells. Nonetheless, two different anti-MuSK antisera specifically immunoprecipitate a ~110 kD doublet that is tyrosine phosphorylated in response to agrin stimulation, we therefore conclude that this polypeptide doublet is avian MuSK.

In previous work we showed that blocking intracellular calcium fluxes inhibits agrin-induced AChR cluster formation but not agrin-stimulated AChR tyrosine phosphorylation (Megeath and Fallon, 1998). Since phosphorylation of MuSK precedes that of AChRs, this observation suggested that agrin-induced MuSK phosphorylation might also be insensitive to blockade of calcium transients. To test this prediction, cells were first treated with either BAPTA or vehicle and then incubated with agrin. As shown in Fig. 3-3b, agrin induced robust MuSK phosphorylation in both control and BAPTA-treated cells. These results place the activation of MuSK tyrosine kinase activity either upstream or parallel to the intracellular calcium-dependent steps in the agrin signaling pathway.

We next examined the relationship between AChR cluster dispersal and MuSK tyrosine phosphorylation. Myotubes were stimulated for 4 hr and then washed to remove the agrin. The level of MuSK phosphorylation was measured immediately, or after 1 hr additional incubation in either BAPTA or vehicle only. MuSK phosphorylation declined
-40% in control cells but ~70% in BAPTA-loaded myotubes (Fig. 3-3c). In other experiments BAPTA was added at 4 hr but the agrin was not washed out. Remarkably, MuSK was similarly dephosphorylated in the BAPTA-loaded cells, despite the continued presence of agrin (data not shown). Thus, clamping intracellular calcium fluxes after AChR clusters have formed results in rapid MuSK dephosphorylation.

A later step in the agrin signaling pathway is the tyrosine phosphorylation of AChR β-subunits. In previous work we showed that the initial agrin-induced tyrosine phosphorylation of AChR was unaffected by BAPTA treatment (Megeath and Fallon, 1998). To test whether intracellular calcium transients play a role in maintaining AChR tyrosine phosphorylation, myotubes were treated with agrin for 4 hr and then loaded with BAPTA or vehicle as described above. In vehicle-treated cells the level of AChR β-subunit tyrosine phosphorylation was only slightly diminished 1 hr after agrin withdrawal (Fig. 3-4, compare lanes 1 and 4). In contrast, after 1 hr of BAPTA treatment phosphotyrosine was undetectable on the AChR β-subunit (Fig. 3-4, lane 5). Similar results were observed when agrin was present throughout the experiment (data not shown). Thus, clamping intracellular calcium fluxes after agrin stimulation results in the dephosphorylation of AChR β-subunits.

The role of tyrosine phosphatase activity in MuSK dephosphorylation and AChR cluster dispersal

The results reported above show that the consequences of clamping intracellular calcium fluxes are a function of the state of AChR clustering. If AChRs (and presumably
MuSK) are not clustered, clamping calcium fluxes does not perturb agrin-induced tyrosine phosphorylation of MuSK or AChR. On the other hand, both MuSK and AChR are rapidly dephosphorylated if calcium transients are clamped after AChR clusters have formed. These observations suggest that there may be a link between agrin-induced intracellular calcium transients and a tyrosine phosphatase(s).

To probe for such a link we used the protein tyrosine phosphatase inhibitor pervanadate. Treatment with pervanadate alone did not alter the rate of AChR cluster dispersal (Fig. 3-5a; see also Wallace, 1988). However, pervanadate treatment blunted the BAPTA-accelerated dispersal of AChR clusters, suggesting that clamping intracellular calcium leads to activation of a tyrosine phosphatase(s). To determine if MuSK is a substrate of such a phosphatase, we assayed MuSK in BAPTA or vehicle-loaded cells. We observed that pervanadate blocked MuSK dephosphorylation in BAPTA treated cells (Fig. 3-5b). These results indicate that MuSK is a substrate of a tyrosine phosphatase that is likely to be selectively concentrated at AChR clusters. Further, our data suggest that agrin-induced intracellular calcium transients tonically inhibit this PTP (Fig. 3-6, and see Discussion).

**Discussion**

The central finding of this work is that AChR cluster maintenance is an active process requiring the suppression of PTP activity by agrin-induced intracellular calcium fluxes. Our results also indicate that distinct molecular mechanisms underlie the formation and the dispersal of agrin-induced AChR clusters. Moreover, these
observations demonstrate that the modulation of intracellular signaling pathways can regulate the maintenance and dispersal of AChR clusters independently of MuSK activation by agrin. These findings suggest mechanisms that could mediate synapse formation and elimination in vivo.

A key technique in these experiments was the use of BAPTA to clamp intracellular calcium concentrations. This reagent was designed to be a specific and rapid calcium chelator (Tsien, 1980) and experiments in several cell systems have confirmed these characteristics (Roberts, 1993) (Whitlock and Lamb, 1999) (Deisseroth et al., 1996). Several lines of evidence indicate that this compound specifically blocks intracellular calcium fluxes in myotubes. Fura-2 imaging confirmed BAPTA’s calcium buffering activity in chick myotubes (Fig. 3-1). In a previous study we showed that this compound is non-toxic and its action is reversible in myotubes (see Megeath and Fallon (1998) for discussion). The most salient control is the selective action of BAPTA: while this compound suppressed agrin-induced AChR cluster formation, it did not inhibit the agrin-induced tyrosine phosphorylation of MuSK or the AChR β-subunit. Finally, none of the effects reported here were observed when EGTA, a calcium buffering compound with slower binding kinetics, was loaded into the myotubes in its AM-ester form (unpublished observations).

The dephosphorylation of clustered MuSK and AChR following calcium buffering could be due to inhibition of kinase activity or to increased PTP activity. We favor the PTP explanation for a number of reasons. First, as noted above BAPTA treatment of naïve myotubes has no effect on agrin-induced MuSK or AChR tyrosine
phosphorylation. Thus clamping intracellular calcium does not appear to affect directly the tyrosine kinase activity of either MuSK or the enzyme that phosphorylates AChRs (which is likely to be distinct from MuSK (Fuhrer et al., 1997)). Second, the rapid dephosphorylation of clustered MuSK and AChR in BAPTA-treated cells is observed even when cells remain exposed to agrin. Moreover, previous work has shown that agrin binding to the cell surface is not altered by BAPTA treatment (Megeath and Fallon, 1998). Finally, the PTP inhibitor pervanadate inhibits both the MuSK dephosphorylation and the AChR dispersal that is triggered by calcium transient clamping. Taken together, these results suggest that the regulation of a tyrosine phosphatase(s) is a key element in the maintenance of agrin-induced AChR clusters.

A striking aspect of our results is that the consequences of clamping intracellular calcium fluxes are a function of the state of AChR clustering: the tyrosine phosphatase activity is manifested only after AChR aggregates have formed. A simple and attractive explanation for this dichotomy is the recruitment of a calcium-regulated PTP to agrin-induced AChR clusters. However, it is possible that a more complex mechanism is at work in which a PTP may be generally distributed but regulated differently at AChR clusters. Interestingly, Dai & Peng (1998) also report evidence of PTP activity in the dispersal pathway. They studied AChR clusters induced by HB-GAM-coated beads. Pervanadate treatment inhibited dispersal of these clusters, while injection of a constitutively active PTP drove their dispersal (Dai and Peng, 1998). They propose a model that includes a ubiquitous PTP that serves as a sink for signals derived from localized tyrosine kinases. We observe that pervanadate alone did not alter the rate of
AChR cluster dispersal (see also Wallace (1995)). We feel that the data presented here best fit a model where both tyrosine kinases and phosphatases are localized to agrin-induced AChR clusters.

On the basis of the results presented here, we propose a new model for the maintenance of postsynaptic specializations (Fig. 3-6). Agrin-induced MuSK phosphorylation plays a central role in AChR cluster formation (DeChiara et al., 1996) that may include establishing a 'primary scaffold' (Apel et al., 1997; Bowen et al., 1998). We propose that at some point in the clustering pathway (certainly before 4 hr) a PTP becomes associated with the MuSK/AChR clusters. Thus agrin may also induce the aggregation of a PTP to postsynaptic specializations. From the time that the PTP joins a cluster, its activity is held in check by agrin-induced intracellular calcium fluxes. Although the characteristics of these fluxes are not known, the simplest explanation is that they result from MuSK activation. Indeed, MuSK remains phosphorylated longer than many other receptor tyrosine kinases (Fuhrer et al., 1997), even after agrin withdrawal. Following agrin washout, this persistent activation of MuSK may perpetuate the calcium fluxes, thereby maintaining AChR clusters as well as preserving tyrosine phosphorylation.

An important feature of our model is that elimination of AChR clusters does not occur via reversing the formation pathway. Rather, the mechanisms regulating AChR cluster formation are distinct from the mechanism of maintenance and dispersal. Cluster formation relies upon increased kinase activity, whereas cluster dissipation requires PTP...
activation. It is interesting to consider how synaptic architecture might be sculpted by balanced regulation of these kinases and PTPs.

Two important directions for future work are the identification of the PTP(s) and the characterization of the intracellular calcium fluxes. Our attempts to visualize agrin-induced intracellular calcium transients with Fura-2 imaging have been unsuccessful, possibly because such calcium fluxes are highly localized or beyond the resolution of our methods (Megeath and Fallon, 1998). Candidate PTPs include the synapse-enriched non-receptor PTP from *Torpedo* electric organ that shows high specificity for AChR β-subunits (Mei and Huganir, 1991) and PTPs activated by binding phosphotyrosine residues on receptor tyrosine kinases, such as SHP-2 (Stein-Gerlach et al., 1998).

There may be a mechanistic connection between the dispersal of AChR clusters observed *in vitro* and the selective postnatal synaptic elimination observed during development of mammalian muscle (Nguyen and Lichtman, 1996; Sanes and Lichtman, 1999). Studies of synapse elimination *in vivo* revealed that the dispersal of AChR clusters occurs prior to withdrawal of presynaptic boutons (Rich and Lichtman, 1989; Balice-Gordon and Lichtman, 1993), and that synaptic maintenance is activity-dependant (Nguyen and Lichtman, 1996). We suggest that synapse disassembly may require PTPs situated within every NMJ. Such a localized effector could be regulated by synaptic activity, extracellular factors like agrin, and by intracellular signals. In our studies the protective effect of agrin could be overridden by BAPTA treatment, and similarly an activity-driven signal from one synapse may destabilize a neighboring synapse *in vivo*.
Materials and Methods

Myotube Culture

Embryonic chick myotube cultures were prepared as previously described (Nastuk et al., 1991). Briefly, myoblasts from embryonic day 11 chick embryos were dissociated and plated in medium containing Minimal Essential Medium (alpha medium, Gibco, Gaithersburg MD) supplemented with 2% chick embryo extract, 10% horse serum, 100 U/ml penicillin G, and 2 mM L-glutamine. For ligand binding, immunoprecipitations, and AChR phosphorylation assays, cells were grown on plastic coated with 100 µg/ml gelatin (Sigma, St. Louis MO). For AChR clustering assays and calcium imaging, cells were grown on glass coverslips coated with 20 µg/ml poly-D-lysine (Sigma, MW>300,000) and gelatin. Myotubes were used 3-4 days after plating.

Drug treatment

Stock solutions of the aminomethoxy ester of BAPTA (tetraacetoxyethyl-1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate; BAPTA-AM; Molecular Probes, Eugene OR) were prepared in DMSO (vehicle) (see Megeath and Fallon (1998)). BAPTA-AM or vehicle was diluted in serum free medium (SFM) consisting of Minimal Essential Medium (alpha medium; Gibco), 2 mM L-glutamine (Gibco), 0.5% bovine serum albumin (BSA), 100 U/ml penicillin G, 5 µg/L insulin, 5 µg/L transferrin, and 5 µg/L selenium (all from Sigma). To load cells with BAPTA, cells were incubated in BAPTA-AM for 1 hr 37°C (final DMSO concentration 0.5%), then rinsed with SFM. Cells were loaded with 50 µM BAPTA-AM except where otherwise noted.
Pervanadate was prepared as previously described (Wallace, 1995). One part 500 mM hydrogen peroxide (Mallinckrodt) was added to 50 parts 10 mM Na orthovanadate (Sigma) in modified Tyrodes solution (145 mM NaCl, 5 mM KCl, 5.5 glucose, 40 μM CaCl₂, 1 mM MgCl₂, and 1 M Hepes pH 7.4). The mixture was incubated for 10 min at room temperature and diluted in SFM for a final concentration of 100 μM immediately before use.

**Calcium Imaging**

To load cells with Fura-2, myotubes were incubated in 0.5 μM Fura-2-AM (Molecular Probes) for 1 hr at room temperature and then rinsed in SFM. Acetylcholine (Sigma) was applied to myotubes for 6 sec via a pressure ejection micropipette system (General Valve, Parker Instruments). Fluorescence measurements were made with a high speed dual wavelength imaging system (IonOptix, Milton, MA) with a custom built image intensifier (Intevac) and modified OEM CCD camera module (Philips Photonics). Calibrations were performed in solutions using standard techniques and adjusted to values obtained using in-cell measurements in cells permeabilized with ionomycin.

To calculate resting calcium levels, fluorescence measurements were made for 2 sec prior to the addition of acetylcholine. 2-5 regions were randomly chosen on cells in a field, and measurements from each field were averaged. Means from 4-6 fields were pooled, and values are expressed relative to intracellular calcium levels in cells loaded with Fura-2 only.
AChR Clustering Assays

Recombinant rat agrin, containing inserts of 12, 4, and 8 amino acids at the x, y and z splice sites, respectively, was produced in COS cells as previously described (O'Toole et al., 1996). Recombinant agrin was used at a concentration of 25 pM SFM.

Cells grown on coverslips were incubated with or without agrin for 4 hr at 37°C. Immediately after this incubation, cultures were rinsed twice in SFM, then BAPTA treatment began. Cultures were rinsed twice in fresh media 1 hr after the addition of BAPTA-AM. In some experiments BAPTA treatment was preceded by 10-20 min in EGTA rinse to quantitatively remove any bound agrin (Bowe et al., 1994). The rates of AChR cluster dispersal did not change following this treatment, indicating that residual agrin was not influencing cluster stability (data not shown).

To detect AChRs, 1 μg/ml rhodamine conjugated α-bungarotoxin (α-BTx) was added to the media 1 hr before each culture was fixed. At different time points, coverslips were fixed in methanol at -20°C for 5 min, mounted in Citifluor (Pella, Redding CA), and viewed on a Nikon Eclipse microscope. For quantitation of AChR clustering, 20-30 myotube segments (200 μm in length) were randomly chosen from 2-3 coverslips. AChR clusters (defined as AChR aggregates ≥ 4 μm in diameter) were scored under rhodamine optics (Nastuk and Fallon, 1991) at 400X. The numbers of clusters in each condition were normalized relative to the amount of clusters observed after cells were stimulated with agrin for 4 hr.
Ligand Binding Assays

Binding of α-BTx to myotubes was quantitated as previously described (Bowe et al., 1994). Myotubes grown on gelatin-coated removable 96 well strips (Immulon 4, Dynatech, Chantilly VA) were blocked for 1 hr in MEM-H with 1% BSA and 10% horse serum, and incubated for 30 min with 10 nM $^{125}$I-α-BTx (10-20 μCi/μg, DuPont NEN, Boston MA). Wells were washed in MEM-H, immersed twice in Hank's balanced salt solution with 1% BSA and 1 mM calcium, dried, and counted. Nonspecific binding was determined by including 1 mM EGTA (in agrin binding experiments) or 100-fold excess competing unlabeled α-BTx. In each experiment, six individual wells were counted for each condition, and then results of multiple experiments were pooled.

AChR Extraction and Isolation

AChRs from cultured myotubes were purified according to the method of Wallace et al. (1991) with minor modifications. Biotinylated α-BTx (Molecular Probes) was purified on an ImmunoPure Immobilized Monomeric Avidin column (Pierce). Myotube cultures were incubated for 4 hr in agrin, loaded with BAPTA or vehicle in the presence of 1 μg/ml biotinylated α-BTx, washed twice in cold PBS, and harvested in extraction buffer (5 mM EDTA, 5 mM EGTA, 20 mM Tris, pH 7.5, 20 mM glycine, 1% Triton X-100, 150 mM NaCl, 40 mM Na pyrophosphate, 50 mM NaF, 10 mM Na molybdate, 1 mM Na orthovanadate, 5 mM benzamidine, 1 mg/ml bacitracin, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 50 μg/ml each chymostatin, pepstatin, aprotinin, leupeptin, and antipain). Cell lysates were briefly
centrifuged, and the cell pellet resuspended in extraction buffer by trituration through a 23 gauge needle. Samples were sonicated 20 s with a Branson 450 Sonifier at maximum power, incubated 15 min at 4°C, then spun for 20 min at 3000 x g. Solubilized AChR-biotinylated α-BTx complexes were incubated with streptavidin-sepharose beads (Sigma) for 1.5-2 hr with constant mixing at 4°C. Beads were washed 4 times in extraction buffer containing 1M NaCl, twice in extraction buffer lacking NaCl and Triton X-100, and eluted in reducing SDS-PAGE sample buffer at 60°C for 10 min.

Isoleted AChRs were electrophoresed on 5-15% gradient SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were then blocked in PBS supplemented with 1% BSA. Tyrosine phosphorylated polypeptides were detected with anti-phosphotyrosine mAb 4G10 (diluted 1:1000) (Upstate Biotechnology Inc., Lake Placid NY). After incubation with primary antibody, blots were washed in PBS, incubated for 2 hr with horseradish peroxidase-linked sheep anti-mouse IgG (Amersham Pharmacia Biotech Inc., Piscataway NJ), and immunolabeled bands were visualized with enhanced chemiluminescence. Blots were prepared for successive Western blotting according to the method of Sugiyama (1997). The blots were liberally rinsed in PBS, stripped with 0.2 M glycine, pH 2.5, and 0.1% Tween 20, then reprobed with mAb 61 (generously provided by J. Lindstrom, Univ. Pennsylvania) to detect AChR β-subunits. Bound antibodies were visualized using alkaline phosphatase conjugated goat anti-mouse IgG (Boehringer Mannheim, Indianapolis IN) and an alkaline phosphatase-based kit (Promega, Madison WI).
MuSK Extraction and Isolation

Myotube cultures were stimulated with agrin for 4 hr, loaded with BAPTA, EGTA, or vehicle, washed twice in cold PBS with 1mM Na orthovanadate, and harvested in scrape buffer (50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.1 M NaF, 10 mM Na pyrophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM Na orthovanadate, 1 mM benzamidine, 0.1 mg/ml PMSF, 21 ug/ml aprotinin, and 1 ug/ml each leupeptin and pepstatin A). Cell lysates were spun briefly, resuspended by trituration through a 23 gauge needle in lysis buffer (scrape buffer supplemented with 10% glycerol and 1% Triton X100), incubated for 20 min at 4°C with gentle mixing, and spun for 22 min at 16,000g. Either rabbit antisera pAb Cyt-MuSK or pAb Ecto-MuSK (Hopf and Hoch, 1998a; Hopf and Hoch, 1998b) was then added to the supernatants. Except where otherwise noted, pAb Cyt-MuSK was used throughout. Cell extracts were mixed with Protein A beads (RepliGen Corp., Cambridge MA) for 1 hr, the beads washed with lysis buffer, and the immunoprecipitates eluted in reducing SDS-PAGE sample buffer at 60°C for 10 min. Samples were probed with mAb 4G10 by Western blotting as described above with modifications. Secondary antibodies, goat anti-mouse IgG conjugated to alkaline phosphatase (Boehringer Mannheim, Indianapolis IN), were preadsorbed by incubation with 10% normal rabbit serum (JR Scientific) overnight with agitation, followed by centrifugation (30 min, 16 000g). Immunolabeled bands were visualized using alkaline phosphatase.
Figure 3-1. Intracellular calcium fluxes are clamped in BAPTA-loaded myotubes.

Cultured myotubes were loaded with either BAPTA or vehicle as described in Methods. Intracellular calcium concentrations were monitored by Fura2 imaging. A 6 sec pulse of acetylcholine (ACh; bar) was applied using a pressure pipette. In vehicle-treated cells (○), ACh caused a rapid, transient increase in intracellular calcium. In contrast, ACh-induced intracellular calcium fluxes are greatly diminished in BAPTA-loaded cells (●). Representative traces are presented. Similar results were obtained in 7 other experiments.
<table>
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<tr>
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<th>Post-Agrin Treatment</th>
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<td>A</td>
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<td>None</td>
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<tr>
<td>B</td>
<td>+</td>
<td>None</td>
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<tr>
<td>C</td>
<td>+</td>
<td>BAPTA 1 hr</td>
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<td>D</td>
<td>+</td>
<td>vehicle 1 hr</td>
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Figure 3-2 A-D.
Figure 3-2 E-F.
Figure 3-2. Blocking intracellular calcium fluxes accelerates AChR cluster dispersal.

(a-d) Cultured myotubes were stimulated with agrin as indicated. In (a) and (b) the cells were fixed immediately after the 4 hr incubation. In (c) and (d) the agrin was washed out after 4 hr and the cells incubated for an additional 1 hr in the presence of BAPTA or vehicle alone. AChRs were labeled with rhodamine α-bungarotoxin and their distribution determined by fluorescence microscopy as described in Methods. Note the reduction in the number of AChR clusters in the BAPTA-treated cells. Scale bar, 20 μm.

(e) Time course. Myotubes were incubated with agrin for 4 hr to induce AChR clustering. The agrin was then washed out and the cultures treated as indicated. AChR clusters were then counted as described in Methods. In control cells AChR clusters gradually disperse following agrin withdrawal (t1/2~3 hr). Clamping intracellular calcium concentrations with BAPTA accelerates the rate of cluster dispersal (t1/2<1 hr). Values are mean ± SEM averaged from 3 separate experiments.

(f) Dispersal of AChR clusters in cells loaded with varying concentrations of BAPTA-AM. After a 4 hr agrin incubation, the agrin was removed and cells were treated with the indicated concentrations of BAPTA-AM. The most rapid dispersal is observed in cells loaded with 50μM BAPTA-AM, the concentration used for all other experiments. Mean ± SEM.
Figure 3-3.
Figure 3-3. MuSK is rapidly dephosphorylated when calcium fluxes are clamped after agrin stimulation. Primary chick myotubes were stimulated with agrin and treated with BAPTA and vehicle as indicated. The cell extracts were then immunoprecipitated and probed with anti-phosphotyrosine antibody mAb 4G10.

(a) Identification of avian MuSK. Two different antisera to rodent MuSK (pAb Ecto-MuSK and pAb Cyt-MuSK, directed against the extracellular (ecto) and intracellular (cyto) domains of MuSK, respectively) specifically immunoprecipitate a tyrosine phosphorylated ~110 kD polypeptide doublet (arrow) from cultured chick myotubes. This doublet was not immunoprecipitated by normal rabbit serum (NS). Agrin (25 pM agrin for 15 min) induced the tyrosine phosphorylation of the doublet. The large band at ~90 kD is non-specific. No specific signal was detected in the absence of mAb 4G10 (no primary).

(b) Initial agrin-induced MuSK phosphorylation is insensitive to BAPTA treatment. Myotubes were loaded with either BAPTA or vehicle for one hour, then stimulated with agrin for 4 hr. Agrin induced similar levels of MuSK phosphorylation in both BAPTA- and vehicle-treated cells.

(c) MuSK dephosphorylation is accelerated when intracellular fluxes are clamped after agrin-induced AChR clusters have formed. Myotubes were incubated in the presence or absence of agrin for 4 hr. Cells were either assayed immediately (lane 1), or washed and
treated for 1 hr with vehicle (lanes 2,3) or BAPTA (lane 4). Similar results were observed in 3 other experiments.
Figure 3-4.

Agrin_0→4 hr
BAPTA_4→5 hr

anti-P-tyr

anti-AChRα

+ - - + +
- + - +
Figure 3-4. AChRs are rapidly dephosphorylated when calcium fluxes are clamped after agrin-induced AChR clusters have formed.

Myotubes were treated with or without agrin for 4 hr. The cultures were then washed and the levels of AChR β-subunit tyrosine phosphorylation was then assessed immediately (lane 1) or following a 1 hr treatment with BAPTA or vehicle as indicated (lanes 2-5). In vehicle-treated cells, agrin-induced AChR β-subunit tyrosine phosphorylation remained elevated 1 hr hour after agrin withdrawal (compare lanes 1 and 4). In contrast, tyrosine phosphorylation of AChR β-subunit was undetectable following 1 hr of BAPTA treatment (lane 5). The basal level of AChR tyrosine phosphorylation was unchanged by BAPTA treatment alone (compare lanes 2 and 3). AChR β-subunit tyrosine phosphorylation was assessed as described in Methods. Equivalent loading of samples was verified by stripping the blots and reprobing with antibodies specific for the AChR α-subunit (bottom panel).
Figure 3-5.
Figure 3-5. MuSK is a substrate of a calcium-regulated PTP that regulates AChR dispersal.

(a) A PTP inhibitor occludes BAPTA-induced AChR dispersal. Myotubes were stimulated with agrin for 4 hr. The agrin was then washed out and the cells were treated with vehicle (control), pervanadate (100 µM), BAPTA, or pervanadate and BAPTA. Pervanadate alone did not alter the number of AChR clusters remaining 1hr post-agrin. However, pervanadate inhibited the dispersal of AChR clusters induced by BAPTA treatment. Values are mean ± SEM averaged from 3 separate experiments.

(b) MuSK is a substrate of a calcium-regulated PTP. Pervanadate treatment rescues BAPTA-induced MuSK dephosphorylation. Myotubes were incubated with agrin or control media for 4 hr and then treated for 1 hr with BAPTA, pervanadate, and/or vehicle as indicated. Cell extracts were then immunoprecipitated with anti-MuSK and Western blotting was performed with anti-phosphotyrosine mAb 4G10. Pervanadate treatment had no effect on MuSK phosphorylation (lane 1 and compare lanes 2 and 5), while agrin induced robust MuSK phosphorylation (lane 2). The agrin-induced MuSK phosphorylation was reduced to baseline levels when cells were treated with BAPTA alone (lane 3). In contrast, MuSK phosphorylation remained high when cells were treated with a combination of pervanadate and BAPTA (compare lanes 2 and 4).
Figure 3-6.
Figure 3-6. Proposed model for the interactions of intracellular calcium fluxes and protein tyrosine phosphatases (PTP) in AChR cluster formation, stabilization and dispersal. Formation is triggered agrin activation of MuSK kinase activity (Glass et al., 1996). Intracellular calcium fluxes are necessary for the progression from MuSK activation to AChR aggregation (Megeath and Fallon, 1998). We propose that the maintenance of AChR clusters requires the calcium-dependent suppression of a PTP that is selectively localized at them. Relief of this suppression leads to PTP activation, of MuSK (and AChR) dephosphorylation, and AChR cluster dispersal. Note that dispersal can occur in either the presence or the absence of bound agrin. See text for further details.
CHAPTER IV
DISCUSSION

We investigated a fundamental component of synapse formation: the assembly of molecules necessary for synaptic transmission. In particular, we examined postsynaptic differentiation at the NMJ. In vivo this differentiation is orchestrated by agrin, a neuronally secreted ECM molecule. Agrin triggers the assembly of intracellular, transmembrane, and ECM molecules via a complex signaling pathway. Formation of postsynaptic specializations can be modeled in culture by stimulating myotubes with agrin and monitoring the redistribution of a key synaptic component, the AChR.

Using this model of synaptic differentiation, I discovered a calcium-dependent step necessary for the formation of AChR clusters. Characterization of this step included determining its relative position in the agrin signaling pathway. Intracellular calcium fluxes were shown to be essential for the maintenance as well as the formation of agrin-induced AChR clusters. Blocking calcium transients after agrin stimulation led to the activation of a PTP activity and the rapid dispersal of AChR clusters. Calcium-dependent suppression of this PTP activity is a key component of the molecular mechanism that actively maintains AChR clusters. Finally, the formation and dispersal of postsynaptic specializations occur by distinct pathways.

Agrin triggers a signaling cascade in which only a few steps are known. The data presented in Chapters II and III demonstrate that intracellular calcium fluxes are
necessary elements of the agrin signaling pathway. Though a role for extracellular calcium had been previously found (Wallace, 1988; Nastuk et al., 1991), the requirement for intracellular calcium fluxes was unknown. Our understanding of the agrin signaling cascade would be significantly enhanced by further characterization of these intracellular calcium fluxes. Two aspects are of particular interest: the calcium source and the calcium target.

Identification of the calcium source—be it an intracellular store or the extracellular pool—would suggest additional molecules that might be involved in the agrin signaling pathway. For example, if agrin induces an influx of calcium from the outside of the myotube, then agrin must regulate molecules that allow calcium to cross the plasma membrane. Such regulation is probably indirect since an exhaustive search for agrin binding partners failed to detect agrin binding to any calcium channels. However, if agrin triggers calcium release from an intracellular pool, then the most likely candidates are those channels frequently implicated in signaling cascades, the IP3R and RyR.

Identification of other molecules whose functions are regulated by agrin would allow us to further piece together this signaling cascade and ultimately enhance our understanding of synaptic differentiation. Furthermore, finding the relevant calcium source may assist in identifying the phosphatase involved in dispersal of AChR clusters (see below).

There is evidence to suggest that the agrin signaling pathway relies upon both intracellular calcium stores and the extracellular pool. Extracellular calcium plays a role in the very first step of this pathway: the binding of agrin to the cell surface (Nastuk et al., 1991). The importance of this first step may explain the observation that extracellular
calcium is required for the formation of agrin-induced AChR clusters (Wallace, 1988). The extracellular pool may also supply calcium for the intracellular fluxes, though some studies suggest otherwise. Wallace (1988) screened several compounds that interfere with calcium channels. Compounds that block voltage-gated calcium channels did not inhibit agrin-induced AChR cluster formation. Neither A23187, a calcium ionophore, nor Ruthenium red, an inhibitor of CICR, had any effect in these assays. In preliminary experiments, thapsigargin, an inhibitor of SERCA, partially inhibited agrin-induced AChR clustering (unpublished observations). Together these data intimate that agrin utilizes intracellular calcium stores.

The issue of the calcium source might be resolved by further experimentation with pharmacological compounds or with an alternative method of triggering the agrin signaling pathway. Treatment of cells with antibodies that recognize MuSK will induce MuSK phosphorylation and initiate AChR clustering (Xie et al., 1997; Hopf and Hoch, 1998a). Cells could be placed into calcium-free media prior to antibody treatment. If AChR cluster formation ensues, then intracellular calcium stores are the relevant source.

A rapid intracellular calcium buffer (BAPTA) inhibited agrin-induced AChR cluster formation whereas a slower calcium binding buffer (EGTA) did not (Chapter II). I concluded from these experiments that the target(s) of the calcium flux was located close to the calcium source. In order to place the calcium target in close proximity with the calcium source, agrin may direct the localization of the target, the channel that provides the calcium fluxes, or both. As yet, the only channels known to redistribute in response to agrin are sodium channels and AChRs. Sodium channels will not pass
calcium ions and receptor aggregation is not inhibited when AChRs are blocked with BTx, indicating that these channels are not the source of the intracellular calcium fluxes. Therefore agrin may induce the aggregation of more molecules than those we are currently aware of.

It is possible that agrin does not induce any intracellular calcium signals and instead relies upon constitutive calcium fluxes. Perhaps the myotubes use ongoing calcium transients for homeostasis and the agrin signaling pathway makes use of this mechanism. This model would predict that AChR clusters would preferentially form close to a calcium source. However, postsynaptic specializations can form at any place in the plasma membrane (Sanes and Lichtman, 1999), suggesting that agrin can assemble all necessary components. Therefore, if agrin does rely upon constitutive calcium fluxes, these fluxes are re-located to AChR clusters.

In addition to their origin, other aspects of these intracellular calcium transients warrant further investigation. I demonstrated that the maintenance of AChR clusters required inhibition of a tyrosine phosphatase by intracellular calcium fluxes (Chapter III). These calcium fluxes were required in mature clusters (>12 hr agrin stimulation) as well as in newly formed AChR clusters (4 hr agrin stimulation). How long must this calcium signaling persist? Perhaps the postsynaptic apparatus must be maintained indefinitely and localized PTPs are vigilantly poised to disperse the postsynaptic apparatus should the calcium signals ever cease. Alternatively, AChR aggregates may be anchored and preserved from the PTP activity after a longer maturation period. Should this be the case, is the PTP permanently inactivated or perhaps removed from the cluster? Experiments
designed to address this issue in cultured cells may prove to be difficult. Myotubes detach from the substrate after prolonged time in culture. In addition, such experiments lack other relevant factors such as synaptic input. In order to explore further the mechanism that maintains postsynaptic specializations, *in vivo* experiments may be required.

Visualization of agrin-induced calcium signals may eventually be able to shed light onto some other interesting questions. I attempted to visualize agrin-induced intracellular calcium fluxes using the calcium indicator Fura-2 but was unsuccessful, probably due to the highly localized nature of these fluxes. Should such visualization be achieved, basic questions regarding the amplitude, duration, and timing of agrin-induced calcium signals could be answered. Furthermore, this would provide a means of evaluating how other pathways modulate the agrin signaling pathway. For example, laminin also induces AChR clustering and thus may also rely on intracellular calcium. This pathway is too slow to be investigated with BAPTA since cells recover from BAPTA treatment more rapidly than laminin-induced AChR clusters can form (unpublished observations). Since the effects of laminin and agrin are additive, laminin might enhance some aspect of agrin-induced signaling that could be detected by calcium imaging. Finally, calcium imaging might provide information on the functions of other agrin isoforms.

Intracellular calcium fluxes are necessary for proper functioning of the agrin signaling pathway, but what is the target of these fluxes? As yet, no one knows. Of the molecules known to be localized at the NMJ, none appear to be directly regulated by
calcium. In particular, neither MuSK nor the kinase responsible for phosphorylating AChRs requires intracellular calcium for activation (Chapters II and III). Cells contain numerous calcium binding proteins and thus provide us with numerous candidates. Pharmacological inhibition of two candidate molecules, calmodulin and calcium/calcmodulin kinase II, did not alter agrin-induced receptor clustering (Chapter II and Wallace (1988)). Hopefully the relevant calcium binding molecule will be discovered as the agrin signaling pathway is further characterized.

Though the study of calcium signaling is complicated by the large number and variety of pathways in which calcium participates, there is an inherent benefit as well. A common signaling mechanism provides a potential means of integrating signals. Intracellular calcium is so highly regulated that information is conveyed not only by increases or decreases in calcium concentration, but by the amplitude, duration, and frequency of a calcium signal as well. Though no evidence is yet available, it is interesting to consider how another signaling pathway could impact the formation and maintenance of a NMJ. Synaptic activity, for example, results in a steep rise in cytosolic calcium. Does calcium derived from an action potential contribute to inhibition of phosphatase activity at postsynaptic specializations? Such contributions might be key ingredients in the competition between synapses on multiply innervated muscle fibers.

My discovery that maintenance of AChR clusters requires active inhibition of a PTP leads to many further questions. Of primary importance is ascertaining which PTP is involved. Identification of the relevant PTP is complicated by the possibility that multiple PTPs may be involved. This far, little is known about this particular
phosphatase. It targets phosphorylated tyrosine residues and two of its substrates are MuSK and the AChR β subunit. Furthermore, this PTP is probably localized to AChR clusters.

The PTP responsible for dispersal of AChR clusters is regulated by calcium, either directly or indirectly. Since calcium directly activates some PTKs, it is conceivable that calcium also directly regulates some PTPs. If the PTP in the agrin signaling pathway is directly regulated by calcium, then it is most likely to be localized near the calcium source. Determining whether the calcium is from the extracellular pool or from an intracellular source might aid in the identification of this PTP. For example, if agrin utilizes calcium fluxes from the ER, then the PTP is less likely to have a transmembrane domain in the plasma membrane. Identifying the PTP that regulates AChR dispersal will be a challenging task that may become easier as more of the signaling pathway is pieced together.

Studies of other pathways may discover mechanisms relevant to the formation and dispersal of postsynaptic specializations. One potentially related pathway controls the metabolic stabilization of AChRs. Prior to innervation or following denervation, AChRs turn over quite rapidly (t_1/2 ~1 day) compared to AChRs at mature NMJs (t_1/2 ~10 days) (Vaca, 1988). Research into the regulation of metabolic stabilization has identified two key elements: calcium influx and serine/threonine phosphorylation (Rotzler et al., 1991; Caroni et al., 1993). Though AChR cluster stabilization and metabolic stabilization are gauged on different time scales (a few hours compared to days), intriguing parallels can now be drawn between them. I found that intracellular calcium
fluxes are necessary for the stabilization of AChR clusters. Similarly, Rotzler et al. (1991) observed that calcium influx through DHPRs increases the metabolic stabilization of AChRs. My results demonstrate that activation of a PTP leads to rapid dispersal of AChR clusters. Caroni et al. (1993) found that inhibition of serine/threonine phosphatases promoted the metabolic stabilization of AChRs and that inhibition of serine/threonine kinases had the opposite effect. Furthermore, rapsyn plays an important role in both AChR cluster formation and metabolic stabilization (Gautam et al., 1995; Wang et al., 1999b). The similarities between cluster stabilization and metabolic stabilization may reflect conservation of signaling mechanisms or an intersection between these two processes.

The molecular mechanisms underlying synapse elimination are not well understood. The first morphological sign that a synapse will be eliminated is the dispersal of postsynaptic components, including AChRs (Rich and Lichtman, 1989; Balice-Gordon and Lichtman, 1993; Culican et al., 1998). Does the formation pathway operate in reverse to disperse these molecules? My results indicate that this is not the case. Activation of a PTP is a necessary event in the dispersal pathway, but there is no evidence of PTP activity in the formation pathway. The activity of this PTP may therefore be specific to AChR cluster dispersal. Such a distinction between two opposing pathways might allow cells to more precisely regulate the modification of synapses in neighboring areas. Contemporaneous remodeling is observed in vivo as axonal branches segregate on multiply innervated myotubes (Gan and Lichtman, 1998). My findings indicate that distinct and localized molecular mechanisms regulate the pathways that
shape postsynaptic specializations during development. These same mechanisms may be responsible for changes in synaptic structure driven by experience in the mature animal.

The nervous system relies upon intercellular communication that is rapid, efficient, and malleable. Synapses are the structures through which such communication occurs, thus the composition of synapses is of great importance. All of the molecules necessary for sending and receiving information must be localized to the synapse. In addition, a synapse must be capable of incorporating long-lasting changes. Synaptic plasticity is the means by which previous experience modulates future synaptic output.

Synaptic plasticity is one of several processes that shape the nervous system. Synapse formation, elimination, and regeneration also determine the architecture of the nervous system. Understanding these components will ultimately help us to comprehend the workings of the brain.
# APPENDIX 1

## LIST OF ABBREVIATIONS

**Neuromuscular junction nomenclature**

<table>
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<th>Abbreviation</th>
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</tr>
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<tbody>
<tr>
<td>α-DG</td>
<td>alpha-dystroglycan</td>
</tr>
<tr>
<td>ARIA</td>
<td>acetylcholine receptor inducing activity</td>
</tr>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>AChR</td>
<td>acetylcholine receptor</td>
</tr>
<tr>
<td>AChE</td>
<td>acetylcholinesterase</td>
</tr>
<tr>
<td>β-DG</td>
<td>beta-dystroglycan</td>
</tr>
<tr>
<td>BTx</td>
<td>alpha-bungarotoxin</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>MuSK</td>
<td>muscle specific kinase</td>
</tr>
<tr>
<td>MASC</td>
<td>myotube associated specificity component</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
</tr>
<tr>
<td>PTP</td>
<td>protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RATL</td>
<td>rapsyn associated transmembrane linker</td>
</tr>
<tr>
<td>R-PTP</td>
<td>receptor protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
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### Calcium nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CICR</td>
<td>calcium induced calcium release</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DHPR</td>
<td>dihydropyridine sensitive receptor</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol triphosphate</td>
</tr>
<tr>
<td>IP3R</td>
<td>receptor for inositol triphosphate</td>
</tr>
<tr>
<td>I-CRAC</td>
<td>calcium release-activated current</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol (4,5)-bisphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PM</td>
<td>plasma membrane</td>
</tr>
<tr>
<td>PMCA</td>
<td>plasma membrane calcium-ATPase pump</td>
</tr>
<tr>
<td>SERCA</td>
<td>smooth endoplasmic reticulum calcium-ATPase pump</td>
</tr>
<tr>
<td>SOC</td>
<td>store operated channels</td>
</tr>
<tr>
<td>SOCaE</td>
<td>store operated calcium entry</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>ROC</td>
<td>receptor operated calcium channel</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>VOC</td>
<td>voltage operated calcium channel</td>
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</table>
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