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MOLECULAR MECHANISMS THAT REGULATE NEUROMUSCULAR SYNAPSE FORMATION

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Getu Gonfa Teressa

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Getu Gonfa Teressa

We, the dissertation committee for the above candidate for the
Doctor of Philosophy degree
Hereby recommend acceptance of this dissertation.

Joav Prives Ph.D., Professor
Department of Pharmacological Sciences
Dissertation Advisor

Holly Colognato Ph.D., Assistant Professor
Department of Pharmacological Sciences
Chairperson of Defense

Michael Frohman M.D. Ph.D., Professor and Interim Chair
Department of Pharmacological Sciences

Marian Evinger Ph.D., Associate Professor
Departments of Pediatrics and Neurobiology and Behavior

Michael Hayman Ph.D., Professor
Department of Molecular Genetics and Microbiology

This dissertation is accepted by The Graduate School

Lawrence Martin
Dean of the Graduate School

Abstract of the Dissertation

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A key feature of postsynaptic specialization at the neuromuscular junction (NMJ) is the accumulation of high densities of acetylcholine receptors (AChR) immediately under the nerve ending. The molecular mechanisms responsible for this phenomenon are poorly understood. This study investigated the role of the Focal Adhesion Kinase (FAK) in mediating the formation of AChR-enriched membrane regions on skeletal muscle cells in response to the signaling molecules agrin and laminin. Our findings show that clustering of AChR on the surface of cultured muscle cells by either agrin or laminin is preceded by increased phosphorylation of FAK at tyrosine residues. Moreover, inhibition of FAK by either siRNA-induced FAK knockdown or pharmacological blockers of FAK catalytic activity abolishes AChR cluster formation. Our results indicate that FAK utilizes both kinase-dependent and –independent mechanisms in coupling agrin and laminin stimulation to AChR aggregation. In addition, FAK forms functional complexes with

RhoA, which was also shown to participate in AChR aggregation, as well as surface AChR. Activated FAK colocalizes with AChR aggregates both in cultured myotubes and *in vivo* at intact mouse diaphragm NMJs.

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List of Abbreviations

ACh	Acetylcholine
AChR	Acetylcholine receptor
α-DG	α-Dystroglycan
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
FAK	Focal Adhesion Kinase
FITC	Fluorescein isothiocyanate
FRNK	FAK-related-non-kinase
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GST	Glutathione S-transferase
GST-PBD	GST fused to p-21 binding domain
GST-TRBD	GST fused to Rho-binding domain
GTPases	Guanosine triphosphatases
MASC	Myotube-associated specificity complex
MuSK	Muscle-specific kinase
NMJ	Neuromuscular junction
ROCK	p160 Rho kinase
TMR-Bgt	Tetramethylrhodamine α-bungarotoxin

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Chapter I

General Introduction

I.1. The Neuromuscular Junction as a Model Chemical Synapse

The functional characteristics of the nervous system are dependent on the rapid, precise, and ordered communication between neurons and their target cells. The primary site of this information relay is at cell-cell contact sites called chemical synapses. At chemical synapses, information from the presynaptic nerve terminal is passed on to the postsynaptic cell by means of neurotransmitters which selectively bind to their corresponding receptors on the postsynaptic cell membrane. These neurotransmitter receptors are typically oligomeric channels which undergo conformational change upon ligand binding, resulting in the opening of the ligand-gated ion channel and allowing the movement of ions across the plasma membrane. There are different types of ligand-gated ion channels which differ from each other with respect to the specific neurotransmitter they recognize, the type of ions they are permeable to and the direction of ion movement through the channel. Based on these characteristics, the opening of ion channels at a chemical synapse may result in membrane depolarization (excitatory synapses) or membrane hyperpolarization (inhibitory synapses).

The vertebrate neuromuscular junction (NMJ) is a prototype chemical synapse that has been extensively studied and has been instrumental in elucidating key aspects of synapse structure, function, and assembly (Fig.I.1). It is composed of the presynaptic motor neurons and the postsynaptic skeletal muscle cell. The cell bodies of the motor neurons lie in the ventral horn of the spinal cord and the axons extend to skeletal muscle where they branch into 20-100 unmyelinated axon terminals each of which innervate a restricted area of a single skeletal muscle cell membrane. Synaptic transmission at the vertebrate NMJ involves the release from the motor terminal of the neurotransmitter acetylcholine (ACh), which diffuses across the synaptic cleft to bind to muscle type

nicotinic acetylcholine receptors (AChR) on the skeletal muscle cell surface. AChRs respond by allowing the influx of cations, producing local depolarization and activation of voltage-gated sodium channels resulting in action potential and muscle contraction.

ACh was the first neurotransmitter to be identified, and AChR was the first neurotransmitter receptor to be purified and cloned (Duclert and Changeux, 1995). The discovery of the deadly snake toxin α-Bungarotoxin which binds with high affinity to muscle AChR made possible the purification of AChR and its chemical and structural characterization. These hallmarks, combined with its large size, accessibility, and relative simplicity compared to neuronal synapses made NMJ the leading model synapse in the elucidation of the mechanisms of synapse assembly.

The presynaptic nerve terminal, the postsynaptic membrane as well as the synaptic basal lamina at the NMJ are each characterized by their unique biochemical composition and structural specialization. Specializations at the presynaptic terminal include concentration of neurotransmitter-containing vesicles at the tip of the nerve terminal in specialized regions called active zones, while the postsynaptic terminal is characterized by the presence of membrane folds and the concentration of high densities of AChR aggregates immediately under the nerve terminals at the crests of these membrane folds (Huh and Fuhrer, 2002; Sanes and Lichtman, 1999; Sanes and Lichtman, 2001). The synaptic basal lamina is composed of key molecules that are deposited in a developmentally regulated fashion to direct synaptic differentiation including the formation of AChR aggregates at the crests of postsynaptic membrane folds. This specialization of postsynaptic membranes represents an integrated response to complex

signaling pathways that originate in the presynaptic nerve ending and are mediated by the synaptic extracellular matrix (ECM). During NMJ formation, these signals cause the localized transformation of a restricted region of the muscle cell surface into a region specialized in both morphology and biochemical composition for the efficient reception of nerve impulses and initiation of action potential across the muscle membrane.

While the architecture of the synapse as well as the molecular machinery of synaptic transmission are relatively well characterized, the molecular mechanisms that are responsible for the formation of topologically restricted sub-cellular specializations at synapse are poorly understood. This thesis presents several findings that identify signaling molecules and pathways that regulate the surface redistribution of AChR in response to synaptogenic cues.

I.2. Acetylcholine Receptor Clustering

Skeletal muscle AChR is a heteropentamer comprised of two α subunits and single β, δ, and γ (or ε in adult NMJs) subunits arranged symmetrically around a central ion pore. Before the formation of nerve-muscle contact, AChRs on the skeletal muscle cell surface are diffusely distributed at a density of<1000/μm^2. A crucial event during NMJ formation is the establishment of high density aggregates of AChR (>10000/μm^2) and associated proteins on the muscle cell membrane region directly underlying the motor nerve ending (Burden, 1998; Sanes and Lichtman, 1999; Sanes and Lichtman, 2001).

A key neural factor that transforms the localized regions of postsynaptic membrane into high density AChR-containing patches was identified to be agrin- a heparan sulfate proteoglycan that is released from motor neurons and incorporated into synaptic muscle basal lamina. In addition to the redistribution of pre-existing surface AChR, the establishment and maintenance of high density of AChR aggregates at synaptic sites (>1000 fold compared to extrasynsaptic sites) involves at least three additional mechanisms: increased expression of AChR subunit genes by specialized subsynaptic myonuclei; suppression of AChR subunit gene expression by extrasynaptic myonuclei; and increased metabolic stability of synaptic AChRs (Sanes and Lichtman, 2001). In addition to agrin, the ECM glycoprotein laminin appears to be crucial for normal development of the NMJ (Patton et al., 2001; Patton et al., 1997) and also causes clustering of AChR on cultured muscle cells (Patton et al., 1997; Sugiyama et al., 1997).

The early events in the induction of AChR clustering can be mimicked by applying a soluble recombinant form of neural agrin (Burden, 1998; Colledge and Froehner, 1998) or soluble laminin to cultured muscle cells (Patton et al., 1997; Sugiyama et al., 1997). The clusters induced by both soluble agrin and laminin are virtually identical in their oval appearance-reminiscent of a partially mature postsynaptic apparatus in embryonic NMJ. In contrast, it has recently been shown that myotubes cultured on laminin-coated substrates form elaborated branched AChR-rich domains remarkably similarly to fully mature postsynaptic apparatus seen *in vivo* (Kummer et al., 2004).

The intracellular molecular pathways mediating the effects of agrin and laminin are not well understood. However, the formation of stable AChR clusters was shown to depend on tyrosine kinase signaling (Ferns et al., 1996; Wallace, 1994) as well as on focal changes in actin cytoskeleton (Dai et al., 2000). We have investigated the mechanism by which tyrosine phosphorylation signaling and cytoskeletal dynamics are coupled to the clustering of AChR. Our findings characterize the specific contributions to the formation of AChR aggregates on skeletal muscle surface of key intracellular signaling molecules, monomeric RhoGTPases and Focal Adhesion Kinase (FAK), all of which are crucial regulators of actin cytoskeletal dynamics.

I.3. Agrin

Agrin is a single chain, heparan sulfate proteoglycan with a predicted molecular weight of ~225 KDa. It was originally isolated from the basal lamina of the torpedo electric organ and identified by virtue of its ability to induce AChR aggregation on cultured myotubes (McMahan, 1990; Nitkin et al., 1987) . Agrin is synthesized in the cell body of motor neurons, transported down the axons, and released from the nerve terminal into the synaptic basal lamina where it becomes a stable component of the ECM. It orchestrates the specialization of the presynaptic and postsynaptic membrane mediated by the activation of a muscle-specific receptor tyrosine kinase MuSK. Agrin deficient mice show both presynaptic and both synaptic defects before dying perinatally- AChR aggregates are deficient in these animals and their presynaptic axon terminal shows abnormally extensive arborization.

Agrin is comprised of highly conserved domains that are also shared by a number of basal lamina proteins. The N-terminal region contains a domain necessary and sufficient for binding of agrin to basal lamina proteins- particularly laminins. The C-terminal region (~95KDa) containing the three laminin-G-like domains (encompassing the α-dystroglycan binding site) is necessary and sufficient for the full extent of agrin's AChR cluster-inducing activity (Nitkin et al., 1987). The smallest C-terminal fragment that retains agrin's ability to form AChR aggregates is a 21KDa fragment, but this fragment has a higher EC50 compared to the 95KDa fragment. Agrin has several alternative splicing sites. The insertion of exons (encoding 8 and/or 11 amino acids) at the B/Z splicing site located within the 21KDa C-terminal end gives rise to AChR-aggregating isoform of agrin that is expressed only in motor neurons (Gesemann et al., 1995; Hoch et al., 1994a).

The early events in postsynaptic membrane assembly can be mimicked by applying soluble full-length neural agrin or recombinant C-terminal fragment to myotube cultures. This induces the redistribution of AChRs into ovoid aggregates, while at mature NMJ these aggregates are further differentiated into a pretzel-like morphology.

I.4. Laminin

Laminin is a glycoprotein component of the basal lamina of all tissues. It is a heterotrimer composed of three subunits -α, β, and γ - each of which is encoded by separate genes. There are at least 11 different types of laminin as determined by their subunit compositions (Burgeson et al., 1994; Timpl et al., 1982), and their expression and

distribution in the muscle basal lamina is under tight developmental control. Laminins 4, 9, and 11 are found exclusively in synaptic basal lamina while the remaining subunits are either found throughout the muscle basal lamina or completely excluded from the synaptic basal lamina. Laminin isoforms have wide range of functions in NMJ assembly including the regulation of the size of the NMJ, alignment of the pre- and post-synaptic apparatus (Patton et al., 2001), pre-synaptic differentiation, and membrane folding (Noakes et al., 1995).

In cultured myotubes, laminin-1, -2, and -4 have been shown to induce ovoid AChR aggregates (Sugiyama et al., 1997) that are more or less indistinguishable from AChR clusters induced by agrin under the same conditions. However, there is compelling evidence indicating that agrin and laminin utilize different pathways to induce AChR aggregation. In marked contrast to agrin, laminin can induce AChR aggregation independent of MuSK in MuSK-/- myotubes (Montanaro et al., 1998; Sugiyama et al., 1997) indicating that laminin uses other surface protein(s) as its receptor(s). Two laminin-binding skeletal muscle surface proteins, α-dystroglycan (α-DG) and α7β1 integrin, have been implicated as potential laminin receptors since both are shown to mediate laminin-induced AChR clustering in cultured myotubes. α-DG as well as a specific alternatively spliced variant of α7β1 co-distribute with AChR in response to laminin, and monoclonal blocking antibodies directed against either of these proteins block laminin-induce AChR aggregation (Burkin et al., 1998). Furthermore, myotubes lacking functional α-DG or expressing antisense α-DG mRNA fail to aggregate AChR in response to laminin (Montanaro et al., 1998). Together, these findings led to the

conclusion that laminin utilizes α-DG and α7β1 integrin as surface receptors to induce AChR aggregation on skeletal muscle surface.

I.5. Muscle-Specific Tyrosine Kinase

Muscle-Specific Tyrosine Kinase (MuSK) is a receptor tyrosine kinase (RTK) that is believed to be a component of the functional agrin receptor complex on skeletal muscle cell surface (Valenzuela et al., 1995). It is a single transmembrane receptor containing a large ectodomain with immunoglobulin-like motifs and a cytoplasmic kinase domain characteristic of RTKs. Agrin and MuSK-null mutant mice show identical phenotype, but the synaptic differentiation defects are more pronounced in the latter. MuSK-null mice show no detectable signs of postsynaptic differentiation and the mice die immediately after birth due to respiratory failure. Moreover, muscle cultures derived from MuSK knockout mices are unresponsive to agrin. Additional evidence that establishes ligand-RTK relationship between agrin and MuSK include findings that agrin triggers MuSK dimerization and tyrosine phosphorylation on three critical tyrosine residues in the kinase activation loop in myotubes. However, these effects are not observed in MuSK-expressing heterologous cell lines. Moreover, despite the mapping of the agrin-responsive elements of MuSK to the first two immunoglobulin-like domains in the MuSK ectodomain, MuSK is unable to bind directly to agrin, leading to the hypothesis that MuSK and another yet unidentified Muscle-Associated Specificity Component (MASC) make up a functional agrin receptor in myotubes (Glass et al., 1996).

I.6. The Cytoskeleton and AChR Clustering

AChR clustering pathways initiated by agrin and laminin share fundamental features that point to central roles for tyrosine kinase signaling and cytoskeletal dynamics in regulating the redistribution of surface AChR. AChR aggregation is accompanied by increased anchorage of AChRs to the cytoskeletal framework and loss of lateral mobility (Prives et al., 1982; Stya and Axelrod, 1983). Moreover, both tyrosine kinase inhibitors (Ferns et al., 1996; Wallace, 1994) and cytoskeletal toxins (Dai et al., 2000) were shown to inhibit AChR aggregation. Therefore, it is believed that ECM-cues initiate tyrosine kinase signaling resulting in surface redistribution of AChR by a mechanism mediated by focal changes in the peripheral actin-based cytoskeleton(Dai et al., 2000; Hoch et al., 1994b). The localization of the focal adhesion proteins vinculin, talin, paxillin, and α-actinin at AChR clustering sites suggest a shared molecular mechanism of focal adhesion assembly and AChR clustering. It is notworthy that these proteins each interact with peripheral actin cytoskeleton pointing to the central role of the actin cytoskeleton in these dynamic events.

The small monomeric RhoGTPases and the non-receptor tyrosine kinases FAK (Focal Adhesion Kinase) are two prominent intracellular signaling molecules that regulation actin cytoskeletal dynamics and the assembly of focal adhesions in diverse biological context in different cell types. The roles of these signaling molecules in the regulation of agrin- and laminin-triggered AChR redistribution on skeletal muscle cell surface are examined in our laboratory and this thesis reveals their crucial contributions to AChR cluster assembly.

I.7. Rho Guanosine Triphosphatases (RhoGTPases)

Monomeric G-proteins of the Rho family are molecular switches that cycle between active GTP-bound form and inactive GDP-bound form. They have been shown to integrate a variety of extracellular signals and link these signaling events to dynamic changes in actin cytoskeleton organization. In cultured fibroblasts, Rac1 activation induces actin polymerization at the plasma membrane causing the appearance of membrane ruffles and lamellipodia. RhoA exerts its effects by stimulating actin–myosin interactions, stress fiber appearance and focal adhesion complex formation. Through these effects on actin dynamics RhoGTPases can serve as molecular switches to direct focal changes in cell surface composition and properties(Bishop and Hall, 2000; Hall, 1998; Ridley, 2001; Takai et al., 2001). Findings that localized reorganization in the peripheral cytoskeleton can underlie the aggregation of AChRs at NMJs (Dai et al., 2000; Hoch et al., 1994b) suggested that RhoGTPases could have important roles, and led us to investigate this possibility. Studies carried out in my current laboratory demonstrated that agrin triggers the activation of Rac1 and Cdc42, and that this activation is necessary but not sufficient for formation of full-sized AChR clusters(Weston et al., 2000). Subsequent studies, part of which is presented in Chapter IV of this paper, show that RhoA plays a crucial role in agrin- and laminin-initiated signaling that is subsequent and complementary to the contribution of Rac1/Cdc42.

I.8. Focal Adhesion Kinase (FAK)

Focal Adhesion Kinase (FAK) is a nonreceptor tyrosine kinase that has been demonstrated to be a downstream integrator of signals from receptor tyrosine kinases, integrins, and G-protein coupled receptors in different cell types (Schlaepfer et al., 1999; Sieg et al., 2000). It is a prominent constituent and regulator of focal adhesion-macromolecular complexes involved in cell-substrate adhesion and signaling (Burridge and Chrzanowska-Wodnicka, 1996; Hanks et al., 1992; Schaller et al., 1992). The focal adhesion proteins vinculin, α-actinin, filamin, talin, and paxillin are also localized at AChR clustering sites (Bloch and Geiger, 1980; Shadiack and Nitkin, 1991) suggesting a shared molecular mechanism between focal adhesion assembly and AChR clustering. Furthermore, some proteins that are reported to play roles in AChR aggregation in skeletal muscle cells such as Src kinases (Fuhrer and Hall, 1996; Marangi et al., 2002; Mohamed et al., 2001; Smith et al., 2001), PI3 kinase (Nizhynska et al., 2007), Shc (Jones et al., 2007) and calpain (Chen et al., 2007) are known to interact with FAK in other cell types (Mitra et al., 2005; Schaller et al., 1994). Our evidence on the crucial role of FAK as well as its mechanistic relationship to RhoA in the context of AChR aggregation is described in Chapter V of this thesis.

I.9. General Significance

Neuromuscular Disorders: Congenital Myasthenic Syndromes (CMS) and Myasthenia Gravis (MG) are heterogeneous groups of NMJ disorders arising from mutations and autoantibodies, respectively, targeting NMJ components. These disorders are characterized by inefficient transmission and reception of electrical impulses at the NMJ. The identification and characterization of the structural and signaling components

of the NMJ is providing a better understanding of the molecular basis for these NMJ disorders. AChR was the first for mutations or autoantibodies in these disorders to be identified. However, with an increasing understanding of the molecular mechanisms of synaptic differentiation, several additional targets are now being recognized. Mutations in CMS are now identified in the genes encoding MuSK as well as rapsyn (Ohno et al., 2002), a scaffolding protein that is essential for AChR clustering (Antolik et al., 2006; Apel et al., 1997; Banks et al., 2003; Fuhrer et al., 1999; Gillespie et al., 1996; Marangi et al., 2002; Mittaud et al., 2001). Soon after its discovery as a scaffolding postsynaptic protein essential for synaptogenesis at NMJ, Dok-7(Okada et al., 2006) has also very recently been shown to be mutated in subset of patients with CMS (Beeson et al., 2006). MuSK is also identified as an autoantibody target in a third of patients with AChR antibody-negative MG. Autoantibodies against various intracellular targets such as rapsyn are also shown to emerge in association with either AChR antibody-positive MG (Agius et al., 1998b) or other autoimmune disorders such as lupus (Agius et al., 1998a) where NMJ disorder may not be the predominant clinical presentation.

The most frequent targets of mutations (80%) and autoantibodies in CMS and MG are components of the postsynaptic apparatus, reflecting the structural and molecular complexity of the postsynaptic specialization. Our studies focusing on the characterization of the molecular mechanisms of postsynaptic membrane assembly therefore has the potential to contribute to a better understanding of molecular targets as well as mechanisms of pathogenesis associated with NMJ disorders.

CNS synapse function and regulation: Although the central synapses are far more diverse and experimentally inaccessible than the NMJ, they share several key features that point to conserved general mechanisms in synapse assembly, maintenance, and remodeling. The synaptic apparatus of both neuronal and nerve-muscle synapses possess similar structural features such as high concentrations of neurotransmitter receptors, postsynaptic densities, cortical cytoskeletal networks, and specialized synaptic basal lamina. The similarities are most apparent at synapses involving receptors belonging to the genetically conserved superfamily of ligand-gated ion channel (Karlin and Akabas, 1995; Olsen and Tobin, 1990; Sargent, 1993; Seeburg, 1993), which includes neuronal AChRs, Glycine Receptors, GABA, and type 3 serotonin receptors. Therefore, our studies of the developmental mechanisms of NMJ can increase understanding of mechanisms involved in the assembly and plasticity of the postsynaptic apparatus in the CNS, including regulatory events underlying learning and memory.

I.10 Figures

Figure I.1 The neuromuscular junction. The NMJ is composed of a presynaptic motor nerve terminal and the postsynaptic muscle cell. ACh packaged in vesicles at the presynaptic terminal is exocytosed when an action potential reaches the nerve terminal. ACh binds to AChR found at high concentration on the postsynaptic cell, causing cation influx resulting in action potential and muscle contraction. High density of AChR aggregates are formed primarily by neural agrin-MuSK signaling.

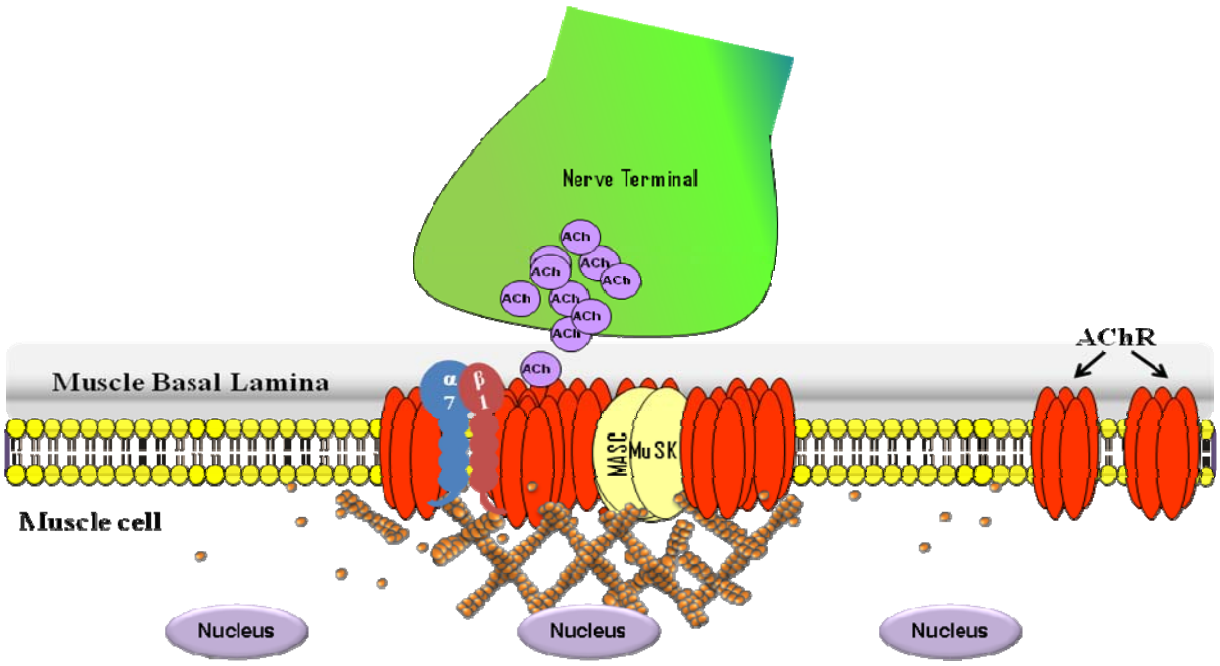

Nerve Terminal
ACh
Muscle Basal Lamina
α 7
β 1
MASC
MuSK
AChR
Muscle cell
Nucleus
Nucleus
Nucleus

Figure I. 2 Domain structure of agrin. Functional domains of agrin include laminin G-like (G) domains, EGF-like domain, and laminin-binding N-terminal domain (NtA). The C-terminal region containing G3 and insert(s) at the alternative splicing site is necessary and sufficient for AChR clustering.

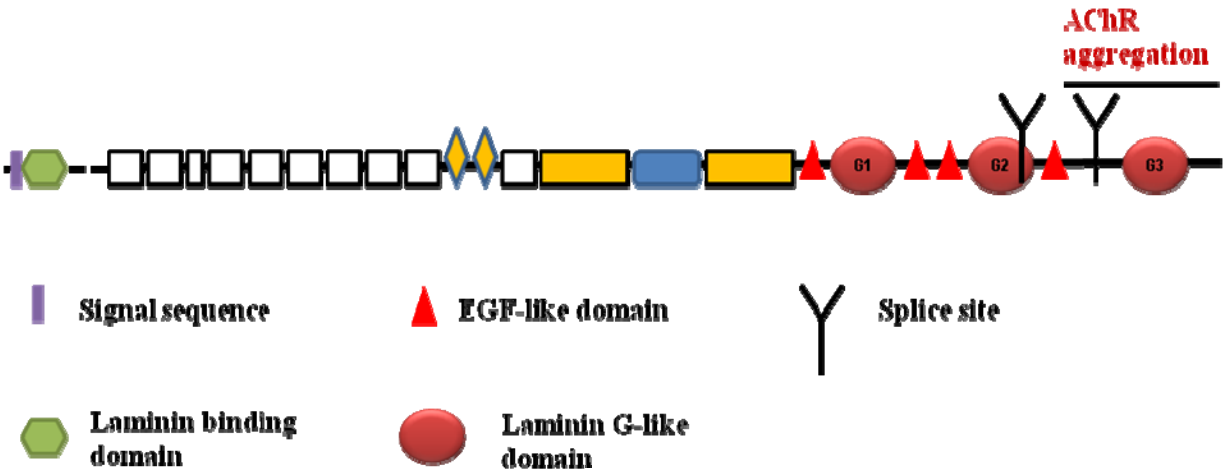

AChR
aggregation
G1
G2
G3
Signal sequence
EGF-like domain
Splice site
Laminin binding
domain
Laminin G-like
domain

Figure I. 3 Subunit composition and structure of laminin. Laminin is composed of α, β and γ chains. The agrin binding site is located in the coiled-coil region, whereas, the α7β1 integrin and α-dystroglycan binding sites are located in the laminin globular domains (G-domains).

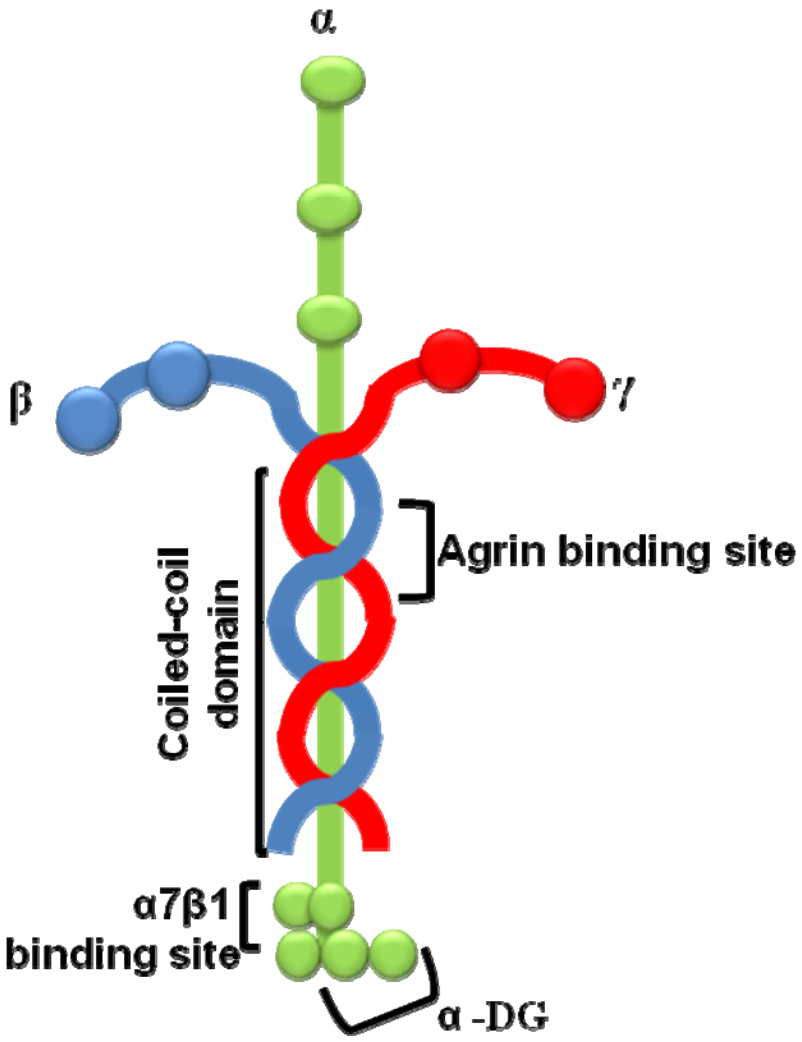
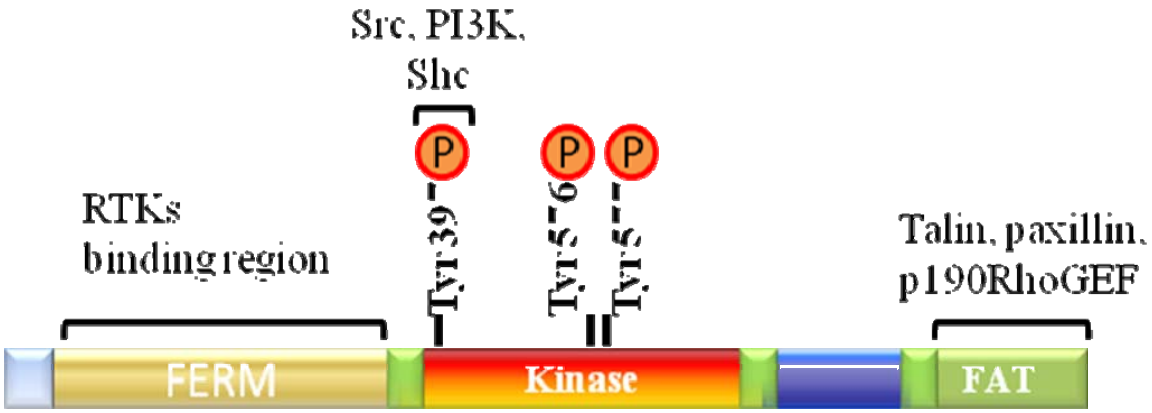

α
β
γ
Agrin binding site
Coiled-coil domain
α7β1 binding site
α -DG

Figure I. 4 Domain Composition of FAK. FAK is composed of a FERM (protein 4.1, ezrin, radixin and moesin homology) domain, a central kinase domain and a focal adhesion targeting (FAT) domain. The FERM domain mediates FAK's interactions with receptor tyrosine kinases (RTKs) such as the epidermal growth factor (EGF) receptor and platelet-derived growth factor (PDGF) receptor. The FAT domain targeted FAK to focal adhesion sites via its interactions with integrin-associated proteins such as talin and paxillin. The FAT domain also links FAK to Rho GTPases activation by binding to guanine nucleotide-exchange factors (GEFs) such as p190 RhoGEF.


Src, PI3K,
Shc
P
P
P
RTKs
binding region
Tyr397
Tyr576
Tyr577
Talin, paxillin,
p190RhoGEF
FERM
Kinase
FAT

Chapter II

Materials and Methods

II.1. Antibodies and Reagents

Expression plasmid encoding GST fused to the Cdc42/Rac (p-21) binding domain of PAK (GST-PBD) as well as to the Rho-binding domain (TRBD) of the Rho effector protein Rhotekin were gifts from S. Moores and J. Brugge (Harvard University, Boston, MA) and M. Schwartz (University of Virginia, Charlottesville, VA), respectively. Expression plasmids encoding dominant negative EGFP-tagged Rho (GFP-RhoN19) were generously provided by M. Frohman and G. Du (Stony Brook University, Stony Brook, NY). C3 transferase proteins were generously provided by D. Bar-Sagi (Stony Brook University, Stony Brook, NY). Laminin-1 was purchased from Invitrogen. A peptide encoding the c-terminal half of agrin was purchased from R & D Systems. Nucleofector V transfection Kit was purchased from Amaxa. FAK siRNA was purchased from Dharmacon. Tyrophostin 25 (AG82) was purchased from Calbiochem. PF-573,228 was supplied by Pfizer Inc. (Central Research Division, Groton, CT). Tetramethylrhodamine-α-bungarotoxin was purchased from Molecular Probes) Mouse monoclonal antibody against RhoA and mouse monoclonal antibody against FAK were purchased from BD Transduction. Rabbit monoclonal p-FAK (Tyr397) was purchased from Biosource. Mouse antiphosphotyrosine antibody 4G10 was purchased from Upstate Cell Signaling. P-Tyr (PY99), p-Tyr (PY20), as well as p-AChRβ1 (Tyr390) and p-FAK (Tyr 576/577) were purchased from Santa Cruz Biotechnology. Other reagents were obtained from Sigma-Aldrich, unless otherwise stated.

II.2. Cell Culture

C2C12 mouse myoblasts were plated on 100 mm culture dishes or 12mm coverslips in Growth Medium consisting of Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 5% calf serum and 100 µg/ml penicillin-streptomycin at 37°C with air/5% CO_2. To stimulate muscle differentiation, two days after plating Growth Medium was replaced with Differentiation Medium consisting of DMEM containing 2% horse serum and 100 µg/ml penicillin-streptomycin. Under these conditions the majority of myoblasts fused to form multinucleated myotubes during the subsequent 3 days.

II.3. Secondary Culturing (Replating)

Where indicated, differentiated myotubes were replated as follows: 12mm glass coverslips were coated with laminin for microscopy by first coating with 2 µg/ml polyornithine in sterile distilled water for 30 minutes and air dried for 30 minutes. Next, a 100 µg/ml solution of laminin in DMEM was applied to the surfaces for 3 hours at 37°C, prior to replating myotubes over these surfaces. The newly fused myotubes are detached by mild trypsinization, centrifuged at 5000rpm, and gently resuspended in Differentiation Medium before replating onto the laminin-coated coverglasses. 3-4 days after initial contact with the laminin-coated substrate, elaborate pretzel-shaped AChR aggregation structures reminiscent of postsynaptic regions of innervated muscle are observed.

II.4. Transfection

For experiments utilizing transient transfections, 1 day post-plating C2 myoblast cultures were transfected with the indicated plasmids at a final concentration of 5 µg of DNA/ml using either LipofectAMINE reagent (Invitrogen) or Amaxa electroporation system for C2C12 cells (Nucleofector Kit V solution, Amaxa) . The transfection medium was replaced with differentiation medium for 3 days prior to addition of 5 nM soluble recombinant neural agrin

II.5. RhoGTPase Activity Assay

C2 myotubes were treated with 5 nM agrin for 15 min and then rinsed with ice-cold Tris-buffered saline supplemented with 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The cells were then lysed by incubation for 5 min on ice with either lysis buffer A (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) or lysis buffer B (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1% Nonidet P-40, 10% glycerol, 100 mM NaCl, 1 mM benzamidine, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 0.5% sodium deoxycholate) and centrifuged for 5 min at 21,000 × *g* at 4 °C, and the supernatants were utilized as cell lysates.

To measure Rho activation, an affinity precipitation method was (Ren and Schwartz, 2000) in which cell lysates prepared with lysis buffer A were incubated with GST fused to the Rho-binding domain from the effector protein Rhotekin (GST-TRBD) bound to glutathione-coupled Sepharose beads for 45 min at 4 °C. The beads were washed four times with wash buffer (50 mM Tris, pH 7.2, containing 1% Triton X-100,

150 mM NaCl, 10 mM $MgCl_2$, 10 µg/ml each of leupeptin and aprotinin, and 0.1 mM phenylmethylsulfonyl fluoride). Bound Rho proteins were eluted with sample buffer (Laemmli, 1970) and detected by Western blotting using a monoclonal antibody against RhoA (Santa Cruz Biotechnology). The blots were developed using goat anti-mouse antibody coupled to horseradish peroxidase (1:1,000 dilution) and visualized with the ECL detection system (Amersham Biosciences).

In a similar manner Rac activation was measured by affinity precipitation of cellular GTP-bound forms of Rac (Sander et al., 1998). In this case cell lysates were prepared with lysis buffer B and incubated with GST fused to the Cdc42/Rac (p21)-binding domain of PAK (GST-PBD) bound to glutathione-coupled Sepharose beads for 30 min at 4 °C. The fusion protein beads with bound proteins were then washed three times in an excess of lysis buffer, eluted in sample buffer, and then analyzed by Western blotting with a mouse monoclonal antibody against human Rac1 (Transduction Labs) at a 1:1,000 dilution. The blots were developed using sheep anti-mouse coupled to horseradish peroxidase (1:1,000 dilution) and visualized with the ECL detection system (Amersham Biosciences).

II.6. Rho Pathway Inhibitors

Myotubes were treated with either soluble C3 exotransferase from *Clostridium botulinum* (Aktories et al., 1989; Sekine et al., 1989b; Wilde and Aktories, 2001b; Wilde et al., 2001) or Y27632 (Ishizaki et al., 2000) at varying time points and were monitored for clustering ability in response to agrin or laminin. C3-transferase was applied to C2 myotubes at a concentration of 50 µg/ml and preincubated for 30min at 37 °C prior to

agrin or laminin treatment. The cultures were then incubated for an additional 8 h in the presence of both C3-toxin and agrin. For Y-27632, myotubes were treated with Y-27632 at 20 μM concentration, and the cells were incubated at 37 °C for 2 h prior to the addition of agrin or laminin. For experiments involving replating onto immobilized laminin, Y-27632 was added immediately after replating of differentiated myotubes. 20 μM of Y-27632 was then subsequently added every 2 h for 8 h to ensure a continuous presence of Y-27632 at a pharmacologically effective concentration. The effect of Y-27632 on agrin- or laminin-induced AChR clustering was measured by labeling AChR clusters with the AChR ligand tetramethylrhodamine-conjugated α-bungarotoxin (Molecular Probes). For all biochemical experiments involving Rho pathway inhibitors (Y-27632 and Toxin-B), myotubes were pretreated with the indicated concentrations of the inhibitors 30min prior to agrin addition.

II.7. Cell Staining

For visualizing the surface distribution of AChRs, the cells were incubated with 10nM tetramethylrhodamine-α-bungarotoxin (TMR-Bgt) dissolved in DMEM with 1 mg/ml of BSA for 1 hour at 37°C, rinsed with PBS, and fixed with 3.7% formaldehyde for at least 30min at 4°C. To detect the cellular distribution of activated FAK on myotubes replated onto immobilized laminin, cells were first detergent extracted with saponin (Bloch, 1984). Briefly, TMR-Bgt labeled myotubes were washed with PBS supplemented with 10mM MgCl2, 1mM EGTA, and 1% BSA, and further incubated at room temperature while shaking in 0.2% saponin solution in PBS supplemented with 10mM MgCl2, 1mM EGTA, and 1% BSA. Cultures shaken for 20min were washed in

PBS, and fixed with 3.7% formaldehyde for at least 30min at 4°C. The cells were then rinsed with PBS and individual coverslips were incubated with rabbit anti-phospho FAK antibody for 1 hour at 37°C. The coverslips were rinsed with PBS and stained by incubating with an FITC-conjugated goat anti-rabbit antibody. Cover slips were mounted on slides using Aqua-Mount (Lerner Laboratories).

II.8. Immunohistochemistry

Diaphragm muscles were dissected from P22 mice, fixed in 1% paraformaldehyde in PBS , pH7.3 at 4°C for overnight, rinsed three times for 10 min in PBS, washed for 30 min in PBS supplemented with 0.1 M glycine, washed for 5 min in PBS, permeabilized for 30 min with 0.5% Triton X-100 in PBS (PBT). The muscles were then incubated overnight at 4°C with rabbit antibodies specific to the phosphorylated FAK (Tyr397and Tyr576/7 mixture) in PBT with 2% BSA, 2% goat serum. The muscles were subsequently rinsed in PBT, washed three times for 1 hr in PBT, incubated with FITC-conjugated donkey anti-rabbit IgG and TMR-Bgt in PBT overnight at 4°C, washed three times for 1 hr with PBT, washed once for 10 min in PBS, post-fixed for 10 min in 1% formaldehyde and mounted.

II.9. Confocal Fluorescence Microscopy

Samples were scanned with a Zeiss LSM 510 laser scanning confocal device attached to an Axioplan 2 microscope using a 63x Plan-Apochromat oil objective (Carl Zeiss, Inc.), and processed using Adobe Photoshop. For double staining experiments, each dye was scanned independently using the multitracking function of the LSM 510

unit to eliminate emission crosstalk, and colocalization analysis was conducted after linear unmixing of fluorescence signals using Zeiss Automatic Component Extraction (ACE) software to remove possible excitation bleed-through. AChR clusters were easily distinguished in TMR-Bgt-labeled cultures as discrete patches of intense fluorescence, and have characteristic appearances and dimensions, dependent on whether induced by soluble agrin, or substrate laminin, or intact motor neurons at the NMJ. For AChR cluster quantitative determination, clusters in 50-100 randomly-selected myotubes in at least two separate experiments were viewed by fluorescence microscopy and counted. Clusters 2-5μm in their longest diameter are categorized as 'microclusters' while larger clusters are designated as 'full-sized clusters'. For colocalization analysis, the Zeiss LSM510 Aim3.2 colocalization software was used to measure the weighted colocalization and overlap coefficients of 21 randomly selected NMJ endplates from 3 separate experiments and results are expressed as mean+/- standard deviation. The weighted colocalization coefficient is the sum of intensities of colocalizing pixels in the two channels relative to the overall sum of pixel intensities above the threshold in each channel. The advantage of the weighted colocalization analysis is that differences in pixel intensity are taken into account so that brighter pixels contribute more to the coefficient.

II.10. Statistical analysis

For statistical analysis, Student's *t*-test was used to compare data between two groups. Values are expressed as mean ± sem, or sd where specified. $*p < 0.05$ was considered statistically significant, and #*p* value between *0.05 and 0.1* was regarded as a statistical trend.

Chapter III

The Role of RhoA in Agrin- and Laminin-Induced AChR Aggregation

III.1. Introduction

Focal changes in the peripheral actin-based cytoskeleton are thought to underlie the aggregation of AChR at neuromuscular junctions (Dai et al., 2000; Hoch et al., 1994b; Phillips, 1995). The monomeric G proteins Rac1 and RhoA function to link extracellular signals to dynamic changes in actin cytoskeleton organization leading to the assembly of actin polymers and actin-myosin filaments, respectively (Bishop and Hall, 2000; Hall, 1998; Ridley, 2001; Takai et al., 2001). Rac1 activation induces the appearance of lamellipodia with resultant stimulation of cell spreading and motility (Nobes and Hall, 1999; Ridley et al., 1992). RhoA stimulates actin stress fiber and focal adhesion complex formation to promote cell adhesion and contractility (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992). As several recent studies have shown, Rho GTPases have coordinate actions in the formation of focal complexes and adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Ridley and Hall, 1992). Rac1 and Cdc42 regulate the formation of small focal complexes, whereas RhoA is required for the formation of focal adhesions by the aggregation of focal complexes (Nobes and Hall, 1999; Ridley and Hall, 1992; Rottner et al., 1999).

AChR clustering induced on the surface of cultured myotubes by agrin involves two stages; (1) the aggregation of diffusely distributed receptors into numerous microclusters (<5 μm diameter) within 1-2 hours; (2) the subsequent coalescence of microclusters into full-sized typically ovoid AChR clusters (>5 μm diameter) after 4 to 6 hours of exposure to agrin [ ]. Although soluble laminin-induced clusters appear to have a slightly increased density of receptors compared to agrin-induced clusters (Lee et al.,

2002), they nevertheless closely resemble agrin clusters in the progression through intermediate microclusters as well as in their final ovoid appearance and dimensions.

Our laboratory has shown that agrin triggers the activation of Rac1 and Cdc42 and that this activation is necessary but not sufficient for formation of full-sized AChR clusters (Weston et al., 2000). Evidence is presented in this chapter showing that RhoA plays a crucial role in agrin-initiated signaling by mediating the coalescing of microaggregates into full-sized AChR aggregates thereby providing a missing link to the mechanisms of AChR clustering subsequent to the contributions of Rac1/Cdc42.

III.2. Results

III.2.1. Agrin and laminin induce RhoA activation in myotubes

Our laboratory has recently shown that Rac1 mediates the formation of AChR microclusters but is insufficient to induce full- sized AChR clusters (Weston et al., 2000), suggesting that other regulatory components might be required. In view of these findings and the role of the actin cytoskeleton in AChR surface redistribution events (Colledge and Froehner, 1998), the potential contribution of RhoA was investigated. To this end, the level of active Rho was determined in myotubes treated with agrin or laminin by an affinity precipitation assay based on the selective binding of active Rho (RhoGTP) to a peptide derived from the Rho binding domain of Rhotekin (RBD peptide). As shown in Fig.III.1, both agrin and laminin activate RhoA in myotubes as shown by increased levels of RhoA precipitated with RBD conjugated to GST.

III.2.2. Dominant negative RhoA impairs agrin-induced AChR clustering

The potential contribution of Rho was investigated by testing the effect of a dominant negative mutant of Rho, RhoN19, on agrin-induced AChR clustering. EGFP-tagged RhoN19 plasmid was transfected into myoblast cultures, and 2 days after shift to differentiation medium myotubes were incubated overnight in the absence or presence of agrin. Surface AChR on myotubes was visualized by labeling with TMR-Bgt. Transfected myotubes expressing the EGFP-RhoN19 construct were found to have an impaired ability to cluster AChR in response to agrin compared to untransfected myotubes in the same field (Fig.III.2A). Moreover, as shown in Fig.III.2B, the decrease in numbers of agrin-induced full-size AChR clusters was accompanied by an increase in the number of AChR microclusters in the myotubes expressing RhoN19.

III.2.3. A specific RhoA inhibitor blocks agrin- induced AChR aggregation

The role of RhoA was further verified by selectively inhibiting its activation by C3 exotransferase from *Clostridium botulinum*, which ADP-ribosylates Rho (Aktories et al., 1989; Sekine et al., 1989a; Wilde and Aktories, 2001a). Myotubes pretreated with C3 exotransferase showed decreased numbers of full-sized clusters when treated with agrin (Fig.III.3). As in the case of RhoN19 expression, the number of microclusters increased in the RhoA-inhibited cultures. Combined, these data suggest that RhoA plays an essential role in the formation of full-sizedAChR aggregates and is not necessary for initiation of microcluster formation. Furthermore, these results indicate that microclusters

are precursors of full-sized clusters. In earlier studies in our laboratory microcluster formation was found to be mediated by Rac1-another member of the RhoGTPase family that is crucial for the formation of lamellopodia and membrane ruffles in motile cells.

III.2.1. A pharmacological inhibitor of the Rho downstream effector Rho kinase (ROCK) inhibits agrin - induced AChR aggregation

To investigate if ROCK is the downstream effector of RhoA in AChR clustering, a pharmacological inhibitor of ROCK , Y-27632 (Ishizaki et al., 2000; Uehata et al., 1997), was used. Myotubes were pretreated with Y-27632 30min prior to agrin or laminin stimulation and the surface distribution of AChR was examined by labeling myotubes with TMR-Bgt. As shown in Figure III.4A and B, Y-27632 inhibits the agrin-induced aggregation of AChR into full-sized clusters, with a consequent increase in the number of microclusters per myotube. This result is identical to those observed with direct inhibition of Rho using a dominant negative mutant (Fig.III.2) or C3 exotransferase (Figure III.3), suggesting that ROCK is the downstream effector that couples RhoA activation to the formation of AChR clusters.

III.3. Discussion

A crucial feature of synaptogenesis is the establishment at postsynaptic membranes of a well delineated surface region that displays sharply elevated sensitivity to neurotransmitter. In the case of the neuromuscular synapses, this is accomplished by the clustering of AChR, initiated by spatial cues originating at the motor nerve ending and

acting on the adjacent patch of muscle membrane. The postsynaptic sensor component of this focal signaling mechanism includes the receptor tyrosine kinase, MuSK, which activates a signaling pathway to couple the binding of neurally derived agrin to the assembly of subsynaptic complexes and to the aggregation of diffusely distributed surface AChR into high density clusters. The previous findings that activation of Rac1/Cdc42 is essential for AChR clustering and is sufficient for mediating agrin-induced AChR microclustering but not full-size cluster formation (Weston et al., 2000) suggests that additional regulatory steps are necessary for the maturation of microclusters into full-sized clusters. This chapter furnishes the missing link in our understanding of the molecular mechanism of full-sized cluster assembly and implicates RhoA as a crucial mediator of this process.

Rho GTPases serve crucial roles in the formation of focal complexes and adhesions. The multiprotein cytoskeletal complexes that assemble at postsynaptic membrane sites at the neuromuscular junction and in AChR cluster regions in cultured muscle cells closely resemble the focal adhesions involved in cell-substrate adhesion and signaling (Bloch and Geiger, 1980; Bloch and Pumplin, 1988; Mittaud et al., 2001). Rac and Cdc42 regulate the formation of small focal complexes, whereas Rho is required for the formation of focal adhesions by the aggregation of focal complexes (Nobes and Hall, 1999; Rottner et al., 1999).

Earlier studies have suggested that microclusters and clusters represent distinct stages of neural factor-induced AChR aggregation, with microclusters serving as the precursors of full-size clusters in cultured muscle cells (Kunkel et al., 2001; Luther et al.,

1994; Pumplin and Bloch, 1987; Ross et al., 1988), as well as in developing neuromuscular synapses (Sanes and Lichtman, 2001). Conversely, the dispersal of clusters in response to specific pharmacological agents (protein kinase C activators and phosphoprotein phosphatase inhibitors) similarly proceeds via microclusters as intermediates (Nimnual et al., 1998; Ross et al., 1988). Under the experimental conditions used in the present experiments, exposure of C2 myotubes to agrin results in the appearance of numerous microclusters within 2 h and the accumulation of full-sized AChR clusters in the subsequent 4-8 h (data not shown). The findings presented in this chapter indicate that RhoA participates in the later stage of cluster assembly.

It was shown that agrin or laminin treatment activate RhoA in differentiated myotubes and that inhibition of the RhoA-ROCK pathway impairs agrin-induced AChR clustering. Inhibition of Rho-mediated signaling in agrin-treated myotubes was seen to selectively impair AChR aggregation into full-sized AChR clusters but does not inhibit the formation of AChR microclusters. In fact, the number of microclusters increases when RhoA or ROCK is blocked. These finding are identical to the recent observation that RhoA inactivation blocks focal adhesion assembly and is accompanied by the persistence of punctuate focal complexes (Rottner et al., 1999). Thus, the findings presented in this chapter demonstrate a role of RhoA in AChR cluster formation that is critical, complementary and subsequent to the formation of micro-aggregates by Rac1/Cdc42.

III.4. Figures

Figure III.1 Agrin and laminin induce activation of RhoA in differentiated myotubes. Agrin-and laminin-induced activated RhoA (RhoGTP-bound RhoA) was precipitated by its selective binding to RBD. Cultures were treated with agrin (5 nM) or laminin (20 μM), and lysates were incubated with GST-RBD. As shown, agrin or laminin treatment causes increased binding of Rho to GST-RBD in myotubes, reflecting increased levels of RhoGTP-bound RhoA i.e. activated RhoA in these conditions.

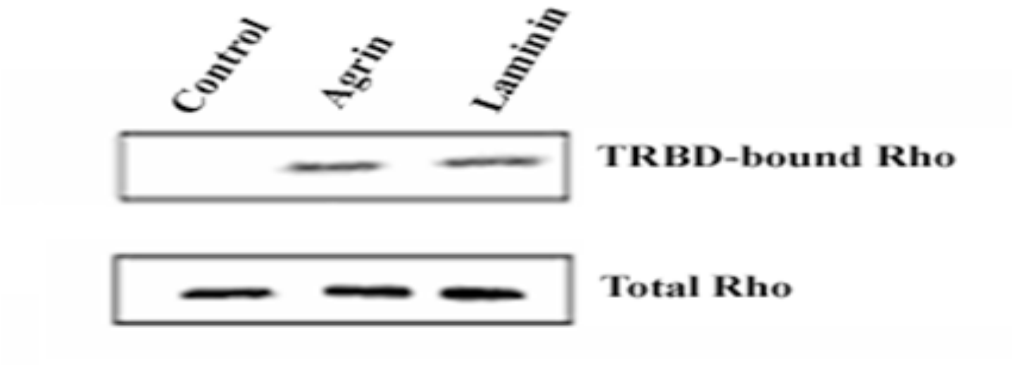
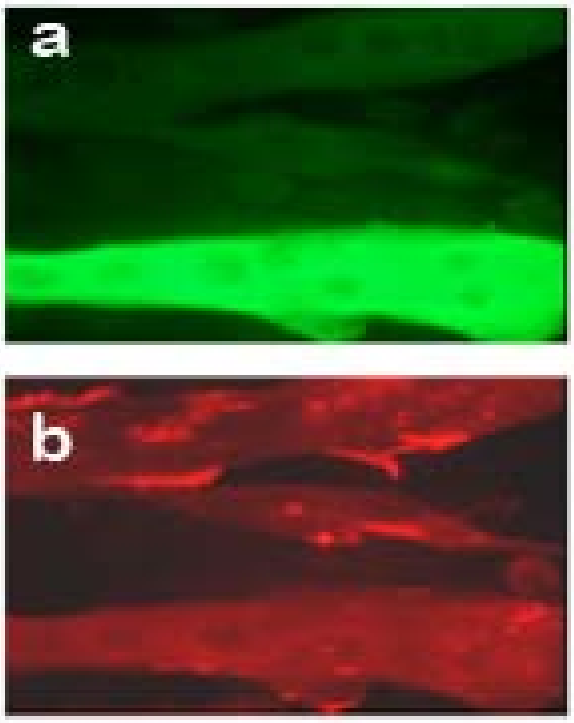

Control
Agrin
Laminin
TRBD-bound Rho
Total Rho

Figure III.2 Dominant negative RhoA impairs agrin-induced AChR clustering. (A) Myotubes expressing EGFP-tagged RhoN19 are unable to form full-sized AChR clusters in response to an overnight agrin treatment but display elevated numbers of microclusters. In contrast, untransfected myotubes form full-sized clusters of AChR after agrin treatment. (B) Quantitative comparison of the number and size of AChR clusters on the surface of transfected myotubes expressing RhoN19 versus untransfected myotubes. (*n = 40; the error bars represent the S.E.M*).

(A)


a
b

(B)

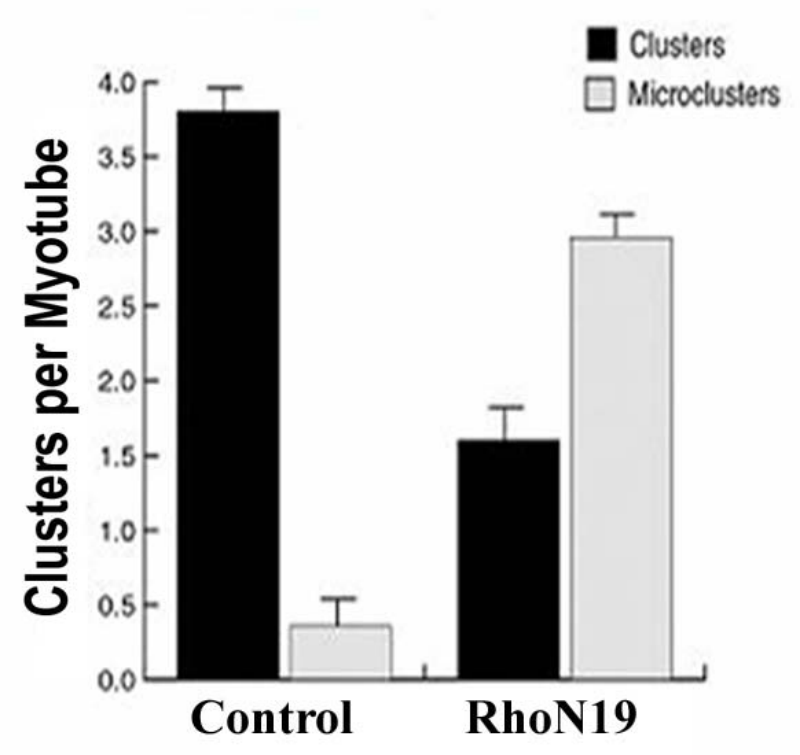
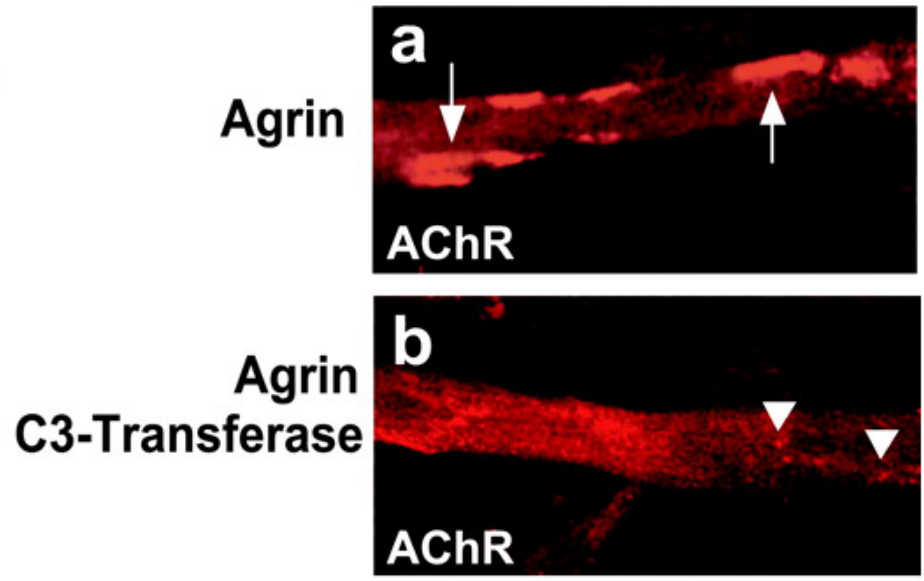

Clusters
Microclusters
Clusters per Myotube
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
RhoN19

Figure III.3 The effect of the Rho inhibitor, C3 transferase, on agrin-induced AChR aggregation. (a) Myotubes were treated with C3 transferase 30min prior to agrin treatment treatment (16h in the presence of inhibitor) and TMR-Bgt staining show a significantly decreased full-sized AChR cluster formation (lower panel) compared with control cells treated only with agrin (upper panel). Arrows and arrow-heads indicate full-sized clusters and microclusters, respectively. (b) Quantitative comparison of the number and size of AChR aggregates per myotube. (*n = 40; the error bars represent the S.EM.*).

(A)


a
Agrin
AChR
b
Agrin
C3-Transferase
AChR

(B)

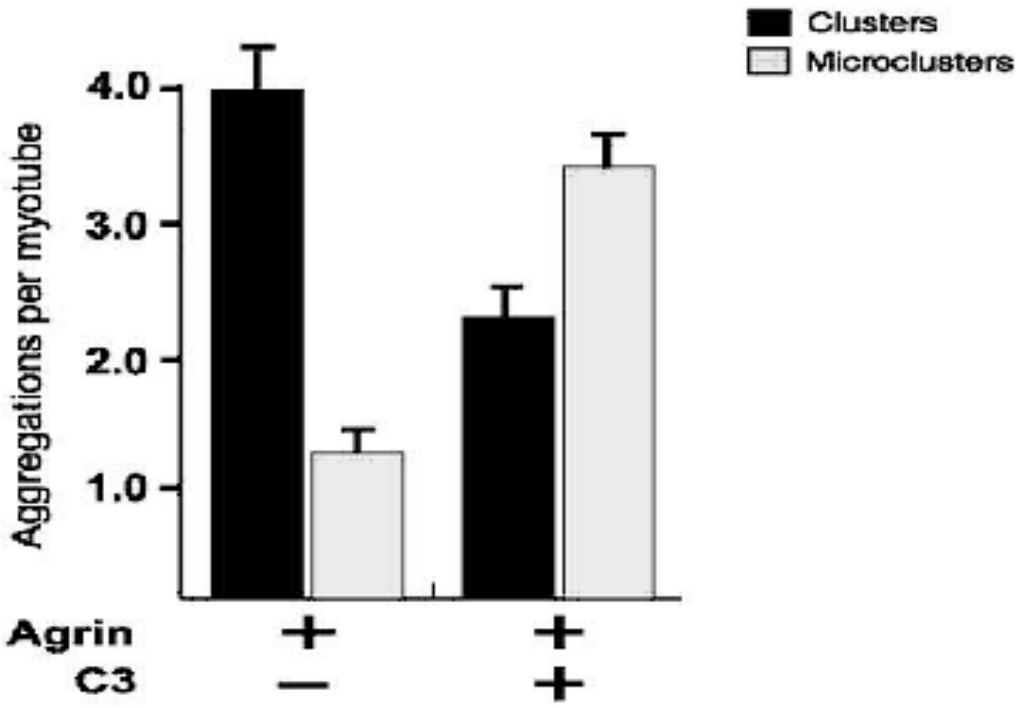
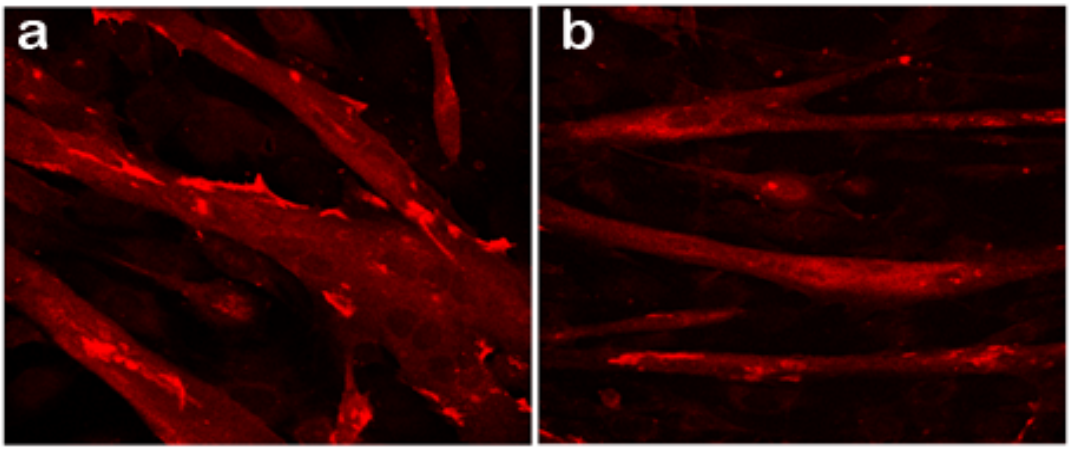
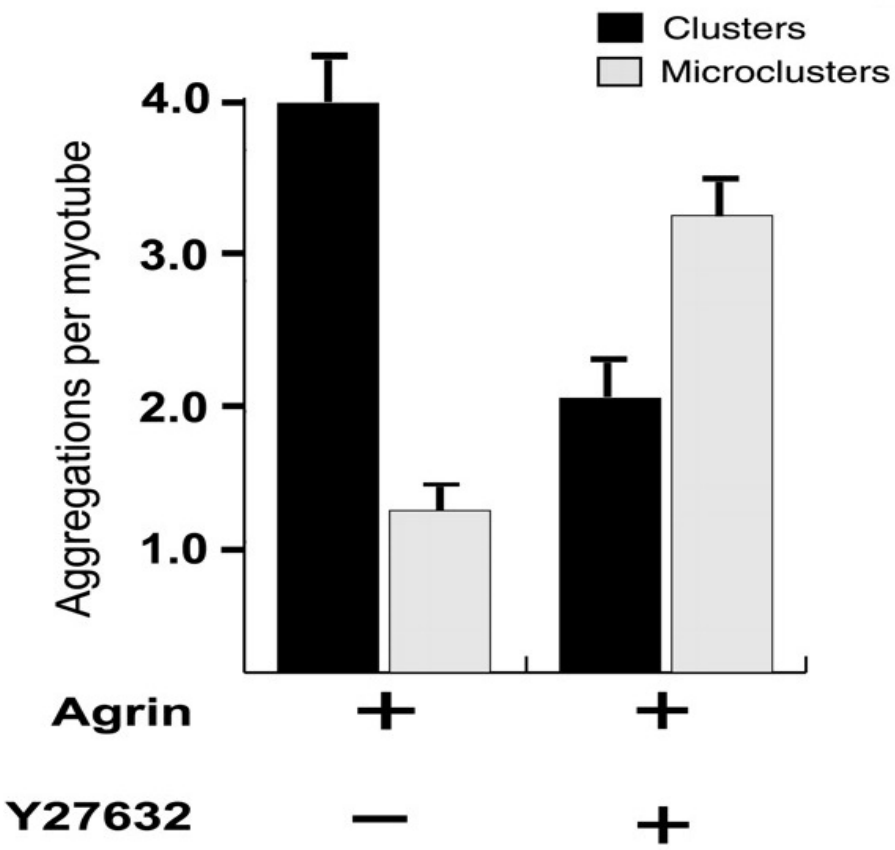

Clusters
Microclusters
Aggregations per myotube
4.0
3.0
2.0
1.0
Agrin
C3
+
—
+
+

Figure III.4 The effect of the Rho kinase (ROCK) inhibitor, Y-27632, on agrin-induced AChR aggregation. (a) Myotubes pretreated with C3 transferase 30min prior to agrin treatment (16h in the presence of the inhibitor) show significantly impaired full-sized AChR cluster formation (right panel) compared with control cells treated only with agrin (left panel). (b) Quantitative comparison of the number of AChR aggregates per myotube shows that agrin-induced formation of full- sized AChR clusters (black bars) is inhibited by more than 50% in Y-27632-pretreated myotubes compared with control myotubes. However, the number of agrin-induced microclusters (white bars) increases in Y-27632 pretreated myotubes (*n = 40; the error bars represent the S.E.M*).

(A)


a
b

(B)


Clusters
Microclusters
Aggregations per myotube
4.0
3.0
2.0
1.0
Agrin
+
+
Y27632
—
+

Chapter IV

Integration by Focal Adhesion Kinase of Signaling Pathways that Regulate Neuromuscular Synapse Formation

IV.1. Introduction

Focal Adhesion Kinase (FAK) is a nonreceptor tyrosine kinase that has been demonstrated to be a downstream integrator of signals from receptor tyrosine kinases, integrins, and G-protein coupled receptors in different cell types (Schlaepfer et al., 1999; Sieg et al., 2000). FAK is a prominent constituent and regulator of focal adhesion-macromolecular complexes involved in cell-substrate adhesion and signaling (Burridge and Chrzanowska-Wodnicka, 1996; Hanks et al., 1992; Schaller et al., 1992). The focal adhesion proteins vinculin, α-actinin, filamin, talin, and paxillin are also localized at AChR clustering sites (Bloch and Geiger, 1980; Shadiack and Nitkin, 1991) suggesting a shared molecular mechanism between focal adhesion assembly and AChR clustering. Furthermore, some proteins that are reported to play roles in AChR aggregation in skeletal muscle cells such as Src kinases (Fuhrer and Hall, 1996; Marangi et al., 2002; Mohamed et al., 2001; Smith et al., 2001), PI3 kinase (Nizhynska et al., 2007), Shc (Jones et al., 2007) and calpain (Chen et al., 2007) are known to interact with FAK in other cell types (Mitra et al., 2005; Schaller et al., 1994).

Therefore, it was of interest to investigate the role of FAK in regulating AChR aggregation on the skeletal muscle cell surface. I found that in cultured myotubes FAK is activated by either agrin or laminin, and that inhibition of FAK expression or enzymatic function blocks the ability of agrin to induce full-sized AChR clusters. My results indicate that FAK utilizes both kinase-dependent and –independent mechanisms in coupling agrin and laminin stimulation to AChR aggregation. Moreover, FAK was seen to form functional complexes with RhoGTP and AChR, as well as to colocalize with

AChR aggregates *in vivo* at intact mouse diaphragm NMJs. In conclusion, this chapter presents a novel finding that FAK is a crucial mediator of agrin- and laminin-induced AChR clustering.

IV.2. Results

IV.2.1. Activation of FAK by Agrin and Laminin

To determine if FAK participates in the events that integrate agrin and laminin signaling leading to AChR clustering in muscle cells, I first investigated if FAK is activated in myotubes by these signaling molecules. Intact muscle cells were treated with agrin (5nM) or laminin (20μM) for 30 minutes and FAK activation was assessed by measuring its tyrosine phosphorylation in cell lysates. As shown in Fig.IV (top panel), both agrin and laminin cause marked increases in FAK tyrosine phosphorylation, as revealed by immunoprecipitation with antiphosphotyrosine antibodies followed by blotting with anti-FAK antibody. In order to assess the functional significance of this phosphorylation, I next determined which tyrosine residues are targeted. Tyrosine 397 is a FAK autophosphorylation site that is triggered by ligand binding to receptors upstream of FAK (Toutant et al., 2002), and phosphorylation of tyrosines 566 and 577 is known to induce maximum FAK kinase activity (Hanks et al., 2003). Tyrosine phosphorylation sites were identified in cell lysates from agrin or laminin treated myotubes by Western blotting using antisera specifically directed against phosphotyrosine 397 or phosphotyrosines 576 and 577. The results show that tyrosine phosphorylation of FAK at Tyr576/577 (Fig.IVA, middle panel) as well as at Tyr397 (Fig.IVB) are markedly increased by both agrin and laminin. These results suggest that binding of laminin and

agrin to their respective surface receptors induces autophosphorylation of FAK and maximal activation of its kinase activity.

IV.2.2. FAK siRNA Blocks AChR Aggregation by Agrin

To determine the contribution of FAK to the aggregation of surface AChR in response to agrin and laminin FAK siRNA was transfected into myoblasts to knock down FAK expression. As shown in Fig.IV.2A, FAK siRNA was effective in sharply reducing FAK levels, while not affecting muscle cell survival or differentiation as determined by counting the number of myotubes per field and the number of nuclei per myotube (data not shown). Under these conditions, FAK knockdown sharply reduced the ability of agrin to induce full-sized clusters (>5μm in diameter, Fig.IVB). These effects are quantified in Fig.IVC, which shows that the number of full-size clusters is decreased by ~2.7 fold while the number of microclusters is increased by ~ 1.6 fold. These results indicate that normal FAK levels are essential for the induction of full-sized AChR clusters by agrin. In contrast, the numbers of microclusters were seen to increase when FAK levels were reduced, consistent with the interpretation that FAK is dispensable for microcluster formation but essential for the coalescence of microclusters into full-sized clusters.

IV.2.3. Inhibitors of FAK Tyrosine Kinase Activity Block Agrin-Induced Formation of Full-Sized AChR Clusters

Since FAK has been shown to have both kinase-dependent and –independent functions (Segarra et al., 2005; Sieg et al., 2000), it was of interest to determine the extent to which AChR clustering is dependent on the kinase activity of FAK. I examined the effects of blocking the enzymatic activity with pharmacological tyrosine kinase inhibitors

AG82 and a novel FAK specific kinase inhibitor PF-573,228 (Slack-Davis et al., 2007) (hereafter referred to as PF-228). Myotubes were treated for 16h with agrin (5nM) in the presence or absence of AG82 (20μM) or PF-228 (0.5 μM). AG82 treatment is shown to result in the inhibition of agrin-induced FAK activity, as measured by autophosphorylation using anti pY-397 FAK antibody (Fig. IV.3A). Under these conditions, I observed that the number of full-sized AChR clusters induced by agrin was reduced significantly (Fig. IV.3B). Moreover, as in the case of FAK siRNA knockdown, microclusters were found to increase in number in the presence of AG82 or to a lesser extent in the presence of PF-228 (Fig. IV.3C). In the case of PF-228, significant number of small clusters less that 2μm in diameter were observed and were not counted as microclusters. These findings suggest that the kinase activity of FAK is crucial for the formation of AChR full-sized clusters from microclusters by agrin.

IV.2.4. FAK Interacts with Activated Rho in a Kinase-Independent Manner

I have demonstrated in Chapter III that RhoA mediates agrin- and laminin-induced aggregation of AChR from microclusters into full-sized clusters. The similarity of these earlier findings with the present observations made with FAK led to the investigation of whether RhoA and FAK participate in a common signaling pathway initiated by agrin and laminin. To examine this possibility, I asked whether RhoA and FAK form a signaling complex. To this end, RhoGTP pulldown assay was carried out exploiting the selective binding of activated RhoA to the Rhotekin Rho-binding domain (TRBD) (Ren and Schwartz, 2000), and monitored the level of associated FAK by immunoblotting using FAK specific antibody. As shown in Fig.IV.4, FAK is readily

detected in the activated RhoGTP precipitates indicating the physical association of FAK with RhoGTP. Importantly, this interaction is markedly enhanced by agrin as well as laminin.

To determine if the extent of the interaction between FAK and RhoGTP is dependent on the tyrosine kinase activity of FAK, I repeated this assay in the presence and absence of AG82. As can be seen in Fig.IV.4B, AG82 did not diminish the agrin-dependent association of FAK with RhoGTP indicating that FAK kinase activity is not necessary for the activation and recruitment of active RhoA by agrin. Thus, FAK appears to serve as a scaffolding protein in the context of agrin-induced RhoA activation.

IV.2.5. Activated FAK Colocalizes with AChR Aggregates at the Neuromuscular Junction

In view of our findings suggesting a role for FAK in AChR aggregation, I investigated the cellular distribution of FAK in relation to AChR aggregates in intact NMJs diaphragm from adult mouse by double-labeling for surface AChR and phosphorylated FAK. As shown in Fig.IV.5A, a high degree of colocalization between TMR-Bgt labeled AChR and activated FAK was observed at intact NMJs. Using the Zeiss LSM 510 colocalization analysis software, the weighted colocalization coefficients for phosphorylated FAK and AChR were found to be 0.87+/-0.15 and 0.88+/-0.12, respectively with an overlap coefficient of 0.85+/-0.07. In contrast to phosphorylated FAK, total FAK shows a more diffuse distribution with only a marginal localization at AChR aggregation regions (data not shown), in agreement with an earlier finding in *Xenopus* (Baker et al., 1994).

IV.2.6. Activated FAK Interacts with AChR β Subunit

To determine if the colocalization between active FAK and aggregated AChR reflects physical association between these components, coimmunoprecipitation experiments were carried out using antibodies directed against phosphotyrosine FAK. Immunoprecipitates were blotted with antibody directed against tyrosine phosphorylated AChR β subunit. As can be seen in Fig.IV.6A, tyrosine phosphorylated AChR β subunits coimmunoprecipitate with tyrosine phosphorylated FAK, indicating that active FAK and AChR coexist in a complex. Moreover, the formation of this complex appears to be stimulated by agrin.

In view of their proximity, I asked if AChR β subunit phosphorylation is dependent on FAK kinase activity. Myotubes were treated with agrin in the presence or absence of AG82 and levels of AChR β subunit tyrosine phosphorylation were compared. AChR pulldown assays were carried our using biotinylated-α–bungarotoxin followed by immunoblotting with AChR β subunit phosphotyrosine specific antibody. As shown in Fig.IV.6B, agrin-induced increase in AChR β subunit tyrosine phosphorylation is reversed under conditions in which FAK kinase activity is blocked by AG82, suggesting that FAK activity mediates agrin-induced AChR β subunit tyrosine phosphorylation.

IV.3. Discussion

The focal aggregation of AChRs in muscle membrane regions immediately subjacent to the motor nerve ending is a defining event in neuromuscular synapse formation that is regulated by specific signaling molecules in the synaptic extracellular

matrix. The neural isoform of agrin was shown to be essential for neuromuscular synapse formation, and the contribution of laminin to AChR clustering *in vitro* is also well documented. In a recent study we presented evidence demonstrating that agrin and laminin direct AChR clustering by utilizing pathways that converge to activate the RhoGTPases Rac1 and RhoA (Weston et al., 2007). The present study identifies the critical role of FAK at the point of convergence of the agrin- or laminin-dependent signaling pathways. I have demonstrated that agrin- and laminin- triggered transmembrane signals are integrated by FAK as shown by the ability of these ECM components to induce FAK tyrosine phosphorylation. Secondly, both the blockade of FAK expression by siRNA and inhibition of its kinase activity by the FAK tyrosine kinase inhibitors AG82 or PF-228 block the ability of agrin to induce AChR aggregation. Furthermore, I have shown that FAK forms complexes with both RhoA and AChR in an agrin-dependent manner, and that activated FAK is colocalized with AChR aggregates at intact mouse diaphragm NMJ.

The present study shows that both agrin and laminin induce the tyrosine phosphorylation of FAK at at least two different sites; Tyr397 and Tyr576/Tyr577. Phosphorylation at Tyr397 reflects autophosphorylation associated with activation of integrin, growth factor receptors, and G-protein coupled receptors in several cell types (Schlaepfer et al., 1999; Sieg et al., 2000). Phosphorylation at this site has been shown to recruit Src kinases, PI3 kinase, as well as Shc (Mitra et al., 2005; Schaller et al., 1994) into a signaling complex localized to focal adhesions. Src kinases (Fuhrer and Hall, 1996; Marangi et al., 2002; Mohamed et al., 2001; Smith et al., 2001), PI3 kinase (Nizhynska et

al., 2007), and Shc (Jones et al., 2007) have all been reported to play roles in AChR clustering. Phosphorylation at Tyr576 and Tyr577, which in other cell types has been shown to be mediated by Src kinases, results in maximum FAK kinase activation (Hanks et al., 2003). Thus, in cultured myotubes, agrin and laminin each trigger FAK autophosphorylation, leading to possible recruitment of Src kinases and PI3 kinases, as well as phosphorylation at additional FAK sites.

The contribution of FAK to AChR aggregation is demonstrated by the finding that myotubes from FAK siRNA knockdown cultures display a substantially reduced number of full-sized AChR aggregates on their cell surfaces in response to agrin. Similarly, inhibition of FAK tyrosine kinase activity by either AG82 or PF-228 decreases the ability of agrin to form full-sized AChR clusters. In both cases, however, microclusters persist, and in fact, their numbers increase compared to control, agrin-treated, myotubes. These findings are reminiscent of my observations with RhoA as presented in Chapter III which was shown to mediate the aggregation of microclusters into full-sized clusters such that inhibition of the Rho pathway resulted in increased numbers of microclusters.

The functional similarities in the action of FAK and RhoA in the context of agrin-induced AChR aggregation strongly suggest that these molecules are components of a common pathway that mediates AChR aggregation on the postsynaptic cell membrane. This possibility is consistent with my finding of physical association between FAK and RhoGTP that is markedly enhanced by agrin and laminin. It is noteworthy that an analogous situation has been described at glutaminergic synapses in neuronal cultures in

which ephrinB-EphB signaling-mediated dendritic spine morphogenesis has been shown to be critically regulated by the phosphorylation and recruitment of FAK and the subsequent RhoA activation at the postsynaptic cell membrane (Moeller et al., 2006). Given that the activation of a tyrosine kinase signaling cascade downstream of MuSK is a critical step that precedes the cytoskeleton-mediated redistribution of AChR into focal aggregates, these findings position FAK as a molecular link between early tyrosine phosphorylation events and subsequent RhoGTPase-mediated reorganization of cell surface components during synapse formation.

Interestingly, although both FAK kinase and RhoA activities are essential for the formation of agrin-induced full-sized clusters, the association of FAK with RhoGTP in agrin-treated myotube cultures is not dependent on the tyrosine kinase activity of FAK. This suggests that in addition to its kinase-dependent function, FAK may also have a kinase–independent function in AChR clustering that is manifested via FAK scaffolding mechanisms.

Our results show significant colocalization of tyrosine phosphorylated FAK with AChR in regions of elevated AChR density at intact diaphragm NMJs. In contrast, FAK was not elevated to sufficient levels to be detectable by these methods. In addition to its involvement in neuromuscular synaptogenesis, the localization of activated FAK at adult NMJs suggests an as yet unidentified persisting role in NMJ regulation of function or plasticity. The relationship between activated FAK and AChR is further emphasized by

the finding that surface AChR is physically associated with FAK as shown by coimmunoprecipitation studies.

There is growing evidence that FAK also has a role in the formation of central synapses. FAK is enriched in growth cones and postsynaptic densities in neuronal cells (Bongiorno-Borbone et al., 2005), and has been implicated in axonal path finding (Robles and Gomez, 2006), branch formation (Rico et al., 2004), and dendritic spine morphogenesis (Moeller et al., 2006). In this wider context, the findings presented here could well represent a more general mechanism for the formation and remodeling of neuronal contacts as well as for other cellular interactions which require the dynamic regulation of spatially restricted membrane specializations at sites of cell-cell communication.

IV.4. Figures

Figure IV.1 Agrin- and laminin-induced tyrosine phosphorylation of FAK in cultured myotubes. Myotubes were treated with agrin (5nM) or laminin (20μM) for 30min. Myotubes were then extracted in lysis buffer, and the supernatants were used to measure the levels of tyrosine phosphorylated FAK. Total tyrosine phosphorylation of FAK (pY-FAK) was measured on a Western blot by probing with anti-FAK of protein immunoprecipitated with antiphosphotyrosine antibodies (4G10, PY99, PY20 mixture; A, top panel). The levels of FAK phosphorylated at tyrosine residues 576/7 and 397 were assessed on Western blots of whole cell extracts probed with antisera that recognize FAK phosphorylated at Tyr576/7 (A, middle panel) and Tyr397 (B, top panel), respectively. (C) Densitometry analysis of Western blot experiments showing the extent of agrin- and laminin-induced FAK tyrosine phosphorylation normalized to total FAK. (Mean+/-SEM, n≥5,*$P<0.05$, #p value between 0.05 and 0.1).

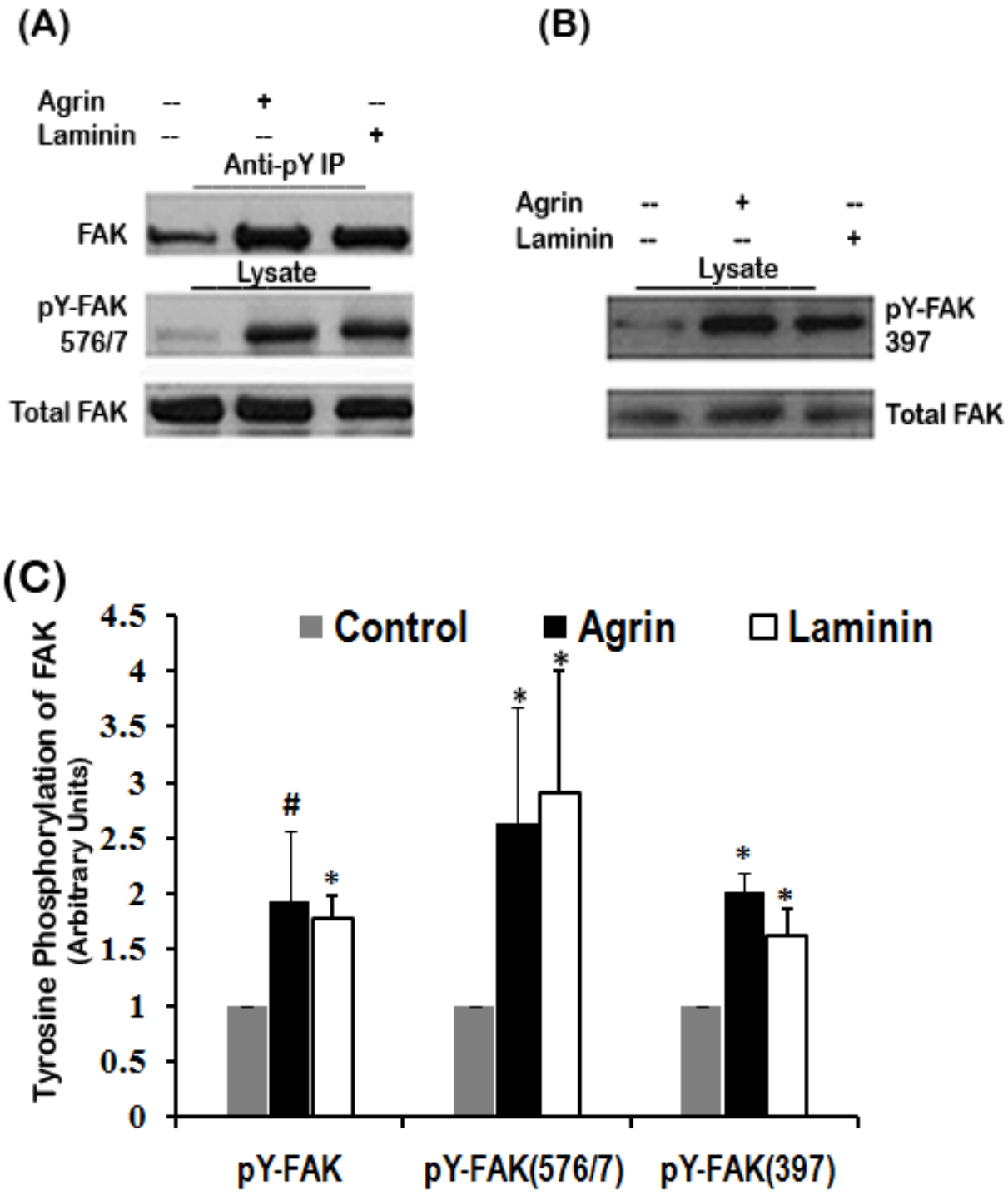

(A)
Agrin -- + --
Laminin -- -- +
Anti-pY IP
FAK
Lysate
pY-FAK 576/7
Total FAK
(B)
Agrin -- + --
Laminin -- -- +
Lysate
pY-FAK 397
Total FAK
(C)
Control
Agrin
Laminin
Tyrosine Phosphorylation of FAK (Arbitrary Units)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
#
*
pY-FAK
pY-FAK(576/7)
pY-FAK(397)

Figure IV.2. FAK siRNA blocks agrin-induced formation of full-sized AChR clusters in cultured myotubes. (A) The levels of FAK protein in FAK siRNA-transfected and untransfected cultures. (B) Distribution of TMR-Bgt-stained AChRs on the surface of agrin-treated myotube cultures from non-transfected controls (a) and FAK siRNA-transfected myotube cultures (b). (C) Number of agrin-induced AChR clusters and microclusters per myotube in FAK siRNA-transfected and untransfected myotube cultures. (Mean +/- SEM, n=100 myotubes per condition. * $P<0.005$. Scale bar, 20μm).

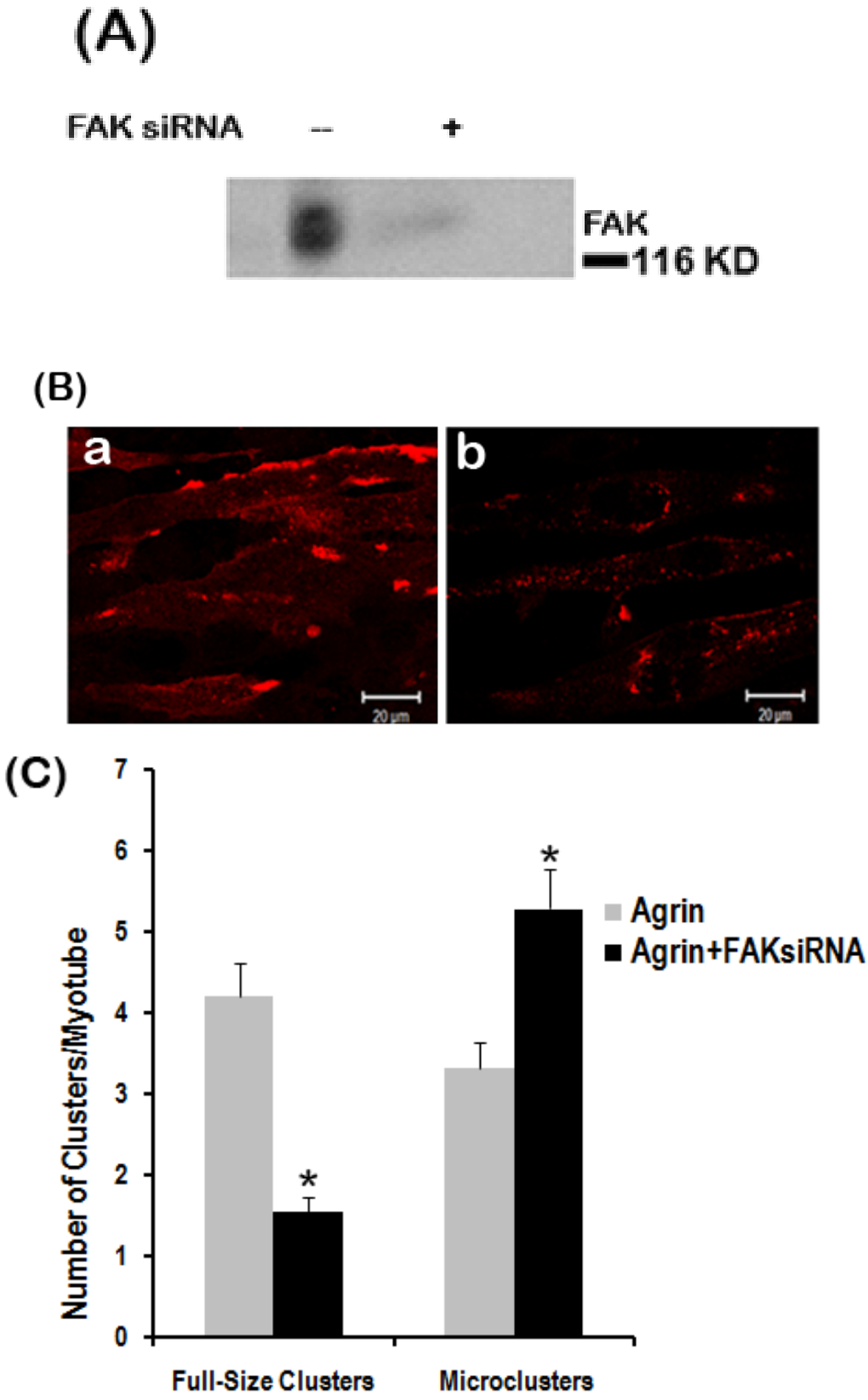

(A)
FAK siRNA
–
+
FAK
116 KD
(B)
a
b
20 μm
20 μm
(C)
Number of Clusters/Myotube
0
1
2
3
4
5
6
7
*
*
Agrin
Agrin+FAKsiRNA
Full-Size Clusters
Microclusters

Figure IV.3. Pharmacological inhibitors of FAK catalytic activity block agrin-induced AChR clustering in cultured myotubes. (A) Inhibition of FAK autocatalytic activity by Tyrophostin A25 (AG82) as monitored by the reversal of agrin-induced increase in pY-FAK Tyr397 phosphorylation. (B) Agrin-induced AChR clustering on myotubes; control (a), 20μM AG82 (b), or 0.5μM PF-228 (c). (C) Quantitation of agrin-induced AChR full-sized cluster and microcluster formation in AG82- or PF-228-treated myotubes relative to agrin-only treated controls. (Mean+/-SEM, n=100 myotubes per condition, from two independent experiments,**P*<0.001.Scale bar 20μm.)

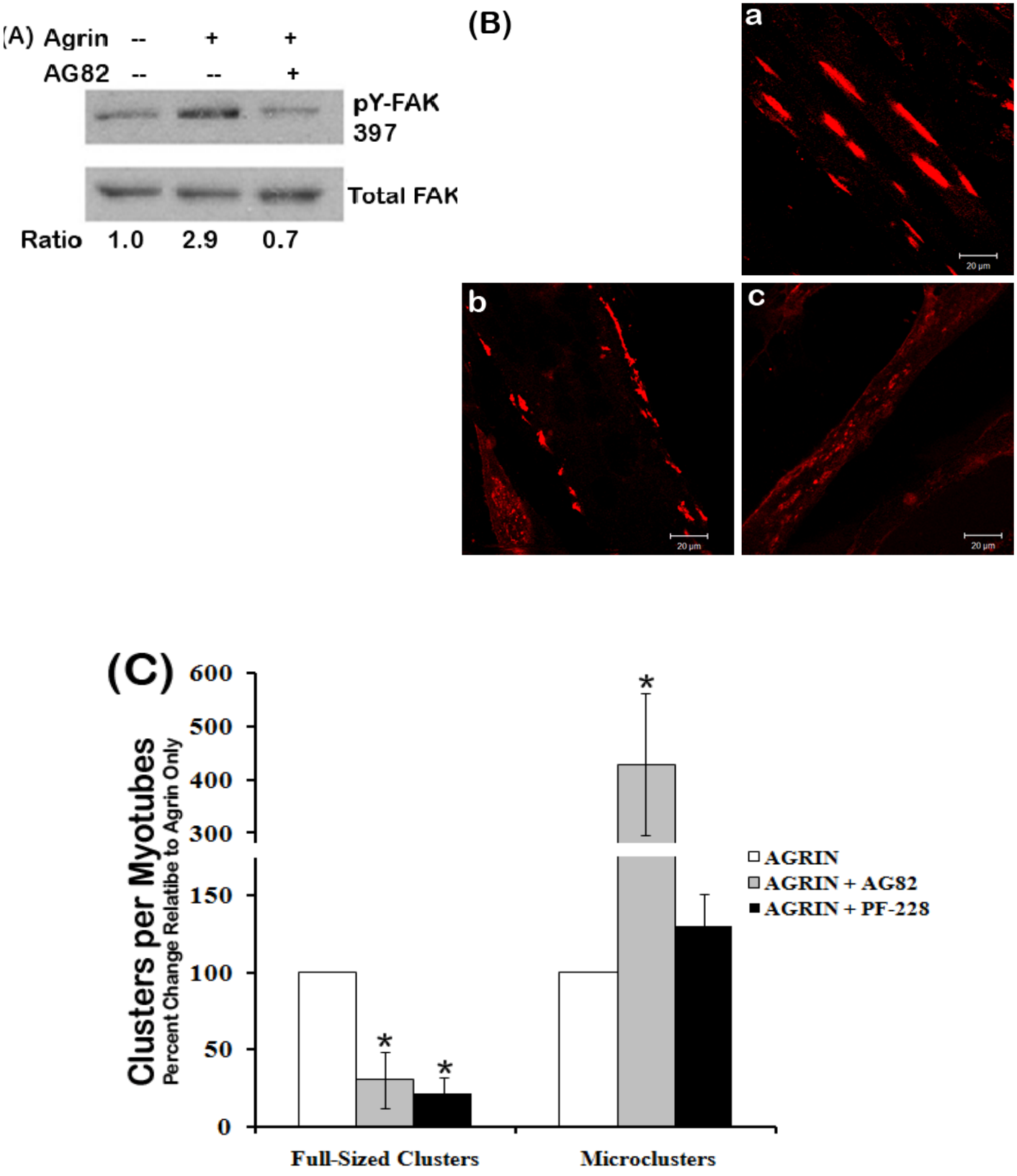
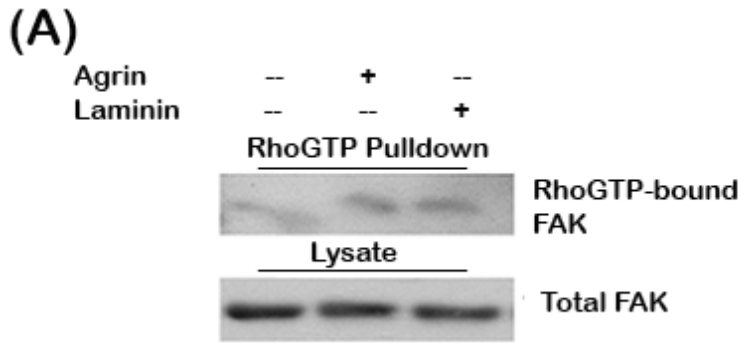

(A)
Agrin -- + +
AG82 -- -- +
pY-FAK 397
Total FAK
Ratio 1.0 2.9 0.7
(B)
a
b
c
20 µm
(C)
Clusters per Myotubes
Percent Change Relatibe to Agrin Only
600
500
400
300
150
100
50
0
*
AGRIN
AGRIN + AG82
AGRIN + PF-228
Full-Sized Clusters
Microclusters

Figure IV.4. FAK interacts with activated RhoA in a kinase-independent manner. Agrin-and laminin-induced activated RhoA (RhoGTP) was precipitated by its specific binding to RBD. Cultures were treated with agrin (5 nM) or laminin (20 μM), and lysates were incubated with GST-RBD and Western blotted with antibodies to FAK. (A) Detection of FAK in RhoGTP precipitates in myotubes treated with agrin or laminin. Both agrin and laminin caused increased association of active RhoA with FAK. (B) Measurement of FAK in RhoGTP precipitates in myotubes treated with agrin in the presence or absence of AG82. FAK kinase activity is not necessary for the association of FAK to activated RhoA.


(A)
Agrin -- + --
Laminin -- -- +
RhoGTP Pulldown
RhoGTP-bound FAK
Lysate
Total FAK

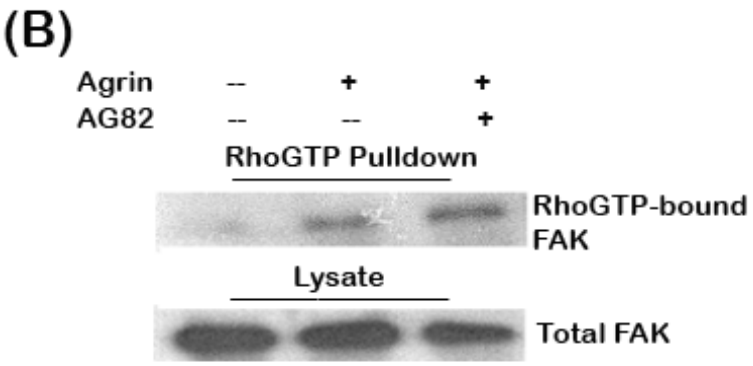

(B)
Agrin -- + +
AG82 -- -- +
RhoGTP Pulldown
RhoGTP-bound FAK
Lysate
Total FAK

Figure IV.5. Tyrosine phosphorylated FAK is concentrated at sites of AChR clustering at the NMJ. The cellular distribution of phosphorylated FAK in relation to AChR aggregates at the NMJ in adult mouse. Diaphragm from adult mouse was stained with TMR-Bgt to visualize AChR (a, a') and with rabbit anti-phosphotyrosine FAK primary antibodies followed by FITC anti-rabbit secondary antibody (b, b'). AChR aggregates and activated FAK colocalize (c, c') with an overlap coefficient of 0.85+/-0.07. (Mean+/-Standard Deviation. n=21 end plates from 3 separate experiments. P=0.031. Scale bar=10μm.)

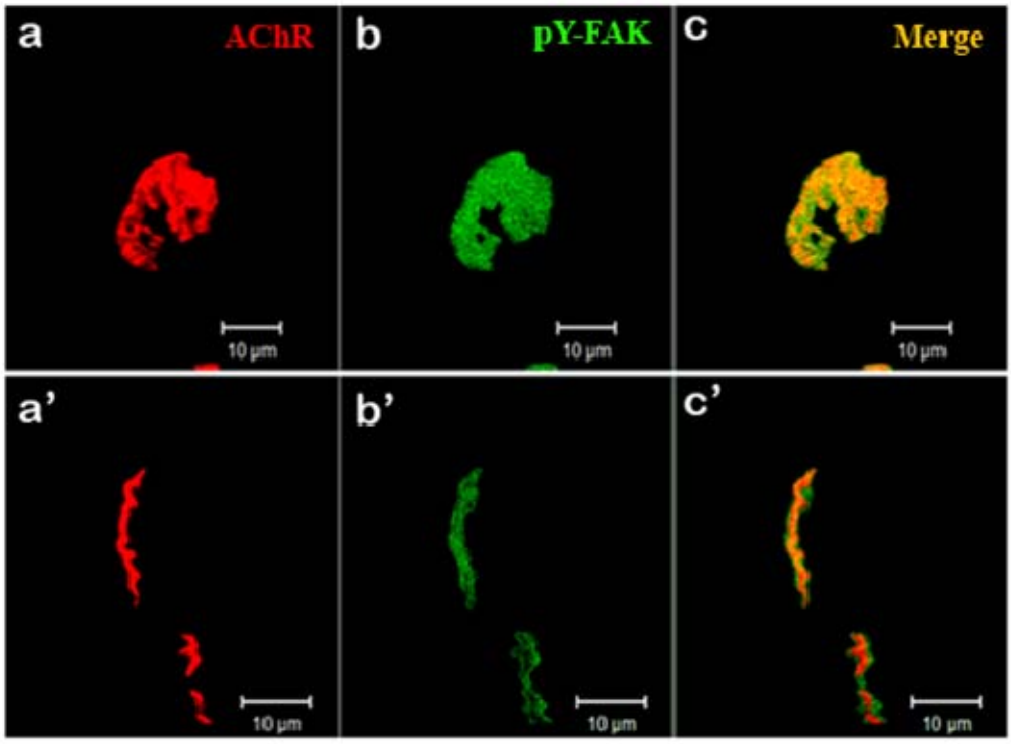
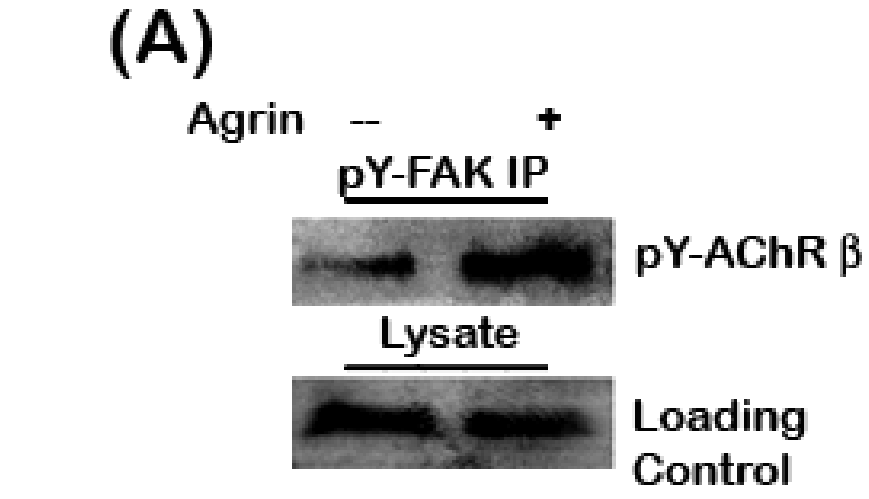
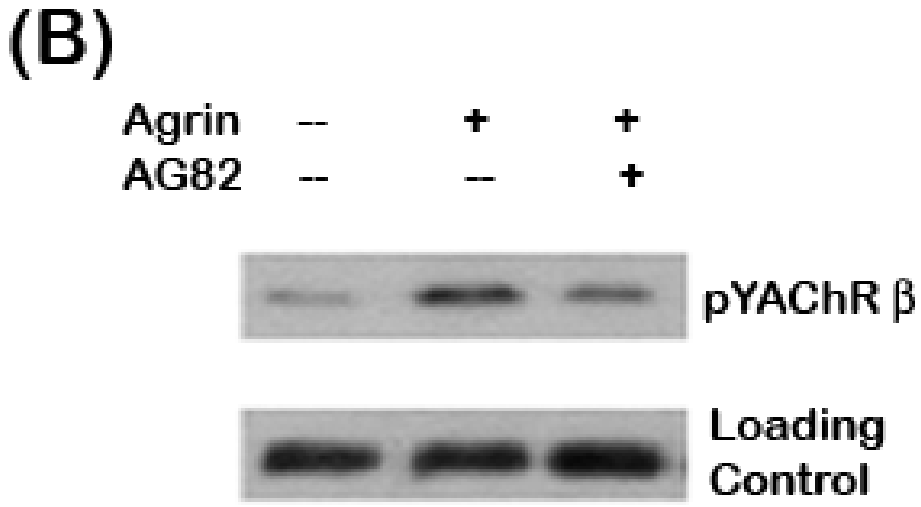

a
AChR
b
pY-FAK
c
Merge
10 µm
10 µm
10 µm
a'
b'
c'
10 µm
10 µm
10 µm

Figure IV.6. Interaction between activated FAK and AChR β subunit in cultured myotubes. (A) Co-immunoprecipitation of pYAChR β subunit by anti-pYFAK antibodies in the presence and absence of agrin (5 nM). (B) Affinity precipitation of AChR using biotinylated α-bungarotoxin followed by immunoblot with antibody directed against phosphorylated AChR β. Extent of AChR β subunit tyrosine phosphorylation is shown in controls and agrin-stimulated myotubes in the presence or absence of AG82.


(A)
Agrin -- +
pY-FAK IP
pY-AChR β
Lysate
Loading
Control


(B)
Agrin -- + +
AG82 -- -- +
pYAChR β
Loading
Control

Chapter V

Molecular Mechanisms of Substrate Laminin-Induced Complex AChR Cluster Assembly

V.1. Introduction

Although soluble laminin-induced clusters appear to have a slightly increased density of receptors and cluster size compared to agrin-induced clusters (Lee et al., 2002), they nevertheless closely resemble agrin clusters in their final ovoid appearance and dimensions as well as in the progression through an intermediate microcluster stage. Upon comparison of these cell culture clustering events with the AChR redistribution induced by innervation of muscle cells, it is clear that soluble agrin- and laminin-induced clustering on myotubes mimics only the early events in postsynaptic membrane assembly. Nerve-induced clustering is significantly more elaborate, passing through several subsequent stages to form a region of elevated AChR density that is markedly more structurally complex than the clusters induced in myotube cultures by soluble agrin or laminin.

Consequently, to date muscle cultures have been invaluable for the elucidation of the initial events in the formation of neuromuscular synapses, but no comparable experimental system has been available for unraveling the sequence of subsequent steps crucial for the maturation of these structurally complex synapses. If aneural muscle cultures could be induced to undergo a more complete postsynaptic membrane differentiation process that is more akin to NMJ formation, this could offer the experimental means for significantly advancing our understanding of the mechanistic basis for postsynaptic membrane specialization during NMJ synaptogenesis.

Recently it was shown that myotubes differentiated on substrate laminin undergo on their adherent surfaces an AChR aggregation process that more closely resembles the postsynaptic specialization induced by innervation at NMJs (Kummer et al., 2004). It is of relevance that in the intact NMJ, both agrin and laminin are present as immobilized components of ECM rather than soluble molecules. Similar to the *in vivo* situation, the formation of these aneural aggregates has been reported to be dependent on MuSK.

Our recent findings show that the sequential activation of the small GTPases Rac1 and RhoA is essential in signaling pathways leading to AChR clustering by soluble forms of agrin and laminin. We have therefore identified a potential molecular mechanism for the organization of a partially mature postsynaptic membrane. The goal of experiments presented in this chapter is to decipher the roles of RhoGTPases as well as FAK in the context of the multistage assembly of more mature AChR aggregates.

In order to determine the mechanisms by which substrate laminin induces these complex AChR clusters, it was important to distinguish the direct focal actions of extracellular laminin at sites of cluster formation from the generalized stimulation by substrate laminin of the muscle differentiation process, since immobilized laminin has previously been shown to enhance skeletal myogenesis *in vitro*(Foster et al., 1987). Thus, the appearance of complex AChR clusters on these myotubes might reflect an indirect effect of the more extensive differentiation process.

Consequently, I have developed culture conditions that make it possible to bypass the effects of substrate laminin on muscle differentiation, in order to investigate the possibility that contact with immobilized laminin directly induces cluster formation and maturation. I found that myotubes differentiated in the absence of exogenous laminin can be induced to form morphologically complex AChR aggregates when replated onto immobilized laminin coated coverslips. Furthermore, the cluster assembly by immobilized laminin on these replated myotubes recapitulates the stages of AChR aggregation observed at the NMJ. This finding shows that acute contact with immobilized laminin is sufficient to induce complex clustering. This method can be employed to determine the signaling events connecting substrate laminin stimulation to the clustering of AChR and associated components of postsynaptic membranes. Using this technique, I have shown the potential roles of FAK and RhoGTPase in the assembly of highly complex AChR aggregates in myotubes replated onto immobilized laminin.

V.2. Results

V.2.1. *Acute contact of myotubes to immobilized laminin induces a multistage complex AChR cluster assembly*

Soluble laminin is known to induce ovoid-shaped AChR clusters on muscle cells (Patton et al., 2001; Patton et al., 1997) reminiscent of early stages of postsynaptic membrane differentiation upon innervation (Sanes and Lichtman, 1999). In contrast, muscle cells plated and differentiated in contact with immobilized laminin display far more complex clusters of cell surface AChR resembling mature postsynaptic membranes of innervated muscle cells (Kummer et al., 2004). A secondary plating method was developed, and was utilized to determine if acute contact with substrate laminin can

induce similarly complex AChR clusters on myotubes. In this method, cultures are allowed to differentiate into multinucleated myotubes on culture dishes in the absence of exogenous laminin substrate. The newly fused myotubes, which express AChR that is diffusely distributed on the cell surface, are then detached by mild trypsinization and replated onto laminin-coated coverglasses (Fig.V.1). Upon contact with the laminin-coated substrate, ovoid AChR clusters appeared on the underside of reattached myotubes within 8-12 hours as an initial stage of AChR aggregation (Fig.V.2Aa; Fig.V.2B left panel). Subsequently, cluster morphology continued to increase in complexity (Fig.V.2A, B) until elaborate pretzel-shaped AChR aggregation structures reminiscent of postsynaptic regions of innervated muscle were achieved 2-3 days after ovoid clusters first appeared(Fig.V.2Ad; Fig.V.2B right panel). Therefore, this finding indicates that complex AChR clustering by immobilized laminin is a consequence of the direct inductive role of laminin on AChR aggregation.

V.2.2. Acute contact of myotubes to immobilized laminin induces Rac1 activation

The replating method provides a suitable experimental approach to investigate the early molecular events triggered by contact of fully differentiated myotubes to substrate laminin as well as the subsequent signaling events that regulate the maturation of AChR cluster. Our laboratory has previously shown that activation of Rac1, a small GTPase that regulates actin polymerization and plays crucial roles in cell membrane dynamics (Bishop and Hall, 2000; Hall, 1998; Ridley, 2001; Takai et al., 2001), is critical for AChR aggregation into ovoid clusters in response to soluble agrin or laminin (Weston et al., 2003; Weston et al., 2000; Weston et al., 2007). By replating myotubes onto laminin-coated 100mm culture dishes, I observed that cellular Rac1 is activated within 8h of

plating, before the appearance of AChR complex clusters on the adherent muscle cell surface (Fig.V.3), consistent with the potential role of Rac1 in AChR aggregation by immobilized laminin.

V.2.3. Assembly of complex AChR aggregates by immobilized laminin is dependent on the RhoA-ROCK pathway

To assess the contribution of the RhoA effector pathway to AChR complex cluster formation triggered in myotubes replated onto laminin-coated surfaces, the effects on this process of a dominant negative mutant of Rho, RhoN19, as well as Y- 27632, a pharmacological inhibitor of the Rho effector p160ROCK (Ishizaki et al., 2000; Uehata et al., 1997) were measured. As shown in Figure V.4A, myotubes transfected with RhoN19 using Lipofectamine and replated onto substrate laminin displayed a drastically reduced number of clusters as compared to controls, measured 3 days after replating. Similarly, addition of Y-27632 (20μM, added immediately upon replating and subsequently re-added every 8h) was seen to block the aggregation of AChR induced by immobilized laminin at 3 days after replating. Figure V.4B is a quantitative measurement verifying the inhibition by RhoN19 of cluster formation. As shown in Fig.V.4C, N19RhoA-expressing myotubes display significantly reduced number of clusters of all morphologies. These findings further define the role of Rho GTPases in the events that couple contact of myotubes to substrate laminin contact to complex AChR cluster formation on cell surface.

V.2.4. Myotubes replated onto immobilized laminin activate FAK

In view of the findings indicating the critical role of FAK in AChR clustering by soluble agrin (Chapter IV), it was important to test if FAK also participates in a more

complete AChR clustering process observed in myotubes replated onto substrate laminin. This was examined by monitoring the association of FAK activation with the appearance of clusters in myotubes replated onto immobilized laminin. As shown in Fig.V.5, substrate laminin activates FAK in myotubes as shown by elevated phosphorylation at tyrosine residues, Tyr576 and Tyr577.

V.2.5. Activated FAK colocalizes with AChR aggregates induced by immobilized laminin

To further investigate the participation of FAK in the formation of complex AChR aggregation induced by immobilized laminin, the cellular distribution of FAK in relation to these AChR aggregates in cultured myotubes was assessed. To determine the distribution of FAK in these structures, after surface labeling of AChR the cells were detergent extracted with saponin with mild shaking and the detergent-resistant cellular fraction, consisting of substrate adherent cell membrane and associated cytoskeleton was probed with antibody directed against phosphorylated FAK. As seen by indirect immunofluorescence (Fig. V.6), increased levels of tyrosine phosphorylated FAK are detected at regions of high AChR density in the fractionated myotubes.

V.3. Discussion

Whereas the AChR aggregation state achieved by soluble agrin and laminin is limited to the formation of ovoid AChR clusters - an early stage in the overall postsynaptic differentiation process - the replating of differentiated myotubes onto immobilized laminin induces the aggregation of AChR into clusters that are highly extensive, and more reminiscent of the situation *in vivo* than is achievable with soluble laminin or agrin.

Consequently this procedure allows a more complete characterization of the molecular mechanisms that direct aggregation of surface AChR into complex clusters. Because in the case of soluble laminin and agrin the clustering process is arrested at the ovoid cluster stage, the subsequent differentiation of high density AChR membrane regions has not heretofore been accessible to *in vitro* studies of this type. As demonstrated in this chapter, this experimental technique should allow elucidation of the signaling pathways underlying the multistage process that produces elaborately branched AChR aggregates that approximate in complexity the motor end-plate of innervated muscle.

The secondary plating approach derives from the interesting recent finding that myoblasts grown on substrate laminin differentiate into myotubes that display morphologically complex AChR clusters(Kummer et al., 2004). Immobilized laminin, but not soluble laminin, has been shown to enhance general skeletal muscle differentiation in *vitro* (Foster et al., 1987) and thus it appeared possible that the complexity of AChR clusters reflects the extent of muscle differentiation. When myoblasts differentiated in the absence of exogenous laminin were subsequently replated onto laminin-coated substrate, they formed complex clusters indistinguishable from those observed on cells grown on laminin. This shows that this clustering response is due to the direct inductive role of immobilized laminin rather than reflecting an indirect effect of the enhancement of muscle differentiation by substrate laminin. Thus acute contact with immobilized laminin is sufficient to induce complex clustering.

This approach has proven valuable in the investigation of the participation of the RhoGTPases as well as the FAK in coupling substrate laminin signaling to the formation of branched AChR aggregates. It is observed that AChR aggregation by acute contact of myotube membranes to substrate laminin is associated with the activation of Rac1 and FAK prior to the appearance of complex clusters on the adherent surfaces of myotubes. In addition, the dominant negative Rho mutant RhoN19 as well as the Rho kinase inhibitor Y-27632 block cluster formation on myotubes replated onto immobilized laminin . Furthermore, as is the case at an intact NMJ (Chapter IV), activated FAK is readily detectable at AChR aggregation sites induced by immobilized laminin.

In conclusion, the replating of myotubes grown on uncoated dishes onto surfaces coated with laminin has been shown here to be sufficient to stimulate the assembly of complex AChR aggregates and to provide a means for the elucidation of the roles of immobilized ECM components and signaling molecules in the formation of specialized AChR-rich membrane regions closely resembling postsynaptic membranes at the NMJ. Evidence derived using this method demonstrates a shared biochemical pathway of AChR cluster assembly between immobilized laminin, soluble agrin, and soluble laminin. This method could also be used to dissect the specific contribution of signaling molecules in the morphological transformations of AChR aggregates induced by immobilized laminin.

V.4. Figures

Figure V.1: Schematic representation of the secondary-plating method. C2C12 myoblasts were plated on uncoated plastic culture dishes in Growth Medium, and after two days, the medium was replaced by Differentiation Medium to induce myoblast fusion. Where indicated, myoblasts were transfected a day after plating. After 3 or 4 days in Differentiation Medium, myotubes were detached by mild trypsinization and replated onto laminin-coated 100mm dishes for biochemical assays, or 12mm laminin-coated coverslips for microscopy.

Figure-1

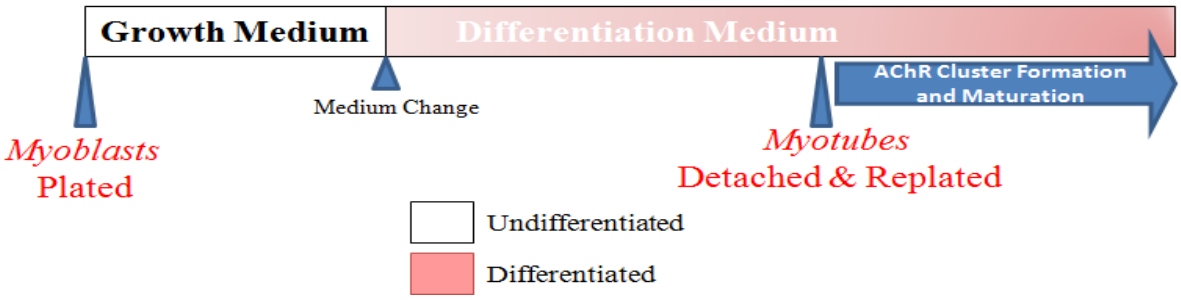
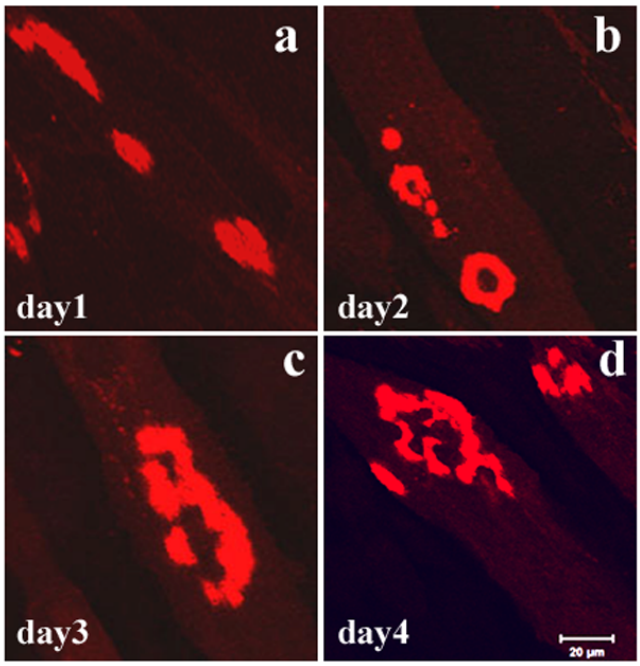

Growth Medium
Differentiation Medium
AChR Cluster Formation and Maturation
Medium Change
Myoblasts
Plated
Myotubes
Detached & Replated
Undifferentiated
Differentiated

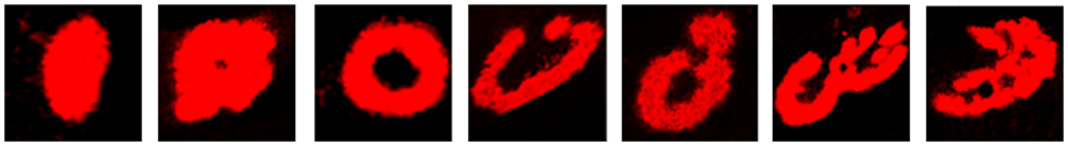
Figure V.2 Effects on AChR clustering of secondary plating of differentiated myotubes onto substrate-coated laminin. Differentiated myotubes replated on laminin-coated coverslips were seen to form increasingly complex and heterogeneous AChR clusters, as visualized by labeling AChRs on intact myotubes with TMR-Bgt (10nM for 1h at 37°C). The morphological maturation of AChR aggregates proceeds through the ovoid stage (2Aa; 2B) on day 1 to pretzel-like aggregates by day 4 post-replating (2Ad; 2B). Scale bar, 20μm.

Figure-2

A



B



8-12h 12-16h 16-24h 24-36h 30-50h 50-72h 72-96h

Figure V.3 Effect of substrate laminin on Rac1 activity in replated myotubes. Myoblasts were cultured and replated as described in Fig.1. Rac GTPase activity was measured in extracts of differentiated myotubes by means of a pulldown assay 8h after replating. Contact with substrate laminin increases the amount of GTP-bound Rac as measured by selective binding of Rac-GTP to GST-PBD.

Figure-3

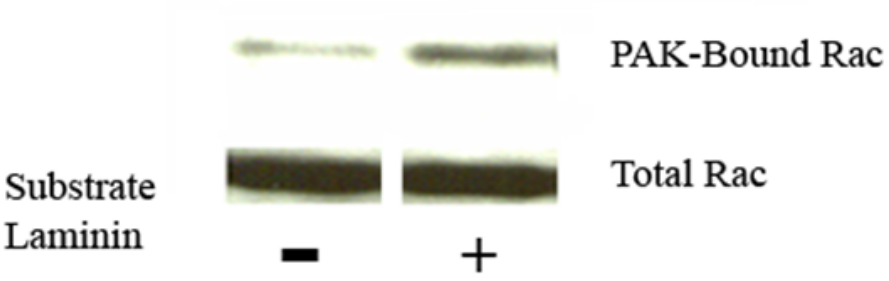
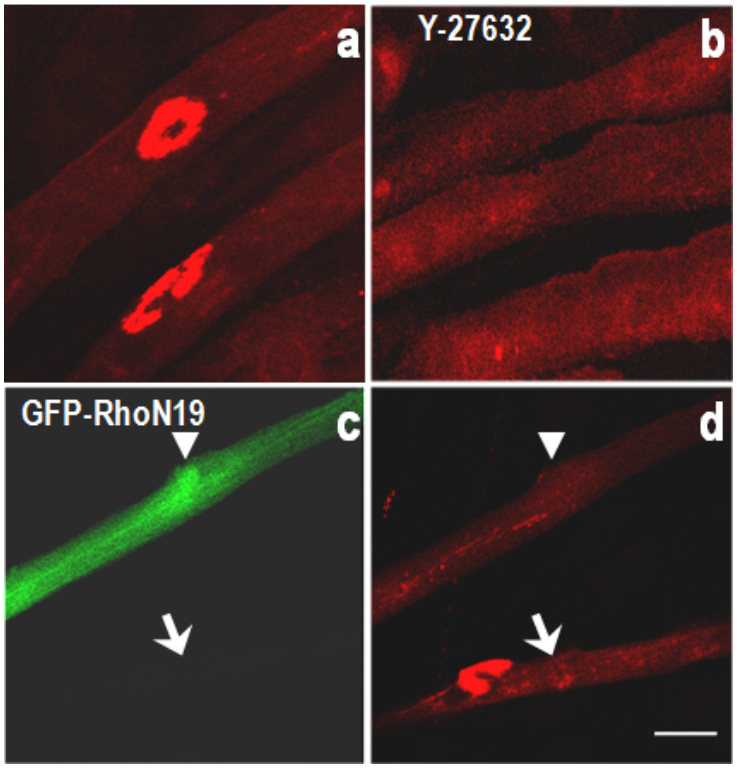


Figure V.4 Effects of blocking Rho-mediated signaling on AChR clustering induced by substrate laminin. Confocal microscopy of replated cultures labeled with TMR-Bgt was conducted to visualize the induction by substrate laminin of complex clusters (Fig.4A panel a). Inhibition of the Rho effector, ROCK, using a pharmacological inhibitor Y-27632, is seen to block assembly of complex AChR clusters (Fig.4A panel b). Expression of a dominant negative Rho mutant (GFP-RhoN19) in muscle cells (Fig.4A panel c, arrowhead) can be seen to block the formation of AChR clusters after replating onto immobilized laminin (Fig.4A panel d, arrowhead) compared to non-transfected myotubes in the same field (Fig.4A panels c,d arrow). Figure 4B and 4C are quantitative comparisons of substrate laminin-induced clusters of various morphologies in these RhoN19 expressing myotubes versus untransfected cells. The total number of clusters is significantly reduced in RhoN19 expressing myotubes. Scale bar, 20μm. (n= 100, Error bars represent +/- SEM).

Figure-4A


a
Y-27632
b
GFP-RhoN19
c
d

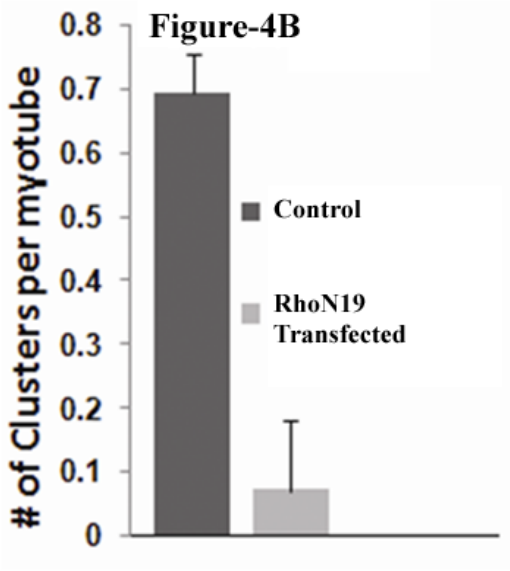


Figure-4B
# of Clusters per myotube
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
RhoN19
Transfected

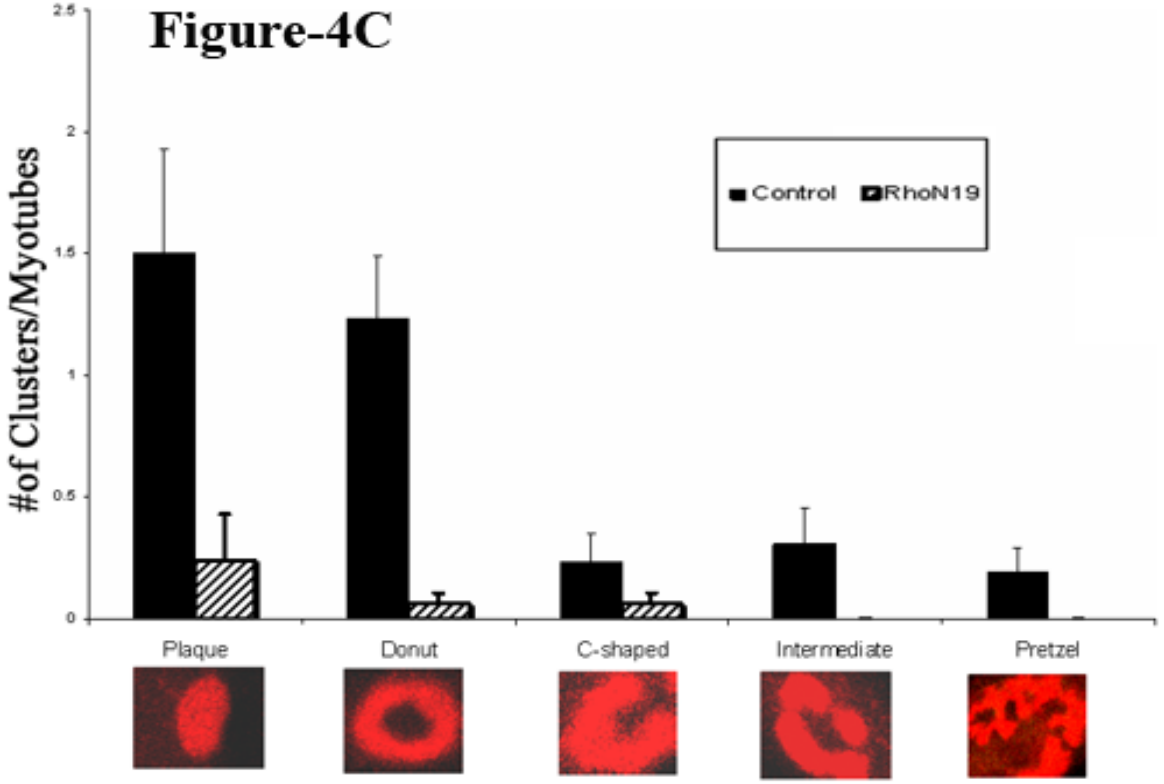


Figure-4C
#of Clusters/Myotubes
2.5
2
1.5
1
0.5
0
Control
RhoN19
Plaque
Donut
C-shaped
Intermediate
Pretzel

Figure V.5 Activation of FAK by immobilized Laminin. Myoblasts were cultured and replated onto polyornithine- or laminin-coated 100mm tissue culture dishes. Activated FAK was measured by immuneblotting membrane with pYFAK (576/7) antibody.

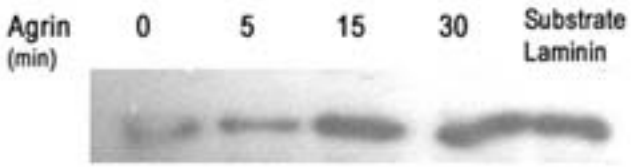
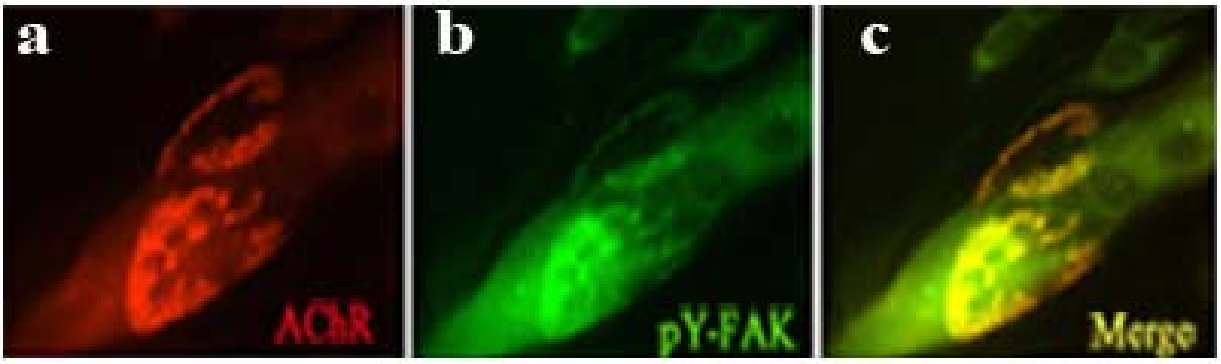

Agrin
(min)
0
5
15
30
Substrate
Laminin

Figure V.6. Tyrosine phosphorylated FAK is concentrated at sites of AChR clustering in cultured myotubes. The cellular distribution of phosphorylated FAK overlaps with AChR aggregates in differentiated myotubes replated onto laminin-1 coated coverslips. AChR distribution was visualized by labeling live cells with TMR-Bgt (a), and activated FAK was visualized by immunolabeling detergent-permeabilized myotubes using rabbit anti-pY-FAK primary antibodies, and FITC anti-rabbit secondary antibody (b). Phosphotyrosine FAK is concentrated at sites of complex AChR clusters induced by immobilized laminin (c).


a
AChR
b
pY-FAK
c
Merge

Chapter VI

Diversity and Convergence of Signaling Pathways that Mediate AChR Cluster Assembly

VI.1. Introduction

The formation of the neuromuscular junction (NMJ) involves combinatorial inputs from multiple extracellular matrix (ECM) components, most prominently agrin and laminin. All currently available data suggest that these inputs have distinctive but overlapping contributions. The initial point of distinction between soluble agrin- and soluble laminin-induced signaling is their utilization of distinct surface receptors: agrin is known to utilize a MuSK-containing receptor complex while soluble laminin is not dependent on MuSK but may utilize integrin and/or dystroglycan as its surface receptor(s). In addition, as we have observed, only agrin can induce AChR β subunit phosphorylation and this is believed to be due to a MuSK-pathway specific activation of another tyrosine kinase downstream of MuSK. Despite these differences, however, both soluble agrin and laminin induce AChR aggregates that are similar in their overall ovoid appearance- reminiscent of the early stages in NMJ formation. Moreover, agrin and laminin have synergistic effects when applied in cultured myotubes in combination.

Since the formation of NMJ is critically dependent on MuSK, the MuSK-independent induction of AChR aggregation in cultured myotubes by laminin has been discounted as a reliable model system for *in vitro* studies (Kummer et al., 2004). Agrin and MuSK-deficient mice show similar phenotypes: their NMJs lack any signs of postsynaptic differentiation and the animals die perinatally (DeChiara et al., 1996; Gautam et al., 1996). Recently, however, it was shown that myoblasts plated onto immobilized laminin acquire complex AChR aggregates remarkably more similar to NMJs than is achievable with soluble agrin or laminin both in terms of morphological

complexity and dependency on MuSK (Kummer et al., 2004).

Experiments were carried out to study the separate and combinatorial effects of soluble and immobilized forms of agrin and laminin as manifested in the redistribution as well as post-translational modification of surface AChR. These studies show the unexpected finding that signals initiated by soluble recombinant neural agrin cause regression of immobilized laminin-induced complex AChR clusters into simple ovoid aggregates. Moreover, despite the reported dependency on MuSK of substrate-laminin induced complex cluster formation, there was no detectable induction of AChR β subunit tyrosine phosphorylation in myotubes replated onto immobilized laminin. Therefore, this data is consistent with the interpretation that MuSK activation may not directly link the agrin-induced transmembrane signal to AChR β phosphorylation. Moreover, data are presented showing that FAK and the RhoA-ROCK pathways have opposite effects on the tyrosine phosphorylation of AChR β subunit while playing complementary roles in the redistribution of surface AChR. Together, the results presented in this chapter highlight the diversity and convergence of the mechanism of post-synaptic membrane assembly that requires a precise integration of local nerve and ECM-derived signals.

VI.2. Results

VI.2.1. Combinatorial Signaling by Immobilized and Soluble forms of Agrin and Laminin in the Induction of AChR Aggregates

The myotube replating protocol was utilized to study the combined actions of soluble and immobilized forms of agrin and laminin on AChR aggregation. Immobilized COOH-terminal fragment as well as full-length agrin failed to induce AChR aggregates

when immobilized alone, and also had no effect on the complex AChR aggregation patterns induced by immobilized laminin. In contrast, soluble agrin radically modified the aggregation pattern of AChR induced by immobilized laminin. Exposure to soluble agrin reorganized the complex clusters into a simpler, ovoid morphology characteristic of agrin clusters (Fig.V.1). Moreover, soluble agrin produced the subsequent displacement of these aggregates to the myotube periphery by 2 days after the initial exposure to agrin. Surprisingly, these effects were manifested even under conditions where the exposure duration of replated myotubes to agrin was as brief as 15min. Contrastingly, soluble laminin did not affect the AChR aggregation pattern induced by its immobilized counterpart.

VI.2.2. AChR β subunit tyrosine phosphorylation is a soluble agrin-signature event

The separate nature of the signaling pathways initiated by soluble agrin and soluble laminin is documented in findings regarding the tyrosine phosphorylation of AChR β subunit. An agrin-pathway signature event is a significant increase in β subunit tyrosine phosphorylation. In contrast, we have not observed a detectable increase in β subunit phosphorylation induced by soluble laminin (Fig.VI.2A), as well as immobilized laminin (Fig.VI.2B) .

VI.2.3. Regulation of AChR β subunit tyrosine phosphorylation by FAK and RhoA-ROCK pathways

The phosphorylation of AChR β subunit on tyrosine has been correlated with agrin-induced clustering as well as to the linkage of AChR aggregates to the cytoskeleton (Meier et al., 1995; Mohamed and Swope, 1999). As shown in the preceding chapters, the condensation of microaggregates of AChR into full-sized AChR clusters is inhibited by

FAK or Rho-pathway inhibitors, which were seen to decrease the number of full-sized AChR clusters while increasing the number of microclusters upon agrin stimulation. Moreover, it was shown by co-immunoprecipitation studies that these proteins form signaling complexes upon agrin or laminin stimulation. Furthermore, I have shown that activated FAK colocalizes with AChR aggregates both *in vitro* and *in vivo*. Therefore, in view of their functional interactions, it was of interest to see if the AChR clustering activities of FAK and RhoA are associated with AChR β subunit tyrosine phosphorylation.

First, it was tested if FAK catalytic activity correlates with the extent of AChR β subunit tyrosine phosphorylation in the context of agrin-induced AChR clustering. In this experiment, myotubes were treated with agrin for 45min in the presence or absence of AG82 (20μM), and levels of AChR β subunit tyrosine phosphorylation were compared. AChR pulldown assays were carried out using biotinylated Bgt followed by immunoblotting with AChR β subunit phosphotyrosine specific antibody. As shown in Fig.VI.3, agrin-induced increase in AChR β subunit tyrosine phosphorylation was reversed under conditions in which FAK kinase activity was blocked by AG82, suggesting that FAK activity mediates agrin-induced AChR β subunit tyrosine phosphorylation.

Next, the effect of blocking Rho GTPases or the RhoA effector pathway on agrin-induced AChR β subunit phosphorylation was determined. The result shows that pre-treatment of myotubes with the general Rho GTPase inhibitor toxin B (Fig.VI.4A) or

with the Rho Kinase (ROCK) inhibitor Y-27632 (Fig.VI.4B) significantly increases the level of agrin-induced AChR β-subunit tyrosine phosphorylation, suggesting that the Rho-ROCK pathway attenuates AChR β subunit tyrosine phosphorylation.

VI.2.4. RhoA pathway inhibition enhances FAK autocatalytic activity

The enhancement of agrin-induced AChR β subunit tyrosine phosphorylation by Rho pathway inhibition, combined with the finding indicating that FAK mediates AChR β subunit phosphorylation, suggests the possibility that the RhoGTPase pathway regulates FAK activity. This notion is further supported by the observed formation of a physical complex between RhoA and FAK. To test this possibility, the level of agrin-induced FAK phosphorylation was assessed under different conditions by blotting membranes with anti-FAK (pY397) antibody which is a measure of the extent of FAK autocatalytic activity. As shown in Fig.VI.5, agrin-induced FAK autocatalytic activity is augmented in myotubes incubated with the ROCK inhibitor Y-27632 compared to agrin-only treated myotubes. This finding suggests the negative regulation of FAK activity by ROCK.

VI.3. Discussion

While soluble agrin and, to a lesser extent, soluble laminin have been used extensively to investigate mechanisms of AChR clustering, these model systems mimic only the initial stages of AChR clustering. Using these approaches, the present studies demonstrate that dependent mechanisms. Recently, it was shown that substrate laminin induces an NMJ-like highly complex AChR aggregation in myotubes in a MuSK-dependent manner (Kummer et al., 2004). Using the secondary plating method developed

in our laboratory (Teressa and Prives, 2008), it was shown that acute contact of myotubes with immobilized laminin recapitulates the *in vivo* multistage AChR cluster assembly process by a mechanism identical to those elucidated for soluble agrin and laminin.

While the preceding chapters have focused on the shared molecular mechanism of cluster assembly by agrin, laminin, and immobilized laminin, the present chapter emphasizes their distinct contributions. The formation of specialized postsynaptic membrane regions on the muscle cell surface involves signaling via multiple pathways, each with distinct inductive cues. The diverse nature of the signals initiated by soluble and immobilized agrin and laminin is manifested in the distinctiveness of the AChR aggregation patterns as well as by the disparate AChR β subunit tyrosine phosphorylation responses to these ligands. Furthermore, the intracellular signaling molecules, FAK and Rho GTPases, involved in the assembly of AChR aggregates are shown to oppositely regulate AChR β subunit tyrosine phosphorylation

The experiments using soluble and substrate agrin and laminin in combination illustrate the separate nature of signals initiated by agrin and laminin. This is most clearly demonstrated by the dramatic effect of soluble agrin on substrate-laminin induced complex clusters. Exposure to soluble agrin reorganizes the complex clusters into a simpler, ovoid morphology characteristic of agrin clusters as well as their subsequent displacement to the myotube periphery by 2 days after the initial exposure to agrin. Surprisingly, this effect is manifested even under conditions where the exposure duration of replated myotubes to agrin is as brief as 15min. Moreover, this effect is achieved

regardless of whether the C-terminal fragment of agrin (which lacks laminin binding domain) or full-length of agrin is used, suggesting that the effect of soluble agrin is not via direct interaction with laminin. In contrast, soluble laminin did not affect the AChR aggregation pattern induced by its immobilized counterpart. Another point of divergence between agrin and laminin is their dissimilar effects on AChR β subunit tyrosine phosphorylation; agrin enhances phosphorylation whereas laminin has no effect.

While FAK and RhoA-ROCK both mediate the aggregation of AChRs into full-sized clusters, these two molecules modulate AChR β subunit phosphorylation in an opposite manner: FAK mediates agrin-induced tyrosine phosphorylation of AChR β subunit and the RhoA-ROCK pathway attenuates this response. This suggests a possible negative regulation of FAK kinase activity by ROCK thereby explaining the apparent opposite influence of the FAK and Rho-ROCK pathways on AChR phosphorylation. This interpretation is consistent with the finding that ROCK inhibition amplifies agrin-induced FAK autocatalytic activity. The regulation of a tyrosine kinase, FAK, by a serine-threonine kinase, ROCK could be either direct or indirect. ROCK is reported to directly phosphorylate FAK but the functional significance of this is not fully understood (Le Boeuf et al., 2006). ROCK could indirectly exert its effect on AChR β phosphorylation by regulating other tyrosine kinases or tyrosine phosphatases.

VI.4. Figures

Figure VI.1 Combinatorial effects of agrin and laminin in replated myotubes
Replated myotubes were used to analyze AChR aggregation patterns induced by soluble and immobilized agrin and laminin. AChR clusters on myotubes replated onto substrate coated with laminin are shown in panel b. Agrin has no effect when immobilized either alone (panel a) or in combination with substrate laminin (panel c), while soluble agrin redistributes substrate laminin-induced complex AChR aggregates (panel e) into a pattern more characteristic of soluble agrin (panel f). Unlike soluble agrin, soluble laminin does not have any discernible effect on substrate laminin induced complex AChR cluster formation (panel d). Scale bar, 20μm. *Immob=Immobilized; Sol=Soluble.*

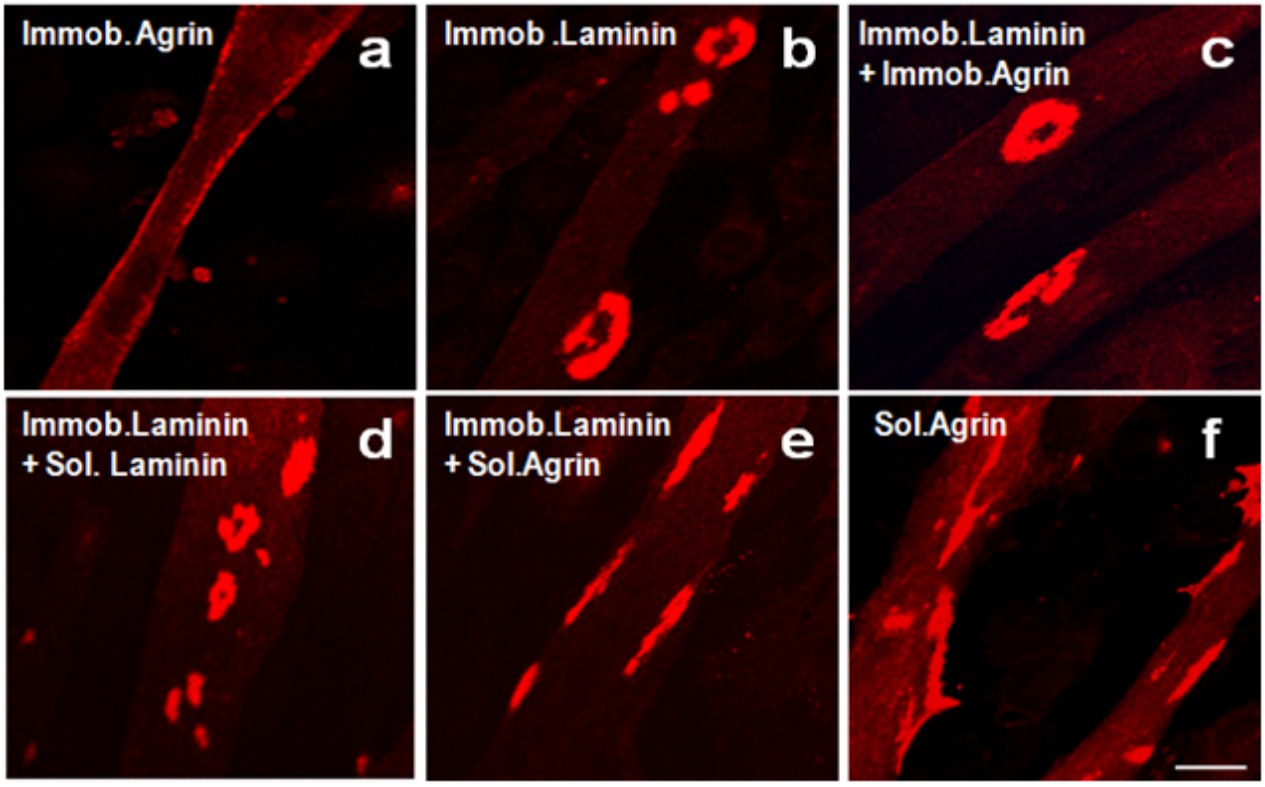
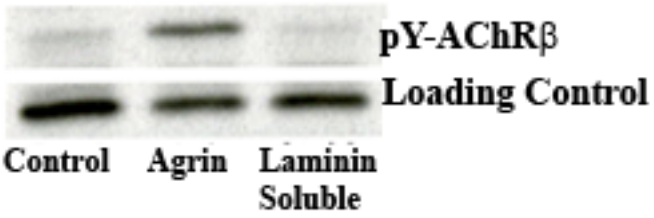

Immob.Agrin
a
Immob .Laminin
b
Immob.Laminin
+ Immob.Agrin
c
Immob.Laminin
+ Sol. Laminin
d
Immob.Laminin
+ Sol.Agrin
e
Sol.Agrin
f

Figure VI.2 AChR β tyrosine phosphorylation is an agrin-pathway specific event. Myotubes were treated with either soluble agrin or laminin for 45 minutes (A), or replated onto immobilized laminin (B). Surface AChR in these myotubes was then purified using biotinylated-.Bgt. Tyrosine phosphorylated AChR β subunit was measured by immunoblotting with pY-AChR β antibody.

(A)


pY-AChRβ
Loading Control
Control
Agrin
Laminin
Soluble

(B)

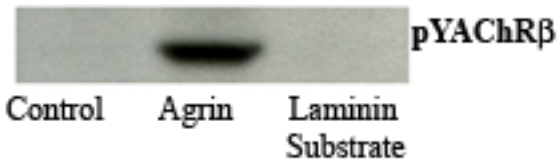

pYAChRβ
Control
Agrin
Laminin
Substrate

Figure VI.3. Inhibition of FAK by tyrophostin A25 (AG82) interferes with Agrin induced AChR β subunit tyrosine phosphorylation. Myotubes were treated with 5nm agrin for 45min in the presence or absence of 20 μm AG82. Agrin induces AChR β tyrosine phosphorylation compared to the controls, and this phosphorylation is significantly reduced in myotubes pretreated with FAK kinase inhibitor AG82 30min prior to agrin addition.

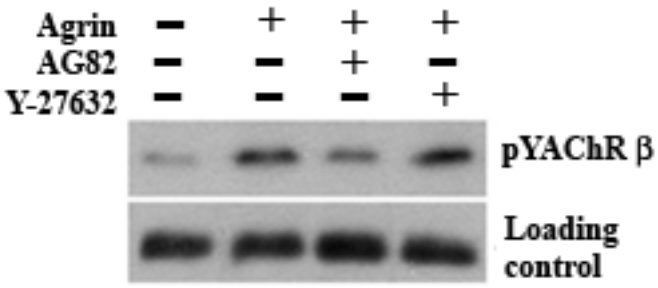
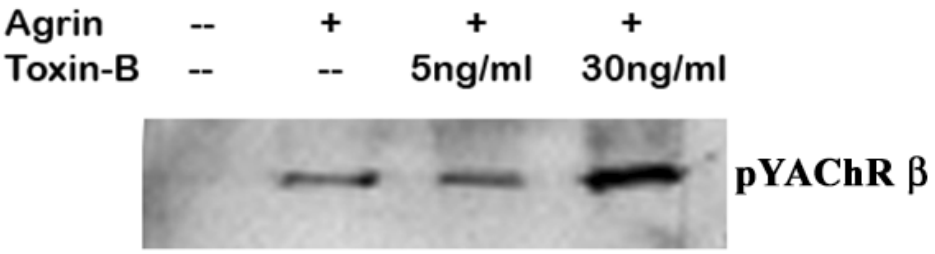

Agrin
AG82
Y-27632
pYAChR β
Loading control

Fig.VI.4 Inhibition of the Rho GTPase pathway causes increased AChR β subunit tyrosine phosphorylation. Myotubes were treated with 5nM agrin for 45min in the presence or absence of toxin B (A) or Y-27632 (B). Agrin induced AChR β subunit tyrosine phosphorylation compared to the control, and this phosphorylation is significantly increased in myotubes pretreated with 30ng/ml toxin B or 20 μM Y-27632 30min prior to agrin addition.

(A)


Agrin -- + + +
Toxin-B -- -- 5ng/ml 30ng/ml
pYAChR β

(B)

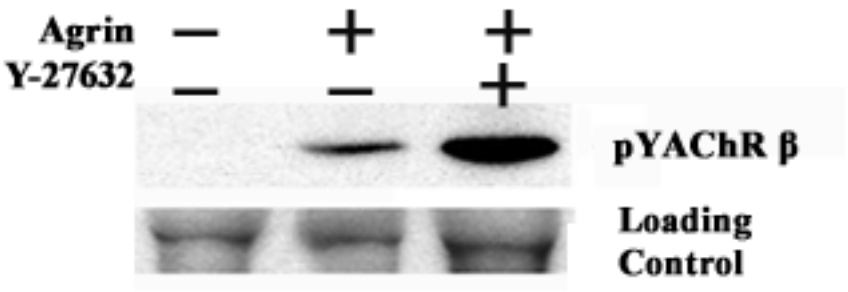
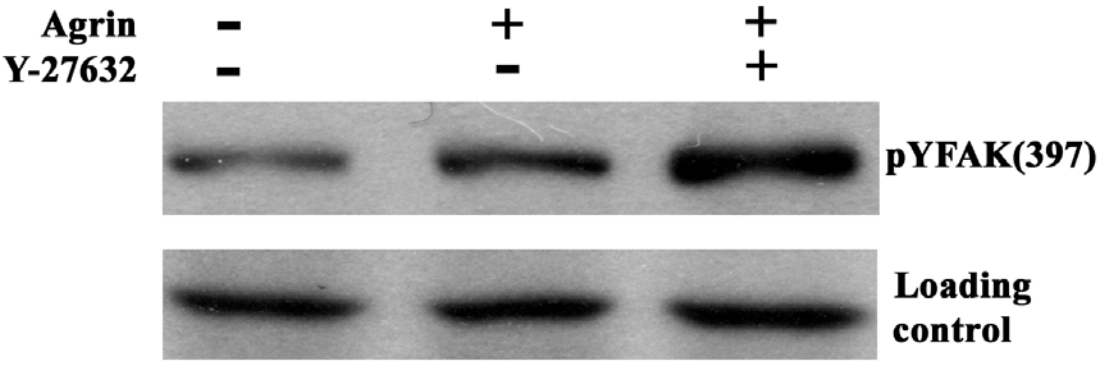

Agrin — + +
Y-27632 — — +
pYAChR β
Loading
Control

Fig.VI.5 Rho-pathway inhibition enhances FAK autocatalytic function. Myotubes treated with agrin (5nM) for 30min in the presence or absence of Y-27632 (20 μM) and level of FAK phosphorylated at Tyr397 was assessed by immunoblotting with pY-FAK (397) antibody.


Agrin
Y-27632
-
-
+
-
+
+
pYFAK(397)
Loading
control

Chapter VII

Concluding Remarks

During the past few decades, considerable progress has been made towards understanding the signaling pathways that regulate the redistribution of AChR on the postsynaptic membrane at the NMJ. The discovery that a soluble neural factor residing in the synaptic basal lamina, later on isolated and identified to be agrin, induces the aggregation of AChR on aneural muscle culture, led to the formulation of the "agrin hypothesis" (McMahan, 1990; McMahan et al., 1992; Reist et al., 1992). The agrin hypothesis predicted that agrin was synthesisized in motor neuron cell body, transported to nerve terminals where it is released and incorporated into the basal lamina, and interacts with receptors on myotube surface to initiate AChR clustering. This hypothesis was subsequently validated and led to the identification of MuSK (Jennings et al., 1993) as a component of the agrin receptor, and rapsyn as a crucial linker protein (Block and Froehner,1987). Despite the tremendous progresses that followed these major discoveries, the molecular mechanism of AChR cluster assembly is far from being completely understood. For instance, the identity of molecules that make up the functional agrin receptor along with MuSK is not understood. Moreover, crucial questions such as what directs the site of synapse formation on the skeletal muscle membrane have not been yet settled.

While the agrin hypothesis have provided a helpful theoretical framework for research, evidence is mounting in recent years that challenge the originally envisioned roles of agrin as encapsulated in this hypothesis. These findings include: 1) Synapse formation appears to begin before nerve-muscle contact, where, at the postsynaptic membrane, a number of synaptic molecules are presumed to be prepositioned by a

mechanism completely unknown. This notion stems from a finding that nascent aneural AChR aggregates on skeletal muscle cell surface form before the arrival of the motor neuron or without prior exposure of muscle to agrin (Lin et al., 2001; Yang et al., 2001; Yang et al., 2000). 2) While the postsynaptic receptor aggregates are highly branched and mirror the branching patterns of the nerve terminal, agrin induces AChR aggregates *in vitro* whose maturation is arrested at the ovoid-stage. In contrast, mature NMJ-like AChR structures can be formed on myotubes plated on immobilized laminin or fibronectin, thereby challenging the paradigm that the nerve terminal dictates the site of AChR aggregate formation, and that the transformation of AChR aggregates into branched patterns is dictated by the site of agrin release from the nerve terminal branches. 3) The finding that agrin knockout phenotype is rescued in agrin and ChAT (choline acetyltransferase, the enzyme that synthesizes ACh) double knockouts (Misgeld et al., 2005) suggests that the primary function of agrin is to counter the cluster destabilizing effects of synaptic transmission mediated by ACh.

Thus, the past decade has registered scores of advances toward understanding synaptogenesis, but has also raised many more questions. The findings presented in this thesis answer the following crucial questions: 1) By identifying RhoGTPase as crucial mediators of AChR aggregation, we have defined the molecular mechanism that couple agrin and laminin signaling to cytoskeleton-mediated cell surface AChR redistribution. 2) By elucidating the multiple functions of FAK in AChR aggregation, we have identified a tyrosine kinase signaling that was postulated to be the hallmark of the early signaling events that precede the redistribution of AChR. Additionally, functional and physical

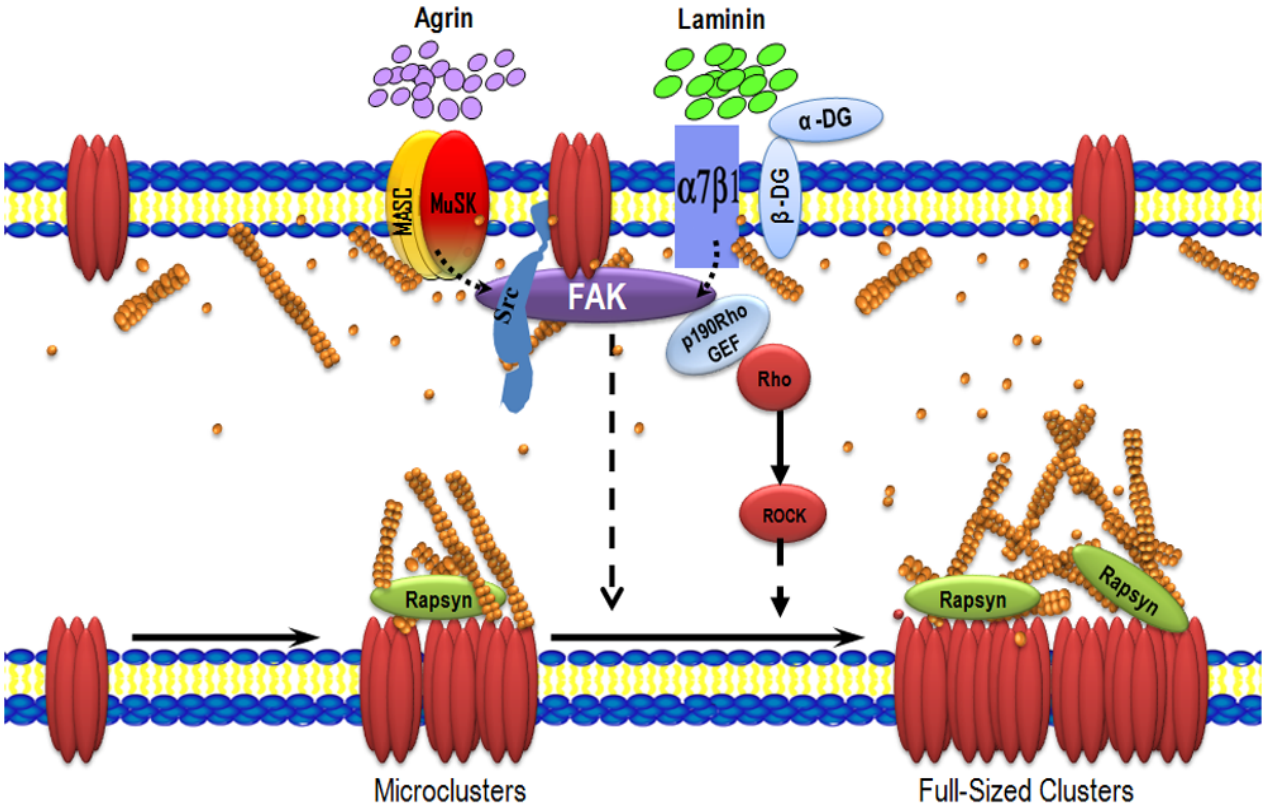
relationships between RhoA as well as FAK were also defined. These findings together reveal that the kinase-dependent and –independent functions of FAK position it at the nexus of tyrosine kinase signaling and RhoGTPase-mediated AChR redistribution events.

How might FAK integrate incoming signals from agrin and laminin receptors and transduce these signals into the redistribution of surface AChR into clusters? It is well documented that RhoA can cause reorganization of cortical cytoskeleton by stimulation of actin-myosin interaction leading to formation of focal adhesions. It was shown in Chapter III that the RhoA-Rho Kinase pathway mediates the formation of full-sized AChR clusters from microclusters in response to agrin. The findings in this thesis are summarized in the proposed scheme shown in Fig.VII.1, which indicates that agrin and laminin each induce a highly localized activation of FAK that is coupled to AChR clustering via two independent, complementary pathways. The first pathway depends on FAK kinase activity, whereas the second pathway is dependent on FAK but not on its kinase activity. The FAK kinase-independent pathway utilizes the capacity of FAK to form functional complexes with RhoA, and most probably additional as yet unidentified components. Furthermore, although both pathways are essential, neither is sufficient in itself to drive full-sized cluster formation.

These findings can serve as starting points for a more complete characterization of the relationships between FAK and RhoGTPases as well as for the investigation of the missing regulatory steps that couple the agrin and laminin signaling pathways to AChR clustering. Future experiments should be aimed at identifying a GEF (Guanine nucleotide

exchange factor) that is activated and/or a GAP (GTPase activating protein) that is inhibited in order to activate RhoA in the context of agrin- and laminin-induced AChR clustering. Although we have identified a physical association between FAK and RhoA in response to agrin and laminin, these interactions are likely to be mediated by GEFs and/or GAPs, as is the case in other biological contexts. It is also important to note that a number of signaling molecules identified to play roles in AChR clustering, including Src kinases (Fuhrer and Hall, 1996; Marangi et al., 2002; Mohamed et al., 2001; Smith et al., 2001), PI3 kinase (Nizhynska et al., 2007), Shc (Jones et al., 2007) and calpain (Chen et al., 2007) are each recruited to AChR aggregation sites and all are known to interact with FAK in other cell types (Mitra et al., 2005; Schaller et al., 1994). Currently, the relationship between these signaling molecules in the context of AChR aggregation remains unexplored. Thus, it is important to address this question with the hypothesis that FAK may serve as focal organizer to recruit and activate these signaling molecules in response to synaptogenic cues.

Figure VII.1 Scheme showing integration of signals mediating AChR surface redistribution. Stemming from the findings presented in this thesis, the scheme depicts a hypothetical model for agrin- and laminin-induced AChR clustering. Synaptogenic signals originating from the presynaptic nerve terminal and ECM are integrated by FAK, which couples agrin- and laminin- induced signaling to AChR aggregation on myotube surfaces by kinase-dependent and -independent mechanisms. FAK kinase activity is necessary but not sufficient for the formation of full-sized clusters. The RhoA-ROCK pathway mediates the redistribution of AChR microaggregates through its effect on localized cytoskeletal dynamics. FAK is recruited to AChR aggregation sites, and in turn recruits RhoA by a mechanism independent of FAK kinase activity, underscoring its additional function as a scaffolding protein in the assembly of the structural and signaling components of the NMJ.


Agrin
Laminin
α-DG
α7β1
β-DG
MASC
MuSK
Src
FAK
p190Rho GEF
Rho
ROCK
Rapsyn
Rapsyn
Rapsyn
Microclusters
Full-Sized Clusters

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