

The Neuromuscular System: Postsynaptic Regulation

by

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DEDICATION

To my parents, Hsiu-Chuan and Ching-Kun,
my husband, Shih-Kang,
and our expected daughter.

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ABSTRACT

Synapses are specialized structures for neural functions at both the peripheral and central nervous system. The efficiency of synaptic transmission between the presynaptic and postsynaptic cells depends on the maintenance of an appropriate receptor density at postsynaptic sites. Despite extensive studies on synaptic plasticity, the mechanisms that regulate the density of postsynaptic neurotransmitter receptors is not fully understood. In this thesis, I used the peripheral neuromuscular junction (NMJ), a chemical synapse made between a spinal motor neuron and skeletal muscle fibers, as a synapse model to investigate the interplay between postsynaptic proteins and acetylcholine receptors (AChRs).

A high density of AChRs at postsynaptic membranes is the hallmark of NMJs. Two major groups of proteins are critical for the tethering and stabilization of AChRs: the agrin-Lrp4/MuSK/rapsyn signaling pathway and the auxiliary proteins [dystrophin-glycoprotein complex (DGC), neuregulin signaling molecules, and the non-kinase muscle-specific anchoring protein (α kap)]. Recent work from our lab has shown that α kap forms a complex with AChRs and promotes their stability by an ubiquitin-dependent mechanism. However, it remains unclear the interplay between the postsynaptic protein network and the stability of AChRs. To understand the connection between the stability/function of AChRs and postsynaptic proteins, I have used a multi-disciplinary approach, including biochemical and molecular tools, mouse models and cultured cell lines.

First, in collaboration with Dr. Martinez, we explored the mechanism by which rapsyn, a core protein for AChR clustering, is explicitly targeting the synaptic membrane for its function. By electroporating rapsyn domain deletion mutants-GFP into sternomastoid muscles, we found that

the targeting of rapsyn is independent of synaptic activity neither the N-terminal myristoylation nor the RING-H2 domain of rapsyn. However, when the AChR-binding coiled-coil domain is deleted, rapsyn fails to associate with AChRs at NMJs of living mice, providing evidence for an active role of AChRs in the targeting of rapsyn to NMJs.

I also investigated α -dystrobrevin, a cytoplasmic protein of the DGC, on the expression of α kap and its association with AChRs. α -Dystrobrevin is required for the maintenance of a high postsynaptic receptor density and maturation of NMJs. My results established a strong link between the expression levels of α kap and α -dystrobrevin on the expression of AChRs. Particularly, I showed that α -dystrobrevin forms a complex with α kap and promotes its expression in a dose-dependent manner, while the expression of α -dystrobrevin is independent of α kap. Importantly, α -dystrobrevin and α kap promote AChR accumulation and enhance the size of agrin-induced AChR clusters, which suggests the protective role of α kap on AChR is mediated by α -dystrobrevin. To further explore how α kap/ α -dystrobrevin stabilizes AChRs, I investigated the deubiquitinating enzyme Usp9x, an α -dystrobrevin binding protein. My results showed that Usp9x promotes the α kap/ α -dystrobrevin stabilizing effect on AChRs and its expression is highly correlated with the maturation of synapses.

Finally, Dr. Martinez and I generated α -syntrophin/ α -dystrobrevin null mouse to investigate signaling and structural functions of these proteins in the maturation and stability of NMJs. Analyses of these mice revealed that these two proteins do not complement each other and suggested that they may function in the same signaling pathway.

Collectively, my results showed a mechanistic link between the intracellular proteins, rapsyn, α -dystrobrevin, α -syntrophin, α kap, and Usp9x and the stability of the postsynaptic AChR and ultimately the structural integrity of the NMJ. Such findings are potentially important for adding new insights into discovering new therapeutic targets of NMJ-related disorders.

CHAPTER 1

1. Introduction

1.1 Overview of synaptic function and structure

The human's brain uses complicated and interconnected neural circuits to control all sensations, activities, reflections, emotions, learning and memory, and movement throughout the body. The building block of these neural circuits are individual neurons, receiving signals from previous neurons and passing signals forward to the next neuron or responding cells. To facilitate this communication between neurons or a neuron and its target cells, the connective terminals of the projecting and receiving cells develop into specialized structures, known as synapses.

Synapses consist of three spatial areas: presynaptic compartment, synaptic cleft, and postsynaptic compartment. Signals are transmitting from the presynaptic site to the postsynaptic cell via synaptic cleft. In the central nervous system, several types of synapses have been described (dendro-dendritic synapses, axo-axonic synapses, axo-somatic synapses, and axo-dendritic synapses). Axo-dendritic synapses are the most common and classical synapse in the mammalian nervous system (Palay 1956, Gray 1959, Blackstad and Kjaerheim 1961). Upon the arrival of action potentials to the presynaptic nerve terminal, the neurotransmitter molecules are released and diffused across the synaptic cleft, bind with specific receptors on the postsynaptic membrane, causing depolarization or hyperpolarization of the postsynaptic cell. In the central nervous system, there are at least three types of synapses: excitatory synapses, inhibitory synapses, and modulatory synapses, which are introduced in the following section.

The complex interrelationship between the neurotransmitters and receptors of chemical synapses help integrate information and govern activities in the central nervous system. For example, learning and memory are significantly attributed to the modification of the synaptic strength by the most abundant neurotransmitter in the central nervous system, glutamate (Erecinska and Silver 1990). Glutamate is primarily involved in excitatory synapses, which propagate signals and activate downstream neurons. Two major glutamate receptors, AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor and NMDA (N-methyl-D-aspartate) receptor, are known to change postsynaptic superficial expression levels, subunit composition, and phosphorylation status by activity-dependent regulations, which result in long-term efficacy changes in synaptic transmission, so-called long-term potentiation (LTP) and long-term depression (LTD) (Lee, Kameyama et al. 1998, Beattie, Carroll et al. 2000, Heynen, Quinlan et al. 2000, Malinow 2003, Malenka and Bear 2004, Luscher and Malenka 2012, Chater and Goda 2014, Nicoll 2017). LTP significantly increases the size and number of dendritic spines; in contrast, LTD is involved in synapse elimination, which results in a lower number of dendritic spines. The ability to shape and modify glutamate-related LTP and LTD according to experience-dependent activities, result in synaptic plasticity, and a more significant result, learning and memory.

While glutamate is required for LTP and LTD excitatory synaptic transmission, it is also a precursor of GABA (gamma-aminobutyric acid), a typical neurotransmitter associated with inhibitory synapses. Unlike excitatory synapses, inhibitory synapses decrease firing action potentials, have no spines and are less protein-enriched at postsynaptic sites. GABA is the most common inhibitory neurotransmitter, and GABAergic receptors are expressed throughout the brain (Watanabe, Maemura et al. 2002), particularly playing the role of a modulator in interneurons (Ferando and Mody 2014, Tremblay, Lee et al. 2016). Among GABAergic receptors, ionotropic ones (type A and type C, GABA_A receptor and GABA_C receptor) are chloride (Cl⁻) channels, while metabotropic ones (type B, GABA_B receptor) couple with G-protein to open potassium (K⁺) channels. The opening of ion channels in inhibitory synapses leads to hyperpolarization, which prevents action potential from occurring. As an inhibitory synapse, GABAergic synapses often gate signal flow; any deficiency within the GABA signal

transmission or imbalance between glutamate excitatory and GABA inhibitory synapses leads to a spectrum of disorders, such as epilepsy (Treiman 2001) and schizophrenia (Nakazawa, Zsiros et al. 2012). Also, strengthening GABA sensitivity or the downstream signals of GABA receptor has a tranquilizing and sedative effect, which results in GABAergic receptors functioning as therapeutic targets for broader applications in therapies for chronic pain, insomnia, and stroke (Rudolph and Knoflach 2011, Wu and Sun 2015).

In addition to GABA dominating inhibitory neurons in the brain, glycine is another major inhibitory neurotransmitter in the brainstem and spinal cord (Bechade, Sur et al. 1994). Glycinergic receptors (GlyRs) are ionotropic chloride channels, which are similar as GABAergic ionotropic receptors, that lead to hyperpolarization with an inhibitory function. When mutations on GlyRs occurs, that reduces glycine sensitivity, chloride conductance, or receptors' surface expression. These reductions cause an exaggerated response to unexpected auditory or tactile stimuli and transient muscle stiffness, so-called hyperekplexia or startle disease (Muller, Le-Corrone et al. 2008, Dutertre, Becker et al. 2012).

The above-mentioned receptors glutamate, GABA, and glycine have been introduced according to the abundance of neurotransmitters in the central nervous system. Among these receptors, the ionotropic ones (NMDAR, AMPAR, GABA_AR, and GlyR) are also referred to as ligand-gated ion channels. In the ligand-gated ion channel family, glutamate receptors (NMDAR and AMPAR) are excitatory and consist of four subunits, GABA_AR and GlyR are inhibitory and consist of five subunits, and have a pentameric structure. Each subunit consists of four transmembrane (TM) domains. The linker between the third and the fourth TM is characterized as a large intracellular Cystine-loop, which contains multiple potential intracellular protein binding sites for trafficking and regulation. In addition to these receptors, two other five-subunit, excitatory ligand-gated ion channels expressed in the central nervous system: nicotinic acetylcholine receptor (nAChR) and serotonin type-3 receptor (5-HT₃R, 5 hydroxytryptamine type-3 receptor).

The nicotinic acetylcholine receptor (nAChR) plays an essential role in the CNS. nAChR is activated by acetylcholine (ACh) and a potent agonist, nicotine. It has been shown that nicotine activation of nAChR trigger arouses visual attention and perception as well as enhances cognitive and memory (Paterson and Nordberg 2000). The benefits of nAChR activation explain why tobacco is both attractive and addictive. The other ligand-gated ion channel, serotonin type-3 receptor (5-HT₃R) mediates nausea and vomiting in the brainstem (Rojas and Slusher 2012, Navari 2015), anxiety, and memory (Gargiulo, Viana et al. 1996). Besides ionotropic receptors, ACh and serotonin also activate metabotropic receptors, such as muscarinic acetylcholine receptor (mAChR) and other types of 5-HTR. Among all seven 5-HT receptors, only 5-HT₃ receptor is an ionotropic type, but all the rest are metabotropic. Each subtype of 5-HT receptors has different, but overlapping functions and distributions in the CNS. Serotonin has been the focus of considerable research on happiness, reward systems, and sleep modulation (Jacobs and Azmitia 1992, Ursin 2002, Mohammad-Zadeh, Moses et al. 2008, Kringelbach and Berridge 2009).

Complexity arises when discussing the interactions between different neurotransmitters and receptors. It is commonly understood that the long-term plasticity of the neural network is regulated by the strength modulation of glutamatergic excitatory synapses, and inhibitory synapses gate the threshold for the changes. However, more and more evidence suggests that GABAergic and glycinergic synapses participate in the shaping of LTP and LTD (Gaiarsa, Caillard et al. 2002), and glycine becomes an excitatory neurotransmitter that serves as a co-agonist of the NMDA excitatory receptor (Johnson and Ascher 1987, Thomson 1990, Chatterton, Awobuluyi et al. 2002). Some receptors modulate the release of other neurotransmitters, such as serotonin (5-HT) which activates GABA release and either increases or reduces glutamate release by different subtypes of 5-HT receptors at distinct structures (Ciranna 2006), while nAChR mediates glutamate, GABA, ACh, 5-HT in the brain (Tang, Karson et al. 2011, Dani 2015, Wu, Cheng et al. 2015). The crosstalk of neurotransmitters and receptors layer up and lead to critical balances between excitatory and inhibitory neurons in different regions of the central nervous system.

The efficiency of synaptic impulses depends largely on the high density of postsynaptic receptors and their associated proteins in the cell membrane. The stability of the postsynaptic density of a given synapse is regulated by different post-translational modifications (PTMs) on postsynaptic proteins, which are required for postsynaptic membrane protein homeostasis between protein targeting and degradation, and protein-protein interaction for protein stability.

Protein targeting and degradation

Individual PTMs change protein properties and provide more information for the regulation of complex cellular organization. In protein tagging and sorting systems, PTMs guide proteins to designated locations and eliminate undesirable problems, a process which requires interaction and crosstalk between various PTMs. For protein targeting and trafficking, myristoylation and palmitoylation signal membranes, and glycosylation is the quality control mechanism for ER to Golgi. The quality control screens unassembled and misfolded proteins in the ER lumen, and triggers ubiquitin conjugation for protein degradation (Hiller, Finger et al. 1996, Ellgaard and Helenius 2003, Hebert and Molinari 2007). Ubiquitin conjugation is not only for secretory proteins in the ER, but for cytosolic and membrane proteins. For these proteins, the ubiquitin system uses polyubiquitin chains, such as K48 and K63, for proteasome and lysosome-dependent degradation (Schrader, Harstad et al. 2009).

Protein-protein interaction for protein stability

PTMs modify side chains, N or C termini of proteins and change protein surfaces for recognition. These modifications, including phosphorylation and ubiquitination, generate new binding sites for protein-protein interactions (Seet, Dikic et al. 2006). PTMs are involved in protein-protein interactions in various ways. For example, PTMs modify residues to create new binding recognition, change protein conformation to expose or cover potential PTM sites, and alter affinity and specificity of protein-protein interactions. These changes often lead to sequential events, such as when the newly exposed PTM sites trigger different protein binding activities, the binding events change protein enzymatic abilities or targeting location, the binding protein

covers the original ubiquitinated lysine sites of the target protein, or changes of the binding affinity between proteins alter protein complex stabilities. These sequential events often change protein stability and are essential for much cellular organization, such as regulation of cell cycles, initiation of signaling cascades, and membrane protein endocytosis.

Membrane protein homeostasis

To sustain regular cellular functions, membrane proteins have unique turnover mechanisms and homeostasis. The level of membrane protein expression is determined by the balance between insertion and internalization, which are largely accounted by ubiquitin-dependent signaling. Upon the initiation of endocytosis, membrane proteins are monoubiquitinated at their cytoplasmic domains and recruit vesicle-related coated proteins for internalization (Dunn and Hicke 2001, Hicke 2001, Haglund, Di Fiore et al. 2003). The internalized membrane proteins are further processed in one of two ways. Either the membrane proteins are deubiquitinated and recycled back to surface or the vesicles go through lysosome-dependent degradation. One well-studied example of membrane protein homeostasis is the growth factor EGF and its receptor tyrosine kinase endocytosis, in which the binding of EGF and its receptor trigger tyrosine autophosphorylation of the cytoplasmic domain of the receptor, recruits phosphorylation-dependent binding partners for cell signaling and ubiquitination events, leading to the initiation of the internalization of the receptor (Seet, Dikic et al. 2006, Mukhopadhyay and Riezman 2007, Lemmon and Schlessinger 2010). This example not only demonstrates the crosstalk of phosphorylation and ubiquitination, but also indicates the essential roles of PTMs in the membrane protein homeostasis, especially with regard to receptors.

1.2 Chemical synapses in the peripheral nervous system

While the central nervous system processes information and governs activities, the communication between the CNS to the rest of the body occurs through the peripheral nervous system (PNS). The PNS includes both the autonomic nervous system and the sensory-somatic nervous system, which are distinguished in term of whether the control is conscious or not. For

sympathetic "fight or flight" and parasympathetic "rest and digest" functions, the unconscious control by the autonomic nervous system targets internal organs, including heart, lung, glands, and smooth muscles (McCorry 2007). Unlike the unconscious control, the conscious control of the sensory-somatic nervous system mediates the information flow from sensory organs to the CNS and back to skeletal muscles to produce sensations (sight, smell, taste, touch, sound) and actions.

After the sensory neurons are stimulated, the response is then carried out by motor neurons. Motor neurons innervate skeletal muscles to give rise to movements. Movements are coordinated by specific muscle contractions between joints (Sasaki, Neptune et al. 2009). To achieve simplicity and efficiency, motor neurons in vertebrates use only one neurotransmitter, acetylcholine (ACh). ACh has various uses and wide distribution in the nervous system. ACh enhances perception in the CNS and "rest and digest" response in the autonomic system of the PNS. In the somatic system, ACh is the excitatory neurotransmitter for skeletal muscle contraction, and it is received by nicotinic AChR (nAChR). Muscular nAChR is composed of multiple subunits: two α_1 , one each of β_1 , δ , and fetal γ or adult ϵ (Figure 1.1A) (Albuquerque, Pereira et al. 2009). The sites where ACh activates nAChR for muscle contraction is called the neuromuscular junction, which is the focus of this dissertation work.

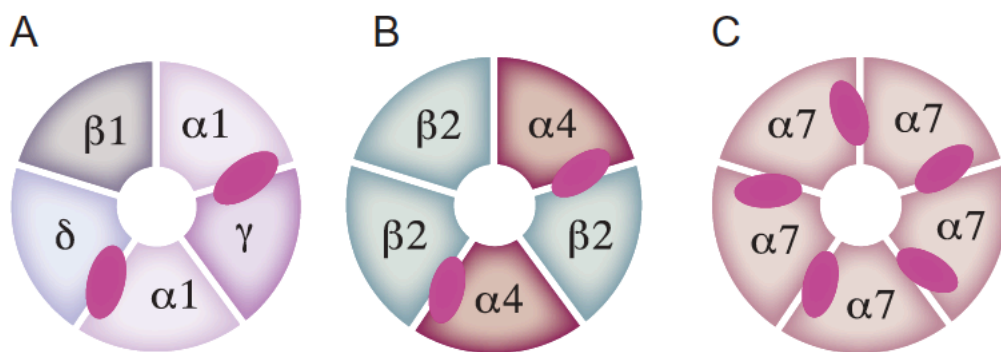


Figure 1.1 nAChR from the top-down view.

(A) nAChR from skeletal muscle, (B)(C) two of nAChR composition from the brain. Purple ovals indicate the ACh binding sites on each receptor. (Waxham 2014)

1.2.1 The Biology of the neuromuscular system

Neuromuscular junction (NMJ) is the site where motor neurons control muscle contraction. When motor neurons are activated, action potentials propagate through the axon until the terminal to trigger vesicles releasing ACh. ACh diffuses across the synaptic cleft and binds to nAChRs expressed at the muscle side. Once nAChR is activated, nAChR opens its ion channel for sodium influx and potassium outflow to generate depolarization, which then initiates muscle contraction. The health and efficacy of muscle contraction in term of neuronal transmission can be discussed spatially: the presynaptic motor neuron, the synaptic cleft, and the postsynaptic muscle.

At the presynaptic motor neuron, the release of ACh from synaptic vesicles is controlled by the activation of voltage-gated calcium channel via action potentials and by the coordination of the vesicle-related proteins, such as synapsin and SNARE complex (Catterall 1999, Hilfiker, Pieribone et al. 1999). Besides ACh, motor neurons also release agrin to communicate across synapses, as agrin triggers nAChR clustering (Reist, Werle et al. 1992). When ACh crosses the synaptic cleft, a 50 nm distance between motor neuron and muscle, ACh can either be hydrolyzed by acetylcholinesterase (AChE) or diffuse away before reaching muscle. It is worth noting that AChE is critical for ACh recycling and transmission control (Mesulam, Guillozet et al. 2002). Therefore, before ACh reaches postsynaptic muscles, any defects in the presynaptic channels, vesicle proteins, agrin, AChE, or related configuration, affects the neuronal transmission of muscle contraction (Naguib, Flood et al. 2002, Hirsch 2007).

For nAChR to receive ACh properly, nAChR must be highly concentrated at the postsynaptic site. In a mature neuromuscular synapse, nAChR is 10,000 fold more concentrated than it is a few micrometers away in nearby membranes (Fambrough 1979, Matthews-Bellinger and Salpeter 1983, Balice-Gordon and Lichtman 1993, Sanes and Lichtman 2001). A lower density of nAChRs at the NMJ is a hallmark for NMJ related disorders, often associated with muscle

fatigue and weakness. Concentrating membrane proteins at a specific location requires the coordination of multiple cellular components. In the case of nAChR at the NMJ, concentration requires both signal and structural interaction from pre and postsynaptic sites. To further elucidate nAChR at NMJ synapses, synapse formation, synapse maturation, and synapse maintenance are discussed in the followed sections.

1.3 Synapse formation and maturation in neuromuscular junctions

Neuromuscular junctions are the communication platform for motor neurons that control skeletal muscle contraction. NMJs, as peripheral synapses, are easily accessible and larger than central nervous synapses. These features make NMJs the most characterized synapse, studies of which have shed light on synaptogenesis.

1.3.1 Synapse formation in NMJ

Prepatterning

Before motor neurons reach skeletal muscle fibers, muscle fibers already exhibit AChR clusters on the central region. This event is known as prepatterning, which prepares AChRs to become highly concentrated at postsynaptic sites for proper synapse formation. Prepatterning, which is nerve-independent, happens before motor neurons arrival, and still appears in motor neuron deficient mice (Yang, Li et al. 2000). The critical time frame for innervation in mice is embryonic day 12 to 13.5 (E12 – E13.5). To determine the genes required for the prepatterning, mouse knockout models have been generated to examine mice after this stage. Mouse knockout data suggest that MuSK, a receptor tyrosine kinase expressed at the muscle site, is indispensable for the prepatterning. MuSK knockout mice lose AChR centralization at E14.5 (Lin, Burgess et al. 2001, Yang, Arber et al. 2001) and die at birth (DeChiara, Bowen et al. 1996). Apart from MuSK, the centralization of AChR requires AChR expression and composition. The loss of the fetal AChR subunit, AChR γ , results in a failure to fold AChR channels and express prepatterning AChRs, but still produces a normal MuSK distribution (Takahashi, Kubo et al. 2002, Liu,

Padgett et al. 2008). These results indicate that MuSK and AChR γ are two essential factors for the prepatternning independently. The prepatternning exhibits the initiation of neuromuscular synaptogenesis before innervation.

Innervation

Nerves arrive at muscles around E12 – E13.5, and enlarge prepatterned AChRs, but eliminate unconnected ones by E18.5 in mice. This innervation event is essential for neuromuscular synaptogenesis. Prepatternning requires MuSK at the initiation stage in the muscle, and to sustain the AChR clusters a MuSK activator, the neural-derived ligand agrin is required (Yang, Arber et al. 2001). Agrin is a heparan sulfate proteoglycan, which often plays a role in cell-cell and cell-matrix interactions (Poulain and Yost 2015). Without agrin, the knockout mice fail to cluster AChRs and to stabilize synaptic contact, causing the pups to die in utero (Gautam, Noakes et al. 1996). When discussing motor neuron effects on neuromuscular synaptogenesis, it is necessary to talk about ACh, the neurotransmitter released from motor neurons. To stop ACh release from motor neurons, the gene called choline acetyltransferase (ChAT) is manipulated, the biosynthesis enzyme for ACh. ChAT knockout mice have aberrant innervation, including a broader (not concentrated) region of AChRs, and excessive branches of motor neurons, but each nerve terminal is smaller and less connected to muscles. These knockout mice cannot survive after birth (Misgeld, Burgess et al. 2002, Brandon, Lin et al. 2003). Other factors that contribute to innervation are associated with either motor neuron or ACh release, such as Hb9, a motor neuron transcriptional factor (Lin, Burgess et al. 2001, Yang, Arber et al. 2001), or VACHT, a vesicular ACh transporter that loads ACh into synaptic vesicles so that it can be released (de Castro, De Jaeger et al. 2009). The innervation process starts the communication between pre and postsynapses, which is crucial for synaptogenesis.

Agrin-Lrp4-MuSK signaling

MuSK and agrin knockout mice lose prepatternning or have aberrant innervation, and neither of the knockout mice can survive at birth. The link between agrin activated MuSK is established

when agrin promotes tyrosine phosphorylation of MuSK. Agrin cannot cluster AChRs on MuSK deficient myotubes (Glass, Bowen et al. 1996). To further elucidate the physiological role of MuSK in adult mice, knockout of MuSK expression at later stages using the conditional knockout Cre/loxP system suggested that the kinase activity of MuSK is essential throughout the synaptic formation and postsynaptic integrity (Hesser, Henschel et al. 2006). The signaling pathway between agrin and MuSK exhibits a critical foundation of neuromuscular synaptogenesis.

While agrin is essential, it does not bind directly to MuSK, which led to the proposal that other MuSK binding partners may serve as the bridge (Glass, Bowen et al. 1996). The missing link was not found until 2008, with the identification of Lrp4 as the direct receptor for agrin, and that binds to MuSK (Kim, Stiegler et al. 2008). Lrp4, low-density lipoprotein (LDL) receptor-related protein 4, belongs to the LDLR family, which is a group of large transmembrane proteins containing multiple binding domains for complex interactions (Hussain 2001). Lrp4 not only utilizes different domains to bind with agrin and MuSK, but also serves as a ligand to trigger MuSK phosphorylation by agrin promotion (Zhang, Coldefy et al. 2011). Moreover, Lrp4 has a distinct role in both presynaptic differentiation and postsynaptic development (Wu, Lu et al. 2012). This has been demonstrated in Lrp4 knockout mice, which die at birth similar to the phenotype of MuSK knockout mice (Weatherbee, Anderson et al. 2006).

The agrin-Lrp4-MuSK signaling pathway is critical for NMJ synaptic formation and AChR clustering. A malfunction in any of the molecules in this pathway leads to muscle weakness. In the cases of the congenital myasthenic syndrome, patients have deficient genes in agrin, Lrp4, MuSK, or MuSK downstream Dok7 and rapsyn (Engel, Shen et al. 2015). To provide a better therapeutic framework for neuromuscular disorders, studies have examined the agrin-Lrp4-MuSK pathway (Ohno, Ohkawara et al. 2017). Research shows that MuSK, a tyrosine kinase receptor, is a key organizer once it is activated by agrin and Lrp4 (Barik, Xiong et al. 2012). One of the MuSK downstream cytoplasmic effectors, Dok7, is activated by MuSK phosphorylation and further facilitates MuSK autophosphorylation (Bergamin, Hallock et al. 2010). This

feedback phosphorylation reinforcement is critical for MuSK kinase activity. Given this relationship, Dok7 knockout mice exhibit similar deficiencies as MuSK knockout mice and die shortly after birth (Okada, Inoue et al. 2006). For NMJ synaptogenesis, research has investigated agrin-Lrp4-MuSK signaling crosstalk with other signaling pathways (Wu, Xiong et al. 2010) and the organization of the mouse knockout models (Witzemann, Chevessier et al. 2013).

Rapsyn is required for AChR clustering

Agrin-Lrp4-MuSK signaling ultimately activates rapsyn, clustering AChRs. Rapsyn, a 43 kD receptor-associated protein at synapses, is required for AChR cluster formation *in vivo*, as rapsyn knockout mice lack of AChR synapses, and *in vitro*, as rapsyn *-/-* cultured myotubes lack AChR clusters (Frail, McLaughlin et al. 1988, Gautam, Noakes et al. 1995). In order to understand the role of rapsyn in AChR clustering, previous studies of heterologous systems have defined the domains of rapsyn. These include an N-terminal myristoylation site (glycine 2) to anchor to the membrane (Phillips, Maimone et al. 1991); seven tetratricopeptide repeats (TPR1-7, residue 6-279) to mediate self-association; a coiled-coil domain (residue 298-332) to associate with the cytoplasmic loop of AChR subunits (Ramarao, Bianchetta et al. 2001); and a RING-H2 domain (residue 363-403) to link to dystrophin-glycoprotein complex (DGC) (Bartoli, Ramarao et al. 2001).

To trigger rapsyn to cluster AChRs, motor neurons-derived agrin, binds to LRP4 to activate MuSK for the downstream signaling cascade to phosphorylate tyrosine 390 (Y390) of the AChR β subunit. This agrin-activated signaling leads to increase affinity between rapsyn and AChR (Moransard, Borges et al. 2003, Borges, Yechikhov et al. 2008, Lee, Rudell et al. 2008, Barik, Lu et al. 2014). Altogether, these previous works indicate that rapsyn is an essential mediator of AChR clustering.

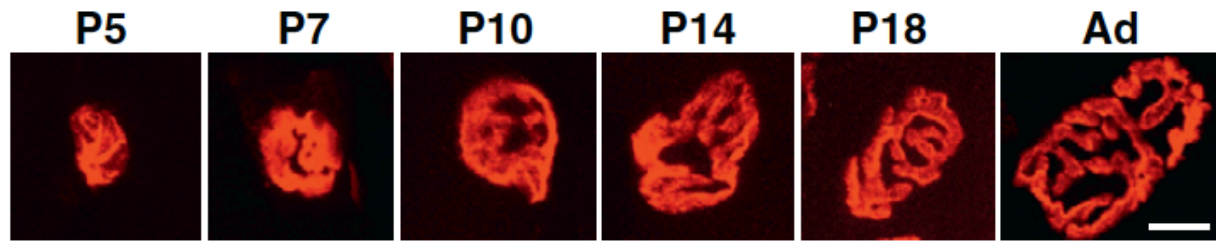
1.3.2 Synapse maturation in NMJ

From plaque to pretzel: structural change for synapse maturation

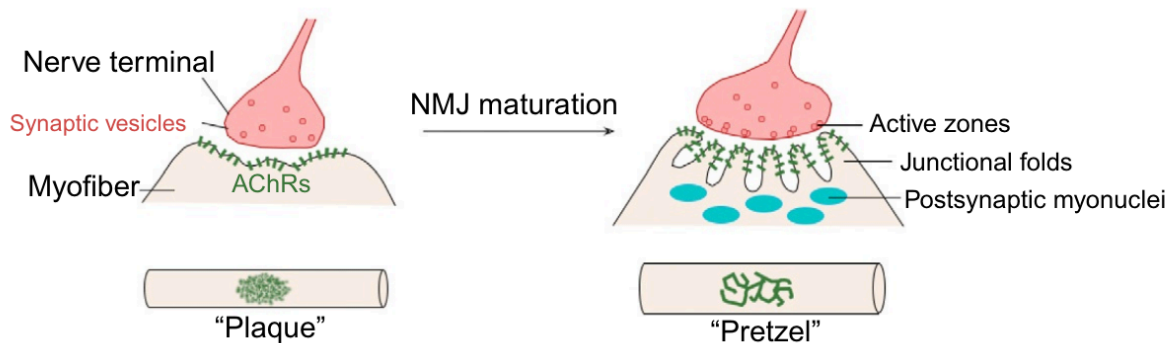
After synapses are formed, neuromuscular synapses go through several changes to enhance synaptic transmission for maturation. In AChR composition, the embryonic AChR γ subunit is gradually substituted by the AChR ϵ subunit in the early postnatal stage (Mishina, Takai et al. 1986, Numberger, Durr et al. 1991, Missias, Chu et al. 1996). The switch from γ to ϵ increases the calcium permeability of the endplate channels (Villarroel and Sakmann 1996). For innervation, the early synapses are polyinnervated by small branches of multiple motor neurons, and the mature synapses are monoinnervated by a single strong motor neuron (Brown, Jansen et al. 1976, Akaaboune, Hantai et al. 1998, Wyatt and Balice-Gordon 2003). In term of morphology, neuromuscular synapses change from plaque-like to pretzel-like structure (Figure 1.2) (Sanes and Lichtman 2001).

The plaque-like synapses, which in the early stage are flat and small, are characterized by a high level of AChR expression at the surface of postsynaptic muscles. During postnatal development, several structural proteins form a complex, and the postsynaptic muscle membrane undergoes invagination. During invagination, AChRs settle on the crest of the junctional folds, forming bigger and better structural support for pretzel-like synapses. The pretzel-like mature synapses have a unique structural feature. No two identical synapses can be found just as with fingerprints in humans. To understand the synaptic structure studies have combined fluorescent tags with α -bungarotoxin (α -BTX), a high and irreversible binding affinity chemical to AChR derived from snake venom, to trace the same AChR synaptic structure and density over time. This approach has become a powerful tool to study NMJs (Akaaboune, Culican et al. 1999). The protein complex that mediates the structural support and maturation of AChR at NMJs is referred to as the dystrophin glycoprotein complex.

Figure 1.2 NMJ synapses go through significant structural change for synapse maturation.



(A) NMJ synapses are images during developing from postnatal day 5 (P5) to adult (Ad) in wildtype mice. Synapses are labeled by red-bungarotoxin (BTX), scale bar 10 μm . (Shi, Fu et al. 2012)



(B) With junctional folds appearance, AChRs form distinct and clear-edged structure from plaque to pretzel shapes during maturation. (Liu and Chakkalakal 2018)

The dystrophin glycoprotein complex for synapse maturation

The dystrophin glycoprotein complex (DGC) is a group of membrane-associated proteins that support AChRs by a link from the intracellular cytoskeleton to the extracellular matrix (Figure 1.3) (Ervasti and Campbell 1991, Ohlendieck, Ervasti et al. 1991). The anchor of this complex is dystroglycan, which is encoded by a single gene and cut into two proteins: extracellular α -dystroglycan and transmembrane β -dystroglycan. Extracellular α -dystroglycan is heavily glycosylated and binds to the extracellular matrix, which includes laminin. α -Dystroglycan also noncovalently attaches to the transmembrane β -dystroglycan (Henry and Campbell 1999). β -Dystroglycan has other transmembrane partners, such as sarcoglycans (α , β , γ , δ) and sarcospan, and links to cytoplasmic dystrophin or its homolog utrophin. Dystrophin or utrophin, both major cytoplasmic scaffolding proteins, binds not only to the soluble proteins α -dystrobrevin and syntrophins, but to the intracellular cytoskeleton, which includes F-actin (Ervasti and Campbell 1993). β -Dystroglycan is also the anchor for rapsyn clustering AChRs. Together, AChRs are

clustered by rapsyn and anchored to β -dystroglycan within the dystrophin glycoprotein complex. Synapses are stabilized by linking to both inside (dystrophin associates with F-actin) and outside (α -dystroglycan links to laminin) structural support.

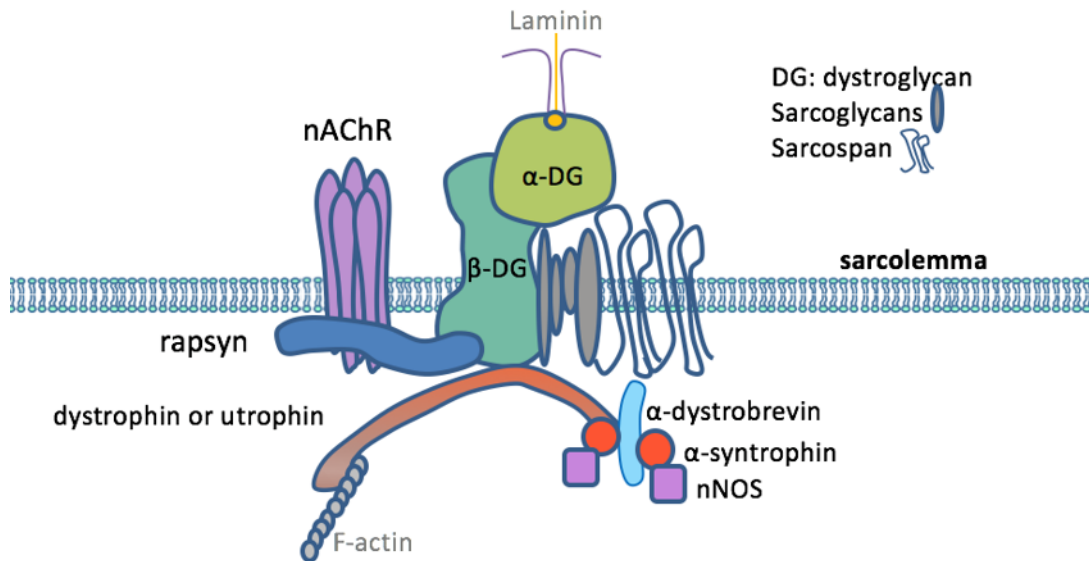


Figure 1.3 The composition of the dystrophin glycoprotein complex.

DGC is the anchor for rapsyn clustering AChR and stabilizes synapses by linking to the intracellular cytoskeleton and extracellular matrix.

Several human muscular dystrophies are linked to mutations in DGC components. For instance, Duchenne muscular dystrophy and Becker muscular dystrophy are both associated with dystrophin mutations, while limb-girdle muscular dystrophy is associated with sarcoglycans (Durbeej and Campbell 2002). All of these muscular dystrophy patients have a severe reduction of α -dystrobrevin in their muscles (Metzinger, Blake et al. 1997). To investigate each of the function of the DGC components, scientists have turned to mouse models.

When the central component of DGC, dystroglycan, is absent, the deficient mice are embryonic lethal. Transmembrane sarcoglycan deficient mice, depending on the isoform, have moderate to severe muscular dystrophies, and γ -sarcoglycan deficient mice have a shorter life span. The

cytoplasmic linker, dystrophin, is naturally found in mdx mice with dystrophin deficiency (Dangain and Vrbova 1984). However, mdx mice cannot perfectly represent the pathology of muscular dystrophy patients. These patients have severe skeletal dystrophy and an approximately 20-year life expectancy. Yet, mdx mice have only moderate skeletal dystrophy and generally have a more than one-year life span (Manning and O'Malley 2015). Scientists suspect the milder syndrome for mdx mice might be due to the compensation effect between dystrophin and its homolog utrophin (Tinsley, Deconinck et al. 1998). This is based on utrophin deficient mice have no muscular dystrophy and a normal life span. With utrophin/dystrophin double deficiency, mice have a severe muscle phenotype and only a 4 – 20 week life span. Still, the mdx model indicates that without the cytoplasmic linker dystrophin, the other intracellular components, α -dystrobrevin and α -syntrophin, lose their synaptic colocalization. When the c-terminal domain of dystrophin is restored, this increases the recruitment of α -dystrobrevin and α -syntrophin, leading to partial improvement in mdx mice muscular pathology (Koo, Malerba et al. 2011). α -Dystrobrevin deficient mice have only mild dystrophy and average life span, while α -syntrophin deficient mice seem completely normal. This restoration data suggests both α -dystrobrevin and α -syntrophin play essential roles in NMJ transmission. More details of all the DGC related mouse models can be found in the review article by (Durbeej and Campbell 2002).

Despite the absence of dystrophy and normal life span, careful examination of α -syntrophin deficient mice shows that synapses form normally in the first postnatal week, but mature abnormally afterward, with faster AChR turnover rate. In these mice most of the removed AChRs are targeted to degradation (Martinez-Pena y Valenzuela, Mouslim et al. 2011). The loss of α -syntrophin also leads to neuronal nitric oxide synthase (nNOS) losing its synaptic localization (Adams, Anderson et al. 2010). nNOS synthesizes nitric oxide (NO) to modulate blood flow and generate a second messenger cyclic GMP (cGMP). These nNOS signaling pathways are crucial for the health of skeletal muscles and dystrophic pathology (Balke, Zhang et al. 2019). α -Syntrophin deficient mice have a lower expression level of α -dystrobrevin-1, the major isoform for maintaining postsynaptic integrity, but the overexpression of GFP- α -dystrobrevin-1 partially rescues the α -syntrophin deficient phenotype. This data suggest that α -

syntrophin may contribute to synaptic function via α -dystrobrevin-1 (Martinez-Pena y Valenzuela, Mouslim et al. 2011).

As with α -syntrophin deficient mice, α -dystrobrevin deficient mice have normal synaptic formation, but develop synapses that turn aberrant in morphology with faster AChR turnover rate during maturation (Grady, Grange et al. 1999). In order to link this finding with previous research, scientists sought to understand the nature of the interaction of a cytoplasmic protein such as α -dystrobrevin within DGC components for synaptic maturation (Grady, Zhou et al. 2000). This research has shown that without α -dystrobrevin, the depth of junctional folds shrink and the structural support to AChR is weaker. Of all the α -dystrobrevin isoforms, α -dystrobrevin-1 is the biggest protein product, which connects to the postsynaptic membrane specifically, and contains a C-terminal with three distinct tyrosine phosphorylation sites (Wagner, Cohen et al. 1993, Balasubramanian, Fung et al. 1998, Grady, Akaaboune et al. 2003). By changing the three tyrosine sites to non-phosphorylatable phenylalanine, the mutated α -dystrobrevin-1 fails to fully rescue synaptic abnormality in α -dystrobrevin deficient mice (Grady, Akaaboune et al. 2003). This data reveals tyrosine phosphorylation on the α -dystrobrevin-1 is essential to NMJ synaptic integrity.

NRG-ErbB signaling for phosphorylated α -dystrobrevin

Regulation of tyrosine phosphorylation on α -dystrobrevin has become a focus of interest in investigations of NMJs. Dephosphorylation of α -dystrobrevin-1 has been found to associate with the abolishment of the neuregulin/ErbB signaling (Schmidt, Akaaboune et al. 2011). Neuregulins (NRGs) are part of the EGF family and known to regulate multiple functions in synaptic development in both CNS and PNS (Mei and Nave 2014). NRGs bind and activate ErbB receptors, which are tyrosine kinase receptors. At NMJs, NRG1 is released from motor neurons and turns on AChR gene expression (Schaeffer, de Kerchove d'Exaerde et al. 2001). Moreover, NRG1 utilizes ErbB2/ErbB4 heterodimers as receptors at postsynaptic NMJs (Trinidad, Fischbach et al. 2000). Therefore, it is no surprise that the NMJ synapses have lower transmission efficacy in *erbb2/4* deficient mice. The lower transmission efficacy is caused by the

dephosphorylation of the tyrosine sites on α -dystrobrevin-1, and this signaling pathway selectively targets the AChR recycling pool (Schmidt, Akaaboune et al. 2011). Together, the NRG/ErbB signaling pathway stabilizes and maintains AChR synaptic transmission through phosphorylation of α -dystrobrevin.

1.4 Synapse maintenance in NMJ

1.4.1 AChR homeostasis at NMJ

AChR half-life at NMJ

After NMJ synapses are formed and matured, healthy synaptic transmission relies on the homeostasis of AChR at postsynaptic sites. AChRs are highly dynamic with a 9 - 14 day half-life at fully functional synapses (Akaaboune, Culican et al. 1999). The blockade of neurotransmission through either pharmacological (BTX to AChR or TTX to endplate potential) or surgical (denervation) produces a rapid loss of AChRs by greatly increasing the turnover rate, which decreases to 2-4 days. Direct muscle stimulation counteracts the blockade, as the dispersed AChRs are reaccumulated (Fumagalli, Balbi et al. 1990, Akaaboune, Culican et al. 1999). The activity blockade also selectively upregulates the expression of AChR γ , the fetal AChR subunit (Wang, Engisch et al. 2006). This upregulation of AChR γ after the developmental stage is a feature for muscle regeneration, in which synapses undergo a mimic ontogenetic progress (Gaspersic, Koritnik et al. 2001).

AChR half-life is greatly reduced at other types of compromised synapses. For instance, many DGC deficient mouse models show an AChR half-life of around three days. Examples include the deficient models of α -dystrobrevin, utrophin, α -dystrobrevin/utrophin, α -dystrobrevin/dystrophin, and α -syntrophin (Akaaboune, Grady et al. 2002, Martinez-Pena y Valenzuela, Mouslim et al. 2011). Although these DGC deficient mouse models have different levels of muscular dystrophy, all of them have reduced levels of α -dystrobrevin, suggesting that α -dystrobrevin is associated with the regulation of AChR turnover.

The existence of three pools of AChR at the postsynaptic sites: preexisting, recycled, and newly synthesized

To distinguish the contribution of AChRs at synaptic sites, scientists in the Akaaboune lab developed a sequential labeling method (Bruneau, Sutter et al. 2005). This method consists of three components: biotin-bungarotoxin (BTX), streptavidin-fluorophore, and time-lapse imaging. BTX has a strong and almost irreversible affinity to AChR; streptavidin binds to biotin, but the binding is lost once the receptor is internalized. Combined with different fluorescent tags conjugated with streptavidin, labeling AChRs at different time points can distinguish the composition of AChRs at synapses: those that are preexisting, those once internalized, but reinserted (recycled), and those that are newly synthesized. Surface AChRs can be internalized, while some are targeted to degradation, and still others are recycled back to the surface. The recycled pool of AChR contributes a substantial amount of the surface AChRs, which have a four times shorter half-life than the preexisting ones (Bruneau and Akaaboune 2006). These pools of AChR maintain the healthy homeostasis of AChR at synaptic sites. At denervated or compromised NMJs, the rate of AChR removal from the surface is increased, and the internalized AChRs are less likely to recycle back to the surface (Bruneau and Akaaboune 2006, Martinez-Pena y Valenzuela, Mouslim et al. 2011). The recycled pool of AChR is tyrosine phosphorylation dependent, while inhibition of tyrosine phosphatase, more AChRs are accumulated at the surface nearby synaptic sites (Bruneau and Akaaboune 2006). Although nAChR dynamics at the NMJ have been extensively studied, it is not clear how AChR internalization occurs. It is well established that many membrane receptors are endocytosed in response to ubiquitin signals (Haglund, Di Fiore et al. 2003, Mukhopadhyay and Riezman 2007).

α Kap protects AChR from proteasomal-dependent degradation

Recent work from our lab has shown that a non-kinase muscle-specific anchoring protein (α kap) encoded within the calcium/calmodulin kinase II (CaMKII) α gene (Bayer, Harbers et al. 1998) plays an important role in regulating the equilibrium dynamics of the postsynaptic AChR density. Specifically, it was demonstrated that α kap co-purifies with AChR in cultured myotubes, in living mice, and in heterologous HEK cells. Knockdown of α kap endogenous expression in

either cultured muscle cells or living animal promotes AChR degradation (leading to fewer receptor clusters on the cell surface). More importantly, it was found that α kap promoted AChR α stability by a ubiquitin-dependent mechanism. In the absence of α kap, AChR α was heavily ubiquitinated and the number of AChR α was increased by proteasome inhibitors (Mouslim, Aittaleb et al. 2012).

Ubiquitin is a small protein (76-amino acid) that can be attached to lysine residues of proteins targeted for degradation. There are seven lysines (K) as K6, K11, K27, K29, K33, K48, and K63. Of these, the K48 and K63 linkages are the most well-known ubiquitin chains, with K48 targeting to 26S proteasome degradation and K63 for membrane protein internalization or the lysosome pathway (Pickart and Fushman 2004, Marx, Held et al. 2010). Apart from single (mono-) or multiple (poly-) ubiquitin on a lysine residue, ubiquitin modification can also occur at multiple lysine residues on a target protein. With the combination of mono or poly-ubiquitin, different types of poly-ubiquitin linkages, on one or several lysine residues of the target proteins, ubiquitin tags contain a considerable amount of information that is involved in complicated protein targeting, sorting, and degradation (Laney and Hochstrasser 1999).

In the presence of α kap, AChR α was less ubiquitinated and proteasome inhibitors had almost no effect on AChR α accumulation. However, when α kap is knocked-down, AChR α is highly ubiquitinated and degraded rapidly. The intracellular loop of AChR α contains four lysines (K359, K374, K388 and K393) and mutations of these lysine residues significantly increased the stability of AChR in the absence of α kap, suggesting that the effect of α kap on AChR α stability is mediated by controlling AChR α ubiquitination.

The ubiquitin-proteasome system (UPS) has been involved in regulating synaptic protein turnover and synaptic efficacy at a variety of synapses (Ehlers 2003, Speese, Trotta et al. 2003, Christianson and Green 2004, Mabb and Ehlers 2010). In UPS, target recognition is achieved by the E3 ubiquitin ligase and the DUB deubiquitinating enzyme, which attach or remove ubiquitin,

respectively. A considerable body of literature exists on E3 and DUB expression at NMJs, which has shed light on UPS in regulating AChR homeostasis. Some DUB deubiquitinating enzymes involved in synapse development were discovered in mouse disease models. For instance, a mouse model with a homologous mutation on the ataxia (ax) gene, ax^J mice, have hindlimb paralysis and shorter lifespans (only 6 – 10 weeks). Studies have mapped the mutation on the ax gene and found that it fails to properly encode a deubiquitinating enzyme, ubiquitin-specific protease 14 (Usp14) (Wilson, Bhattacharyya et al. 2002). Usp14 not only cleaves the mono-ubiquitin from the target protein, but controls the gate opening of the 20S proteasome (Peth, Besche et al. 2009). The loss of Usp14 leads to a decrease in muscle mass, a lack of muscle flexibility, and defects in the developmental motor neuron endplates. Since the muscle and motor neuron endplate are altered, the postsynaptic sites of neuromuscular junctions are focused. The normal postsynaptic sites of neuromuscular junctions are characterized by a high density of acetylcholine receptors (AChRs), the shape of which has been altered from plaque to pretzel, and by AChR subunit replacement from fetal γ to mature ϵ during development. Ax^J mice have immature plaque-shaped AChR synapses and higher γ mRNA expression. In these mice the neuronal-specific Usp14 transgene rescues the abnormal phenotypes (Chen, Qin et al. 2009) but a male-specific reproductive problem remains (Crimmins, Sutovsky et al. 2009). The loss of Usp14 also leads to a decrease in synaptic free ubiquitin levels, and this can be rescued by the transgenic of neuronal-specific ubiquitin to fertilized egg stage (Chen, Bhattacharyya et al. 2011). This indicates the importance of DUB deubiquitinating enzymes and ubiquitin levels in the development of neuromuscular junctions.

E3-ubiquitin ligases, essential ubiquitin regulators, are also involved in the formation of AChR clusters and synaptic development of the NMJ. For instance, in C2C12 muscle cells, it was found that the expression levels of PDZ domain containing RING finger 3 (PDZRN3), an E3 ligase increased during the muscle differentiation and its knockdown by siRNA has totally obstructed muscle differentiation from myoblasts to myotubes (Ko, Kimura et al. 2006). In mice, PDZRN3 expression is associated with the formation of AChR clusters where it colocalized with them (Lu, Je et al. 2007). When PDZRN3 is overexpressed in muscle cells, the number and size of AChR clusters were significantly reduced (Lu, Je et al. 2007). Further studies have shown that

the effect of PDZRN3 on the formation of AChR clusters is mediated by regulating the expression levels of muscle-specific receptor tyrosine kinase (MuSK), a protein that is required for synapse formation. Mechanistically, it was proposed that PDZRN3 tags ubiquitin to MuSK PDZ domain (via its RING), leading to an increase in MuSK cell surface endocytosis (Lu, Je et al. 2007) and consequently a reduction in the formation of AChR clusters. This suggests that E3-ubiquitin ligases are crucial for membrane protein homeostasis at neuromuscular junctions.

Although it is known that rapsyn clusters AChRs through the activation of the agrin-Lrp4-MuSK pathway, a new property of rapsyn has been identified. Specifically, the RING-H2 domain of rapsyn, previously known as anchoring AChR to β -dystroglycan, contains the E3 ligase ability. The RING-type E3 ligases have a conserved motif consisting of a series of cysteines (C) and histidines (H), C3H2C3, in which the RING-H2 of rapsyn is aligned with the known RING-type E3s, such as APC11 and Mdm2. When the second cysteine of C3H2C3 of rapsyn is mutated (C366), the E3 ligase activity is abolished. The E3 activity of rapsyn conducts both ubiquitination and neddylation. During the neddylation process, a Ub-like tag, Nedd8 (neural precursor cell expressed, developmentally downregulated 8), tags to a target protein. In the NMJ, rapsyn neddylates the δ subunit of AChR, which then regulates agrin-dependent AChR clustering and NMJ formation. When the C366 of rapsyn is mutated in mice, NMJ formation is aberrant, and the single nucleotide change also gives rise to breathing difficulty, which becomes lethal (Li, Cao et al. 2016). This new role of rapsyn expresses a more complicated interaction with AChR, and shows a new enzymatic ability in a known structural AChR associated protein.

Many additional binding partners have been identified for DGC proteins. For instance, mass spectrometry has recently shown that α -dystrobrevin has several new binding partners and this has been verified by co-immunoprecipitation. This study focused on the α -dystrobrevin major isoform, α -dystrobrevin-1, which has three tyrosine phosphorylation sites. α -Dystrobrevin-1 binds to Grb2 in a phospho-dependent manner and α -catulin in a phospho-independent manner. Other new binding partners are liprin- α 1, PI3K, Arhgef5, and Usp9x (Gingras, Gawor et al. 2016). It is worth noting that Usp9x is belonged to DUB family and has the deubiquitination

ability. Investigations of more potential binding partners and new properties of AChR associated proteins have revealed that the UPS dynamics regulate AChR synaptic functions.

1.4.2 Dynamic regulation for other synapses

Receptor homeostasis and ubiquitination

The most studied synaptic receptor homeostasis regulated by ubiquitin is glutamatergic AMPA receptors, especially the GluA1 and GluA2 subunits of the four GluA1-4. The major ubiquitin sites of the GluA subunits are located at the carboxyl-terminal tail in the cytosol, K868 for the GluA1, and K870, K882 for the GluA2. The E3 and DUB enzymes for GluA1 and GluA2 have been identified. For the E3 ubiquitin tagging, GluA1 has Nedd4-1, and GluA2 has Nedd4-2 and RNF167 (RING finger protein 167). Nedd4-1 and Nedd4-2 belong to HECT-type E3 and RNF167 belongs to RING-type E3; and for the DUB ubiquitin untagging, the two DUBs Usp46 (ubiquitin-specific protease 46) and Usp8 remove GluA1 ubiquitin to increase the surface level of the AMPA receptors. The different E3s and DUBs for AMPA receptors may respond to different neuronal stimulation and conditions, and provide tight control over the dynamics of AMPA receptors (Lussier, Sanz-Clemente et al. 2015, Widagdo, Guntupalli et al. 2017).

Besides AMPA receptors, other synaptic receptors are also regulated by ubiquitin. The glutamatergic NMDA receptors are composed of NR1 with NR2 or NR3 subunits. The NR2 subunits have four A to D isoforms, all of which contain a longer carboxyl-terminal tail than NR1 has to interact with regulatory proteins in the cytosol. The C-terminal tail of the NR2B subunit is targeted explicitly by a RING-type E3 ligase, Mind bomb-2, in a phosphorylation-dependent manner (Jurd, Thornton et al. 2008). NR2B is also gradually decreased and diluted by NR2A elevation, which changes the time lapse for the gate opening along with the development, in a process called NR2B-NR2A developmental switch (Liu, Murray et al. 2004).

The composition of NMDA receptors and the homeostasis of AMPA receptors at synapses is essential for synaptic plasticity, and further contributes to learning and memory (Liu, Wong et al. 2004, Yashiro and Philpot 2008, Bard and Groc 2011). A ubiquitin imbalance in the receptors leads to several neurodegenerative disorders, such as Alzheimer's disease.

Receptor and clathrin-dependent endocytosis

The ubiquitination of a receptor could yield insights into the receptor internalization. To explore this possibility, studies have looked into a more direct link between receptor internalization and endocytosis. Endocytosis is a cellular process in which substances are taken up into the cell by membrane wrapping. Receptor internalization is primarily clathrin-dependent, which requires specific receptor signals, such as ubiquitin, to recruit adaptor protein 2 complex (AP2) and clathrin. Because they have a unique triskelion structure, many clathrins form a coat-like inward bud that wraps AP2-associated receptors for endocytosis (Collins, McCoy et al. 2002). AP2 has been shown to have a direct association with many neurotransmitter receptors, including AMPAR, NMDAR, and GABA_AR (Kittler, Delmas et al. 2000, Man, Lin et al. 2000, Arancibia-Cárcamo, Fairfax et al. 2006). The AP2 and receptor association is essential for receptor endocytosis, as well as for healthy receptor homeostasis at synaptic sites.

Receptor and its associated proteins

Receptor stability and homeostasis are not only regulated by its modification but by its associated proteins, such as rapsyn to nAChR. For excitatory synapses in the CNS, the highly protein-enriched area at the postsynaptic site, called the postsynaptic density, is essential for the stability of the glutamatergic receptors. The whole postsynaptic density protein complex includes large scaffolding proteins that bring together excitatory receptors, channels, cell adhesive molecules, kinases, phosphatases, regulators, and cytoskeletons (Sheng and Hoogenraad 2007). The main scaffolding protein is PSD-95 in excitatory synapses. When PSD-95 is oligomerized, it is relatively stable in the PSD complex. PSD-95 binds directly to NMDAR and indirectly to AMPAR via stargazin and synapse-associated protein 97 (SAP97) (Kim and Sheng 2004,

Howard, Elias et al. 2010), which results in differential turnover rates. Specifically, NMDARs, bind to PSD-95 directly, facilitating a more sustained localization at synapses compared to AMPAR, which has rapid turnover rate (in 10 minute order) (Noel, Ralph et al. 1999, Luscher and Frerking 2001). In terms of distribution, NMDAR is expressed more homogeneously throughout the PSD membrane, while AMPAR is expressed closer to the edge of the PSD-95, where stargazin and SAP97 bridge AMPAR and PSD-95 for more efficient lateral diffusion (Masugi-Tokita, Tarusawa et al. 2007, Renner, Specht et al. 2008). The differences in the turnover rate and distribution of NMDAR and AMPAR give rise to the plasticity of synaptic strength and are critical for learning and memory.

Comparatively, for inhibitory synapses in the CNS, the main scaffolding protein is gephyrin. Gephyrin binds directly to GABA_AR and GlyR, and anchors them by tubulin stabilization (Langosch, Hoch et al. 1992). Similar to rapsyn clustering nAChR, gephyrin has the ability to cluster GABA_AR and GlyR in the brain and spinal cord. During GlyR synaptic maturation in the spinal cord, presynapses release glycine to gate the GlyR Cl⁻ channel, leading to a Ca²⁺ influx for gephyrin aggregation. The aggregation of gephyrin further clusters the GlyRs for postsynaptic maturation (Kirsch and Betz 1998). In gephyrin deficient mice, not only are GlyR clusters lost in the spinal cord, but GABA_AR clusters containing $\alpha 2$ or $\gamma 2$ subunit are lost in the brain (Kneussel, Brandstatter et al. 1999). The loss of gephyrin has no effect on glutamatergic synapses, which indicates the specificity between gephyrin and inhibitory synapses. Gephyrin itself is regulated by different posttranslational modifications (Choi and Ko 2015). For example phosphorylation, the status of gephyrin phosphorylation leads to conformational changes for clustering (Tyagarajan, Ghosh et al. 2013). Another critical posttranslational modification is palmitoylation, which enhances gephyrin's membrane association and clustering (Dejanovic, Semtner et al. 2014). Both phosphorylation and palmitoylation of gephyrin change the cluster formation, and further, alter the frequency and amplitude of the miniature inhibitory postsynaptic currents.

Receptor-associated scaffolding proteins alter the stability and dynamics of receptors, such as rapsyn to nAChR, PSD-95 to excitatory NMDAR and AMPAR, and gephyrin to inhibitory

GABA_AR and GlyR (Renner, Specht et al. 2008, Bruneau, Esteban et al. 2009). This critical role of scaffolding protein in maintaining receptor dynamics at synapses is essential for synaptic plasticity and neurotransmission.

1.5 Overview of the dissertation

Receptor stability and homeostasis at postsynaptic sites is critical for synaptic plasticity and neurotransmission. Previous research has revealed much about the individual roles of receptor-associated proteins in sustaining receptors. What is lacking, however, is a more systematic view of the dynamics and interactions within a protein complex. Specifically, at neuromuscular junctions, the postsynaptic protein complex of nAChR includes α kap, rapsyn and the dystrophin glycoprotein complex. α Kap protects nAChR from ubiquitin-dependent degradation. Rapsyn clusters nAChRs in response to agrin-Lrp4-MuSK pathway and anchors nAChR to β -dystroglycan, a major component in the dystrophin glycoprotein complex. In the chapter II, III of this dissertation, the nAChR dynamics are investigated from different aspects, as described below.

Chapter II focuses on rapsyn targeting to synapses. Rapsyn, a cytoplasmic protein, is essential for clustering AChRs at synapses. From cytoplasm to synapses, rapsyn must correctly target to the proper location in order to maintain synaptic function. To explore this process, I adapted rapsyn domain deletion constructs from our lab (Aittaleb, Chen et al. 2015) to determine how the rapsyn domain function in targeting to synapses in both living mice and C2C12 mouse muscle cell line. Interestingly, the interaction between AChR and rapsyn via rapsyn coiled-coil domain is the driving force for rapsyn targeting to synapses.

Chapter III examines the relationship between α kap and α -dystrobrevin in sustaining AChRs. α Kap binds directly to AChR and protects AChRs from ubiquitin-dependent degradation in both living mice and cell culture (Mouslim, Aittaleb et al. 2012, Martinez-Pena, Aittaleb et al. 2015).

However, little is known about how α kap interacts within the entire AChR-associated protein complex to sustain AChRs. To tackle this question, I found that α -dystrobrevin, a crucial intracellular component in the DGC, specifically enhanced α kap stability in a dosage-dependent manner. Together, α kap and α -dystrobrevin sustain AChR at postsynaptic sites. To further explore how the complex of α kap/ α -dystrobrevin stabilizes AChRs, in appendix A, I studied the deubiquitinating enzyme Usp9x, which has been shown to bind to α -dystrobrevin-1 in a phosphorylated-independent manner (Gingras, Gawor et al. 2016). My data suggested that Usp9x promotes the stabilizing effect of α kap/ α -dystrobrevin on AChRs, and Usp9x expression is highly correlated with the maturation of synapses in mice.

Appendix B explores the interplay within the α -dystrobrevin and α -syntrophin subcomplex. α -Dystrobrevin and α -syntrophin, the two intracellular components in the DGC, play both structural and signaling roles in sustaining AChRs. The knockout mice of either α -dystrobrevin or α -syntrophin have synapses that are similarly compromised in structure, density, and turnover. Overexpression of α -dystrobrevin-1 in α -syntrophin deficient mice partially rescues the synaptic deficiency, which suggests α -syntrophin acts via α -dystrobrevin (Martinez-Pena y Valenzuela, Mouslim et al. 2011). To investigate the crosstalk signaling pathways between α -dystrobrevin and α -syntrophin involved in the stability of AChRs, Dr. Martinez and I generated the double mutant mice of α -dystrobrevin and α -syntrophin.

Chapter IV offers conclusions and suggestions for future work. This dissertation investigates the stability of the nicotinic acetylcholine receptor and its associated proteins at neuromuscular junctions. By tackling different AChR-associated proteins, rapsyn, α kap, α -dystrobrevin, α -syntrophin, and Usp9x, this work provides new insights into mechanistic interactions at neuromuscular junctions.

CHAPTER 2

2. AChRs are essential for the targeting of Rapsyn to the postsynaptic membrane of NMJs in living mice

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* contributed equally to this work. I prepared the DNA constructs, managed and participated in the animal experiments, which were in charge by I. M-P. V.. I also performed the cell culture experiments, took the spinning disk confocal microscope images, analyzed data, and helped draft the manuscript.

2.1 Abstract

Rapsyn, a 43 kDa scaffold protein, is required for the clustering of acetylcholine receptors (AChRs) at synaptic sites between mammalian motor neurons and muscle cells. However, the mechanism by which rapsyn is inserted and retained at postsynaptic sites at the neuromuscular junction (NMJ) *in vivo* remains largely unknown. We found that neither the N-terminal myristoylation nor the cysteine-rich RING H2 domain of rapsyn is required for its stable association with the postsynaptic membrane of NMJs. When N-myristoylation-defective rapsyn-EGFP mutant (G2A) and RING-H2 domain truncated rapsyn-EGFP were electroporated into

sternomastoid muscles, a strong rapsyn fluorescent signal was selectively observed at synapses, similar to wildtype rapsyn-EGFP. The targeting of rapsyn-EGFP (wildtype and mutants) is independent of synaptic activity as they were inserted at denervated NMJs. However, when the coiled-coil domain (AChRs binding domain of rapsyn) is deleted, rapsyn fails to associate with AChRs at NMJs of living mice. In cultured myoblasts (in which AChRs are absent), myristoylated wildtype rapsyn mostly localizes to lysosomes, but is not associated with plasma membrane. However, in presence of AChR subunits, rapsyn molecules were targeted to cell surface and formed aggregates with AChRs. The targeting of AChRs to the cell membrane, in contrast, does not require rapsyn as expressed AChRs are visible on cells membrane of rapsyn deficient myoblasts. These results provide evidence for an active role of AChRs in the targeting of rapsyn to the NMJ *in vivo*.

2.2 Introduction

The formation and maintenance of AChR clusters in the postsynaptic membrane is the hallmark of a neuromuscular junction (NMJ). The effectiveness of synaptic impulses between nerve terminals and postsynaptic cells depends largely on the stability and the maintenance of a high density of AChRs. Several molecules of the signaling pathway involved in the clustering of AChRs and formation of NMJs have been identified (Sanes and Lichtman 2001, Wu, Xiong et al. 2010, Witzemann, Chevessier et al. 2013). Among these molecules, rapsyn, a 43kDa receptor-associated scaffold protein, which is enriched at postsynaptic membranes (Porter and Froehner 1985), is required for the insertion and maintenance of AChR clusters at synaptic sites in an agrin dependent mechanism (Gautam, Noakes et al. 1995). The exact colocalization of rapsyn and AChRs at the crest of junctional folds of NMJs *in vivo* and their precise accumulations at newly forming clusters strongly suggest that location and function of these proteins may be influenced by each other. Indeed, several studies have shown that rapsyn interacts with the large intracellular loop of all receptor subunits (Miyazawa, Fujiyoshi et al. 1999, Lee, Rudell et al. 2009) and this interaction is likely to be critical for the localization of both AChR and rapsyn at the synapse.

In non-muscle expression system, functional and structural studies on rapsyn have shown that the N-terminal myristoylation site of rapsyn is required for its targeting to the plasma membrane (Phillips, Kopta et al. 1991), that the coiled-coil domain (amino acids (aa) 298–331) is required for its interaction with AChRs and that the cysteine-rich RING domain (aa 363–402) is required for its interaction with β -dystroglycan (Ramarao and Cohen 1998, Bartoli, Ramarao et al. 2001, Ramarao, Bianchetta et al. 2001)). However, it remains largely unknown whether any of these domains are essential for the targeting of rapsyn to postsynaptic membrane of NMJs in living mice.

While it is well documented that rapsyn is essential for the clustering of AChRs on the cell surface of postsynaptic membrane (Gautam, Noakes et al. 1995), increasing evidence suggests that AChRs control the trafficking and localization of rapsyn at synaptic sites. For instance, in the zebrafish mutant *sofa potato*, which lacks expression of AChR, rapsyn remains stuck in the Golgi (Park, Ikeda et al. 2012). Similarly, focal dissipation of AChRs by laser illumination from clusters on cultured myotubes prevents the insertion and accumulation of new rapsyn (Bruneau, Brenner et al. 2008). However, it remains unclear whether AChRs are required for the targeting of rapsyn to synaptic sites in living mice.

In this study, we electroporated rapsyn mutants lacking specific domains into sternomastoid muscles of living mice to test which of functional rapsyn's domains are essential for its targeting and/or association with plasma membrane at the synapse. We also tested whether the presence of AChRs is required for the rapsyn localization at cell surface in myoblasts.

2.3 Materials and Methods

Plasmid constructs and reagents

All rapsyn-EGFP constructs (WT, G2A, Δ RING-H2, and Δ CC) and Lamp1-mCherry were generated in our lab as described in (Aittaleb, Chen et al. 2015). All constructs were driven by a CMV promoter. Rat anti-Lamp1 (1D4B) and rat anti-AChR (mab35) antibodies were purchased from Developmental Studies Hybridoma Bank (University of Iowa).

Mice and muscle denervation

In this study, female non-Swiss albino mice (6-10 weeks old, 20 – 30 g) were used. The original pairs of the mice were purchased from Harlan and breed in animal facility at the University of Michigan. All animal methods and usage are approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA). The sternomastoid muscle of mice was denervated by excising 5 mm of nerves under proper anesthesia.

Plasmids electroporation, AChRs labeling, and NMJs imaging

Innervated and denervated (8 days of denervation) adult mice were anesthetized with an IP injection of a mixture of 80 mg/kg ketamine and 20 mg/kg xylazine). The sternomastoid muscles were surgically exposed and rapsyn constructs (wildtype and mutants, 5 $\mu\text{g}/\mu\text{l}$) were electroporated as described in our previous studies (Bruneau and Akaaboune 2010). 7 days after electroporation, NMJs were labeled with BTX-Alexa594 and then imaged with a confocal microscope (Leica SP5) as described in our previous work (Martinez-Pena, Aittaleb et al. 2015). The z-stacks were then collapsed and the contrast adjusted with Photoshop.

In some experiments, NMJs were imaged with a conventional microscope and the fluorescence intensity of synapses was assayed using a quantitative fluorescence imaging technique, as described previously (Martinez-Pena, Aittaleb et al. 2015).

Cell culture, transfection, and cell imaging

C2C12 myoblasts were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (11995-065; Gibco, NY) supplemented with 20% fetal bovine serum (FBS) (S11050, Atlanta Biologicals, GA).

All cells were grown in the presence of 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C, under a humidified atmosphere and 5% CO₂.

The localization of AChRs and rapsyn was performed on C2C12 myoblasts that were cotransfected with rapsyn-EGFP and four subunits of AChRs as described in our previous studies (Aittaleb, Chen et al. 2015).

To examine whether the insertion of AChR at the cell surface is independent of rapsyn, myoblast cells deficient in rapsyn were transfected with the four subunits of AChR and then fixed, labeled with Anti-AChR antibody (mAb35) and imaged. Images of fixed and live cells were acquired using a 100× objective on an inverted Andor/Olympus IX81 spinning disk confocal microscope. The z-stacks were performed using the ImageJ software (NIH) and the contrast of images was adjusted with Photoshop.

2.4 Results

2.4.1 Neither the N-terminal myristoylation nor cysteine rich RING-H2 domain of rapsyn are required for its targeting to NMJs *in vivo*

Previous studies have shown that N-terminal myristoylation, a lipid modification that anchors proteins to membranes (Carr, Tyler et al. 1989, Resh 2004), is necessary for the association of rapsyn with the plasma membrane in a non-muscle expression system (Phillips, Maimone et al. 1991, Ramarao and Cohen 1998). To test whether the N-myristoylation of rapsyn is also essential for its association with AChR clusters at the NMJs of living mice we electroporated a rapsyn mutant construct (tagged with EGFP), in which a single glycine was changed to alanine (G2A) to prevent NH2-terminal myristoylation, into sternomastoid muscles. If N-terminal myristoylation is essential for the targeting of rapsyn to synaptic sites, the rapsyn-G2A mutant should not be present at synaptic clusters. However, 7 days after electroporation NMJs in all muscle cells electroporated with rapsyn G2A mutant showed a strong fluorescence signal that was perfectly co-localized with AChRs (identified by α -bungarotoxin Alexa594 (BTX-Alexa594) at synaptic clusters (39 NMJs; 6 mice), similar to the localization of wildtype rapsyn-EGFP at NMJs of electroporated muscles (Figure 2.1A, B). This suggests that the NH2-terminal

myristoylation of glycine 2 is not necessary for the targeting and association with the postsynaptic membrane of NMJs.

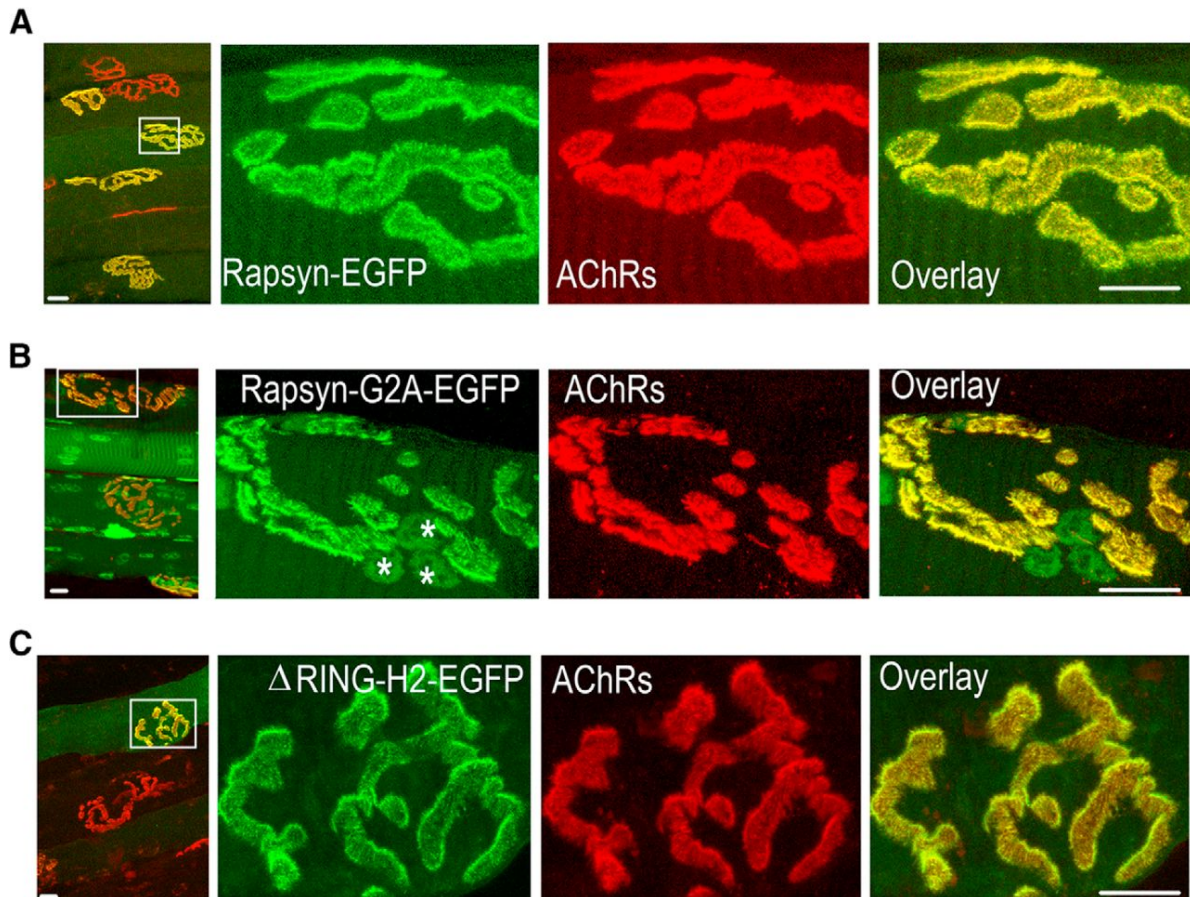


Figure 2.1 Neither the N-terminal myristoylation site nor the cysteine rich domain of rapsyn is required for the targeting and association of rapsyn with the plasma membrane at adult NMJ.

Sternomastoid muscles were electroporated with either wildtype, mutant G2A rapsyn-EGFP, or Δ RING-H2-rapsyn-EGFP and 7 days later AChRs were labeled with BTX-Alexa 594, muscles were fixed and synapses expressing fluorescent rapsyn were imaged with the confocal microscope. **A)** Example of a NMJ from electroporated muscles with wildtype rapsyn-EGFP, **B)** Example of a NMJ expressing G2A mutant rapsyn-EGFP (the N-terminal myristoylation site glycine was changed to alanine), nuclei show also rapsyn fluorescent labeling as indicated by asterisks; **C)** Example of a NMJ expressing rapsyn-EGFP lacking the RING-H2 domain (Δ RING-H2-rapsyn-EGFP). Scale bars are 10 μ m.

Previous studies have shown that the cysteine rich RING-H2 domain of rapsyn binds to β -dystroglycan (β -DG), a component of the dystrophin glycoprotein complex (Bartoli, Ramarao et

al. 2001). To test whether the RING-H2 domain is necessary for rapsyn association with the plasma membrane at synaptic sites, the sternomastoid muscle was electroporated with a rapsyn construct (Δ RING-H2-EGFP) that lacks the RING-H2 domain. As shown in Figure 2.1C, Δ RING-H2-EGFP fluorescence was specifically observed at synapses and perfectly colocalized with AChRs at the crest of junctional folds on all electroporated muscles (31 NMJs; 6 mice), indicating that RING-H2 domain is not required for rapsyn targeting to synaptic sites.

It is also noteworthy to mention that muscle cells electroporated with the rapsyn G2A mutant showed strong GFP fluorescence in their nuclei (Figure 2.1B, asterisks) but this nuclear localization of GFP fluorescence was not observed in muscle cells electroporated with wildtype rapsyn-EGFP or Δ RING-H2EGFP. The reason for the nuclear localization of mutated rapsyn G2A is unknown (see Discussion). Quantification of GFP fluorescence intensity (only high intensity of the GFP signal) of mutants and wild type rapsyn in electroporated muscles showed roughly similar expression levels (WT = 24.42, SEM = 4.73, 4 mice; G2A = 18.69, SEM = 3.21, 4 mice; Δ RING-H2 = 19.16, SEM = 1.86, 5 mice.)

2.4.2 Targeting of rapsyn to synapses does not require synaptic activity

Since neither the N-terminal myristoylation nor RING-H2 appeared to be required for the targeting of rapsyn to synaptic sites, we wondered whether the presence of factor(s) released from the nerve terminal might play a role. To assess this idea, the sternomastoid muscle was surgically denervated and 8 days later, muscles were electroporated with wildtype or rapsyn-EGFP mutant constructs (G2A-EGFP, rapsyn Δ RINGH2-GFP). Seven days after electroporation, AChRs were labeled with BTX-Alexa594 and rapsyn fluorescence was examined. We found that both wildtype rapsyn and the rapsyn mutants colocalized with AChRs at all electroporated denervated NMJs (WT: 29 NMJs, 3 mice; G2A: 8 NMJs, 3 mice; Δ RINGH2: 11 NMJs, 3 mice) (Figure 2.2). The rapsyn G2A mutant also accumulated in the nucleus of denervated muscles. These results showed that the association of rapsyn with the postsynaptic plasma membrane does not require nerve derived factors or muscle activity.

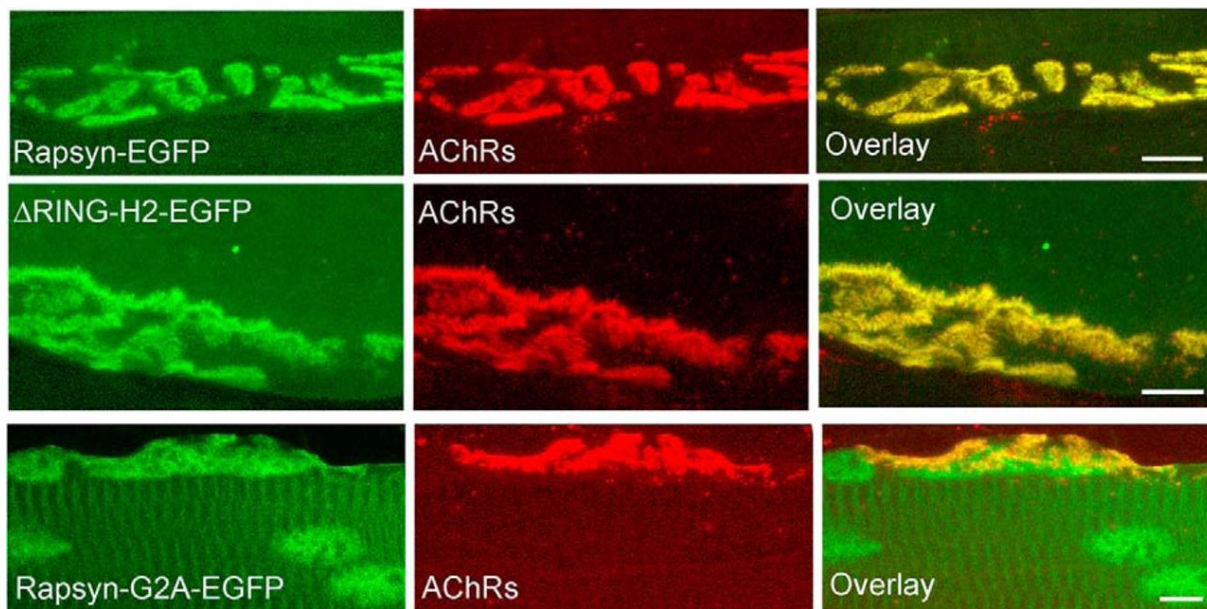


Figure 2.2 Lack of Synaptic activity had no effect on the targeting of rapsyn.

Denervated sternomastoid muscles (8 days) of an adult mice were electroporated with either wildtype rapsyn-EGFP, G2A rapsyn-EGFP, or Δ RING-H2 rapsyn-EGFP. 7 days after electroporation, AChRs were labeled with BTX-Alexa Fluor 594 (red) to identify synapses, fixed, and imaged. Shown are examples of NMJs expressing wildtype rapsyn-EGFP (top panels), Δ RING-H2 rapsyn-EGFP (middle panels), and G2A-rapsyn-EGFP (bottom panels). Scale bars are 10 μ m.

2.4.3 The coiled-coil domain is essential for the targeting of rapsyn to synaptic sites

Previous studies have shown that the coiled-coil (CC) domain was required for rapsyn interaction with AChRs (Ramarao, Bianchetta et al. 2001). To test whether this interaction is relevant for the targeting and/or keeping rapsyn at the plasma synaptic membrane, a rapsyn mutant construct in which the coiled-coil domain was deleted (rapsyn Δ CC-EGFP), was electroporated into sternomastoid muscles. In contrast to the synaptic localization of wildtype, G2A and Δ RINGH2-GFP rapsyn, we found that the lack of CC domain resulted in a complete loss of the exogenous rapsyn localization to synaptic sites of both denervated (NMJ= 16, 3 mice) and innervated muscles (NMJ= 15, 3 mice). Instead the rapsyn CC rapsyn mutant-EGFP exhibited a diffuse distribution throughout the entire innervated and denervated muscle fibers (**Figure 2.3A**). The absence of the CC rapsyn mutant at synapses rule out also the possible association between exogenous and endogenous rapsyn. All together, these results indicate that

the targeting and association of rapsyn with the plasma membrane of synaptic sites required CC domains, and possibly mediated by an interaction with AChR.

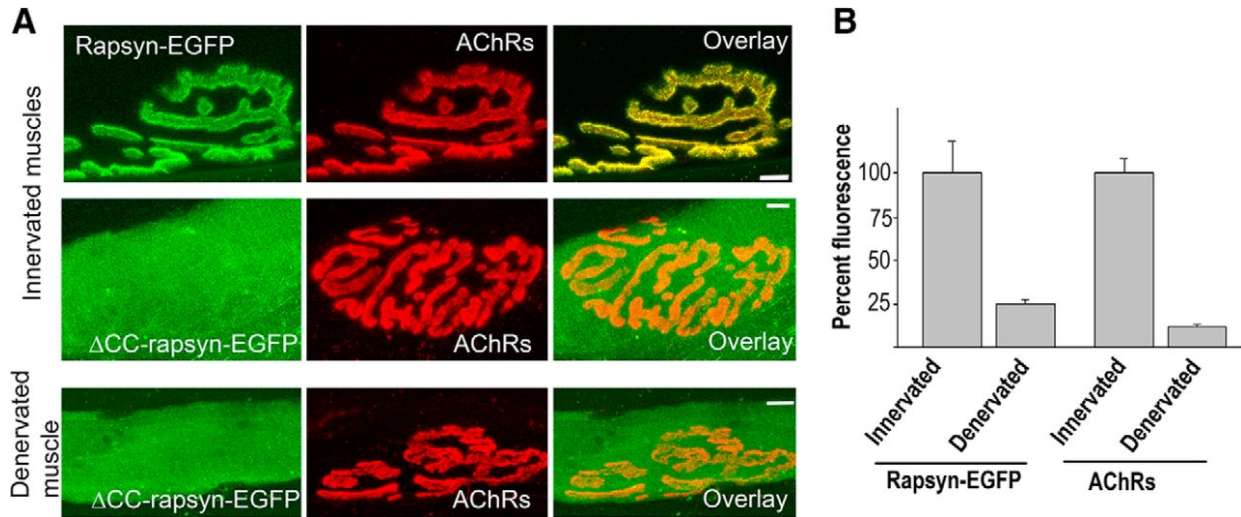


Figure 2.3 The coiled-coil domain is required for the targeting of rapsyn to synaptic sites.

The sternomastoid muscle of adult innervated and denervated mice were electroporated with rapsyn-EGFP lacking CC domain (rapsyn Δ CC-EGFP). At 7 days after electroporation, AChRs were labeled with a saturating dose of BTX-Alexa Fluor 594, and superficial synapses on electroporated (green) muscle fibers were imaged and then fixed and imaged. **A)** Examples of innervated NMJs on muscle expressing wildtype (top panels) and Δ CC-rapsyn-EGFP (middle panels). The bottom panels showed rapsyn Δ CC-EGFP expressing in denervated muscles. Note that rapsyn mutant fails to express at both innervated and denervated NMJ compared to wildtype rapsyn. **B)** Graph summarizing the mean $\pm$ SD percentage of fluorescence intensity of labeled AChRs and rapsyn-EGFP at innervated and denervated NMJs, $p < 0.001$. Scale bars are 10 μ m.

To further delineate the relationship of rapsyn and AChRs, we asked whether the density of rapsyn changes proportionally when the density of AChR is altered. This was examined by measuring fluorescently labeled AChRs and fluorescent rapsyn-GFP at NMJs of denervated muscles, in which the density of AChR decreases significantly upon denervation (Salpeter, Cooper et al. 1986, Akaaboune, Culican et al. 1999, Bruneau and Akaaboune 2006). Quantification of fluorescence intensity of labeled rapsyn and AChRs at NMJs denervated for 15 days showed that AChR and rapsyn declined to the similar extents (76% decrease in rapsyn and 88% decrease in AChR) (Figure 2.3B), indicating that the number/density of rapsyn at synaptic sites is closely associated with the number/density of AChRs.

2.4.4 AChRs are essential for rapsyn association with the plasma membrane

To directly examine whether the interaction between AChR and the CC domain of rapsyn plays a functional role in the targeting and association of rapsyn with AChR clusters in the postsynaptic membrane, we used a myoblast expression system in which rapsyn and AChR subunits were either introduced simultaneously or alone. If AChRs are directly involved in targeting rapsyn on the plasma membrane of muscle cells, co-transfecting AChRs along with rapsyn should promote the targeting of rapsyn to the cell surface. Cultured myoblasts (cells that do not express AChRs at this stage of development (Aittaleb, Chen et al. 2015) were transfected with either wildtype rapsyn-EGFP (along with LAMP-1-m-cherry to make lysosomes visible; data not shown) or co-transfected with rapsyn-EGFP and the four subunits of adult mouse AChR. It is worth mentioning that in cells devoid of AChRs, expressed rapsyn localized to lysosomes and induced their clustering at high density in the juxtannuclear region (Aittaleb, Chen et al. 2015) (see Figure 2.4A). In myoblasts transfected with wildtype rapsyn-EGFP without AChR subunits, after 24 hours we found that rapsyn was mainly localized to Lamp-1 positive lysosomes in the juxtannuclear region as marked by anti-Lamp1, and that there was little or no fluorescent rapsyn signal associated with the plasma membrane (Figure 2.4A and C), consistent with our previous results (Aittaleb, Chen et al. 2015). In contrast, when myoblasts were co-transfected with rapsyn-EGFP and AChR subunits and cells were fixed without permeabilization to show only surface receptors (Figure 2.4B), rapsyn-EGFP was mostly associated with AChRs at the cell surface (yellow and orange in the merged image) but there also were some regions of densely clustered rapsyn without overlying AChRs, which are located inside the cell (green in the merge, collapse image).

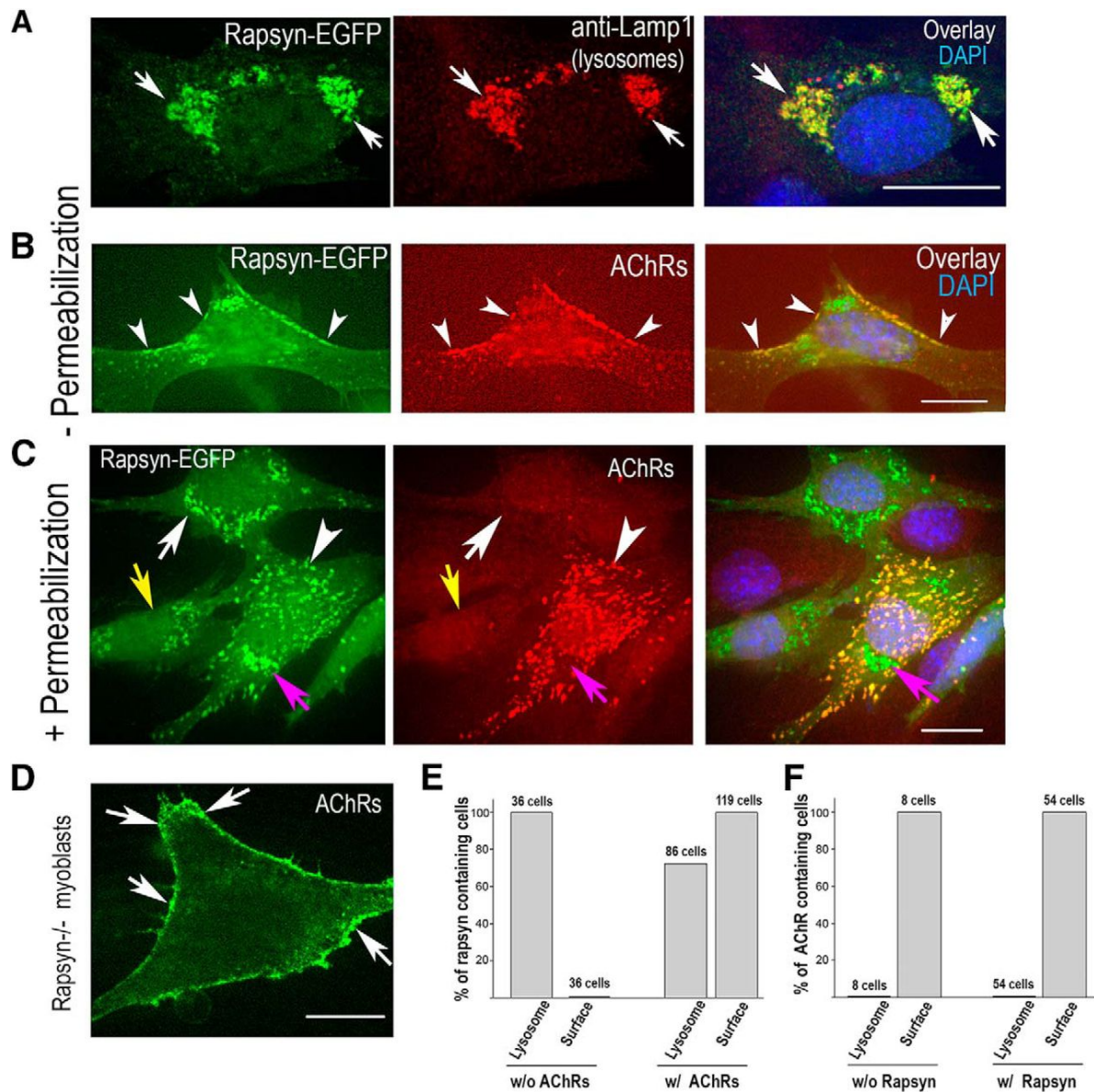


Figure 2.4 AChRs are required for accumulating rapsyn on the cell surface.

C2C12 myoblasts or myoblasts deficient in rapsyn were transfected with rapsyn-EGFP alone, or rapsyn-EGFP and the four subunits of AChRs, or AChRs subunits alone. After 24 hours, cells were fixed and imaged using the confocal spinning disk microscope. **A)** Representative images of C2C12 myoblasts transfected with rapsyn-EGFP, fixed, permeabilized and immunostained with rat anti-Lamp1 are shown. Note that most of rapsyn-EGFP colocalizes with lysosomes (see arrows)(Aittaleb, Chen et al. 2015) and there is a complete absence of rapsyn association with plasma membrane; **B)** Example of images of C2C12 myoblasts co-transfected with rapsyn-EGFP and AChR α , AChR β , AChR δ and AChR ϵ subunits, fixed without permeabilization and stained with AChR antibody (to label only surface receptors). Arrowheads show AChRs and rapsyn co-clusters at the cell surface. **C)** Cells were permeabilized with 1% Triton and immunostained with AChR antibody to label AChRs on the surface and inside the cell. Note that there is no co-localization of intracellular AChRs with rapsyn in lysosomes (see purple arrows). Arrowheads show the colocalization of rapsyn and AChRs. In the absence of AChRs, rapsyn is

mainly concentrated in the lysosomes (white arrows) and not associated with plasma membrane (yellow arrows). **D)** Myoblasts deficient in rapsyn were transfected with AChR four subunits alone and surface receptors were labeled with BTX-Alexa488. **E)** Myoblasts were co-transfected with rapsyn-EGFP, lamp1-mcherry and with or without four subunits of AChRs. **F)** Myoblasts were co-transfected with four subunits of AChRs, lamp1-mcherry and with or without rapsyn-GFP. 24 hours after transfection, cells were fixed with or without permeabilization, stained with anti-AChR antibody and then imaged. Graphs E and F show quantifications of percent of cells containing rapsyn and AChRs on cell surface and lysosomes, respectively. Scale bars are 10 μ m.

Next we asked whether rapsyn promotes the targeting of AChRs at areas rich in rapsyn. If rapsyn is involved in AChRs targeting, we would expect to see the presence of AChRs wherever rapsyn is clustered, particularly in lysosomes at the juxtannuclear region rich in rapsyn (Aittaleb, Chen et al. 2015). To test this, myoblast cells were co-transfected with four AChR subunits and rapsyn-EGFP and 24 hours later cells were fixed, permeabilized, and immunostained with anti-AChR antibody (mAb35) (so both surface and intracellular receptors were labeled). As shown in Figure 2.4C, rapsyn-EGFP was mostly associated with AChRs at surface clusters and intracellular puncta of various size and shape. However, there were regions of highly clustered rapsyn-GFP (green in the merge) that did not show any overlap with AChRs, localized to lysosomes in the juxtannuclear regions (Figure 4C, purple arrows). This suggests that rapsyn is not involved in the targeting of AChRs to cell surface. To further confirm these results myoblasts from mice carrying a null mutation for rapsyn were transfected with the four AChR subunits and 24 hours later, cells were fixed without permeabilization and then stained with BTX-Alexa488 to label surface AChRs. Figure 2.4D shows that in rapsyn^{-/-} myoblasts, there is extensive surface labeling of AChRs on the cell surface, indicating that rapsyn is not required for the targeting of AChR to the myoblast cell surface.

2.5 Discussion

Many lines of evidence indicate that the presence of rapsyn on the plasma membrane is essential for the clustering of AChRs. Most importantly, there is a failure of AChR cluster formation in muscle cell deficient in rapsyn (Gautam, Noakes et al. 1995). Previous work and current study have suggested that the relationship between rapsyn and AChRs can be reciprocal. For instance,

decreased rapsyn expression at the synapse was observed in mice deficient in an AChR subunit (Witzemann, Schwarz et al. 1996, Schwarz, Giese et al. 2000) and the density of rapsyn is significantly reduced in mice deficient in α -syntrophin and α -dystrobrevin, which exhibit a dramatic reduction in total AChR levels compared to wildtype (Adams, Kramarcy et al. 2000, Grady, Zhou et al. 2000, Martinez-Pena y Valenzuela, Mouslim et al. 2011). We had also previously produced results suggesting that that AChRs are required for the maintenance of rapsyn density at receptor clusters on the cell surface of cultured myotubes (Bruneau, Brenner et al. 2008).

This study provided first evidence that AChRs are essential for localizing rapsyn to synapses in adult mouse muscle by interacting with the rapsyn coiled-coil domain. Specifically, we showed that: 1) electroporation of rapsyn mutants that lack either the N-terminal myristoylation or RING-H2 domains are selectively expressed at synaptic sites where they perfectly co-localized with AChRs at NMJs *in vivo*; 2) the specific localization of exogenous rapsyn to synaptic sites is completely lost when the rapsyn coiled-coil domain (known to bind AChRs *in vitro*) is deleted; 3) the association of wildtype rapsyn with the synaptic plasma membrane is not dependent on synaptic activity as evidenced by the presence of a rapsyn signal at surgically denervated NMJs; 4) in the myoblast cell line C2C12 (at a stage of development when AChRs are not yet expressed), when rapsyn is expressed, most of the wildtype rapsyn colocalizes with lysosomes in the juxtannuclear region and rapsyn has no clear association with the plasma membrane; 5) however, when rapsyn co-expressed in C2C12 cells with AChRs, both molecules are predominately found at the cell surface. Altogether, these results strongly suggest that AChR serves as a possible mediator for the targeting and association of rapsyn with the postsynaptic membrane at synaptic sites.

Several studies have shown that myristoylation of proteins serves to target them to the plasma membrane (Pellman, Garber et al. 1985, Rein, McClure et al. 1986). In non-muscle cells the NH2-terminal myristoylation of rapsyn plays an important role for its association with the plasma membrane (Phillips, Maimone et al. 1991). However, the specific co-localization of G2A rapsyn-EGFP mutant with AChRs at crests of junctional folds of NMJs of living mice is strong evidence that the N-terminal myristoylation domain is not essential for membrane targeting of

rapsyn in adult muscle (Figure 2.1B). Interestingly, the G2A rapsyn mutant also accumulated in the nucleus. It is unclear how the loss of N-terminal myristoylation induces the translocation of rapsyn to the nucleus. One possibility is that lack of myristoylation results protein misfolding that uncovers a normally hidden nuclear import signal. Another possibility is that the mutant rapsyn folds normally, but myristoylation prevents nuclear import. Regardless of the mechanism, the nuclear localization raises the possibility that under some conditions rapsyn regulate gene expression, so further exploration of the nuclear role of rapsyn would be of interest. It is worth to mention that Rapsyn mutations have been shown to be associated with the development of Congenital Myasthenia Syndrome. Indeed, multiple forms of the neuromuscular disease of congenital myasthenic syndrome have been linked to several novel mutations in the gene for rapsyn which vary in both pathology and severity (Engel 2012).

The current experiments suggest that the association of rapsyn with the plasma membrane of adult muscle does not require nerve derived factors or muscle activity since in denervated muscles wildtype and mutant rapsyn (Δ RING-H2 or G2A) still localized to the AChR clusters of denervated NMJs (Figure 2.2). The denervation experiments thus suggest that under these conditions, the targeting of rapsyn to synaptic sites is mediated by other postsynaptic proteins. One possibility was β -dystroglycan, because in non-muscle cells, it has been reported that rapsyn clusters β -dystroglycan (a molecule of the dystrophin glycoprotein complex) through its association with the cysteine-rich RING-H2 domain (Bartoli, Ramarao et al. 2001). However, in living mice, it appears that deletion of RING-H2 domain has no effect on rapsyn targeting since deletion of the RING-H2 domain didn't prevent rapsyn's association with the plasma membrane at synaptic sites (Figure 2.1C), arguing against the involvement of DGC in the targeting of rapsyn at synaptic site and this despite their presence in the same intracellular vesicular transport (Marchand, Stetzkowski-Marden et al. 2001). As noted above, another strong candidate to be involved in localizing rapsyn is the AChR itself. In zebrafish, animal carrying a null mutant for AChR fail to localize rapsyn at synapses and expression of muscle type AChR in zebrafish mutant muscle cells restores the synaptic localization of rapsyn (Ono, Mandel et al. 2004). Furthermore AChRs have been shown to enable the transport of rapsyn from the Golgi complex to the plasma membrane (Park, Ikeda et al. 2012). It is also possible that the interaction of rapsyn with calpain which inhibits its activity, allowing the stabilization of AChR clusters at the synapse

might contribute to the regulatory mechanism of AChR insertion (Chen, Qian et al. 2007). In sum, the present experiments are the first to show that in mammals, the postsynaptic membrane localization of rapsyn involves AChRs. However, it is not clear if AChRs are the only molecules that target or retain rapsyn to synaptic sites *in vivo*.

CHAPTER 3

3. The stabilization of the acetylcholine receptor by α kap and α -dystrobrevin

3.1 Abstract

The maintenance of acetylcholine receptor (AChR) clusters at synaptic sites is critical for synaptic function and structure of the neuromuscular junction. α -Dystrobrevin (α dbn, a component of the dystrophin glycoprotein complex) and α kap (a muscle-specific anchoring protein) promote the stability of AChR both *in vivo* and in cultured muscle cells. However, the molecular link between α kap and α dbn on the maintenance of AChRs remains unknown. Here, we reported that α dbn is a key regulator of α kap. Co-transfection experiments demonstrate that α dbn stabilizes endogenous and exogenous α kap expression levels in a dosage-dependent manner, while α kap has no effect on α dbn expression. Furthermore, we showed that when the three phosphorylatable tyrosine residues of α dbn were changed to phenylalanine, the expression of α dbn was significantly reduced, but that expression was completely restored in the presence of a proteasome inhibitor. Moreover, we found that high expression of α kap or α dbn promote the formation of large agrin-induced AChR clusters. This study revealed a mechanistic link between α kap and α dbn, two essential AChR-associated proteins, in maintaining AChR expression.

3.2 Introduction

The maintenance of AChR clusters at high density in the postsynaptic membrane is a hallmark of the neuromuscular junction (NMJ) (Sanes and Lichtman 2001). Extensive studies have shown that several core molecules of a multistep process such as MuSK (muscle-specific tyrosine kinase), LRP4 (low-density lipoprotein receptor-related protein 4), rapsyn, and Dok7 (dedicator of cytokinesis family member 7) are required for the initial steps in the formation of AChR clusters (Gautam, Noakes et al. 1995, DeChiara, Bowen et al. 1996, Okada, Inoue et al. 2006, Weatherbee, Anderson et al. 2006, Kim, Stiegler et al. 2008, Zhang, Luo et al. 2008), and auxiliary proteins such as the dystrophin glycoprotein complex (DGC) are essential for the maturation and stability of AChR clusters.

It was recently demonstrated that α kap, a muscle-specific anchoring protein encoded within the *Camk2a* gene, which is thought to play a role in targeting multiple calcium/calmodulin kinase II (CaMKII) isoforms to specific subcellular locations, also plays an important role in promoting the stability of AChR (Bayer, Harbers et al. 1998, Mouslim, Aittaleb et al. 2012, Martinez-Pena, Aittaleb et al. 2015). When endogenous expression of α kap was knocked down with shRNA in cultured myotubes or in mouse sternomastoid muscles, the postsynaptic receptor density was reduced and the metabolic stability of AChR was impaired as the removal rate of receptors was significantly increased, and the insertion rates of both newly synthesized and recycled receptors into the postsynaptic membrane were depressed (Mouslim, Aittaleb et al. 2012, Martinez-Pena, Aittaleb et al. 2015). These alterations were accompanied by the impairment of synaptic structure. However, the mechanistic link between α kap and AChR stability remains unknown.

On the other hand the dystrophin glycoprotein complex (DGC), a multimolecular protein complex that links the intracellular cytoskeleton to the extracellular basal lamina (Ervasti and Campbell 1993), is not only crucial for the structural integrity of the membrane, but also for the formation and maintenance of AChR clusters on the muscle fiber surface (Grady, Merlie et al.

1997, Cartaud, Coutant et al. 1998, Jacobson, Montanaro et al. 1998, Adams, Kramarcy et al. 2000, Grady, Zhou et al. 2000, Grady, Akaaboune et al. 2003, Martinez-Pena y Valenzuela, Mouslim et al. 2011). For instance, in mice deficient in dystrobrevins or α -syntrophins, the maturation and stabilization of the postsynaptic membrane, specifically the density, the turnover rate, and the pattern of AChR distribution are severely altered (Adams, Kramarcy et al. 2000, Grady, Zhou et al. 2000, Akaaboune, Grady et al. 2002, Grady, Akaaboune et al. 2003). In muscle cells, two isoforms of α dbn (α dbn1 and 2) have been shown to be concentrated at the postsynaptic membrane (Grady, Grange et al. 1999, Grady, Zhou et al. 2000). While α dbn1, a substrate for tyrosine kinases *in vivo* (Wagner, Cohen et al. 1993, Balasubramanian, Fung et al. 1998) that is phosphorylated by Erb receptor (Schmidt, Akaaboune et al. 2011), plays a critical role in the maintenance and the stability of the NMJ (Grady, Akaaboune et al. 2003); α dbn2 is crucial for the maintenance of the structural integrity of the muscle (Grady, Akaaboune et al. 2003).

Because both α dbn1 and α kap are involved in promoting the accumulation of AChRs, we sought to investigate the link between these two components. In the present study, we investigate the ability of α dbn to regulate α kap expression levels in cultured muscle cells and heterologous system, and discovered that the phosphorylatability of the three tyrosine residues of α dbn1 are critical for the expression of α dbn and thus α kap and AChR accumulation.

3.3 Materials and Methods

Plasmid constructs and reagents

The plasmids of the mouse AChR subunits (α , β , δ , ϵ) were generous gifts from Dr. William Green at the University of Chicago. Plasmids of α kap-GFP and α -dystrobrevin-GFP were described in our previous work (Martinez-Pena y Valenzuela, Mouslim et al. 2010, Mouslim, Aittaleb et al. 2012, Martinez-Pena, Aittaleb et al. 2015). α -Dystrobrevin-1-GFP is the full-length phosphorylatable isoform; α -Dystrobrevin-3Y/F-GFP is the mutated isoform in which the three C-terminal phosphorylatable tyrosine residues were changed to phenylalanine (Y705F, Y713F, and Y730F). α Kap-mCherry was cloned by swapping GFP (from the α Kap-GFP) to mCherry (from the pmCherry-N3) with 5' EcoRI and 3' KpnI sites. Both α -dystrobrevin-3Y/F-GFP and α Kap-mCherry were verified by sequencing, transfection, and Western blots.

The proteasome inhibitor MG132 (Enzo Life Sciences) and the lysosome inhibitor leupeptin (Sigma-Aldrich) were used at 2.5 μ M and 10 μ g/ml, respectively (Mouslim, Aittaleb et al. 2012). Cycloheximide (Sigma-Aldrich, St Louis) was used at 60 μ g/ml (Ennis and Lubin 1964, Zinck, Cahill et al. 1995, Ahat, Xiang et al. 2019).

The concentration of MG132 and leupeptin were first based on our previous work in HEK cells (5 μ M for MG132 and 20 μ g/ml for leupeptin) (Mouslim, Aittaleb et al. 2012) but resulted in toxicity in transfected HeLa cells. Therefore, a series of doses were treated (0 – 5 μ M for MG132 and 0 – 20 μ g/ml for leupeptin) in HeLa cells. The final concentration was determined by observing consistent AChR α enhancement effect as the previous work on other cell types without seeing major cell death after 6 hours of the drug treatment. The concentration of cycloheximide was chosen by literature review (Ennis and Lubin 1964, Zinck, Cahill et al. 1995, Ahat, Xiang et al. 2019), where 10 μ g/ml of cycloheximide was sufficient to inhibit protein synthesis by 95% and 10 – 100 μ g/ml were the range for research used. 60 μ g/ml of cycloheximide was used to stop protein synthesis and avoid major cell death.

Cell culture, transfection, and cell imaging

HeLa and HEK 293T cells were purchased from the American Type Culture Collection (ATCC, Manassas), and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (11995-065; Gibco) and supplemented with 10% fetal bovine serum (FBS) (S11050; Atlanta Biologicals). C2C12 myoblasts were purchased from ATCC and maintained in DMEM with 20% FBS. To differentiate C2C12 myoblasts into myotubes, C2C12 myoblasts at 80% confluency were switched to DMEM supplemented with 5% horse serum (16050; Gibco) for 5 to 7 days. All cells were grown in the presence of 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C, humidified atmosphere and 5% CO₂. The transfection was performed when cells were at 80% confluency using Lipofectamine 2000 (1467572; Invitrogen) according to the manufacturer's instructions. The transfection efficiency of cells was checked using an Olympus IX81 inverted fluorescence microscope (10X objective lenses), to make sure there was no visual difference of the transfection indicator within the same set of experiments before sample processing for Western blots.

To assess AChR clustering, cells were grown on 35 mm dishes and treated with agrin (550-AG; R&D Systems) at a concentration of 500 ng/ml overnight. Cells were then fixed by 2% paraformaldehyde in PBS for 20 minutes and then washed with PBS three times. AChR clusters were labeled by BTX-Alexa 647 (B35450; Molecular Probes), incubated in the dark for 2 hours at room temperature, followed by four washes in PBS and DAPI Fluoromount-G mounting media (0100-20; Southern Biotech). The fixed samples were imaged on an Andor/Olympus spinning disk confocal microscope (20X dry lenses). The laser excitation lines were used as following: 405 nm for DAPI, 488 nm for GFP, 561 nm for mCherry, 640 nm for BTX-Alexa 647.

Quantification of AChR clusters

Spinning disk images of myotubes were processed and analyzed using ImageJ (NIH, version 1.49). Image acquisition settings appropriate for each color channel were determined in preliminary experiments. They varied for the three color channels, but for each channel all

images were obtained at the same settings. At least 30 myotubes from 3 independent plates were quantified for each experimental condition (**Table 3.1**).

For each color channel, images from focal plains spanning the top to the bottom of myotubes were collapsed to a single plane (Image → Stack → Z project → project type: median). The scale was set (1.4 pixels to 1 μm in 20X amplification (Analyze → Set scale). The images for the BTX-Alexa 647 stained AChR (640 nm channel) were quantified first, so during this process we were unaware whether the clusters were on transfected or untransfected myotubes. AChR clusters were identified by the following steps. Images were set to 8-bit binary (Image → Type → 8-bit) for and we then set a threshold that clearly identified clusters and excluded background (Image → Adjust → Threshold, dark background). In the majority of cases (over 80%) we set the thresholds to 70 – 255. For a few high background images we used 100 – 255 threshold, and few low background images were used 50 – 255 threshold. The threshold images were used “analyze particles” function and quantified the area (μm^2) with the selection range of 4 – infinity (μm^2) to exclude remaining background. The calculated clusters were used “Outlines” function to indicate the number of quantified clusters, and used “straight line” to manual draw the longest axis of the clusters (μm) and “measure” each length.

Once all clusters had been identified, the images from the 488 nm and/or 561 nm channels of the each image stack were analyzed to determine whether each cluster was on a transfected or untransfected fiber. The approach was similar to the method for detecting AChR clusters; we first thresholded the image, but in this case we simply identified each muscle fiber as having (transfected) or lacking (untransfected) significant fluorescence above threshold. We defined a fiber as transfected by $\alpha\text{Dbn-GFP}$ if when the threshold was set to 45 there was substantial signal over most of the myotube. There was a third group of $\alpha\text{Dbn-GFP}$ plate that did not pass the 45 threshold but contained substantial αDbn signal co-localized with the AChR signals. The data obtained from this group were not included in the data presented for transfected myotubes but the size and the number of the clusters were not significantly different from the more heavily

transfected ones. α Kap-mCherry transfected myotubes were defined by signals above a threshold of 25.

Immunoprecipitation and western blotting

C2C12 myotubes were harvested in lysis buffer containing 100 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 1% Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail (11873580001; Roche), and then processed by sonication and centrifugation (10,000 g for 10 min at 4°C.) The C2C12 lysates were separated into two tubes of the equal amount. In one tube, 2 μ g of α kap antibody (CaMKII α A-1, sc-13141; Santa Cruz, mouse) was added; and in the second tube, 2 μ g of mouse control IgG (12-371; Millipore) was added to incubated on ice for 2 hours. Then the lysate/antibody mixtures were incubated with Protein A/G Plus-Agarose beads (sc-2003; Santa Cruz) overnight at 4°C. Beads were collected by centrifugation (1200 g for 3 min at 4°C) and washed three times with cold PBS, resuspended in LDS reducing sample buffer (NuPAGE 4X lithium dodecyl sulfate, NP0007; Invitrogen), and boiled for 5 minutes.

In another series of experiments, HeLa and HEK cells at 90 – 100% confluence were washed with cold PBS, lysed with lysis buffer (100 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 5 mM NaF, 5 mM Na₃VO₄, 2.5 mM Na₄P₂O₇, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail), and centrifuged for 10 min at 10,000 g. Lysates were resuspended in lithium dodecyl sulfate reducing sample buffer (LDS, NP0007; Invitrogen) and boiled for 5 minutes prior to western blotting. Proteins were separated by SDS-PAGE (10%) and transferred to PVDF membranes. The membranes were stained with Ponceau S for 1 hour, imaged, washed three washes with PBST (PBS with 0.05% Tween 20), and then bathed in a blocking solution (5% dry milk and 0.05% Tween 20 in TBS, 10X TBS: 170-6435; Bio-Rad) for 1 – 3 hours. The membranes were incubated with primary antibodies in blocking solution overnight at 4°C. After three times washing with PBST, the membranes were incubated with secondary HRP-conjugated antibodies and developed using SuperSignalWest

Pico solutions (34077; Thermo Fisher Scientific) or Clarity Western ECL Substrate (1705060; Bio-Rad). Mouse anti-HA (clone 16B12, MMS-101R; Covance) and mouse anti- α kap (CaMKII α A-1, sc-13141; Santa Cruz biotechnology) were diluted at 1:2000; mouse anti- α -dystrobrevin (610766; BD Biosciences) was diluted at 1:4000; rabbit anti-GFP (A6455; Invitrogen), rat anti-AChR α (MAB210; Covance), and mouse anti-tubulin (AA4.3-s; The Developmental Studies Hybridoma Bank) were diluted at 1: 5000. All horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) were diluted at 1:10000.

WB band signals were captured by using either BIO-RAD ChemiDoc MP imaging system or FluorChem M FM0467. We first used the auto-exposure mode to determine the optimum exposure time to avoid pixels' saturation (according to the manufacturers' handbook). Within in that time range, we captured at least two images to use for quantification. Only the non-saturated bands in the images were quantified. Between the two images, the samples were assigned proportional values to ensure the signals fell within the ECL linear exposure range. Because of inconsistencies and variations in signal intensity between the same protein bands in different gel lanes, we normalized the proteins bands to its transfection efficiency indicator, housekeeping proteins or total protein identified by Ponceau S. In this way, errors related to variations in sample loading and transfers were corrected. Western blot bands were quantified with ImageJ (NIH, version 1.49). The intensity of bands was quantified by using the rectangular selection tool and keeping the area uniform which covered each band's active area, where a straight line was used to cut off non-specific bands and the wand tool selected the target peak. These were compared to equal area baseline selections of an empty lane nearby (no protein loaded) to subtract background. If the background was not uniform across the blot, the background subtraction would be individually selected for each lane, which the criteria is a no signal, slightly upper or lower active band area. For all data presented as histograms, results from several blots were averaged, with the number indicated in the figure legend.

3.4 Results

3.4.1 α Kap, α -dystrobrevin, and AChRs are present in the same complex in C2C12 myotubes

Previous studies have shown that α kap is present with AChR α in the same complex, protects AChR α from ubiquitin-dependent degradation *in vitro* (Mouslim, Aittaleb et al. 2012), and is involved in the maintenance of the structural integrity of the neuromuscular system *in vivo* (Martinez-Pena, Aittaleb et al. 2015). On the other hand, α -dystrobrevin (α dbn), an essential component of the dystrophin glycoprotein complex, is also required for the maturation of the neuromuscular junction, the maintenance of a high density of AChRs at the postsynaptic membrane density (Grady, Grange et al. 1999), and in the stability of the lifetime of AChRs (Akaaboune, Grady et al. 2002). Here we sought to test whether there is a crosstalk between the α kap and α dbn pathways. To investigate this, we first analyzed whether α kap and α dbn are present within the same complex with AChRs. Lysates from cultured muscle myotubes were incubated with α kap antibodies to immunoprecipitate α kap and eluates were subjected to Western blot analysis with anti-AChR α and anti- α dbn antibodies. **Figure 3.1 top panel** shows that α kap was pulled down successfully and specifically enriched in the α kap lane compared to the total lysate and the control IgG pulled down. When blots were probed with anti- α dbn antibody, a band corresponding to α dbn was detected in total lysate and the immunoprecipitate α kap but not in the control IgG antibody lane (note that the upshifted of α dbn signal, which most likely due to the presence of the protein A/G agarose beads, leading to changes in the mobility of α dbn on the gel) (**Figure 3.1 middle panel**). Next we examined whether α kap and α dbn are present with AChR α in the same complex (it is noteworthy to mention that neither α kap nor α dbn have been shown to interact directly with AChRs). Consistent with previous work, **Figure 3.1 low panel** shows that AChR was also pulled down by α kap antibody, with the strongest signals among the total lysate and the control. The result indicated that AChR α is present in the same complex as α kap and α dbn.

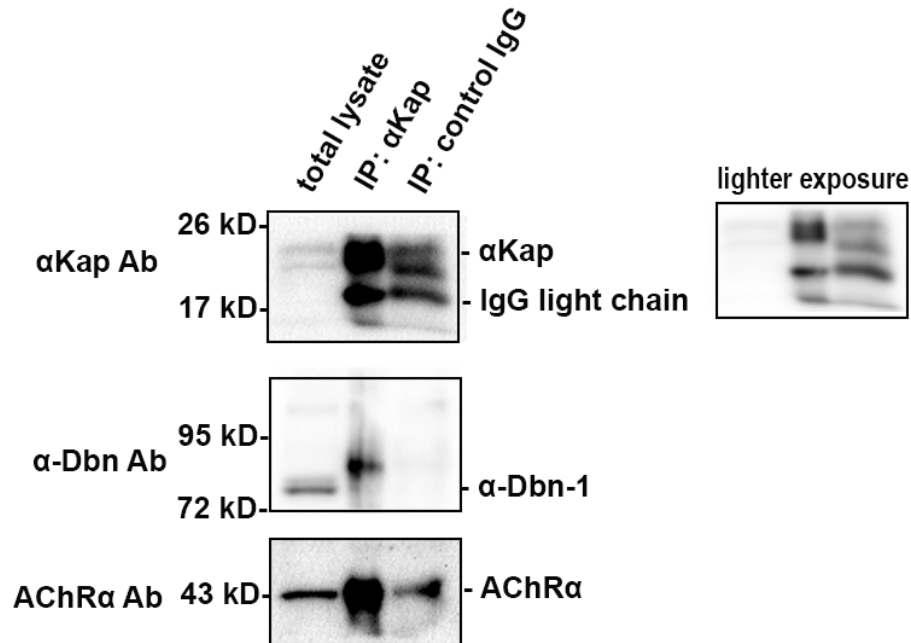
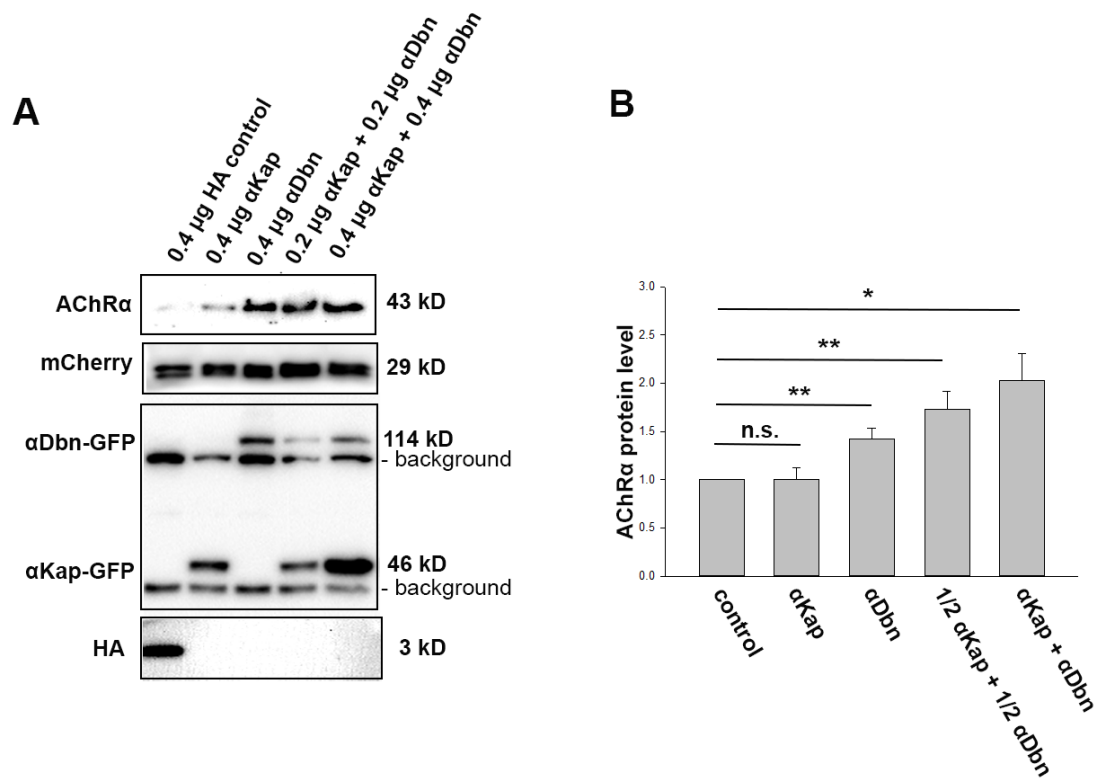


Figure 3.1 The co-immunoprecipitation of α kap is enriched in α -dystrobrevin and AChR α signals in C2C12 myotubes.

C2C12 myotube lysates were incubated with either the α kap antibody or the mouse control IgG antibody, followed by protein A/G agarose beads for immunoprecipitation. The top panel showed that the α kap signals were the most enriched in the α kap IP lane indicating that the pull down was successful. Since the α kap signals in α kap IP lane were too strong to see α kap signal in total lysate within the non-saturated range, the saturated blot was shown for α kap size indication, and the non-saturated blot from the same view was shown in the box to top right to demonstrate two bands for α kap pull down signals. The middle panel showed that α dbn1 signals were specifically enriched in the α kap IP lane but not the control IgG lane. The bottom panel showed that AChR α signals were enriched in the α kap IP lane. Altogether, compared with the control, the α kap pull down was enriched in α dbn1 and AChR α signals. The data was represent one time experiment on the same blot, and the blot was cut twice between (1) 72-56 kD and (2) 34-26 kD for α dbn1 (87 kD, 89 kD upshifted for phosphorylated form or mobility change by agarose beads), AChR α (43 kD), and α kap (21 and 23 kD) antibodies incubation. Similar pull down results were observed in three independent experiments.

3.4.2 AChR accumulation is enhanced in the presence of α -dystrobrevin and α kap

Next, we asked whether the known effect of α kap on AChR α accumulation (Mouslim, Aittaleb et al. 2012) requires the presence of α dbn and vice-versa. First, we used non-muscle HeLa cells (cells that do not express α dbn, AChR, or α kap) that were co-transfected with AChR α (1 μ g) with HA control plasmid (0.4 μ g), AChR α (1 μ g) with α kap-GFP (0.4 μ g) or α dbn-GFP (0.4 μ g), and AChR α (1 μ g) with both α kap-GFP and α dbn-GFP at two different concentrations (0.2 μ g and 0.4 μ g). Twenty-four hours later, lysates from the above co-transfected cells were immunoblotted with anti-AChR α , and anti-GFP antibodies for α kap-GFP and α dbn-GFP. **Figure 3.2** shows that AChR α levels were increased 173% ($P < 0.01$, $n = 4$) in the presence of both α kap-GFP and α dbn-GFP at 0.2 μ g and 202% ($P < 0.05$, $n = 4$) at 0.4 μ g compared to cells treated with control plasmid (the average as 100%) or α kap-GFP alone (103%, n.s., $n = 4$). AChR α levels were slightly elevated by α dbn-GFP alone (143%, $P < 0.01$, $n=4$). Similar results were obtained when all subunits of AChRs (α , β , δ , ϵ) were co-transfected with α kap and α dbn (**Figure 3.2 C**). In the second series of experiment, we used HEK cells (cells that do express endogenous α dbn1, but not α kap or AChR α , **Figure 3.3 B**) that were co-transfected with an increasing concentration of α kap (0, 0.2, 0.4, 0.8 μ g) and a fixed concentration of AChR α plasmid (1 μ g). **Figure 3.3 A** shows that expression levels of AChR α were significantly increased as α kap concentration was increased, while the endogenous expression levels of α dbn remained unchanged. These results suggest that in the absence of α dbn, α kap does not exert its protective effect from ubiquitin-dependent degradation on AChRs and that α dbn is required for maintaining high expression levels of AChR.



	Condition (plasmid added per 35 mm dish)				
μg	Control	αKap	αDbn	½ αKap + ½ αDbn	αKap + αDbn
AChRα	1	1	1	1	1
αKAP-GFP	0	0.4	0	0.2	0.4
αDbn-GFP	0	0	0.4	0.2	0.4
mCherry	0.2	0.2	0.2	0.2	0.2
HA	0.4	0	0	0	0
Total DNA	1.6	1.6	1.6	1.6	2.0

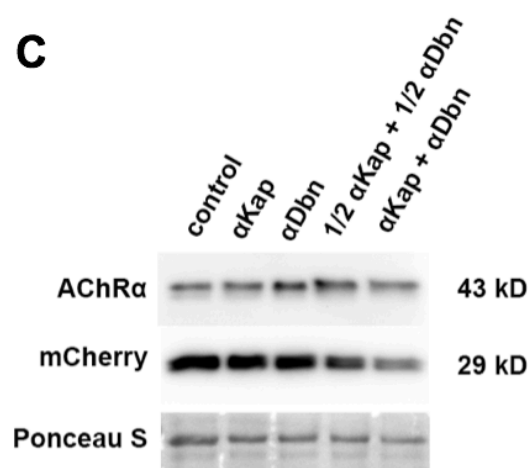


Figure 3.2 The expression of AChRα is enhanced by α-dystrobrevin and αkap

(A) HeLa cells were transfected with the same amount (1 μ g) of AChR α in all conditions, and adding α kap-GFP and α dbn-GFP individually or jointly. The first control lane had 0.4 μ g of HA plasmid to get the same total DNA amount as lanes 2 – 4. mCherry was transfected as the transfection efficiency indicator. The AChR α signals were normalized to mCherry signals before making comparison between conditions. The transfected conditions were listed in the following table.

(B) The level of AChR α was significantly increased in α -dystrobrevin alone and α kap/ α -dystrobrevin groups. This data had four time repeats with the similar AChR α patterns observed, and the error bars were represented standard error, $n = 4$.

(C) A similar experiment as (A), with transfection of all AChR subunits at 1 μ g (2 α , β , δ , ϵ ; α 0.4 μ g, the other subunits 0.2 μ g each). The AChR signals were obtained by anti-AChR α antibody to represent the whole AChR expression. The fifth lane was co-transfected 7 plasmids with high concentration that lower the transfection efficiency. The expression levels of AChRs from the first to the forth lanes were similar to the results from AChR α single subunit in (A), which were 100%, 113%, 141%, and 202%, respectively, $n = 2$.

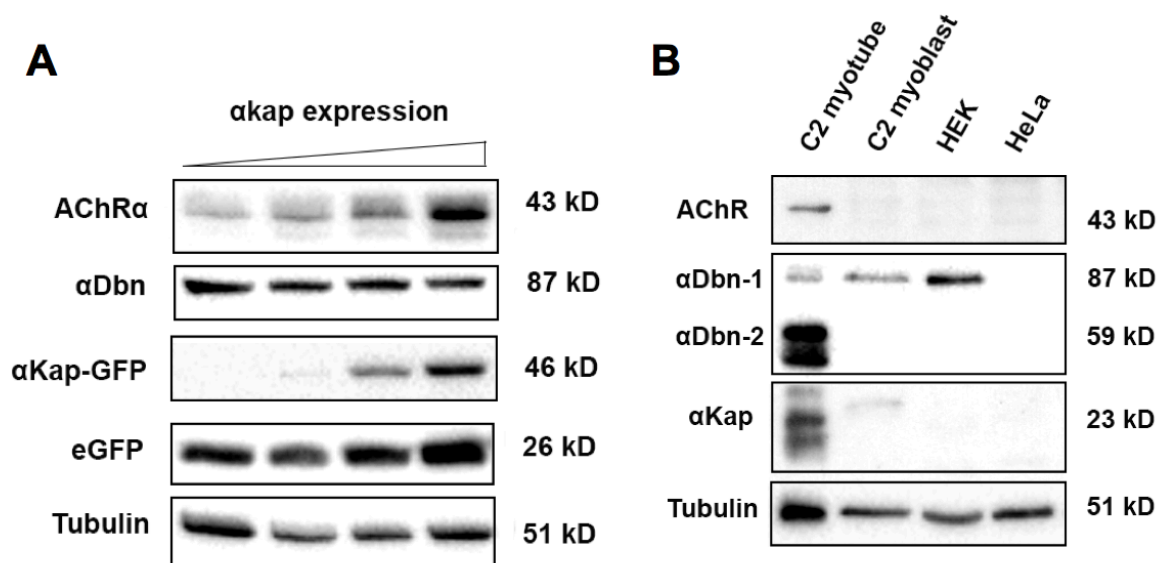


Figure 3.3 The expression of AChRα is enhanced by αkap transfection in HEK cells, which endogenously express α-dystrobrevin-1

(A) HEK cells were transfected with the same amount (1 μg) of AChRα along with increasing amount of αkap-GFP (0, 0.2, 0.4, 0.8 μg). eGFP served as the transfection efficiency indicator, and tubulin was probed to indicate cell confluency.

(B) The protein expression profile of C2C12 myotubes and myoblasts, HEK cells, and HeLa cells. C2C12 myotubes express AChR, αdbn1, αdbn2, and αkap; C2C12 myoblasts express αdbn1 and αkap; HEK cells express αdbn1; HeLa cells do not express any of these proteins. Tubulin was probed to indicate the amount of material input.

3.4.3 α -Dystrobrevin promotes the protein levels of α kap in a dosage-dependent effect

To test the possibility that α dbn increases the protein levels of α kap, we used three cell lines: non-muscle HeLa cells (no endogenous expression of α dbn and α kap), HEK cells (endogenous α dbn1 expression only), and C2C12 myoblasts (endogenous expression of both α dbn1 and α kap) (**Figure 3.3 B**). These cells were co-transfected with α kap-GFP alone or α kap-GFP and α dbn-GFP. Twenty-four hours later cell lysates were immunoblotted with anti- α kap and anti- α dbn for the endogenous proteins, and anti-GFP for α kap-GFP and α dbn-GFP. **Figure 3.4** shows that α kap expression levels were significantly higher in cells transfected with both α dbn and α kap compared to cells transfected with α kap alone; and this was consistent among all lines used. While α dbn-GFP was present, α kap-GFP expression was enhanced by 2.95 fold in HeLa cells ($P < 0.001$, $n = 13$, **Figure 3.4 A,B**), 3.15 fold in HEK cells ($P < 0.01$, $n = 4$, **Figure 3.4 C,D**), and 3.36 fold in C2C12 myoblasts ($P < 0.001$, $n = 8$, **Figure 3.4 E,F**). The overexpression of 0.4 μ g α dbn-GFP did not interfere the endogenous expression of α -dystrobrevin-1 in HEK and C2C12 cells (**Figure 3.4 C and 3.4 E**). In C2C12 muscle cells, the overexpression of α dbn enhances expression levels of not only exogenous α kap-GFP but endogenous α kap by 2.22 fold ($P < 0.01$, $n = 6$, **Figure 3.4 E,F**).

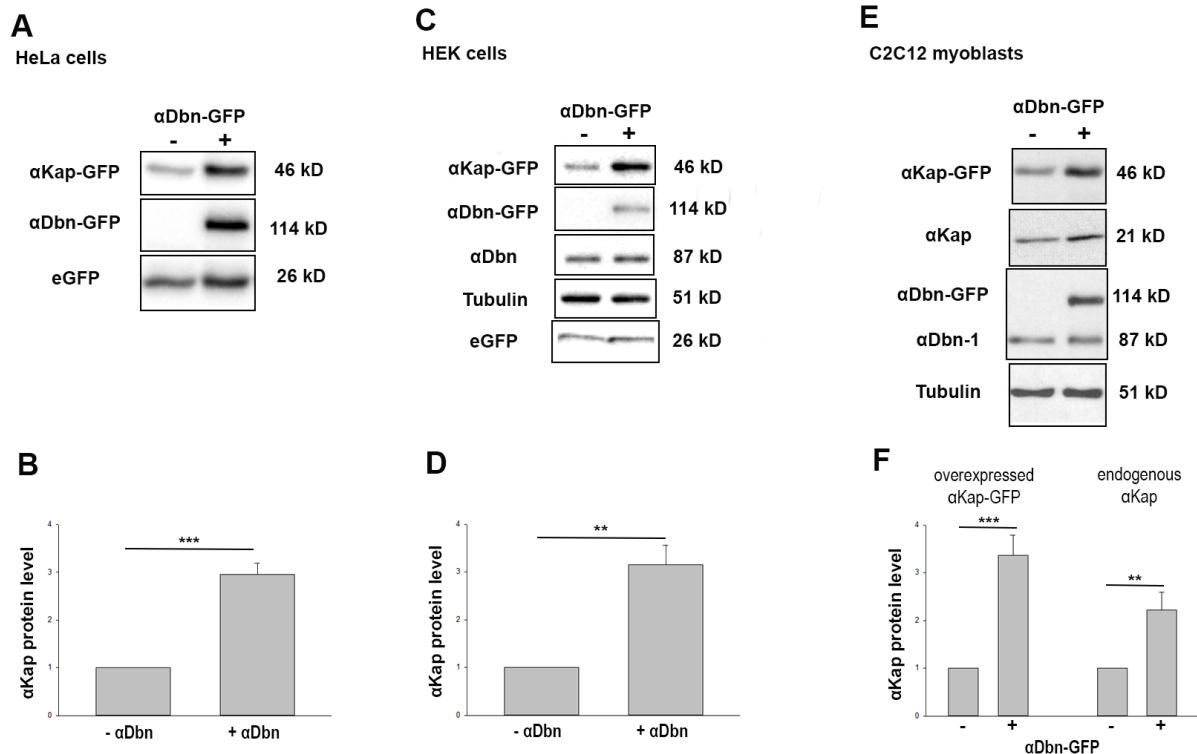


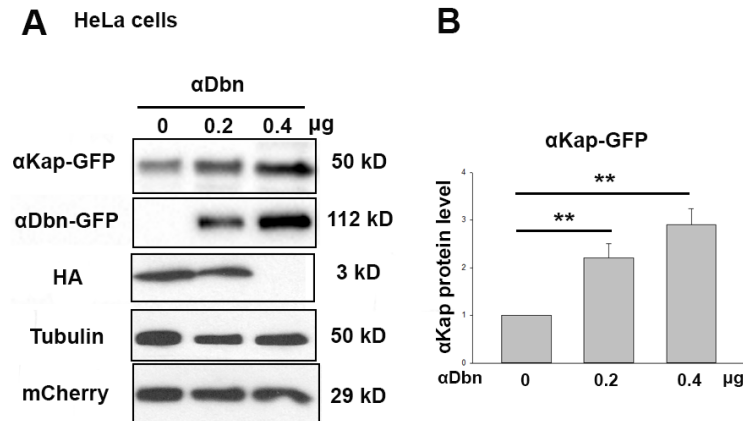
Figure 3.4 The protein level of α kap is enhanced by α -dystrobrevin-1 overexpression in HeLa, HEK, and C2C12 myoblasts

With the same amount (1 μ g) of α kap transfected, while α dbn1 was overexpressed, the expression of α kap was significantly increased in (A,B) HeLa (n = 13), (C,D) HEK (n = 4), and (E,F) C2C12 myoblasts (n = 8). The error bars represent standard error.

(A) Since HeLa cells do not express α dbn or α kap, the α kap-GFP signals were normalized by the transfect efficiency indicator eGFP. (C) Since HEK cells have α dbn1 endogenous expression, though the α kap-GFP was normalized by the transfect efficiency indicator eGFP, the tubulin blot was shown to represent overexpressed α dbn-GFP did not interfere endogenous α dbn expression. (E) Since C2C12 myoblasts have both α dbn and α kap endogenous expression, both α kap-GFP and α kap were normalized by the tubulin signals. The overexpressed α dbn-GFP did not interfere endogenous α dbn1 expression as well.

Next, we asked whether the expression levels of α kap are dependent upon α dbn expression levels. To examine this, we used HeLa cells (no endogenous expression of α kap and α dbn) and C2C12 muscle cells (endogenous expression of both α kap and α dbn) (**Figure 3.3 B**), and co-transfected the same amount (1 μ g) of α kap-GFP with increasing α dbn constructs doses (0, 0.2, 0.4 μ g α dbn-GFP). The dose concentration was determined by preliminary experiments: cells were transfected from 0, 0.1, 0.2, 0.3, 0.4 to 0.5 μ g of α dbn-GFP and all co-transfected with 1 μ g of α kap-GFP, and the result showed a nearly linear increase of α kap expression from 0 – 0.4 μ g α dbn-GFP doses. Between 0.4 and 0.5 μ g α dbn-GFP doses, α kap expression was either similar or slightly lower in the 0.5 μ g dose. We confined our plasmid concentrations because construct doses higher than 0.4 μ g resulted in saturation of expression. Therefore, 0, 0.2, and 0.4 μ g of α dbn-GFP were selected, and the HA control plasmid was co-transfected to make up the same total plasmid amount as 0.4, 0.2, and 0 μ g, respectively. **Figure 3.5** shows that in HeLa cells, α kap expression levels increased significantly as α dbn expression levels increased (2.2 and 2.9 fold, $P < 0.01$, $n = 4$). In C2C12 myoblasts, the α kap levels of both endogenous (α kap: 1.68 and 2.07 fold, $P < 0.05$, $n = 3$) and exogenous (α kap-GFP: 2.43 and 3.02 fold, $P < 0.01$; $n = 4$) expression were increased compared to control plasmid transfected muscle cells. These results strongly suggest that α dbn is involved in promoting the expression of α kap.

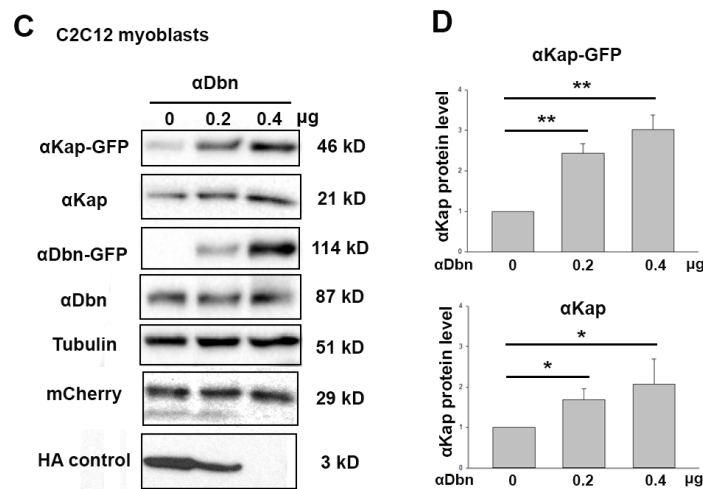
Figure 3.5 The expression of α -dystrobrevin has dose-dependent effect on the expression of α kap



(A) In HeLa heterologous system, the increase of overexpressed α -dystrobrevin showed a dose-dependent effect on α kap expression.

1 μ g of α kap-GFP was transfected in every condition, with 0, 0.2, and 0.4 μ g of α dbn-GFP co-transfected. HA-control plasmid was co-transfected to make up the total plasmid amount (0.4 μ g HA for 0 μ g α dbn-GFP, 0.2 μ g HA for 0.2 μ g α dbn-GFP). The α kap-GFP signals were normalized by mCherry transfection efficiency indicator, tubulin blot was shown to indicate the similar amount of cell confluency were achieved in different conditions.

(B) Quantification from the (A) figure, α kap-GFP expression was enhanced in both 0.2 μ g α dbn-GFP group and 0.4 μ g α dbn-GFP group. The error bars represent standard error, $n = 4$.



(C) In C2C12 myoblasts, a dose-dependent effect of α -dystrobrevin was observed in both exogenous and endogenous α kap. As in (A), the HA control plasmid was co-transfected to make up the same total plasmid amount. α Kap-GFP was normalized to mCherry, and α kap was normalized to tubulin.

(D) Quantification from (C). Both exogenous α kap-GFP ($n = 4$) and endogenous α kap ($n = 3$) were increased. The error bars represent standard error.

Conversely, when cells were co-transfected with the same amount (1 μ g) of α dbn and increasing α kap constructs doses (0, 0.2, 0.4 μ g), the expression levels of α dbn remains unchanged (**Figure 3.6**). The regulatory effect of α dbn on α kap and not the other way around was further observed when cells were co-transfected with AChR α (**Figure 3.2**). When cultured cells were co-transfected with either α kap alone, α dbn alone, or with the same increasing doses of both α kap and α dbn (0.2 and 0.4 μ g of plasmid DNA each), as shown in **Figure 3.2**, α kap expression levels were enhanced when α dbn expression levels were increased, while α dbn expression levels remained unchanged when α kap expression levels were increased. All together, these results indicate that α kap expression depends on α dbn expression while α dbn expression is independent of α kap.

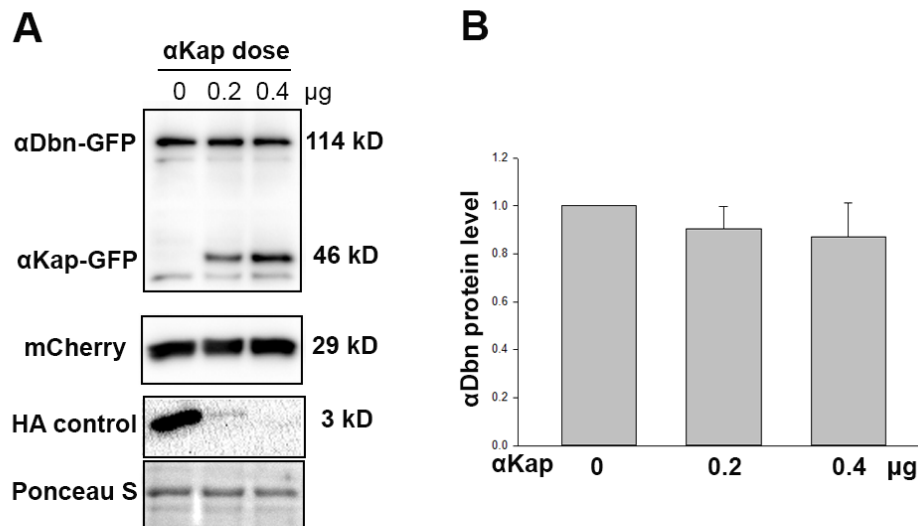


Figure 3.6 α kap had no effect on α -dystrobrevin expression in HeLa cells

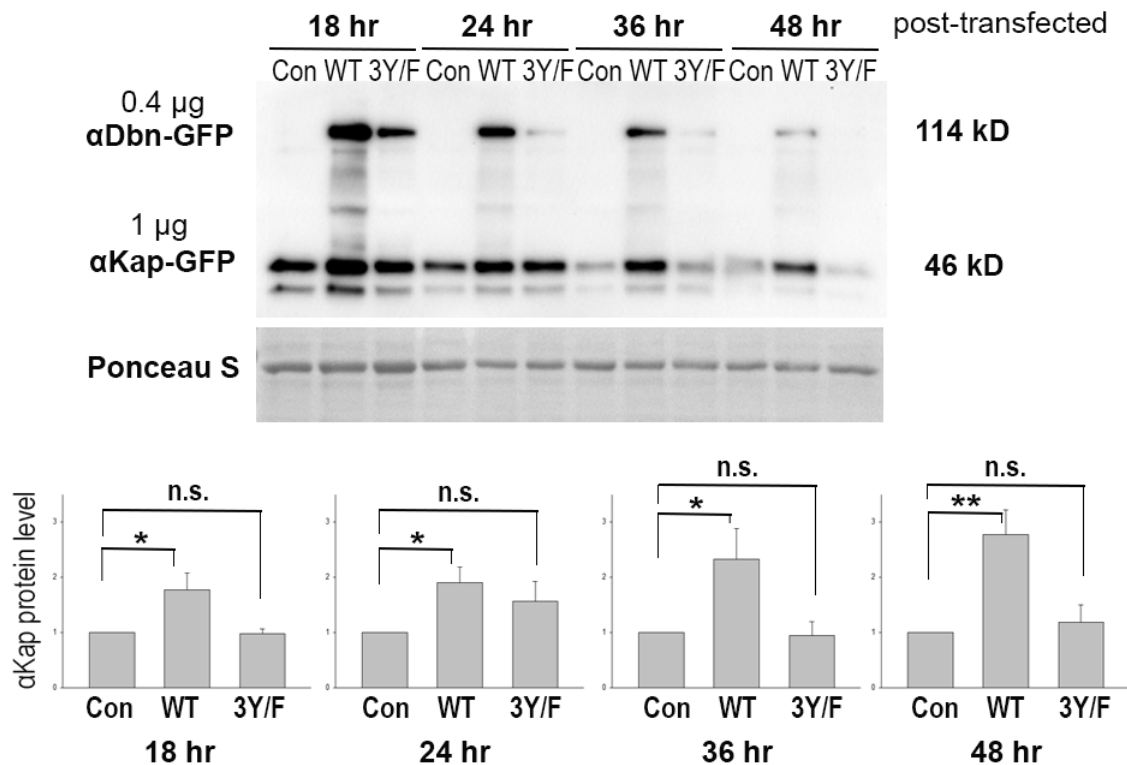
(A) The same amount (1 μ g) of α -dystrobrevin-GFP was transfected along with the increase of α kap (0, 0.2, and 0.4 μ g), and the HA control was co-transfected to make up the same of the total plasmid concentration. The α dbn-GFP signals were normalized by mCherry, which was the transfection efficiency indicator, and Ponceau S indicated the confluency of the cells was similar between conditions.

(B) Quantification from (A). There was no significant difference of α dbn-GFP expression along with α kap dose increase. The error bars represent standard error, n = 3.

3.4.4 Role of phosphorylatable tyrosine residues of α -dystrobrevin on the expression of α -dystrobrevin and α kap

The C-terminal of α -dystrobrevin has three tyrosines, which can be phosphorylated by neuregulin/ErbB pathway and are essential for AChR postsynaptic stability (Grady, Akaaboune et al. 2003, Schmidt, Akaaboune et al. 2011). Here, we tested whether the expression of α dbn itself and its stabilizing effect on α kap is associated with the phosphorylatability of the three tyrosine residues. To examine this, we used heterologous expression in HeLa cells, which lack endogenous α kap and α dbn (so the effect of the endogenous expression of α dbn on α kap would be excluded, **Figure 3.3 B**). In the first set of experiments, we asked whether α kap accumulation is controlled by α dbn phosphorylatability. HeLa cells were co-transfected with nonphosphorylatable α dbn-GFP mutant (the three tyrosines were changed to phenylalanine, 3Y/F) and α kap-GFP, or with phosphorylatable wildtype α dbn-GFP and α kap-GFP. Cells were harvested at 18, 24, 36, and 48 hours post-transfection. Western blot analysis of proteins with anti-GFP antibody showed that α kap protein accumulation was significantly increased only in cells transfected with wildtype phosphorylatable α dbn compared with the control group at every time point (18 hr: 1.77 fold $P < 0.05$, 24 hr: 1.90 fold $P < 0.05$, 36 hr: 2.33 fold $P < 0.05$, 48 hr: 2.77 fold $P < 0.01$), but not in cells transfected with nonphosphorylatable α dbn (all time points did not achieve statistical significance compared with the control group)(**Figure 3.7**). This suggests that phosphorylatability of α dbn plays an important role in the expression of α kap.

Figure 3.7 Phosphorylatable tyrosine residues of α -dystrobrevin are critical for the expression of α -dystrobrevin and α kap



HeLa cells were transfected with the same amount (1μg) of α kap-GFP, with either control plasmid, α dbn-WT-GFP, or α dbn-3Y/F-GFP. Transfected cells were harvested at 18, 24, 36, and 48 hours post-transfection. Both α dbn and α kap were normalized to ponceau S signals, $n = 4$. α Kap-GFP expression levels were only significantly increased while α dbn-WT-GFP present, but not α dbn-3Y/F-GFP nonphosphorylatable mutant, which suggested the phosphorylatability of α dbn plays an important role in the protein level of α kap. Overall, the α kap stabilizing effect by phosphorylatable α dbn was increased along with observation window, which the later stage (48 hours) was more pronounced than the earlier stages. However, at 24 hours post-transfection, α kap-GFP expression had no significant difference between α dbn-3Y/F and α dbn-WT.

Interestingly, the phosphorylatable wildtype and nonphosphorylatable mutant α dbn-GFP only contained difference in three residues, the expression level of α dbn-3Y/F-GFP mutant was always lower compared to wildtype at each time point (**Figure 3.7**). This observation led us to test whether the phosphorylatability of the three tyrosine residues is important for the expression of α dbn. We first tested if the decrease of α dbn-3Y/F-GFP mutant resulted from a change at the transcript level. HeLa cells transfected with α dbn-WT-GFP or α dbn-3Y/F-GFP were harvested and extracted their RNA. When RT-PCR products specific to α dbn1 were analyzed, we found that there was no significant difference in the amount of α dbn mRNA products between cells transfected with the phosphorylatable and nonphosphorylatable isoforms (**Figure 3.8**). The experiment also examined α kap-GFP transcript levels with α dbn-WT-GFP or α dbn-3Y/F-GFP transfected. The result indicated that the increased expression of α kap-GFP by α dbn-WT-GFP was not regulated at the transcript level.

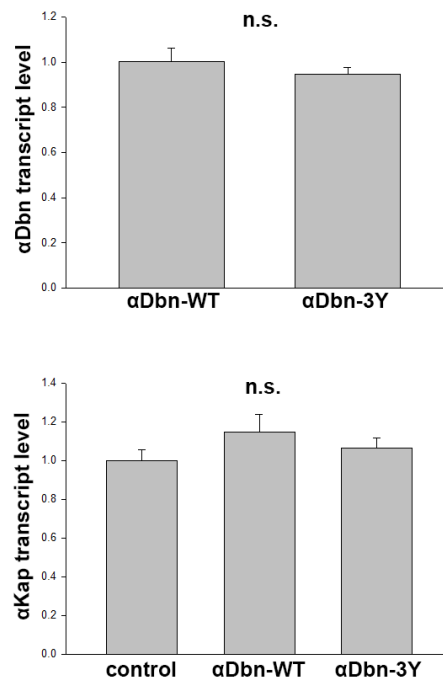


Figure 3.8 The regulation of phosphorylatable α -dystrobrevin on the expression of α dbn and α kap is not altered at the transcript level

Transfected α dbn and α kap have no difference at transcript levels between α dbn wildtype, nonphosphorylatable mutant, or control group.

The transfected cells were harvested, extracted RNA, and reverse-transcribed to cDNA, to examine the expression of α dbn and α kap at transcript levels. The error bars represent standard error, $n = 3$.

Next we tested whether the phosphorylatability of the three tyrosine residues is important for the stability of α dbn. To do this, HeLa cells were transfected with wildtype or mutant α dbn, treated with cycloheximide (CHX), which inhibits protein synthesis, and the lysates were analyzed by western blot. **Figure 3.9** shows that the expression levels of phosphorylatable wildtype α dbn were significantly higher than the nonphosphorylatable mutant, but the decay percentage from four-hour to eight-hour CHX treatment between wildtype and mutant α dbn1 were not significantly different (wildtype $66\% \pm 9.1\%$ and mutant $74\% \pm 9.6\%$, n.s., n=4). This experiment was lacking of a timepoint 0 (before CHX treatment), an improved experimental design is suggested in the discussion section. Although we could not draw conclusion from the current experimental data, we hypothesized that the expression difference between wildtype and mutant α dbn may result from the amount of protein remaining stable throughout cytosol (see discussion).

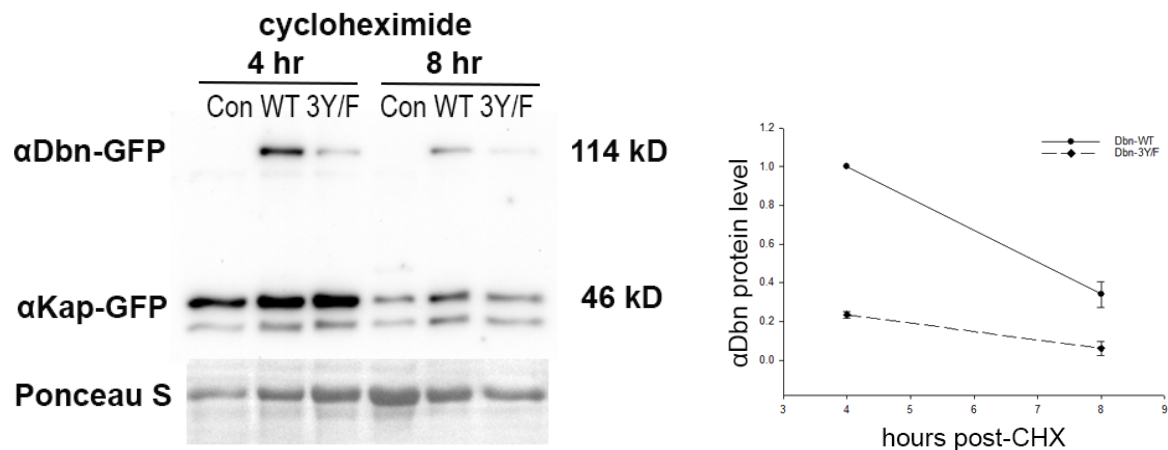


Figure 3.9 The cycloheximide treatment for 4 and 8 hours to analyze the protein decay percentage between wildtype and mutant α -dystrobrevin

Treatment with cycloheximide, which stop protein synthesis, has similar expression levels of α dbn and α kap as Figure 3.7.

The same experiment setting as Figure 3.7, and cells were treated with cycloheximide around post-transfected 18 hours. α Dbn signals were normalized to ponceau S signals and compared to post-CHX 4 hours, n = 4. The decay percentage between wildtype α dbn and non-phosphorylated α dbn was not significantly different.

Then we wanted to know the mechanism by which the nonphosphorylatable α dbn mutant is targeted to degradation. Cells were transfected with phosphorylatable tyrosine wildtype or nonphosphorylatable α dbn, and treated for 6 hours with either MG132 (carbobenzoxy-l-leucyl-l-leucyl-l-leucinal), a widely used proteasome inhibitor that can prevent degradation of many ER-associated degradation (ERAD) substrates (Lee and Goldberg 1998) or with leupeptin, a lysosome inhibitor. Lysates from transfected cells were subjected to western blots using GFP antibodies and their expression levels were analyzed. As shown in **Figure 3.10**, we found that in the presence of MG132, the expression levels of the mutant were significantly increased (93.1% of wildtype, n.s., SEM 0.046, n = 4), comparable to the phosphorylatable wildtype α dbn. However, in the presence of the lysosomal inhibitor leupeptin, the expression levels of α dbn mutant (α dbn-3Y/F-Leu 50.6% of wildtype, $p < 0.01$, n.s. to untreated mutant, SEM 0.043, n = 4) were quite similar to untreated cells (37.3% of wildtype, $p < 0.001$, SEM 0.033, n = 4) (**Figure 3.9**), indicating that the proteasome inhibitor MG132 was able to prevent degradation of α dbn mutant.

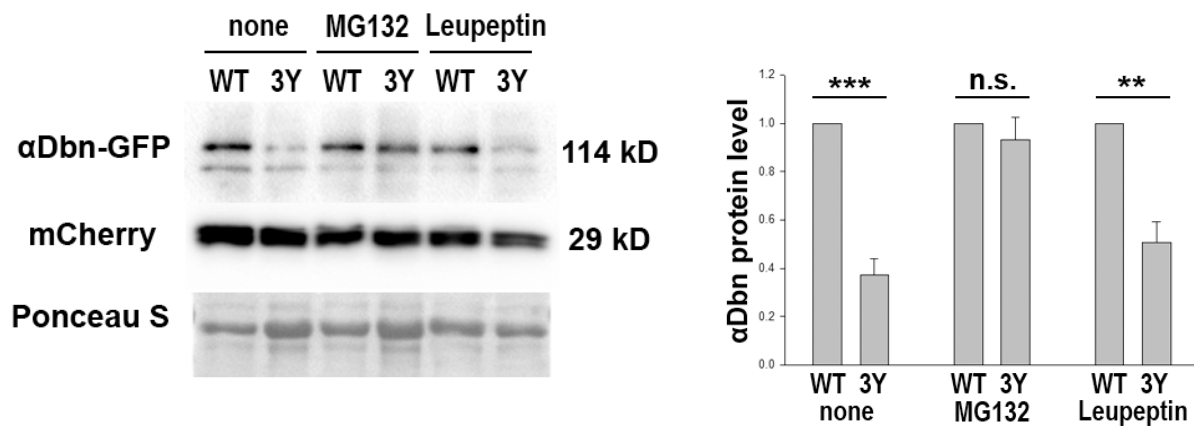


Figure 3.10 The low expression of nonphosphorylatable α -dystrobrevin mutant is increased by MG132 proteasome inhibitor

The treatment of the proteasome inhibitor MG132 for 6 hours is able to increase the expression of nonphosphorylatable α dbn mutant. The α dbn signals were normalized to ponceau S signals and compared with the same treatment group of the wild-type α dbn, n = 4.

3.4.5 α kap and α -dystrobrevin promote the formation of agrin-induced AChR clusters

We previously showed (**Figure 3.2**) that in heterologous HeLa cells, the presence of α kap and α dbn significantly enhanced the expression of AChR subunits. Here, we wanted to know whether the combined overexpression these molecules in muscle cells would increase the number and/or size of AChR clusters. C2C12 muscle cells were transfected with either α kap-mcherry (red), α dbn-GFP (green) or co-transfected with both α kap-mcherry and α dbn-GFP, and differentiated into myotubes. The differentiated myotubes were treated with agrin overnight, fixed, and bathed with fluorescently tagged BTX (Alexa-647) to label only surface AChR. Because transfection efficiency was only about 50%, on each plate, we were able to quantify the number, area, and length of AChR clusters on both transfected and non-transfected myotubes.

Figure 3.11 shows some representative fields and **Table 3.1** shows the quantification. The area and the length of AChR clusters on myotubes cells transfected with either α kap or α dbn was approximately double the number on untransfected myotubes. When comparing the cluster area and length between single transfected and double transfected groups, only the area of α dbn-GFP single transfected group reached a significant difference with α dbn/ α kap double transfected group ($P = 0.0154$). However, the area of untransfected clusters between these two groups also reached a significant difference ($P < 0.0001$). Due to the baseline difference (difference in differentiated levels between myotubes), we do not consider there is a significant difference between α dbn-GFP single transfected and α dbn/ α kap double transfected groups (see discussion). We also analyzed the percentage of agrin-induced AChR clusters that were more than $50 \mu\text{m}^2$ and $15 \mu\text{m}$ (which we called the large and long clusters respectively). Both were significantly increased by either α kap or α dbn, but again, transfection by both molecules had no additional effect. The results were recorded and quantified from three independent plates of each group.

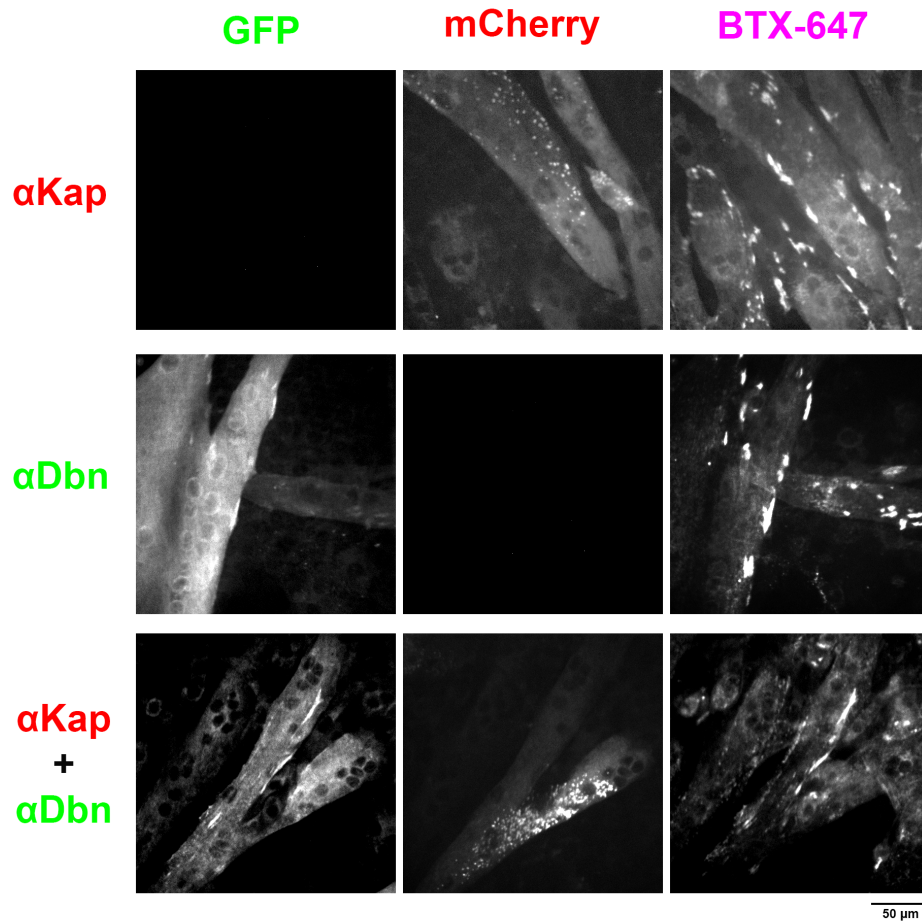


Figure 3.11 The length and area of AChR clusters are increased while exogenous α kap and/or α -dystrobrevin present in agrin-treated C2C12 myotubes

C2C12 were transfected with α kap-mCherry and/or α dbn-GFP in the stage of myoblasts and differentiated into myotubes. Differentiated myotubes were treated with agrin overnight and the superficial AChR clusters were labeled by BTX-Alexa 647. Since each plate of myotubes might contain differences in differentiated levels, the size of AChR clusters were compared within the same plate by transfected and non-transfected group.

Table 3.1 The area and the length of AChR clusters in overexpressed α Dbn-GFP and/or α Kap-mCherry groups

	αDbn-GFP		αKap-mCherry		αDbn + αKap	
Transfected (Tr) or Untransfected (Un)	Un	Tr	Un	Tr	Un	Tr
# of myotubes	48	68	53	33	45	20
# of clusters	137	476	180	221	190	190
clusters/myotube	2.9	7.0	3.4	6.7	4.2	9.5
cluster area (μm^2)	17.5 $\pm$ 1.7	31.9 $\pm$ 1.9	19.4 $\pm$ 1.3	35.1 $\pm$ 2.8	24.6 $\pm$ 2.4	41.2 $\pm$ 3.9
P-value for area	P < 0.0001		P < 0.0001		P = 0.0003	
cluster length (μm)	6.5 $\pm$ 0.4	9.9 $\pm$ 0.4	7.5 $\pm$ 0.4	10.4 $\pm$ 0.6	7.9 $\pm$ 0.4	11.2 $\pm$ 0.7
P-value for length	P < 0.0001		P = 0.0002		P < 0.0001	
clusters > 50 μm^2	8.03%	21.01%	6.11%	21.72%	9.47%	24.74%
clusters > 15 μm	4.38%	19.33%	10.00%	19.91%	8.42%	17.89%

The mean and the SEM of the cluster area and the cluster length, the percentage of the clusters larger than 50 μm^2 and longer than 15 μm , and the total observed clusters and myotubes were quantified and summarized in the table. Note that the size (both area and length) and number of clusters in every transfected group are significantly increased than the neighboring non-transfected group.

When comparing the cluster area and length between single transfected and double transfected groups, only the area of clusters in α dbn-GFP transfected group reached a significant difference with α dbn/ α kap transfected group (P = 0.0154) with a baseline difference, which the untransfected myotubes within these two groups reached a significant difference (P < 0.0001). The other comparison did not achieve significant difference in the length and the area clusters with α dbn/ α kap double transfected group: α dbn-GFP in length, P = 0.2388; α kap-mCherry in length, P = 0.3553; α kap-mCherry in area, P = 0.1980.

3.5 Discussion

The work here has demonstrated that α -dystrobrevin plays an important role in maintaining high expression of α kap and consequently AChRs' expression levels. The major findings from this work include: 1) α dbn and α kap are present within the same complex with AChR; 2) α dbn promotes the expression levels of α kap in a dose-dependent manner while the expression of α dbn is independent of α kap; 3) α dbn is required in α kap promoting AChR α accumulation; 4) α dbn or α kap enhance the formation of large agrin-induced AChR clusters; 5) the tyrosine phosphorylatability of α dbn is critical for the expression of α dbn and thus α kap accumulation. All together, these results demonstrate that the association of α dbn with α kap is essential not only for α kap expression levels but also for the formation of large AChR clusters.

This work shows that α kap is present in the same complex with α dbn. However, at AChR clusters, where α dbn is concentrated, α kap is not colocalized with AChR/ α dbn but expressed throughout cytosol. This suggests that α kap and α dbn may have a direct or indirect interaction in a vesicular complex during their intracellular trafficking, specifically in the ER where α kap has been found to be highly expressed in skeletal and cardiac muscles (Nori, Lin et al. 2003, Singh, Salih et al. 2009, Mouslim, Aittaleb et al. 2012). One unexpected result from this work is that α dbn promotes α kap accumulation in both heterologous non-muscle cells and cultured muscle cells in a dosage-dependent manner (**Figure 3.5**), whereas α kap has no effect on α dbn accumulation (**Figure 3.6**). This raises the question of how might α dbn increases α kap expression? One possibility is that α dbn may serve as a recruiting and signaling center that is important for the stabilization of α kap. For instance, it may recruit proteins such as deubiquitinases to protect α kap from degradation. Indeed, α dbn has been shown to bind to liprin- α 1, Usp9x and α -catulin independently of its phosphorylation status (Gingras, Gawor et al. 2016), which Usp9x is a deubiquitinase that in turn may increase α kap accumulation.

Previous studies have shown that tyrosine phosphorylation of α dbn1 plays an important role in

the maintenance of the structural integrity of the synapse (Grady, Akaaboune et al. 2003) as overexpression of a nonphosphorylatable α dbn-GFP in mice deficient of α dbn resulted in limited rescue of the postsynaptic apparatus. One possibility is that in the absence of the phosphorylatable tyrosine residues, only a few molecules of α dbn are trafficked to the synapse, which may in turn affect the stability of the postsynaptic density, including AChRs. This hypothesis is supported by our current results where we show that the phosphorylatability of α dbn is critical for α dbn and α kap expression (**Figure 3.7**). We also show that a nonphosphorylatable mutant α dbn has low expression that can be selectively increased by the MG132 proteasome inhibition (**Figure 3.10**), and there is no difference at the transcript level between the phosphorylatable wild-type α dbn and our nonphosphorylatable mutant (**Figure 3.8**). Therefore, it seems likely that the low expression of the nonphosphorylatable mutant α dbn is due to translational or post-translational regulation. However, the current cycloheximide experiment can only indicate that there is no significant difference between wildtype and mutant α dbn in the percentage of protein decay between four-hour and eight-hour treatments of the cycloheximide (**Figure 3.9**) (The proposed experiments to better define protein stability will be described in the following paragraphs). Based on these limited results, we speculate that the lower expression of nonphosphorylatable α dbn mutant could be due to less mutant α dbn protein being successfully trafficked, as the mutant still targets to plasma membranes when overexpressed in C2C12 myotubes (preliminary result) and to synapses when electroporated into muscles of wild-type mice (Grady, Akaaboune et al. 2003).

Many regulatory mechanisms could be responsible for the low expression of our nonphosphorylatable α dbn mutant, such as low efficiency of protein synthesis and protein folding, and lack of required associations or binding partners for protein modification and stability. In the case of our nonphosphorylatable α dbn mutant and with the finding that inhibition of the proteasome with MG132 is often associated with aggregation of misfolded protein (Christianson and Ye 2014), we speculate that the low expression of our mutant is a consequence of lowered protein folding efficiency. When MG132 inhibits the proteolytic activity of 26S proteasome, it also inhibits the elimination of misfolded proteins by the endoplasmic reticulum associated degradation (ERAD) pathway (Amm, Sommer et al. 2014, Sun and Brodsky 2018). The accumulation of potentially misfolded mutant α dbn could be cleared through increased time

and assistance from chaperones to fold successfully, but this would also result in more misfolded protein accumulating because MG132 inhibits the proteasome clearance mechanism, but does not inhibit translation. Another possibility is that the wildtype α dbn has certain binding associations required for its proper trafficking, which may depend on its phosphorylatability. A recent study identified several wild-type α dbn binding partners which associate with α dbn through both phosphorylation-dependent and phosphorylation-independent mechanisms (Gingras, Gawor et al. 2016). Phosphorylation-dependent binding partners include scaffold proteins Grb2 and SH3BP2, Rho guanine nucleotide exchange factor Arhgef5, phosphoinositide-3 kinase (PI3K), and the nonreceptor tyrosine-protein kinase Fyn. For instance, Grb2 has one SH2 and two SH3 domains to recruit multiple phosphotyrosine enriched proteins for signaling cascades (Skolnik, Lee et al. 1993) and mediates protein trafficking (Raychaudhuri and Mukhopadhyay 2010, Rajaram, Arnett et al. 2017). These potential binding partners could provide further support to the hypothesis of α dbn as a master regulator that brings in several partners to sustain α kap and AChRs during trafficking.

Proposed experiments to better define protein stability

To better define protein stability, the cycloheximide experiment could be extended as follows. First, it must be confirmed that the dose of cycloheximide blocks protein synthesis sufficiently, which can be demonstrated by two methods. One method would use a high-powered laser to photo-bleach the transfected GFP in the parallel transfected plates (Lippincott-Schwartz, Altan-Bonnet et al. 2003), and observe recovery of the fluorophore in the control plate (vehicle is water) versus the cycloheximide treated plate, where there should be no recovery. The other method would use short half-life endogenous proteins, such as cyclin B (Choi and Zhu 2012), and measure their rate of decrease/disappearance to ensure protein synthesis inhibition.

The second way in which the cycloheximide experiments can be extended is to change the experimental window of cycloheximide treatments. Preliminary experiments indicate that adding cycloheximide after 4 – 6 hours of transfection could interfere transfection efficiency and induce toxicity for HeLa cells, so the experimental window could be delayed to 18 – 42 hours post-transfection. To be specific, 18 hour-post-transfection is time 0 at which cycloheximide

treatments start and cells can be harvested at either 4 or 6 hour intervals up till 24 hours post-treatment, as 24 hours of cycloheximide treated is consider toxic (Kao, Wang et al. 2015). Protein should be extracted from cells after sonication and centrifugation, and the protein lysates should be titrated with the BCA protein assay to achieve similar total protein concentration in each condition. After western blotting analysis, the protein expression at multiple time points can be plotted as a function of exponential decay to determine protein half-life and stability.

Crosstalk between proteasomal and lysosomal degradation

Ubiquitin-dependent proteasomal degradation and autophagolysosomal degradation are two major systems to eliminate unwanted proteins in cells. Ubiquitin acts as a substrate-specific recognition of cytosolic and secretory proteins for proteasomal degradation, and autophagy uses double-layered membrane to engulf unwanted proteins for lysosomal degradation. Although previous studies have considered proteasomal and lysosomal degradation as two independent degradation pathways, recent studies revealed that inhibition of either pathway selectively upregulates the other degradation pathway to reduce accumulated proteins and cellular stress (Korolchuk, Menzies et al. 2010). Our 6-hour MG132 treatment did not observe a compensatory effect between proteasomal and lysosomal degradation, other work has shown that longer treatments, like 12-24 hours of MG132 induced LC3B expression, a key marker of autophagy (Ding, Ni et al. 2007). Ubiquitin modification is known for proteasomal degradation, where the lysine-48 (K-48) polyubiquitin chain is the most frequent ubiquitin modification for proteasomal degradation, but other types of ubiquitin modifications target substrates for different fates. For example mono-ubiquitination on membrane proteins triggers endocytosis for possible reinsertion into the membrane or lysosomal degradation. This is also evidenced by the second most abundant polyubiquitin chain K-63, which has less proteasomal accessibility than K-48 (Jacobson, Zhang et al. 2009) and preferentially associates with autophagolysosomes (Tan, Wong et al. 2008). Therefore, ubiquitin is a common factor for different degradation pathways (Clague and Urbe 2010), and p62 has been shown to shuttle ubiquitin-conjugated proteins between the proteasome and the autophagolysosome (Cohen-Kaplan, Ciechanover et al. 2016, Liu, Ye et al. 2016). The crosstalk and compensatory effect between ubiquitin- dependent proteasome and autophagolysosome systems are crucial for protein quality control and dynamics for cellular functions (Ji and Kwon 2017, Nam, Han et al. 2017). interruption of these

degradation systems leads to increasing cellular stress due to protein waste accumulation, a common mechanism involved in cancer and neurodegenerative diseases (Cecarini, Bonfili et al. 2012, Wang, Yu et al. 2013, Kim, Park et al. 2018).

There is evidence of a relationship between the degradation systems and the α dbn and α kap complex that support AChRs. Previous studies have shown that overexpression of α kap in heterologous cells substantially increases the amount of unassembled AChR α by the protection from a ubiquitin-dependent proteasomal degradation, while knockdown of α kap in muscle cells significantly reduces AChR expression (Mouslim, Aittaleb et al. 2012, Martinez-Pena, Aittaleb et al. 2015). Similarly, the knockout of α dbn in mice dramatically reduces AChR levels (Grady, Grange et al. 1999, Bunnell, Jaeger et al. 2008). Consistent with these observations, the overexpression of either α kap or α dbn in C2C12 muscle cells has significantly increased the number and the size of AChR clusters (**Figure 3.11**). However, when both α kap and α dbn are co-expressed in cultured muscle cells, the size of AChR clusters is not much different from AChR clusters on muscle cells overexpressing either α kap or α dbn (**Figure 3.11, Table 3.1**)(Note that α dbn-GFP transfected group reached a significant difference in the cluster area with the double transfected group, but since there was also significantly different in untransfected cluster area between these two groups, we do not consider the area of clusters different). One possible explanation of the lack of difference in size of AChR clusters between the above treatments is that when α dbn is overexpressed, the endogenous expression of α kap is also significantly increased (See **Figure 3.4 and Figure 3.5**), promoting AChR expression, similarly observed when α kap is overexpressed.

Although it is not clear how the phosphorylatability of α dbn maintains the expression of α kap and ultimately the high density of AChRs at synaptic sites, it is noteworthy to further investigate the association of α kap/ α dbn complex with other postsynaptic scaffold proteins that are required for the formation of the neuromuscular junction.

Current working model

The possible stabilization between α dbn – α kap – AChR α could take place in endoplasmic reticulum (ER) for several reasons: 1) α kap is expressed dominantly in ER (Nori, Lin et al. 2003, Singh, Salih et al. 2009, Mouslim, Aittaleb et al. 2012), 2) the AChR α single subunit is unassembled and accumulated in ER while the AChR α stabilization by α kap – α dbn is observed, and 3) α dbn deficient mice have only 30% of AChR density at NMJs compared to wildtype mice, and this decrease is in both the newly inserted and recycled AChR pools (Grady, Grange et al. 1999, Grady, Zhou et al. 2000, Grady, Akaaboune et al. 2003). Based on what we have observed, we hypothesize that 1) α dbn stabilizes α kap; 2) α kap serves a targeting and anchoring role for the α dbn complex to the membrane while also closely interacting with AChR α ; based on previous work demonstrating that α kap anchors CaMKII to the membrane (Bayer, Harbers et al. 1998); 3) α dbn recruits potential binding partners, such as Usp9x, a deubiquitinase (Gingras, Gawor et al. 2016) to prevent AChR α from ubiquitin-dependent proteasome degradation (Mouslim, Aittaleb et al. 2012). These interactions would stabilize and sustain AChR α throughout the ER trafficking route, and the working model is shown as **Figure 3.12**. Our preliminary data supports this hypothesis where we show that when Usp9x is cotransfected with α dbn/ α kap/AChR α , the expression of AChR α was further increased compared to α kap with AChR α or AChR α alone in HEK cells (with endogenous α dbn1 expressed) (**Figure 3.13**). However, there is no evidence to support direct binding within the α dbn – α kap – AChR α complex. Which other potential interaction partners may be involved, and how the proteins in this complex associate, sequentially or through intermediate association stages still remains to be understood.

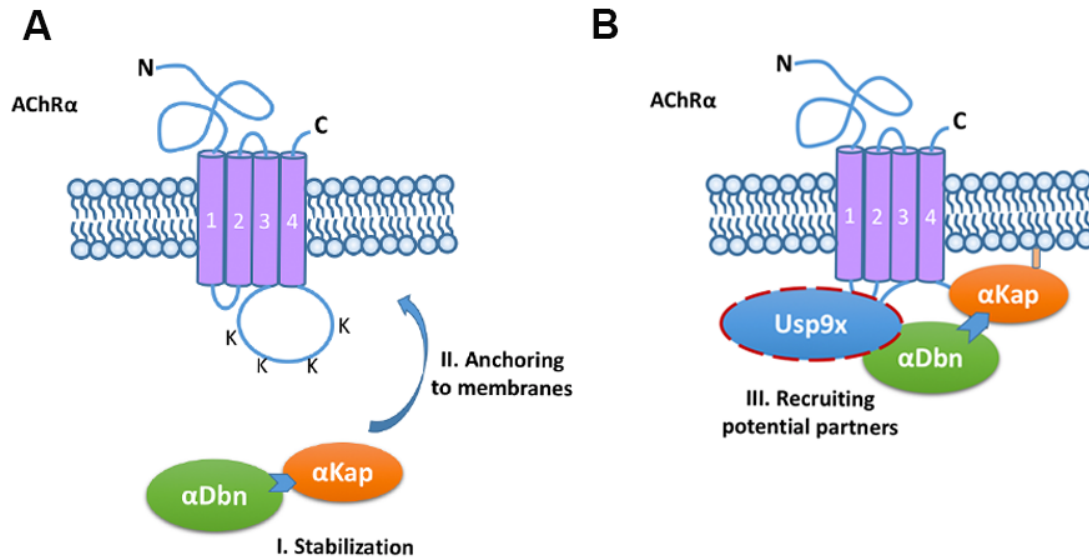


Figure 3.12 The working model for α dbn and α kap promoting the expression levels of AChR α

The current working hypothesis: **(A)** First, α dbn enhances the expression levels of α kap. Second, α kap anchors α dbn closer to AChR α at membrane sites. **(B)** α Dbn recruits potential binding partners, such as Usp9x, a deubiquitinase to promote AChR α expression levels. This recruitment could happen before or after α kap anchoring to membranes.

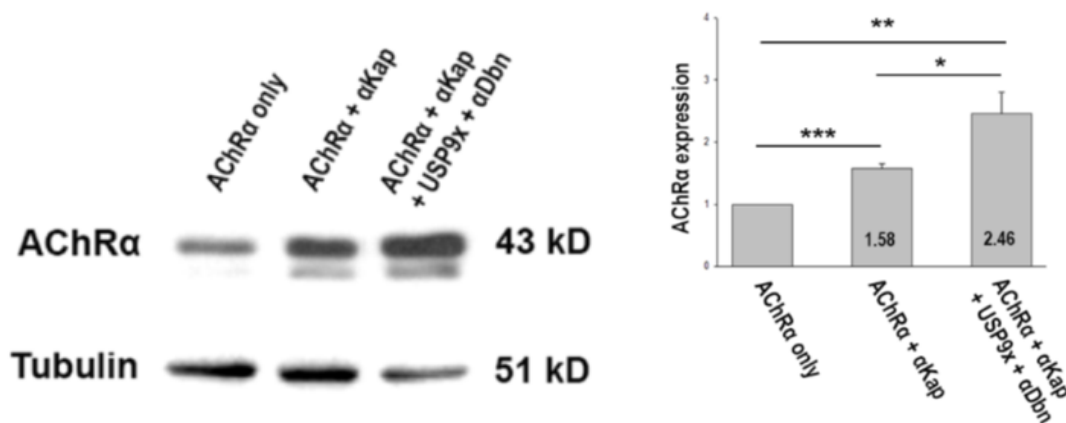


Figure 3.13 Usp9x- α dbn complex promotes the AChR α expression levels in HEK cells

While Usp9x and α dbn are co-transfected with α kap and AChR α , AChR α expression level is further enhanced than AChR α with α kap or AChR α alone condition in HEK cells. The preliminary data suggest that Usp9x could be involved in the effect of α dbn promoting α kap thus AChR α accumulation.

CHAPTER 4

4. Conclusion, discussion, and future works

Research on neuromuscular junctions has shed light on many receptor-associated proteins that play individual roles in synaptic formation, maturation, and maintenance, but there is limited research on the interactions and signaling crosstalks between receptor-associated proteins. This dissertation explored several aspects of interactions between AChR-associated proteins in sustaining AChR postsynaptic homeostasis. Chapter II revealed a new active role of AChR in targeting rapsyn, an effector necessary for clustering AChRs, to synaptic sites. Chapter III discovered α -dystrobrevin, an intracellular DGC component, which works in a dosage-dependent manner to stabilize α kap, a muscle-specific anchoring protein that protects AChR from degradation, and together, α -dystrobrevin and α kap sustain AChR stability. Appendix A explored an α -dystrobrevin binding partner, the deubiquitinating enzyme Usp9x, which promotes the stabilizing effect of α kap/ α -dystrobrevin on AChRs and its expression is highly correlated with the maturation of synapses. Appendix B investigated the relationship between α -dystrobrevin and α -syntrophin, two essential DGC components, and found that α -dystrobrevin and α -syntrophin work within the same pathway to sustain AChR postsynaptic properties. This work elucidates complicated protein interactions at neuromuscular junctions, and hopefully, suggestions for avenues to develop therapeutic targets for the NMJ related diseases. The remainder of this chapter is organized as follows. The first part provides highlights and discussion of chapter II, which investigated the interaction between rapsyn and AChR. The second part discusses α kap and its relationship with DGC components in maintaining AChRs to cover the research area of chapter III, and appendix A and B. Both parts provide some potential research questions for future direction.

Rapsyn at neuromuscular junctions and beyond

Rapsyn has been a focus of interest among scientists beginning when the discovery of its role in clustering AChRs. Rapsyn was first identified by isolation with nAChR from the postsynaptic membranes of *Torpedo* electric organ, and named after receptor-associated protein at synapses (Frail, McLaughlin et al. 1988). Rapsyn clusters AChR in response to agrin-Lrp4-MuSK signaling pathway, and is crucial for neurotransmission at neuromuscular junctions. The crucial role of rapsyn has been shown in rapsyn deficient mice, which cannot survive for more than a few hours after birth due to breathing difficulty (for review, please go to section 1.3-1, synapse formation in NMJ of chapter I introduction). Due to rapsyn's critical role in AChR clustering, I wondered how rapsyn, as a cytoplasmic protein, targets synapses successfully. In chapter II, I incorporated rapsyn domain deletion constructs to examine each rapsyn domain functionality. When rapsyn lacks the coiled-coil domain, which directs interaction with AChR, it loses its synaptic localization. It was not feasible to test a no rapsyn or AChR condition in living mice, so to test if AChR and rapsyn have mutual effects in synaptic targeting, I used C2C12 mouse muscle myoblasts (no AChR expression) and primary myoblasts derived from rapsyn embryonic mutant (neither rapsyn nor AChR). I found that the synaptic targeting effect is in one direction: AChR is essential for rapsyn targeting to synapses, but AChR synaptic distribution is not affected, regardless of rapsyn expression. To generalize, AChR carries rapsyn to meet the demand of rapsyn's clustering function at synaptic sites. This targeting and functionality relationship between AChR and rapsyn is amazingly interesting.

Another noteworthy result in this study is that when the myristoylation glycine 2 site has been mutated, some rapsyn still targets synapses, but the rest is shuttled back to the nucleus (Figure 2.1 B, asterisk sites). Myristoylation is a common posttranslational modification that uses lipidation for membrane association. The absence of a myristoylation site leads to a partial protein reshuttling back to the nucleus, which suggests a link between membrane anchoring and nuclear import machinery (Macara 2001). Although no glycine 2 mutation has been reported in human clinical data, it is known that most of the rapsyn mutations are found in TPR, coiled-coil, and RING-H2 domains (Ohno, Engel et al. 2002, Dunne and Maselli 2003, Maselli, Dunne et al. 2003, Milone, Shen et al. 2009), suggesting that the rapsyn glycine 2 mutation provides either a

novel pathology or an artificial case for study of nuclear import mechanism. To explore this, another potential study of rapsyn reshuttling back to the nucleus is the possibility of controlling gene expression, which has been discussed in the discussion of chapter II.

Recent work has reported the discovery of a new enzymatic function in rapsyn. Specifically, the RING-H2 domain of rapsyn exhibits E3 ubiquitin ligase activity (Li, Cao et al. 2016) for both ubiquitination and neddylation. This finding reveals an unknown function in a known receptor-associated protein, and further emphasizes the influence of the ubiquitin proteasome system (UPS) on AChR homeostasis at synapses. To extend this concept it would be worthwhile, to systematically screen the domain homology between critical components in the UPS and AChR-associated proteins, such as searching for E3 ligase enzymatic domain RING or HECT homology within DGC members and other synaptic proteins.

Although studies of rapsyn have been narrowly focused on AChRs, rapsyn is highly expressed in cells that do not have AChR expression. This is a new discovery from the Akaaboune lab, specifically, we found a novel and AChR-independent role of rapsyn *in vitro*. Rapsyn expresses at junctional sites between lysosomes, not only clusters lysosomes, as well as affects exocytosis (Aittaleb, Chen et al. 2015). Lysosomes are critical digestive organelles in cells, that have a relationship with age-related neurodegeneration (Zhang, Sheng et al. 2009, Lie and Nixon 2019). In light of this, it would be interesting to explore rapsyn functionality outside of the neuromuscular junction, especially in regulating lysosome dynamics in the brain for potential therapeutic targets of neurodegenerative disorders.

α Kap and AChR-associated proteins in the postsynaptic network

α Kap, a non-kinase muscle-specific anchoring protein splicing from calcium/calmodulin kinase II, protects AChRs from ubiquitin-dependent proteasome degradation. When α kap is cotransfected with AChR α subunit in the human embryonic kidney (HEK cells) heterologous system, AChR α is less ubiquitinated and has higher stability. This AChR α enhancement can also

be achieved by MG132 treatment, which is a potent proteasome inhibitor, or via mutation of the four lysine residues of the cytoplasmic-loop of the AChR α , in which AChR α cannot be ubiquitinated (Mouslim, Aittaleb et al. 2012). To explore this further, we investigated α kap function in living mice. We found that when α kap is knocked down by shRNA in the sternomastoid muscles of wild-type mice, the AChR postsynaptic density is reduced with higher turnover rates, leading to impairment of the synaptic structure (Martinez-Pena, Aittaleb et al. 2015). Based on these robust scientific findings, α kap is critical for the AChR postsynaptic stability, but our understanding of how α kap works in the postsynaptic protein network is limited.

To investigate how α kap interacts with the postsynaptic proteins, within the postsynaptic protein network, one protein triggered my attention: α -dystrobrevin. It is known that α -dystrobrevin deficient mice have perforated synapses similar to the α kap knockdown ones, and HEK cells have endogenous α -dystrobrevin-1 (the major α -dystrobrevin isoform) expression. In chapter III, I reported that α -dystrobrevin-1 has a dose-dependent enhancement on α kap stability in HeLa cells (no α -dystrobrevin nor α kap expression) and C2C12 myoblasts (with endogenous α -dystrobrevin and α kap expression). When α -dystrobrevin-1 lacks its three tyrosine phosphorylation sites at C-terminal, α -dystrobrevin has lower stability itself and has lower α kap stability. When overexpressed α -dystrobrevin-1 and α kap to C2C12 myoblasts and differentiated into myotubes, it is more common to observe AChR clusters that are bigger than the non-transfected myotubes within the same culture dish. Together, α -dystrobrevin-1 sustains α kap in a dose-dependent manner and further supports AChR clustering *in vitro*.

Previous research explored α -dystrobrevin-1 binding partners (Gingras, Gawor et al. 2016). One of the α -dystrobrevin-1 binding candidates is Usp9x. Usp9x is a deubiquitinating enzyme (DUB), which removes ubiquitin from substrates. To further explore this, I examined the developmental pattern of Usp9x in wild-type mice. I found that Usp9x expression increases with synaptic maturation, reaching a maximum around P20, after which it gradually decreases. Adult P60 mice have barely detectable levels of Usp9x expression compared with the expression when younger. Usp9x also enhances the effect of α -dystrobrevin sustaining α kap *in vitro*. (All the preliminary

data related with Usp9x have been organized in appendix A.) With the finding of an α -dystrobrevin-1 new binding partner, Usp9x, there is one more puzzle related to the complex AChR postsynaptic protein network. In this regard, it would be interesting to explore new binding partners of the AChR-associated proteins, such as α kap, rapsyn, and DGC components, which would provide opportunities for discovering new proteins participating in AChR postsynaptic dynamics.

The relationship between α Kap and AChR-associated proteins is also discussed in appendix B. While trying to distinguish the interaction between α -dystrobrevin and α -syntrophin subcomplex in sustaining AChRs, my pilot study suggested that α -syntrophin has a synergistic effect in α -dystrobrevin sustaining α kap stability. This synergistic effect on α kap stability is worth investigating in different aspects. First, it would be interesting to examine whether this synergistic effect occurs through better structural support or a signaling pathway. Second, it would be worthwhile to determine whether this effect is specific to α -dystrobrevin and α -syntrophin subcomplex or a more general phenomenon to AChR-associated proteins. Through these in-depth investigations, the interrelationship of α kap within the whole AChR postsynaptic protein network will be more clearly elucidated, and further contribute to the framework of NMJ related therapeutic targets.

Appendix A

A A deubiquitinating enzyme, Usp9x, sustains AChRs through the α -dystrobrevin- α kap pathway

A.1 Introduction

AChRs are highly dynamic at postsynaptic neuromuscular junctions, and the healthy turnover of AChRs is critical for synaptic transmission. The turnover of many neurotransmitter receptors is regulated under the ubiquitin proteasome system (UPS), such as AMPA receptors and NMDA receptors (for review, please go to section 1.4-2, receptor homeostasis and ubiquitination). To investigate the dynamics of AChRs at postsynaptic sites, we turned into the link between AChRs and UPS. Research has shown that AChR is under ubiquitin regulation, which mutating lysine sites at the cytoplasmic loop of AChR α subunit enhances AChR α stability *in vitro*. Besides, an AChR-associated protein, α kap, protects AChRs from ubiquitin-dependent proteasome degradation (Mouslim, Aittaleb et al. 2012). However, α kap has no homology to ubiquitin-related enzymes nor activities reported. To this end, we explored the enzymatic candidates of UPS expressing at neuromuscular junctions.

One enzymatic protein of UPS caught our attention is Usp9x. Usp9x is a deubiquitinating enzyme (DUB) removes diverse ubiquitin chains on substrates (Han, Foster et al. 2012), and binds to a critical AChR-associated dystrophin glycoprotein complex component, α -dystrobrevin, in a phospho-independent manner (Gingras, Gawor et al. 2016). The interaction between Usp9x and α -dystrobrevin led us to investigate the function of Usp9x at neuromuscular junctions.

In this study, I found that Usp9x is highly expressed during developmental stages (P0 - P30) compared to adulthood (P60) in the hindlimb muscles of the wild-type mice. Usp9x expression is independent of α -dystrobrevin regulation, as Usp9x expression has no significant difference between α -dystrobrevin deficient and wild-type mice. Moreover, Usp9x enhances α kap stability via α -dystrobrevin *in vitro* (chapter III). Combined Usp9x, α -dystrobrevin, and α kap, they sustain AChR stability *in vitro*. Altogether, this research discovered Usp9x, a novel deubiquitinating enzyme, sustain AChRs through the α -dystrobrevin- α kap pathway.

A.2 Materials and Methods

Mouse sample preparation

The hindlimbs of mice were harvested, one hindlimb was for protein lysates, and the other was immersed in TRIzol (15596018, Ambion by life technologies) for RNA extraction. For protein lysates, the hindlimb muscle was removed, homogenized on ice in a buffer containing 50 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, and protease inhibitors. Wild-type, and α -dystrobrevin and α -syntrophin single and double deficient mouse models were the same strains from the double knockout study (chapter VI). All animal methods and usage are approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

qPCR

cDNA was prepared by reverse transcription (SuperScript III, 18080-044, Invitrogen) from the RNA muscle samples. The qPCR reagent was Radiant Green 2X qPCR Mix Hi-ROX (QS2020) purchased from Alkali Scientific Inc. The qPCR machine was StepOnePlus Real-Time PCR System by Thermo Fisher Scientific, and the software was StepOne Software, version 2.3. The qPCR primers were designed by NCBI primer blast or PrimerBank database. The internal control of gene expression was GapdH.

The primer sets were: Usp9x-Forward TCCAACAGAATCAGACTTCATCG, Usp9x-Reverse TGGAAATGCAGGTTTCCTCATCT, Fbw7-Forward TGCTGGTCAGTCGTAGCATC, Fbw7-Reverse GGTAGGCGAGTGACAGACAG, GapdH-Forward AACTTTGGCATTGTGGAAGG, and GapdH-Reverse GGATGCAGGGATGATGTTCT.

Antibody information

Anti-Usp9x (A301-351A, 1:4000, Bethyl) and anti-Fbw7 (A301-721A, 1:4000, Bethyl) for mouse muscle protein expression were generous gifts from Dr. Axel Behrens lab at the Francis Crick Institute (Khan, Carvalho et al. 2018). Anti-Usp9x (MABE352, 1:8000) for cell culture was purchased from Millipore. The other antibodies were α -Dystrobrevin (610766, 1:4000, BD Biosciences), α -Syntrophin (PA1-25013, 1:2000, Invitrogen), α Kap (CaMKII α A-1, sc-13141, 1:2000, Santa Cruz), GFP (A6455, 1:5000, Invitrogen), GapdH (hGAPDH-2G7-s, 1:2000, DSHB), and Tubulin (AA4.3-s, 1:5000, DSHB).

Usp9x construct

Usp9x DNA construct was a generous gift prepared by Dr. Qing Zhang lab at the University of North Carolina School of Medicine (Zheng, Zhai et al. 2014), and was obtained permission from Dr. Feng Cong lab at the Novartis Institutes for BioMedical Research (Xie, Avello et al. 2013).

shUsp9x HeLa stable cell lines, cell culture, and transfection

Two HeLa stable cell lines were knockdown Usp9x specifically at different locations (#61 and #64). These two cell lines were generous gifts from Dr. Chang-Wei Liu lab at the University of Colorado School of Medicine (Han, Foster et al. 2012, Han, Foster et al. 2016). HeLa cells were a generous gift from Dr. JK Nandakumar at the University of Michigan. HEK 293T cells were purchased from American Type Culture Collection (Manassas, VA). The HeLa stable cell lines, HeLa and HEK 293T cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (11995-065; Gibco, NY) and supplemented with 10% fetal bovine serum (FBS) (S11050,

Atlanta Biologicals, GA). All cells were grown in the presence of 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C, under a humidified atmosphere and 5% CO₂. The transfect procedure was described in our previous studies (Aittaleb, Chen et al. 2015).

A.3 Results

DUB Usp9x is highly expressed during development in the muscle of wild-type mice

To explore enzymatic candidates of UPS to regulate AChR homeostasis at neuromuscular junctions, we harvested the hindlimb muscles from wild-type mice to examine transcript and protein expression. Our candidate is Usp9x, which has directly associated with α -dystrobrevin-1 (Gingras, Gawor et al. 2016), an essential DGC component support AChRs via its structural and signaling functions. Usp9x is a deubiquitinating enzyme (DUB), and plays an essential regulatory role in many cancers. A recent study in colorectal cancer showed that Usp9x antagonizes Fbw7, which is a E3 ubiquitin ligase (Khan, Carvalho et al. 2018). Since E3 and DUB both serve as substrate recognition to add or remove ubiquitins, we examined the transcript and protein expression levels of Usp9x and Fbw7.

As in Figure A.1, both Usp9x and Fbw7 were expressed in hindlimb muscles. Usp9x had the highest transcript and protein expression level around P10 and P20, respectively, with a generally high protein expression during developmental stages (P0 – P30). Similarly, Fbw7 had the highest transcript and protein expression level around P10 and P20, respectively. However, Fbw7 protein expression levels were gradually increased to a peak at P20 and decreased afterward. Interestingly, both Usp9x and Fbw7 had relatively low protein expression level in the P60 adult stage. The highly expressed pattern specifically around P10 to P20 suggested Usp9x and Fbw7 may play in roles during synaptic maturation. Due to the specific transcript and protein expression of Usp9x and Fbw7 in the hindlimb muscles, we further investigated if these proteins regulate the stability of AChR or AChR associated proteins.

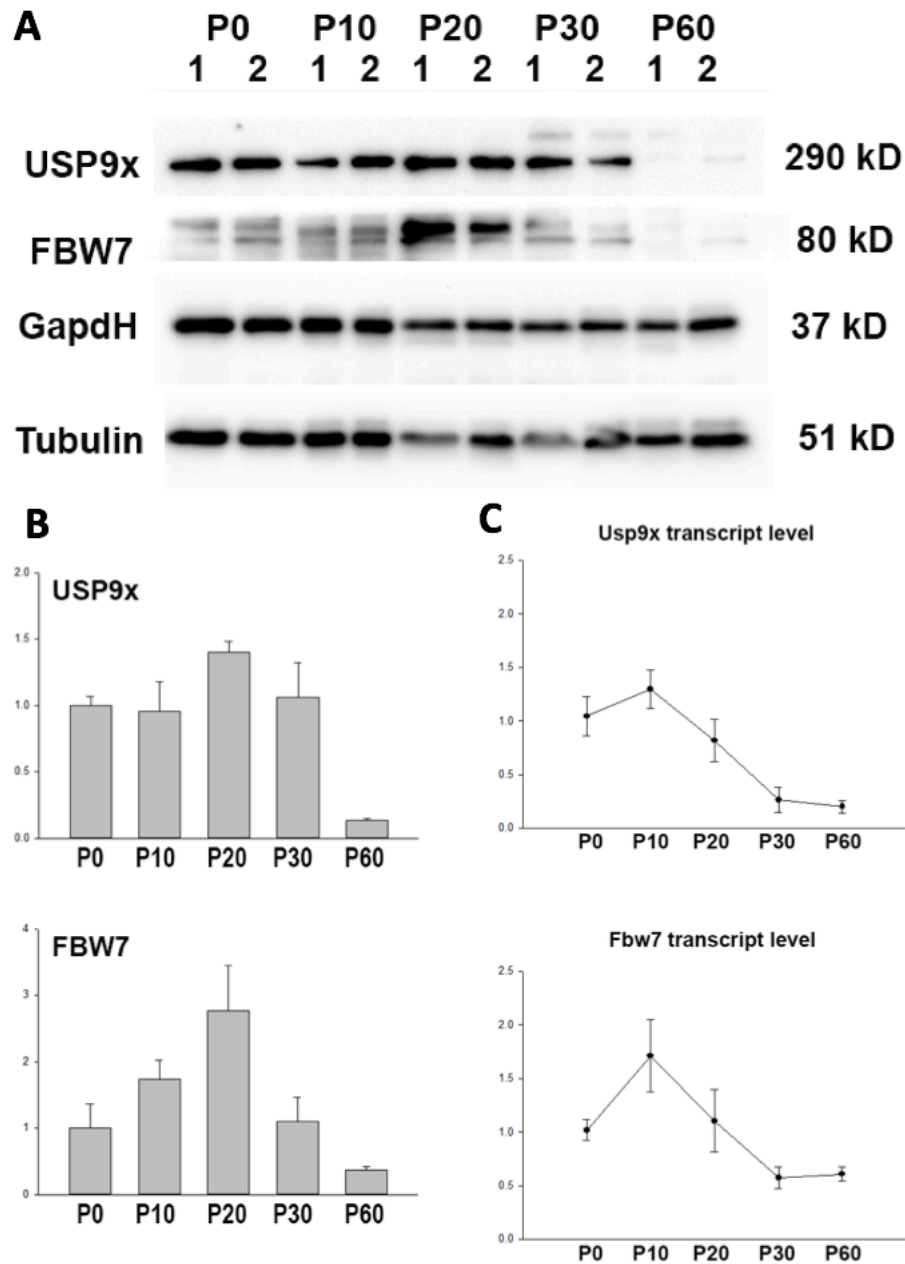


Figure A.1 Usp9x and Fbw7 expression in wild-type mice during development.

(A) Protein expression levels from P0 to P60 wild-type mice, two mice per time point were illustrated in the blot. Usp9x showed high protein expression level during P0 – P30, which reached a peak at P20, and had low protein expression in adult stage (P60). Fbw7 showed a gradual increase in protein expression levels from P0 – P20, and decrease afterward. Both Usp9x and Fbw7 had relatively low expression levels in the adult stage. (B) Quantification of protein expression levels from Western blots. (C) Usp9x and Fbw7 transcript levels were obtained from qPCR. Each time point has 4 – 5 mice. The peak of transcripts happened at P10, and gradually decreased afterward.

Usp9x expression is independent of α -dystrobrevin mutation

Since Usp9x binds to α -dystrobrevin, we wondered if Usp9x expression is dependent upon α -dystrobrevin. α -Dystrobrevin is a cytoplasmic protein within the dystrophin glycoprotein complex (DGC), which stabilizes AChR clusters by linking from the intracellular cytoskeleton to the extracellular matrix. Another DGC cytoplasmic component, α -syntrophin, forms a subcomplex with α -dystrobrevin and has a sequential effect stabilizing AChRs with α -dystrobrevin within the same pathway (appendix B). Therefore, we tested if Usp9x expression level is altered while α -dystrobrevin and α -syntrophin are lost. In α -dystrobrevin and α -syntrophin single and double deficient mouse models, the Usp9x protein levels were fluctuated without a clear association with α -dystrobrevin or α -syntrophin genetic deficiencies (Figure A.2). The similar pattern was observed in Fbw7 as well. Interestingly, Usp9x and Fbw7 had a positive correlation in protein expressions. The data suggested that Usp9x and Fbw7 expression were independent of α -dystrobrevin and α -syntrophin mutations, but there was a positive correlation between Usp9x and Fbw7 expressions in muscles.

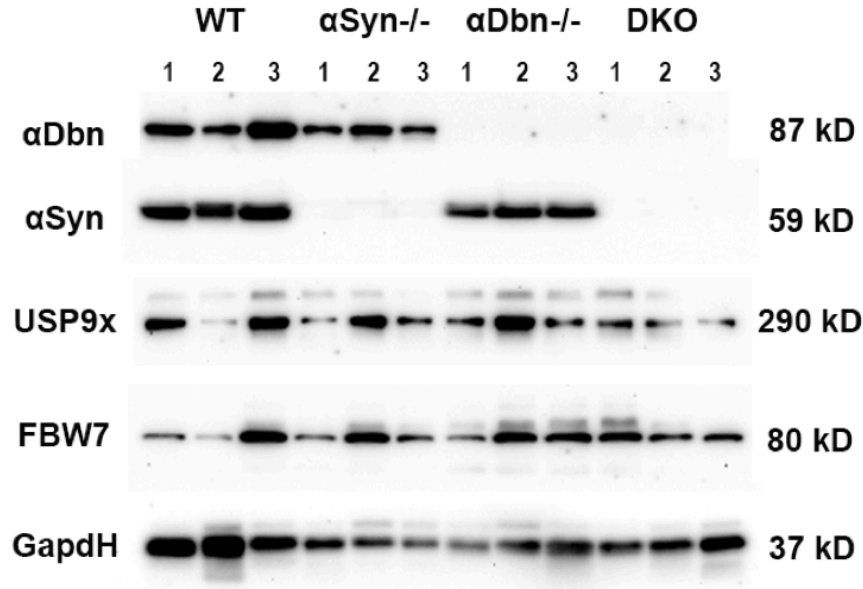


Figure A.2 The protein expressions of Usp9x and Fbw7 do not depend on α -dystrobrevin or α -syntrophin.

There were no specific protein expression pattern of Usp9x or Fbw7 observed along with α -dystrobrevin and α -syntrophin single and double deficient mouse models. Three mice per genotype were illustrated in the blot.

Usp9x sustains α kap stability via α -dystrobrevin

We then examined Usp9x sustains the stability of α -dystrobrevin and AChR-associated proteins. Recently we found that α -dystrobrevin enhances α kap in a dose-dependent manner *in vitro* (chapter III). α Kap is a non-kinase alternative splicing product from calcium/calmodulin kinase II (camk2) and serves as a muscle-specific anchoring protein. α Kap protects AChRs from ubiquitin-dependent proteasome degradation *in vitro* (Mouslim, Aittaleb et al. 2012). When α kap is knockdown in living mice, perforated synapses and higher AChR turnover rate are observed (Martinez-Pena, Aittaleb et al. 2015). α Kap has shown to regulate AChR in a ubiquitin-dependent fashion and α kap stability is enhanced by α -dystrobrevin, all of the evidence led us to examine the relationship between Usp9x and α kap.

Usp9x was highly expressed in every cell line we have tested (including C2C12, HEK, HeLa, and Cho cells, data not shown). Therefore, we tested the relationship between Usp9x and α kap in the HeLa stable cell lines with shRNA-Usp9x knockdown effect (Han, Foster et al. 2012). These two shUsp9x cell lines targeted Usp9x at different gene locations, and achieved Usp9x knockdown significantly (Figure A.3.B). Under the low Usp9x background, there was slightly increased of α kap stability via α -dystrobrevin, and after Usp9x level was rescued, α kap stability was increased to 3.4 fold (Figure A.3.A). To sum up, we found that Usp9x sustains α kap stability via α -dystrobrevin.

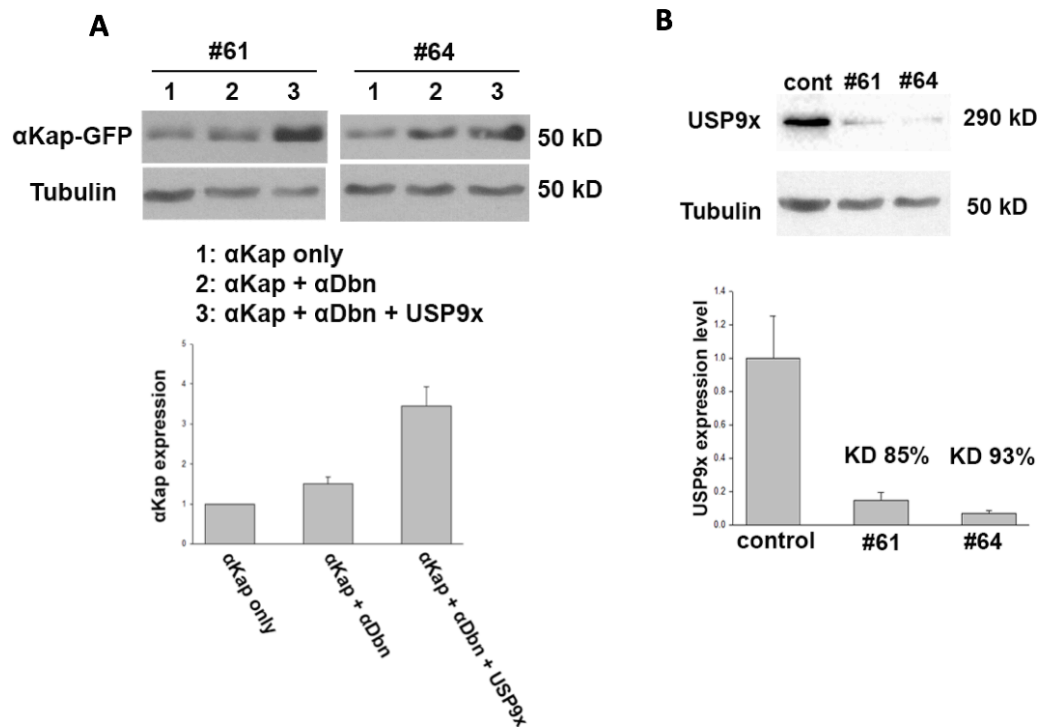


Figure A.3 αKap stability is enhanced by α-dystrobrevin and Usp9x in shUsp9x HeLa cell lines.

(A) αKap stability was increased when co-transfected with α-dystrobrevin alone, and further increased when the Usp9x level was rescued in shUsp9x cell lines. #61 and #64 were two independent shUsp9x cell lines, which targeted Usp9x at different locations. (B) To ensure the Usp9x knockdown performance of the shUsp9x stable cell lines, the parallel untransfected dishes were harvested with the transfected experiments. The (A) experiments knockdown performances were 85% and 93% decreased in #61 and #64, respectively.

Usp9x sustains AChRα stability through the α-dystrobrevin-αkap pathway

It is interesting to know if Usp9x effect can ultimately support AChR stability. The pilot experiments in HEK cells indicated that αkap, α-dystrobrevin, and Usp9x alone had a similar stabilizing effect on AChRα (1.7 fold), and altogether further sustained AChRα stability to 2.6 fold (Figure A.4).

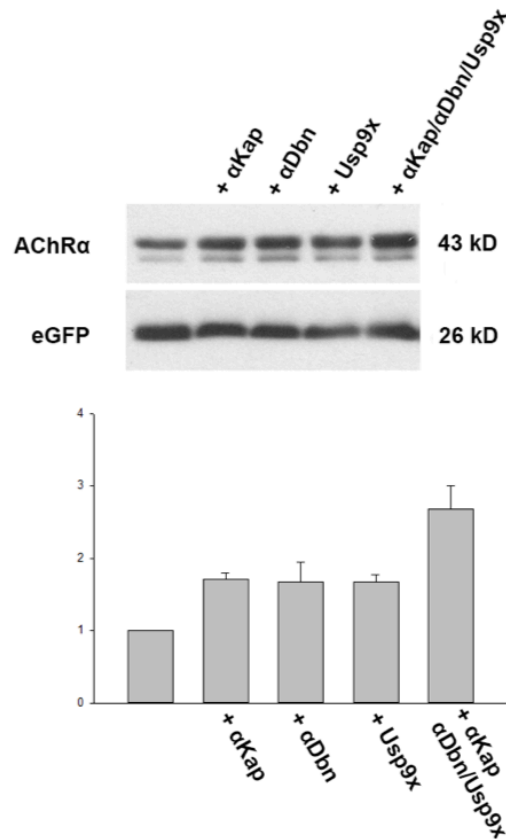


Figure A.4 Usp9x sustains AChR α stability through the α -dystrobrevin- α kap pathway in HEK cells.

α Kap, α -dystrobrevin, and Usp9x alone enhanced AChR α to 1.7 fold and altogether enhanced AChR α to 2.6 fold. eGFP is the indicator of transfect efficiency.

A.4 Discussion

This research found a novel DUB, Usp9x, plays a role in AChR stability. Both Usp9x and its counter E3 partner Fbw7 showed specific expression patterns during developmental stages, which might be critical for synaptic maturation. In cell cultures, we revealed a Usp9x stabilizing effect on α kap through α -dystrobrevin, a direct binding partner of Usp9x and an essential AChR-associated DGC protein. Moreover, the Usp9x stabilizing effect ultimately increased AChR α stability through the α -dystrobrevin- α Kap pathway.

At P15 of wild-type mice, when Usp9x is highly expressed in transcript and protein levels, we examined the Usp9x localization in muscles. In Figure A.5, Usp9x green signals were closely

expressed with AChR synapses (red signals). With only secondary antibody labeling, there was a lack of a specific pattern of green signals (negative control, NC). Although Usp9x expression pattern is independent of α -dystrobrevin mutation, we would like to know if the cellular localization is altered when α -dystrobrevin is lost. To examine Usp9x localization at P15 of the α -dystrobrevin knockout mice will be the next step for establishing Usp9x expression pattern at neuromuscular junctions. The muscle immunostaining experiment will also extend to Fbw7.

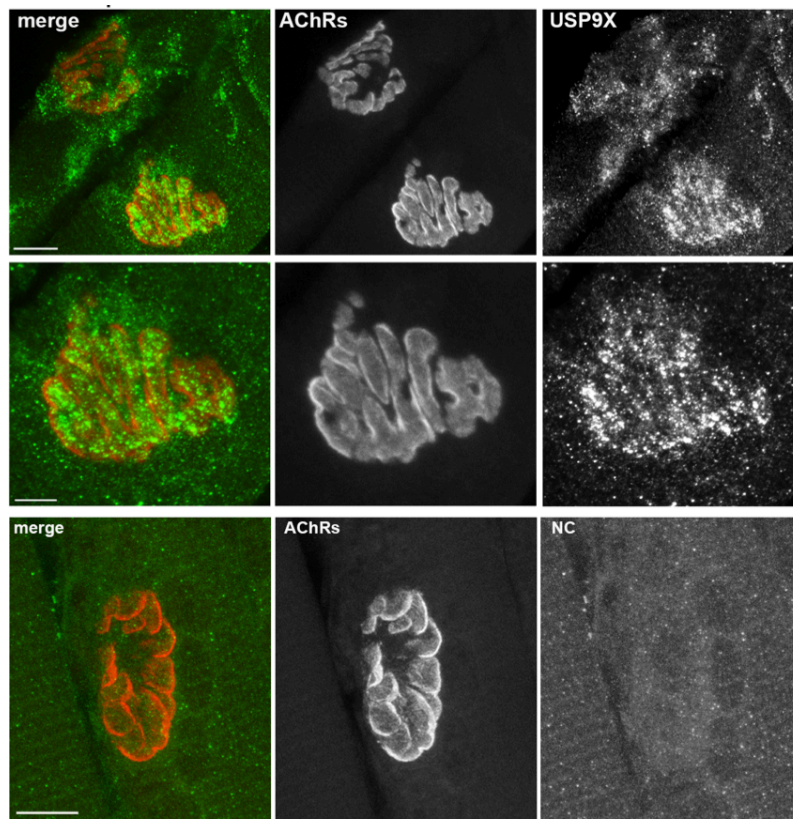


Figure A.5 Usp9x is expressed closely with AChRs at neuromuscular synapses at P15 wildtype mice

(Immunostaining and imaging by Dr. Isabel Martinez-Pena Y Valenzuela)

AChRs were labeled by BTX-Alexa 594 (red), and Usp9x were labeled by anti-Usp9x followed by secondary antibody conjugated with Alexa 488 (green). To rule out the non-specific staining by the secondary antibody, the negative control (NC) was performed under the same procedure without anti-Usp9x primary antibody. Since NC panel was lack of specific green pattern, we concluded that the Usp9x green signals were specific.

There are many interesting but unresolved questions along with this research. First, the UPS enzymes for AChR ubiquitination and deubiquitination are still not yet identified. The E3-DUB pair of enzymes in this study, Fbw7 and Usp9x, could be candidates for future investigation. Second, we found that Usp9x sustained AChR stability through the α -dystrobrevin- α kap pathway, but the detailed mechanism was still unclear.

While keep approaching these interesting questions, there are technical challenges for Usp9x research to bear in mind. Usp9x is such a big protein (290 kD) that is difficult for *in vitro* transfection, not to mention of electroporation on mice. When doing plasmid amplification, the transform efficiency is low with difficult growing bacteria culture, and often the product has undesired homologous recombination. To further study Usp9x in neuromuscular junctions, I propose to generate skeletal muscle-specific Usp9x knockout mouse model. Usp9x total knockout is lethal, but the conditional loxp knockout strain is available, Usp9x^{loxp/loxp} (Stegeman, Jolly et al. 2013). There are also many skeletal muscle-specific or even with the inducible ability of Cre recombinase mice available (McCarthy, Srikuea et al. 2012). Crossing skeletal muscle-specific Cre strain with Usp9x^{loxp/loxp} will be sufficient to generate skeletal muscle-specific Usp9x knockout mouse model. In the studies of Usp9x^{loxp/loxp} mice mating with different tissue-specific Cre recombinase mice, Usp9x has shown in different regulatory roles in many tissues, such as colon and testicle (Kishi, Uchida et al. 2017, Khan, Carvalho et al. 2018).

One of the Usp9x identified substrates is ErbB2 (Marx, Held et al. 2010), and neuregulin/ErbB pathway regulates the development of neuromuscular junction via phosphorylation of α -dystrobrevin (Schmidt, Akaaboune et al. 2011). It is worth investigating that Usp9x might play a role in the regulation between neuregulin/ErbB and α -dystrobrevin during the developmental stages at neuromuscular junctions.

Appendix B

B The role of the sub-complex α -syntrophin and α -dystrobrevin on the stability of postsynaptic AChR dynamics at the NMJ

Poster presented in 2017 Molecular Biology of the Cell Conference by American Society Cell Biology

*I generated the double mutant mice of α -dystrobrevin and α -syntrophin by mating, genotyping, and maintained the stable breeding strain. I performed PCR, qPCR, and Western blot to compare gene expression between wildtype, single, and double mutant mice, and harvested muscles for developing time points (from P0 to P60). I extracted RNA and performed reverse transcription for muscles from all genotypes at all the developing time points, to provide a cDNA platform for future studies. The features of the double mutant mice are characterized by Dr. Isabel Martinez-Pena Y Valenzuela, who is the lead of this project.

B.1 Introduction

A hallmark of healthy neuromuscular transmission is highly concentrated AChRs at postsynaptic sites. The highly concentrated AChRs are sustained by anchoring to the dystrophin glycoprotein complex, which is a multiprotein complex connecting from extracellular matrix to intracellular cytoskeleton (Ervasti and Campbell 1991). Dystrophin glycoprotein complex includes a primary linker transmembrane β -dystroglycan and other transmembrane proteins such as sarcoglycans and sarcospan, binds to extracellular matrix by extracellular α -dystroglycan, and links to intracellular cytoskeleton by a large scaffolding protein dystrophin or its homolog utrophin.

Dystrophin or utrophin binds to intracellular α -syntrophin and α -dystrobrevin for both structural and signaling support.

Within the dystrophin glycoprotein complex, α -syntrophin and α -dystrobrevin are known to play essential roles in synaptic maturation, sustaining AChR density and muscle integrity at NMJs. In mice deficient in either α -syntrophin or α -dystrobrevin, the synapses form normally but mature abnormally during development. The adult synapses have drastically reduced (30%) AChR density and the synaptic morphology appears abnormal, with 4 times faster AChR turnover rate than wild-type synapses (Martinez-Pena y Valenzuela, Mouslim et al. 2011). Based on these similarities between α -syntrophin and α -dystrobrevin deficient mice, with the evidence of α -syntrophin and α -dystrobrevin form a subcomplex, we aim to decipher the relationship between α -syntrophin and α -dystrobrevin in sustaining AChR.

However, α -syntrophin and α -dystrobrevin deficient mice still contain several differences. α -Syntrophin deficient mice have no muscular dystrophy observed, but α -dystrobrevin deficient mice have 50% muscular dystrophy (Durbeej and Campbell 2002). In mice deficient in α -syntrophin, α -dystrobrevin-1, a major isoform of α -dystrobrevin, is barely detected, and it is introduced into sternomastoid muscles of α -syn^{-/-} mice partially rescues the synaptic phenotype. The reverse way, however, electroporating α -syntrophin into α -dystrobrevin deficient mice does not have any rescuing effect (Martinez-Pena y Valenzuela, Mouslim et al. 2011). The feature of α -dystrobrevin-1 contains three tyrosine phosphorylation sites at the C-terminal domain (Grady, Grange et al. 1999, Grady, Akaaboune et al. 2003) , and its phosphorylation is regulated by the neuregulin-ErbB signaling pathway (Schmidt, Akaaboune et al. 2011), which is important for the AChR stability at NMJs. These observations suggest α -syntrophin acts via α -dystrobrevin in supporting AChRs. To know the interplay between α -syntrophin and α -dystrobrevin on the stability of AChR and NMJ structural integrity, we generated the double mutant mice of α -syntrophin and α -dystrobrevin.

B.2 Results

Generation and characterization of α Dbn α Syn double knockout mice

To investigate the interaction between α -dystrobrevin and α -syntrophin in sustaining receptors at synapses, we generated the double mutants of α -dystrobrevin and α -syntrophin mice. We crossed the α -dystrobrevin male knockout mice (F0♂ α Dbn $^{-/-}$, (Grady, Grange et al. 1999)) with the α -syntrophin female knockout mice (F0♀ α Syn $^{-/-}$, (Adams, Kramarcy et al. 2000)), which α -syntrophin knockout mice have better nursing behavior over α -dystrobrevin knockout mice. The first generation was all the double heterozygotes (F1: α Dbn $^{+/-}$ α Syn $^{+/-}$). Then we crossed the different sexes from the F1, and performed PCR on their offsprings to screen the α -dystrobrevin and α -syntrophin double knockout mice (F2: α Dbn $^{-/-}$ α Syn $^{-/-}$). During the PCR screening, we got all the other genotypes in between, we kept the wildtype (α Dbn $^{+/+}$ α Syn $^{+/+}$), α Dbn knockout (α Dbn $^{-/-}$ α Syn $^{+/+}$), α Syn knockout (α Dbn $^{+/+}$ α Syn $^{-/-}$), and double heterozygotes (α Dbn $^{+/-}$ α Syn $^{+/-}$) as the littermate controls to the α Dbn α Syn double knockout mice.

The benefits of keeping the littermate controls are, first we reduced the background differences from the individual strains bred from different labs, and second, we had the best age-matched and housing-control to reduce variation.

To keep the four sustainable strains (wildtype, α Dbn $^{-/-}$, α Syn $^{-/-}$, and α Dbn $^{-/-}$ α Syn $^{-/-}$) in our lab, we mated each genotype from the F2 littermates. Luckily, the double knockout mice are survived and able to breed. To reduce the chances of unwanted mutation accumulated during breeding over generations, we recently performed double knockout mice backcross to C57BL/6 wildtype (B6).

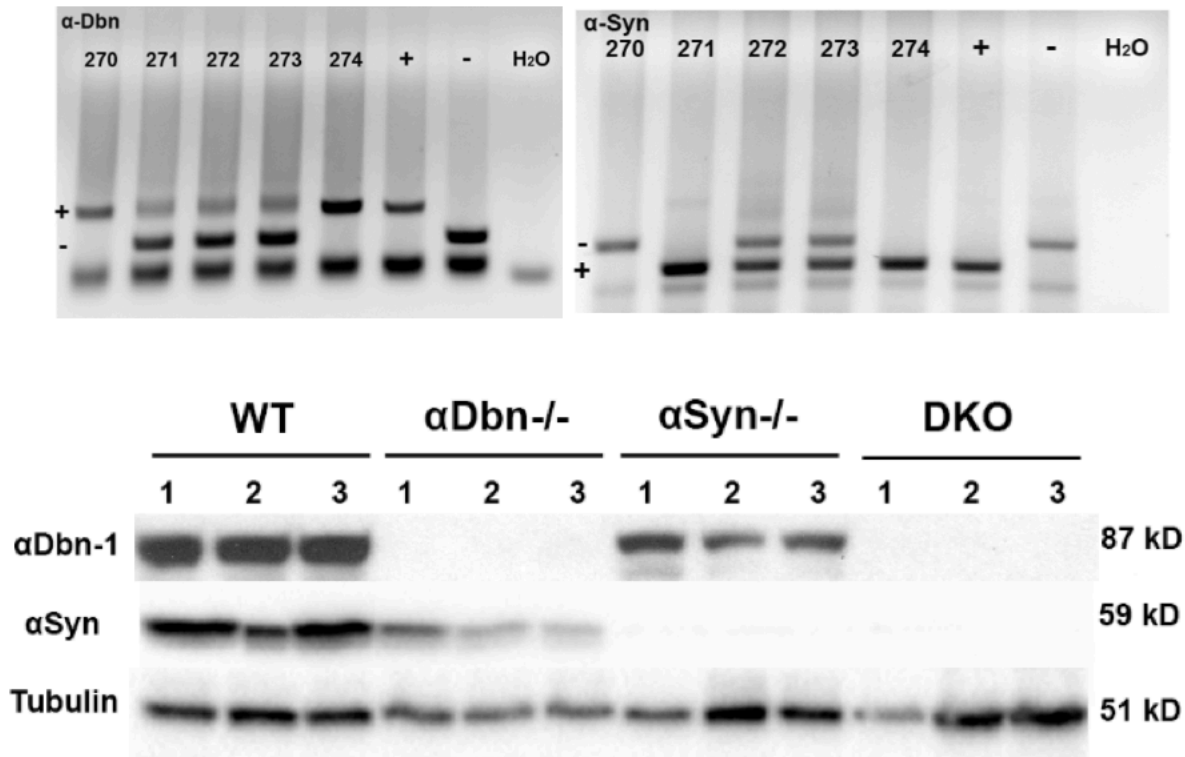


Figure B.1 Verification of the α -dystrobrevin and α -syntrophin double mutants by PCR and Western blots.

The primer sets for examination α -dystrobrevin and α -syntrophin deficiency are listed.

α -Dbn-F: GGGCCTTACCTATGTGACTGAGTGAC

α -Dbn-R: TGCTCGCCCCTACAGCACCCCTTA

α -Dbn-Neo: TGCCAAGTTCTAATTCCATCAGAAGCTG

α -Syn-F: GACTGTCCAGGGAATCATACCGTTCC

α -Syn-R: ACAGGAGCCCAGTCTTCAATCCAGG

α -Syn-Neo: CAAATTAAGGGCCAGCTCATTCTTCC.

Transcript level of AChRs is not affected in single and double knockouts compared to wild-type

The loss of AChR at postsynaptic sites may happen at different stages in gene expression, such as posttranscriptional and posttranslational regulations. To investigate the possibility of posttranscriptional regulation, we harvested hindlimb muscles from all the genotypes and examined their mRNA transcript levels of AChR α subunit. The result (Figure B.2) showed that there is no significant difference of AChR α transcript level between either single or double α -

syntrophin and α -dystrobrevin knockout mice compared to wild-type. Therefore, the loss of AChR does not take place at the posttranscriptional regulation.

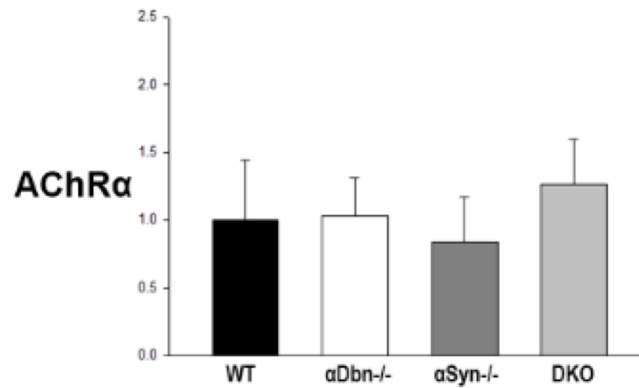


Figure B.2 The mRNA transcript level is not altered in either α -dystrobrevin or α -syntrophin single and double knockout mice.

The hindlimb muscle samples were harvested from at least three adult mice from each genotype. The RNA extraction and reverse transcription were performed, and the cDNA products were further examined by quantitative PCR. The internal control of qPCR is housekeeping GapdH.

B.3 Discussion

α -Syntrophin and α -dystrobrevin work within the same pathway in postsynaptic AChR stability

By generating the double deficient mice of α -dystrobrevin and α -syntrophin, this work partially reveals a long term question about the interaction between different postsynaptic scaffolding proteins in sustaining AChRs. The loss of either α -dystrobrevin or α -syntrophin has significant defects in AChR postsynaptic features, which affirms the indispensable roles of both α -dystrobrevin and α -syntrophin. Incorporating with the data that α -dystrobrevin-1 is able to rescue the defects in α -syntrophin deficient mice partially, but not vice versa, we propose that α -syntrophin works upstream of α -dystrobrevin in the sequential recruitment of proteins involved in postsynaptic AChR stability.

Possible mechanism: α -syntrophin and α -dystrobrevin sustain AChRs through α kap

The mechanism of how α -syntrophin and α -dystrobrevin regulate AChR stability is still not clear. Therefore, we looked into possible interactions between the subcomplex of α -syntrophin and α -dystrobrevin and other AChR associated proteins. One protein that triggered our attention is α kap. α Kap is a muscle-specific anchoring protein, which is a non-kinase and alternative splicing form from calcium/calmodulin kinase II *camk2* gene. α Kap has shown to protect AChR from ubiquitin-dependent proteasome degradation *in vitro* (Mouslim, Aittaleb et al. 2012), while knocking α kap down in mice showed lower AChR postsynaptic density, higher AChR turnover rate, and impaired synaptic structure (Martinez-Pena, Aittaleb et al. 2015). Moreover, we found that α -dystrobrevin has a dosage-dependent manner of enhancing α kap stability *in vitro* (chapter III). We further tested if α -syntrophin has a similar effect on α kap. The pilot study suggested that α -syntrophin alone has no significant enhancement on α kap, but α -syntrophin and α -dystrobrevin together have a synergistic effect on α kap stability. As in Figure B.3, while both α -syntrophin and α -dystrobrevin were overexpressed, was able to increase α kap stability with less amount (0.1 or 0.2 μ g each) of either α -syntrophin or α -dystrobrevin (0.4 μ g). The same effect had been observed in both HeLa heterologous and C2C12 endogenous cell culture system. Altogether, this study indicates that α -syntrophin and α -dystrobrevin work within the same pathway to sustain AChRs through α kap.

**α Syn and α Dbn increase α Kap expression
in a synergistic manner**

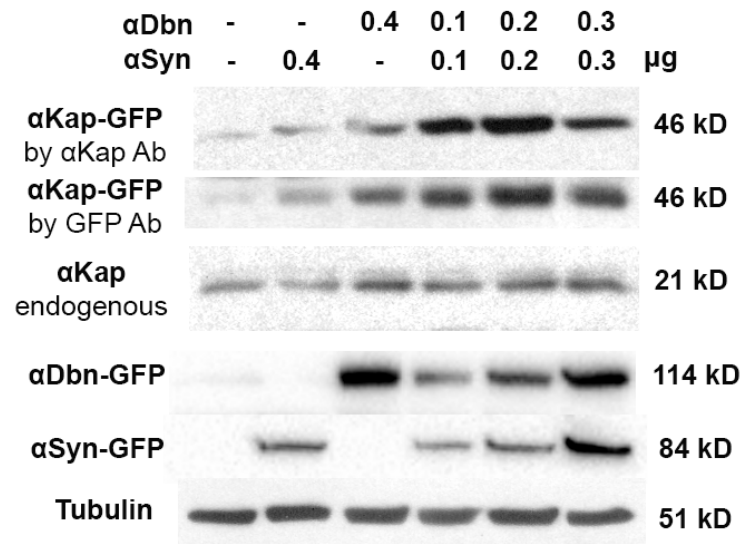


Figure B.3 The synergistic effect in α -dystrobrevin and α -syntrophin sustaining α kap expression level in C2C12 myoblasts.

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