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Enzymatic Activity of the Scaffold Protein Rapsyn for Synapse Formation

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SUMMARY

Neurotransmission is ensured by a high concentration of neurotransmitter receptors at the postsynaptic membrane. This is mediated by scaffold proteins that bridge the receptors with cytoskeleton. One such protein is rapsyn (receptor-associated protein at synapse), which is essential for acetylcholine receptor (AChR) clustering and NMJ (neuromuscular junction) formation. We show that the RING domain of rapsyn contains E3 ligase activity. Mutation of the RING domain that abolishes the enzyme activity inhibits rapsyn- as well as agrin-induced AChR clustering in heterologous and muscle cells. Further biological and genetic studies support a working model where rapsyn, a classic scaffold protein, serves as an E3 ligase to induce AChR clustering and NMJ formation, possibly by regulation of AChR neddylation. This study identifies a previously unappreciated enzymatic function of rapsyn and a role of neddylation in synapse formation, and reveals a potential target of therapeutic intervention for relevant neurological disorders.

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AUTHOR CONTRIBUTIONS

L.L., W.-C.X., and L.M. designed the project. L.L. and Y.C. performed experiments. H.W., X.Y., Z.Z., and G.X. generated plasmids. C.S., A.B., B.Z., and H.S. helped with data interpretation and provided advice. X.X. and L.G. generated C366A mutant mice. W.Z. performed MS analysis. L.L. and L.M. wrote the manuscript.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.10.023.

INTRODUCTION

Neuronal communication, critical for learning and memory, perception, thinking, and reaction, is enabled by synapses where the axonal presynaptic terminal of one neuron aligns with the postsynaptic membrane of another neuron. Efficient synaptic transmission is ensured by a high concentration of neurotransmitter receptors at the postsynaptic membrane (Sheng and Hoogenraad, 2007; Tyagarajan and Fritschy, 2014; Waites et al., 2005). This is believed to be mediated by anchoring of scaffold proteins, such as PSD95 and gephyrin, which interact with glutamate receptors and gamma-amino butyric acid (GABA) receptors, respectively (Sheng and Sala, 2001). How these adaptor proteins work is not completely understood. Evidence suggests that they could bridge neurotransmitter receptors to the cytoskeleton (Kirsch and Betz, 1995; Niethammer et al., 1998).

Neuromuscular junctions (NMJs) are a peripheral synapse formed between motor neurons and skeletal muscles. Large and easily accessible, they have contributed greatly to the understanding of synapse formation and neurotransmission (Sanes and Lichtman, 1999, 2001; Tintignac et al., 2015; Wu et al., 2010). At the NMJ, similar to synapses in the brain, the postjunctional membrane is packaged with acetylcholine receptors (AChRs) at a concentration as high as 10,000 receptors/μm^2 (Fertuck and Salpeter, 1976; Matthews-Bellinger and Salpeter, 1978, 1983). Accumulation of the AChR at the NMJ is mediated by transcribing AChR subunit genes in subsynaptic nuclei (Merlie and Sanes, 1985) and by anchoring the AChR protein to the synaptic cytoskeleton (Sanes and Lichtman, 1999, 2001; Tintignac et al., 2015; Wu et al., 2010). The latter process requires rapsyn (Gautam et al., 1995), a peripheral membrane protein originally identified in Torpedo postsynaptic membranes (Cohen et al., 1972; Sobel and Changeux, 1977). Rapsyn and the AChR colocalize in the electric organ and at developing as well as adult NMJs (Froehner et al., 1981; Neubig et al., 1979; Noakes et al., 1993; Sealock et al., 1984). Expression of rapsyn induces clusters of co-transfected AChRs in *Xenopus* oocytes or in QT6 fibroblast cells (Froehner et al., 1990; Gillespie et al., 1996; Phillips et al., 1991). Rapsyn is believed to function as an anchoring protein because it interacts with the AChR biochemically (Burden et al., 1983; LaRochelle and Froehner, 1986) and also binds actin (Walker et al., 1984). Rapsyn mutations have been identified in congenital myasthenic disorders (Ohno et al., 2002). Molecular mechanisms of rapsyn regulation of NMJ formation are not well understood.

The NMJ formation and maintenance require agrin, a protein released from motor neurons (McMahan, 1990). Agrin stimulates AChR clustering through binding to LRP4 (low-density lipoprotein receptor-related protein 4) and thus activating MuSK (muscle-specific kinase) in muscles (DeChiara et al., 1996; Ferns et al., 1993; Kim and Burden, 2008; McMahan, 1990; Nitkin et al., 1987; Zhang et al., 2008; Zong et al., 2012). Mice lacking agrin, LRP4, or MuSK do not form NMJ (DeChiara et al., 1996; Gautam et al., 1996; Weatherbee et al., 2006). NMJs become unstable when agrin or Lrp4 is mutated in adult animals (Barik et al., 2014; Bogdanik and Burgess, 2011; Samuel et al., 2012). However, intracellular mechanisms downstream of MuSK remain unclear, although activation of the kinase is known to require Dok-7 (Okada et al., 2006).

To better understand how rapsyn regulates NMJ formation, we analyzed its structure in relation to muscular dystrophy in human patients. Rapsyn mutations are most found in the TPRs (tetratricopeptide repeats), the coiled-coil domain, and regions in between (Engel et al., 2003). In contrast with lethality of rapsyn null ($R^{-/-}$) in mice (Gautam et al., 1995), many human mutations are not lethal, suggesting that they may be modulatory to rapsyn function (Engel et al., 2003). Mutation in the RING domain is rare; however, a partial deletion mutation of the RING domain causes fetal akinesia and premature termination of pregnancy (Vogt et al., 2008). Mutant fetuses display fixed positions of hands and feet and no respiratory movement. These observations indicate an essential role of the RING domain in NMJ development. This domain is highly conserved among vertebrates including zebrafish, mouse, and human (Figures 1A and 1B). In vitro data suggest that it could bind to β-dystroglycan (Lim and Campbell, 1998), a transmembrane protein that interacts with dystrophin and utrophin in the subplasmalemmal cytoskeleton (Apel et al., 1995; Bartoli et al., 2001). Mutation of β-dystroglycan impairs muscle development, but its effect on NMJ formation appeared to be mild (Côté et al., 1999; Jacobson et al., 2001), suggesting additional functions of the RING domain.

Here we provide evidence that the RING domain of rapsyn contains E3 ligase activity. Mutation of cysteine 366 in the first characteristic zinc finger abolished the enzymatic activity. We investigated the role of E3 ligase activity of rapsyn in AChR clustering and in NMJ formation by two genetic approaches. Results support a working model where rapsyn, a classic scaffold protein, serves as an E3 ligase to induce receptor clustering and NMJ formation, possibly by increasing neddylation of the AChR. These observations identify a previously unappreciated enzymatic function of rapsyn and reveal neddylation of neurotransmitter receptors as a novel mechanism in synapse formation.

RESULTS

Enzymatic Activity of the RING-H2 Domain

To determine whether the RING domain of rapsyn functions as an E3 ligase, glutathione S-transferase (GST)-Flag-RING fusion protein was purified from bacteria and incubated with the ubiquitin activation enzyme (UbE1) and an ubiquitin conjugation enzyme (Ubc, E2). The E3 ligase activity of the RING domain was assayed by self-ubiquitin conjugation, a character of most E3 ligases (Deshaies and Joazeiro, 2009). As shown in Figure 1C, rapsyn self-ubiquitination was detected when UbcH5A, 5B, 5C, and 6 were used as E2 conjugation enzymes. As control, self-ubiquitination was not detected on GST (Figure 1D) or with other E2 UbcHs (Figure 1C). Moreover, the E3 ligase activity was dose dependent (Figure 1E). To demonstrate that the E3 ligase activity requires the characteristic C2H2C3 motif, we generated C366A mutant by mutating the second cysteine of the motif to alanine. Mutation of the cysteine in RING-H2 E3 ligases has been shown to abolish the E3 ligase activity (Fang et al., 2000; Thien et al., 2001). As shown in Figure 1D, in contrast with wild-type (WT), the C366A mutant did not exhibit any detectable E3 ligase activity. These results indicate that rapsyn may function as an enzyme.

Inability of C366A Mutant to Rescue NMJ Deficits

To determine whether the enzyme activity of rapsyn is involved in NMJ formation, we generated human skeletal α-actin (HSA)-R_{WT} and HSA-R_{C366A} transgenic mice, which express WT or C366A mutant rapsyn under the control of human skeletal α-actin (HSA) promoter (Figure S1A, available online). The HSA promoter drives protein expression specifically in muscle at embryonic day (E) 9.5 (Brennan and Hardeman, 1993; Crawford et al., 2000; Muscat and Kedes, 1987), and it has been widely used to study NMJ formation (Chen et al., 2007; Luo et al., 2003; Wu et al., 2015). The transgenes were expressed in muscles of transgenic mice at mRNA and protein levels (Figures S1B and S1C). Quantitative real-time PCR indicated that the transgene was expressed about 3-fold higher than endogenous rapsyn (Figure S1D). The transgenic mice were viable and fertile, and did not exhibit detectable deficits in NMJ morphology or function (Figures S1E–S1H) (data not shown). We bred HSA-R_{WT} and HSA-R_{C366A} transgenic mice with rapsyn null mutant mice to determine whether the expression of a respective transgene could rescue NMJ deficits caused by loss of rapsyn. As previously reported, rapsyn null ($R^{-/-}$) mice displayed neonatal lethality and did not form the NMJ (Gautam et al., 1995). Expression of WT rapsyn in null mice prevented null mice from dying and the NMJs were observed in $R^{-/-}$;HSA-R_{WT} mice (Figures 2A and 2B), demonstrating that muscle expression of WT rapsyn is able to rescue NMJ deficits of rapsyn null mice. In contrast, $R^{-/-}$;HSA-R_{C366A} mice died soon after birth with cyanosis, a sign of inability to breathe, suggesting that the C366A mutant is unable to rescue. We examined the NMJ morphology in the diaphragm by staining with Alexa Fluor 594-conjugated α-bungarotoxin (BTX) to label AChR and with anti-synaptophysin/neurofilament antibodies to label axons and nerve terminals (Li et al., 2008; Luo et al., 2008; Wu et al., 2012a, 2012b, 2015). In WT mice, AChR clusters were located in the central region, surrounding primary phrenic nerve branches (Figure 2). However, in rapsyn null mice, there were no AChR clusters; primary branches remained in the central region, but there was extensive arborization of secondary and tertiary branches. The defects were rescued by expressing WT rapsyn in muscles (albeit not completely) (Figure 2). However, these rescue effects were not observed in $R^{-/-}$;HSA-R_{C366A} mice, in agreement with the notion that rapsyn E3 ligase activity is necessary for NMJ formation.

Regulation of AChR Aggregation by Rapsyn Enzymatic Activity

The C366A mutation seemed to have little effect on rapsyn protein stability in HEK293T cells (Figure S2A) and on its binding to actin (Figure S2B). Moreover, hemagglutinin (HA)-tagged rapsyn, either WT or C366A, was enriched at the NMJ (Figure S2C). Rapsyn has been shown to interact with AChR subunits (Maimone and Merlie, 1993). To determine whether the C366A mutation disrupts rapsyn binding to AChR, we performed reciprocal co-immunoprecipitation (coIP) experiments in cells expressing differentially tagged rapsyn and AChR subunits. As shown in Figures S2D and S2E, C366A associated with AChR subunits as WT control. Next, we determined whether the enzyme activity of rapsyn regulates AChR cluster formation. Ideally, agrin-induced AChR clusters in muscle cells may serve as a readout; however, increasing rapsyn levels in cultured muscle cells are known to inhibit agrin-induced clusters (Han et al., 1999; Yoshihara and Hall, 1993). Consequently, the effect of C366A mutation, if any, could be blurred by the inhibitory effect of rapsyn overexpression, which would make it difficult to interpret the results. To solve this problem,

we studied the effect of the C366A mutation on AChR aggregates in heterologous cells, a strategy that has been used in previous studies (Borges et al., 2008; Froehner et al., 1990; Gillespie et al., 1996; Lee et al., 2009; Phillips et al., 1991). Rapsyn overexpression causes AChR to form aggregates in non-muscle cells (Froehner et al., 1990; Gillespie et al., 1996; Phillips et al., 1991), and we investigated whether this event requires the enzymatic activity of rapsyn. As shown in Figure 3A, α, β, γ, and δ AChR subunits, when expressed in combination, did not form aggregates in control HEK293T cells that were transfected with GFP. However, co-expression of rapsyn-GFP or rapsyn alone led to the formation of AChR aggregates (Figures 3A, 3B, and S3A). This effect was blocked by the C366A mutation, suggesting a necessary role of E3 activity in rapsyn-induced AChR aggregates (Figures 3A and 3B). Notice that AChR aggregates induced by rapsyn were on the cell surface because they could be labeled by Alexa Fluor 594-conjugated α-BTX in live, non-fixed HEK293T cells (Figures 3C and 3D). These results demonstrate that the E3 ligase activity is necessary for rapsyn to induce AChR aggregates in HEK293 cells.

Rapsyn induces aggregating of individual AChR subunits in non-muscle cells (Maimone and Merlie, 1993). In accord, we found that rapsyn bound to α, β, γ, and δ subunits in HEK293T cells (Figure S3B). We used this assay to determine whether rapsyn E3 activity is necessary for aggregating of individual AChR subunits. HEK293T cells were transfected with each of the AChR subunits, together with GFP or GFP-tagged rapsyn or mutant, and scored for AChR aggregates. As shown in Figure 3E, co-expression of rapsyn increased aggregating of α, β, γ, or δ subunits, in agreement with previous reports (Maimone and Merlie, 1993). This effect was dependent on E3 activity because the subunit aggregates were fewer in cells co-transfected with C366A (Figure 3E). The inhibition by C366A varied from subunit to subunit, with β subunit aggregates being less sensitive.

Neddylation of the AChR by Rapsyn

Having demonstrated the requirement of rapsyn's enzymatic activity for AChR aggregating, we sought to investigate underlying mechanisms. Being an E3-like enzyme, rapsyn may promote ubiquitination of AChR subunits and thus their aggregating. To test this hypothesis, we transfected HEK293T cells with Flag-δ subunit (Flag-δ), a subunit that seemed to bind better with rapsyn (Figure S3B) and whose aggregating response by rapsyn was higher than other subunits (Figures 3E and 3F), and HA-ubiquitin without or with rapsyn-GFP. Ubiquitinated proteins were immunoprecipitated with anti-HA antibody and probed with anti-Flag antibody. Ubiquitinated δ subunit was evident in the absence of rapsyn (Figures 4A and S3C). However, ubiquitinated δ subunit was reduced in cells expressing rapsyn or rapsyn-GFP in a concentration-dependent manner. This unexpected result did not support the hypothesis that rapsyn regulates the clustering of AChR by increasing ubiquitination. Rather, it suggests the involvement of the Ub-independent mechanism.

Nedd8 (neural precursor cell expressed, developmentally downregulated 8) is a Ub-like protein that can be covalently conjugated to substrate proteins by E3 ligases in a reaction called neddylation (van der Veen and Ploegh, 2012). Recent evidence indicates that RING type E3 ligases may catalyze neddylation (Deshaies and Joazeiro, 2009; Watson et al., 2006; Zuo et al., 2013). We determined whether rapsyn regulates neddylation by transfecting

HEK293T cells with Flag-δ subunit and Myc-tagged Nedd8. Neddylated δ subunit was revealed by precipitation with anti-Flag antibody followed by probing with anti-Nedd8 antibody, as previously described (Watson et al., 2006; Zuo et al., 2013). Remarkably, rapsyn dose-dependently increased the amount of neddylated δ subunit (Figure 4B), suggesting that rapsyn may serve as an E3 ligase for neddylation. Besides δ subunit, rapsyn also increased neddylation of α and β subunits (Figures S3D–S3F). To test this hypothesis further, we characterized δ subunit neddylation, and results revealed the following features. First, δ subunit neddylation was inhibited in cells treated with MLN4924, an inhibitor of neddylation-activating enzyme (NAE) (Brownell et al., 2010; Soucy et al., 2009) (Figure 4C). In addition, neddylation of δ subunit was also observed in non-transfected C2C12 cells, indicating the process occurs with endogenous Nedd8 (Figure 4D). Second, mutation of two glycine residues at the C terminus of nedd8, which are required for covalent conjugating ability of Nedd8 (Watson et al., 2006), blocked neddylation of the δ subunit (Figure 4E). Third, δ subunit neddylation was reduced in cells co-expressing NEDD8-specific protease 1 (NEDP1), an Nedd8-specific protease that removes Nedd8 from its substrates (Gan-Erdene et al., 2003; Mendoza et al., 2003). As control, the effect of NEDP1 was not observed in cells co-transfected with a catalytically inactive mutant of NEDP1 (Shen et al., 2005) (Figure 4F). Fourth, we determined whether rapsyn-mediated neddylation of the δ subunit requires its E3 ligase activity. As shown in Figure 4G, neddylated δ subunit was reduced in cells expressing C366A, compared with those expressing WT rapsyn, suggesting a necessary role of the E3 activity of rapsyn. Finally, we determined whether the RING domain of rapsyn is able to catalyze neddylation. Purified WT and mutant RING domain (as in Figure 1C) was incubated with NAE1/Uba3, Ubc12 (an E2 conjugation enzyme) (Gong and Yeh, 1999; Liakopoulos et al., 1998), and Nedd8. As shown in Figure 4H, self-neddylation was detected by the WT RING domain, but not the C366A mutant or GST (as control). This result indicates that rapsyn is able to catalyze neddylation. Together, these observations suggest rapsyn promotes neddylation of the δ subunit through E3 ligase activity.

Neddylation Is Required for AChR Cluster Formation

Next, we investigated the role of neddylation in AChR cluster formation. If neddylation is critical, more neddylated AChR is predicted in the synaptic region, where AChR and rapsyn are enriched, than in the extra-synaptic region. We tested a battery of commercially available anti-nedd8 antibodies; unfortunately, none revealed specific staining in histochemical studies (data not shown). We therefore addressed this question by taking advantage of NMJ localization in the middle of muscle fibers (Figure 5A). As shown in Figure 5B, the neddylated δ subunit was higher in synaptic regions of hemi-diaphragm muscles, compared with that in the extra-synaptic region, suggesting that AChR at the NMJ is neddylated.

To determine whether neddylation is required for AChR clustering, we treated HEK293T cells expressing rapsyn and AChR with the NAE inhibitor MLN4924. Rapsyn-induced AChR aggregates were reduced in treated cells, compared with control (Figures S4A and S4B). A similar effect was observed on agrin-induced AChR clusters in C2C12 myotubes (Figures 5C and 5D). Notice that MLN4924 did not block ubiquitination of the δ subunit (Figure S4C). These results suggest that neddylation may be required for AChR clustering. To further test this hypothesis, we sought to disrupt the neddylation by a different approach:

suppressing the expression of the Ubc12 E2 conjugation enzyme that facilitates rapsyn self-neddylation (Figure 4H). We generated PX330-Ubc12, a clustered regularly interspaced short palindromic repeats (CRISPR) plasmid that suppressed the expression of Ubc12 in C2C12 myotubes (Figures S4D and S4E). Agrin-induced clusters were reduced in myotubes expressing PX330-Ubc12, compared with control C2C12 myotubes (Figures 5E and 5F), suggesting that neddylation is necessary for agrin-induced AChR clustering. To determine that neddylation is important for AChR cluster formation in vivo, we injected MLN4924 into newborn mice (aged postnatal day [P] 0). Diaphragms were isolated and stained whole mount with Alexa Fluor 594-conjugated α-BTX. The number of AChR clusters was similar between control (injected with vehicle) and MLN4924-treated mice. However, the size of AChR clusters was reduced in MLN4924-treated diaphragms (Figures 5G–5J). Together, data from both in vitro and in vivo experiments support the notion that neddylation is necessary for AChR cluster formation.

AChR Neddylation for Clustering and Regulation by Agrin

Neddylation and ubiquitination occur on lysine residues of target proteins. Because the last four amino acid residues of Nedd8 and ubiquitin are identical and contain a trypsin digestion site (Figure S5A), it would be difficult to pinpoint neddylation sites by mass spectrometry (MS) after trypsin digestion. To this end, Flag-tagged δ subunit, co-expressed with Myc-Nedd8 in HEK293T cells, was purified by anti-Flag antibody immobilized on beads and resolved by SDS-PAGE. Isolated bands were digested in situ by lysC protease, which cleaves the peptide bond at the carboxyl side of lysine (Figures S5A and S5B). The first amino acid variant from the C terminus is A72 in Nedd8 and R72 in ubiquitin. The long peptide modification is very likely to be randomly broken down during MS/MS fragmentation steps; therefore, we used ALRGG remnant as Nedd8 conjugation targeting to lysine residues, which adds a mass of 453.2652 (monoisotopic). ALRGG is one of the major fragments and has a suitable length for MS detection (see Experimental Procedures). Three lysine residues (K397, K406, and K462) were identified. The relative counts on Lys397 were eight times higher than the other two lysine residues (Figures S5C–S5E), suggesting that Lys397 may be a major lysine residue for Nedd8 conjugation.

To test this hypothesis, we generated a series of lysine-to-arginine mutations of the δ subunit. Because the AChR contains four transmembrane domains with both N terminus and C terminus outside the plasma membrane (Albuquerque et al., 2009), we focused on lysine residues in the two intracellular loops, between the first and second and between the third and fourth transmembrane domains, respectively, including the three identified by MS. As shown in Figure 6A, mutation of K397 largely reduced δ subunit neddylation, whereas mutations of other lysine residues had little effect, indicating that K397 is a major neddylation site. To determine whether K397 neddylation is critical for clustering, we co-transfected rapsyn with WT or K397R mutant into HEK293T cells. More AChR aggregates were observed in cells expressing WT δ subunit than those expressing K397R (Figure S5F), suggesting that K397 is necessary for clustering. To further test this hypothesis, we used a CRISPR strategy to generate stable C2C12 cells whose δ subunit K397 was mutated to arginine (Figure S5G). The mutation, confirmed by DNA sequencing (Figure S5H), did not change the apparent mobility of δ subunit on SDS-PAGE (Figure S5I). However,

neddylation of the K397R mutant subunit was reduced (Figure S5J), suggesting K397 is a major site for neddylation. Importantly, agrin-induced AChR clusters were diminished in K397R mutant C2C12 myotubes, in contrast with robust clusters in WT controls (Figures 6B and 6C). These data indicate that δ subunit neddylation is required for AChR cluster formation. K397R mutation also diminished the ubiquitination of δ subunit (Figure S5K), suggesting K397 may be a site for regulation by ubiquitination.

Next, we determined whether δ subunit neddylation is regulated by agrin signaling. C2C12 myotubes were treated with agrin, and neddylated proteins were purified by anti-Nedd8 antibody. Immunoblotting (IB) with anti-δ subunit antibody revealed that neddylated δ subunit was enhanced in agrin-treated myotubes (Figure 6D). MuSK null C2C12 cells were generated by CRISPR-Cas9 (Figure S5L) and confirmed by genomic sequencing (Figure S5M) to test whether this event requires MuSK. The MuSK mutant cells were unable to form AChR clusters in response to agrin stimulation (Figure S5N). As shown in Figure S5O, δ subunit neddylation was reduced, indicating a necessary role of MuSK. To test whether the kinase activity of MuSK is involved in regulating δ subunit neddylation, we transfected WT or kinase dead MuSK into HEK293T cells together with rapsyn-GFP, Flag-δ, and Myc-Nedd8. Neddylation of the δ subunit was increased in cells co-expressing WT MuSK, but not the kinase dead mutant (Figure 6E), indicating that MuSK promotes δ subunit neddylation. Moreover, the effect of MuSK was not observed in cells co-expressing the C366A mutant of rapsyn (Figure 6E), indicating the dependence on the E3 ligase activity of rapsyn. Together, these data demonstrate that the agrin-Lrp4-MuSK signaling pathway regulates AChR neddylation by promoting the E3 ligase activity of rapsyn.

Rapsyn E3 Ligase Activity Is Essential for NMJ Formation

To further determine whether NMJ formation requires rapsyn E3 ligase activity, we generated a C366A knockin mutant mouse by a CRISPR-Cas9 strategy (Figure S6A). The guidance RNA was targeted to the indicated region, and the codon TGT (encoding cysteine 366 or C366) in exon 7 of the rapsyn gene was changed to GCC (encoding alanine, A). The third base of the following codon (encoding glycine, G367) was changed from T to C without changing the amino acid (i.e., G367), to generate an NaeI digestion site (TGTGGT to GCCGGC) for convenient genotyping (Figure S6B). The targeted mutation was confirmed by sequencing genomic DNA of C366A mice (Figure S6C). mRNAs of WT and mutant rapsyn were comparable in muscles of respective genotypes, suggesting that the mutation had little effect on the transcription of the gene or mRNA stability (Figures S6E and S6F). In addition, the amount of rapsyn protein was also comparable between WT and $R^{CA/CA}$ mice (Figure S6G).

However, most homozygous mutant ($R^{CA/CA}$) mice died immediately after birth with cyanosis (Figure S6D), apparently caused by breathing difficulty. To characterize the NMJ, we stained muscles of WT and $R^{CA/CA}$ mice whole mount for AChR and nerve terminals, together with rapsyn null ($R^{-/-}$) mice as control. As shown in Figure 7A, $R^{CA/CA}$ mice did not form AChR clusters in diaphragm muscles (Figures 7A and 7B) and in other muscles including tibialis anterior muscles (Figure S6H), resembling the phenotypes of rapsyn null mice (Gautam et al., 1995). On the other hand, motor nerve terminals of diaphragm muscles

in both $R^{CA/CA}$ and rapsyn null mice failed to stop in the central region of muscle fibers and branched extensively (Figures 7A and 7B). Consequently, the length of secondary branches was increased in $R^{CA/CA}$ and rapsyn null mice, compared with WT mice (Figures 7A, 7B, and 7D). At the level of electron microscopy, junctional folds could be frequently identified in WT muscles. Opposed to junctional folds were axon terminals filled with homogeneous synaptic vesicles. Active zones were occasionally visible on the presynaptic membrane where vesicles were clustered (Figure S6I). In muscles of $R^{CA/CA}$ mice, axon terminals were identifiable with synaptic vesicles. However, the size of synaptic vesicles was variable, not as homogeneous as observed in WT muscles (Figure S6L). Nevertheless, some vesicles appeared to be able to concentrate along the membranes that contact the muscle membrane. Noticeably, $R^{CA/CA}$ muscles lack junctional fold-like structures. Basal lamina appeared to form in $R^{CA/CA}$ muscles. Together, these results of light and electron microscopic analysis demonstrate that the E3 ligase activity of rapsyn is critical for NMJ formation.

To eliminate the potential off-target effects of CRISPR, we generated mutant mice that contained one copy of rapsyn null and one copy of C366A mutation ($R^{CA/-}$). If the phenotype of C336A mutation is due to an off-target effect (i.e., mutation of an unknown gene), the C366A/− mice are expected to form the NMJ because the off-target gene is expressed by the chromosome provided by heterozygous null mice. The $R^{CA/-}$ mice died immediately after birth with cyanosis and did not form detectable AChR clusters with increased secondary nerve branches (Figure S7). These results indicate that $R^{CA/-}$ mice duplicated the phenotypes of rapsyn null and $R^{CA/CA}$ mutant mice, excluding the effect of a possible off-target mutation in $R^{CA/CA}$ mice.

Our working model is that rapsyn, as an E3 ligase, increases AChR neddylation for the receptor clustering and NMJ formation. To further test this hypothesis, we characterized neddylated AChR in $R^{CA/CA}$ mice. Neddylated proteins were enriched by immunoprecipitation (IP) with anti-Nedd8 antibody and blotted with anti-δ subunit antibody. As shown in Figure 7E, the amount of neddylated AChR δ subunit was reduced in muscles of $R^{CA/CA}$ mice, compared with that of WT controls. Similar results were observed in muscles of rapsyn null mice (Figure 7F). These results indicate that AChR neddylation requires the enzymatic activity of rapsyn. The association between reduced AChR neddylation and NMJ formation impairment in vivo supports the working hypothesis that rapsyn regulates AChR clustering by E3-dependent neddylation.

DISCUSSION

This study demonstrates that the RING domain of rapsyn contains E3 ligase activity. It provides two lines of genetic evidence that the E3 ligase is critical for NMJ formation. First, rapsyn mutant lacking the E3 ligase activity was unable to rescue NMJ deficits of rapsyn null mutation. Second, knockin mutation of the necessary cysteine residue prevented mice from forming the NMJ, and mutant mice exhibited NMJ deficits similar to those of rapsyn null mice. These results indicate that rapsyn regulates NMJ formation in a manner that requires E3 ligase activity. Our initial studies of downstream mechanisms suggest that rapsyn promotes neddylation of AChR. Suppressing Ubc12, the E2 conjugation enzyme for neddylation, compromised agrin-induced clustering of the AChR. Pharmacological

inhibition of NAE, the E1 for neddylation, attenuated AChR clusters in agrin-stimulated myotubes and in developing muscles in vivo. Based on these observations, we propose that rapsyn serves as an E3 ligase to regulate NMJ formation. Our results suggest that neddylation could serve as a novel mechanism for neural development including synapse formation.

In a current working model, rapsyn promotes AChR clustering by enhancing the neddylation of the receptor. Intriguingly, neddylation of δ subunit was enhanced in agrin-stimulated muscle cells and required MuSK kinase activity. These results support a hypothesis that agrin, via activating Lrp4/MuSK, enhances the E3 ligase activity of rapsyn to regulate AChR clusters, identifying a new signaling mechanism in NMJ formation. There are nine lysine residues in the two intracellular loops of δ subunit, one in the first and eight in the second loop. Mass-spectrometer analysis of δ subunit from HEK293 cells co-expressed with Nedd8 revealed Lys397 in the second loop as a major neddylation site (Figure S5). In agreement, mutation of Lys397 reduced the subunit neddylation (Figure 6A), and K397R mutation blocked rapsyn-induced clustering in HEK293 cells (Figure S5F). Moreover, muscle cells where δ subunit K397 was mutated to arginine failed to form AChR clusters in response to agrin (Figures 6B and 6C). A parsimonious explanation of these data is that K397 is a major neddylation site whose mutation has functional consequences.

How AChR neddylation promotes NMJ formation remains unclear. There appeared to be a reciprocal relationship between ubiquitination and neddylation of δ subunit. Ubiquitination reduction in rapsyn-transfected HEK293 cells was associated with increased neddylation (Figures 4A, 4B, and S3C). Conversely, inhibition of neddylation by MLN4924 increased δ subunit ubiquitination (Figure S4C). These results suggest that neddylation may regulate ubiquitination of the δ subunit and possibly its stability. This notion was supported by the observation that K397 mutation reduced δ subunit ubiquitination as well as neddylation (Figures S6E and S6F), suggesting that K397 could be conjugated with either ubiquitin or Nedd8. By promoting neddylation, rapsyn may effectively reduce ubiquitination and thus increase AChR stability. Ubiquitylation has been implicated in the trafficking and assembly of AChR in culture (Christianson and Green, 2004). Future work will be necessary to determine exactly how neddylation and/or ubiquitination contributes to AChR clustering. Besides AChR, rapsyn may catalyze neddylation of other proteins critical for NMJ formation, maintenance, and/or function. In addition, rapsyn may also modify substrate proteins by mono- or K63-linkage ubiquitination or sumoylation. Posttranslational modification as such has been shown to regulate signaling molecules and structural proteins (Komander and Rape, 2012). We show that, at least in vitro, rapsyn is able to catalyze both ubiquitination and neddylation (Figures 1 and 4H). Future work to reveal underlying mechanisms of rapsyn regulation of the NMJ will benefit from identifying novel substrates of the E3 ligase.

Earlier work suggests that rapsyn may bridge the AChR with the cytoskeleton (Burden et al., 1983; LaRochelle and Froehner, 1986; Ramarao and Cohen, 1998; Walker et al., 1984). Rapsyn coaggregation with AChR and β-dystroglycan requires the coiled-coil and RING domains, respectively, and its self-aggregation depends on the TPR repeats (Bartoli et al., 2001; Ramarao et al., 2001; Ramarao and Cohen, 1998). The interaction between the coiled-

coil domain and AChR is required for rapsyn targeting to the postjunctional membrane (Chen et al., 2016). Our results do not exclude the function of rapsyn as a scaffold protein. Rather, they identify a previously unappreciated function of rapsyn for AChR clustering and NMJ formation. The finding that rapsyn, a classic scaffold protein, is in fact an enzyme would call for revisits of other so-called scaffold proteins that have been implicated in synapse formation.

EXPERIMENTAL PROCEDURES

Generation of Gene-Modified C2C12 Cells by CRISPR/Cas9

Mouse myoblasts and C2C12 cells were propagated and induced to form myotubes, as described previously (Luo et al., 2008). PX330 was purchased from Addgene. The puromycin gene in PX330 was replaced by GFP. The guidance RNA gAAGGA GGAGG AGTCG GCCGG for Ubc12, gCTCAA ACATG AGGTC ACTGC for the AChR δ subunit, and GGTGG CCGTG AAGAT GCTTA for MuSK were inserted into the BbsI site and verified by sequencing. Further details are described in the Supplemental Experimental Procedures.

MS

Immunopurified proteins were separated by SDS-PAGE, and bands were cut and subjected to in-gel lysC digestion. Peptides were analyzed using liquid chromatography (LC)-electrospray ionization (ESI)-MS-MS. Nedd8-conjugated lysine residues were identified using the TurboSequest search engine (reference sequences: UniProt: P02716 [ACHD_MOUSE]). Lysine modification by ALRGG tags was set as variable modifications, which adds a mass of 453.2652 (monoisotopic). ALRGG is one of the major fragments and has a suitable length for MS detection (http://mrm.thegpm.org/~/peak_search/pmass=&pmrange=0.5&fmrange=0.5&unique=&sort=int&pattern=ENSP&seq=ILGGSVLHLVLALRGG&charge=&submit=Search).

Ubiquitination and Neddylation Assay

For in vitro ubiquitination, WT or mutant RING domain of rapsyn in pGEX was transformed in BL21 and purified by glutathione-conjugated beads (Roche). The RING or GST proteins were incubated with UbE1, one of the E2s, and Ub (Boston Biochem) in the ubiquitination buffer containing 20 mM Tris-hydrogen chloride (HCl) (pH 7.5), 5 mM MgCl2, 0.5 mM DTT, and 2 mM ATP for 1 hr at 37° C. The reaction was stopped by the addition of SDS sample buffer. Ubiquitinated proteins were resolved by SDS and probed with anti-Ub antibody.

To characterize ubiquitination and neddylation in cells or in vivo, cultured cells and muscles isolated from mice were lysed in lysis buffer containing 2% SDS, 150 mM sodium chloride (NaCl), 10 mM Tris-HCl (pH 8.0), and protease inhibitors including 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, 1 μg/mL leupeptin, and 2 μg/mL aprotinin. After sonication, samples were diluted by adding 9 vol of the dilution buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, and 1% Triton.

Supernatants were incubated with respective antibodies overnight at 4°C and then with protein G agarose beads. Precipitated proteins were subjected to western blotting.

Mice

Rapsyn null ($R^{-/-}$) mice were generously provided by Dr. Peter Noakes. To generate rapsyn transgenes for transgenic mouse production, we amplified WT and C366A rapsyn by PCR with primers containing NotI and PacI sites. The downstream primer also contained sequences encoding an HA epitope. After digestion, the PCR products were subcloned into NotI and PacI sites of pBSXHSA, as described previously (Luo et al., 2003; Wu et al., 2015). DNA sequences of transgenes were validated by sequencing. To generate HSA-R_{WT} and HSA-R_{C366A} mice, linearized DNA was pronuclear microinjected into one-cell-stage mouseembryos, which were introduced into pseudo-pregnant recipient females.

To generate rapsyn C366A mutant mice, the single guide RNA (sgRNA)-containing targeting sequence GAGCT CTACT GCGGC CTCTG was purchased from Agilent Technologies. The Cas9 mRNA was purchased from TriLink BioTechnologies. The 150 bp oligo donor with indicated mutation was synthesized by Integrated DNA Technologies. The sgRNA, Cas9 mRNA, and oligo donor were pronuclear microinjected into one-cell-stage mouse embryos, which were introduced into pseudo-pregnant recipient females. Offspring were screened for correct genotype by PCR analysis of tail DNA.

Mice were housed in cages in a room with a 12-hr light-dark cycle with ad libitum access to water and rodent chow diet (Diet 1/4″ 7097; Harlan Teklad). P0 pups of either sex were analyzed, unless otherwise indicated. Animal protocols have been approved by the Institutional Animal Care and Use Committee (IACUC) of Augusta University.

Light-Microscopic Analysis

Diaphragms were stained whole mount as previously described (Dong et al., 2006-2007; Li et al., 2008). Further details are described in the Supplemental Experimental Procedures.

Electron Microscopic Analysis

Electron microscopic studies were carried out as described previously (Wu et al., 2012a). Further details are described in the Supplemental Experimental Procedures.

Statistical Analysis

Statistical analysis was done by GraphPad Prism version 5.0 (GraphPad Software). Sample-size choice was based on previous studies (Wu et al., 2012b, 2015), not predetermined by a statistical method. Data were tested for normality by D'Agostino-Pearson normality test and homogeneity of variance; no violations of assumptions were found. One-way ANOVA was used for analysis of data from three or more groups. Student's t test was used to compare data from two groups. All tests were two-sided. A p value <0.05 was considered to be statistically significant. Data were expressed as mean ± SEM unless otherwise indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

Albuquerque EX, Pereira EF, Alkondon M, Rogers SW. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol. Rev. 2009; 89:73–120. [PubMed: 19126755]

Apel ED, Roberds SL, Campbell KP, Merlie JP. Rapsyn may function as a link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex. Neuron. 1995; 15:115–126. [PubMed: 7619516]

Barik A, Lu Y, Sathyamurthy A, Bowman A, Shen C, Li L, Xiong WC, Mei L. LRP4 is critical for neuromuscular junction maintenance. J. Neurosci. 2014; 34:13892–13905. [PubMed: 25319686]

Bartoli M, Ramarao MK, Cohen JB. Interactions of the rapsyn RING-H2 domain with dystroglycan. J. Biol. Chem. 2001; 276:24911–24917. [PubMed: 11342559]

Bogdanik LP, Burgess RW. A valid mouse model of AGRIN-associated congenital myasthenic syndrome. Hum. Mol. Genet. 2011; 20:4617–4633. [PubMed: 21890498]

Borges LS, Yechikhov S, Lee YI, Rudell JB, Friese MB, Burden SJ, Ferns MJ. Identification of a motif in the acetylcholine receptor beta subunit whose phosphorylation regulates rapsyn association and post-synaptic receptor localization. J. Neurosci. 2008; 28:11468–11476. [PubMed: 18987183]

Brennan KJ, Hardeman EC. Quantitative analysis of the human alpha-skeletal actin gene in transgenic mice. J. Biol. Chem. 1993; 268:719–725. [PubMed: 7678010]

Brownell JE, Sintchak MD, Gavin JM, Liao H, Bruzzese FJ, Bump NJ, Soucy TA, Milhollen MA, Yang X, Burkhardt AL, et al. Substrate-assisted inhibition of ubiquitin-like protein-activating enzymes: the NEDD8 E1 inhibitor MLN4924 forms a NEDD8-AMP mimetic in situ. Mol. Cell. 2010; 37:102–111. [PubMed: 20129059]

Burden SJ, DePalma RL, Gottesman GS. Crosslinking of proteins in acetylcholine receptor-rich membranes: association between the beta-subunit and the 43 kd subsynaptic protein. Cell. 1983; 35:687–692. [PubMed: 6652683]

Chen F, Qian L, Yang ZH, Huang Y, Ngo ST, Ruan NJ, Wang J, Schneider C, Noakes PG, Ding YQ, et al. Rapsyn interaction with calpain stabilizes AChR clusters at the neuromuscular junction. Neuron. 2007; 55:247–260. [PubMed: 17640526]

Chen PJ, Martinez-Pena Y Valenzuela I, Aittaleb M, Akaaboune M. AChRs are essential for the targeting of rapsyn to the postsynaptic membrane of NMJs in living mice. J. Neurosci. 2016; 36:5680–5685. [PubMed: 27225759]

Christianson JC, Green WN. Regulation of nicotinic receptor expression by the ubiquitin-proteasome system. EMBO J. 2004; 23:4156–4165. [PubMed: 15483627]

Cohen JB, Weber M, Huchet M, Changeux JP. Purification from Torpedo marmorata electric tissue of membrane fragments particularly rich in cholinergic receptor protein. FEBS Lett. 1972; 26:43–47. [PubMed: 4636749]

Côté PD, Moukhles H, Lindenbaum M, Carbonetto S. Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. Nat. Genet. 1999; 23:338–342. [PubMed: 10610181]

Crawford GE, Faulkner JA, Crosbie RH, Campbell KP, Froehner SC, Chamberlain JS. Assembly of the dystrophin-associated protein complex does not require the dystrophin COOH-terminal domain. J. Cell Biol. 2000; 150:1399–1410. [PubMed: 10995444]

DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, et al. The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell. 1996; 85:501–512. [PubMed: 8653786]

Deshaies RJ, Joazeiro CA. RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. 2009; 78:399–434. [PubMed: 19489725]

Dong XP, Li XM, Gao TM, Zhang EE, Feng GS, Xiong WC, Mei L. Shp2 is dispensable in the formation and maintenance of the neuromuscular junction. Neurosignals. 2006-2007; 15:53–63.

Engel AG, Ohno K, Sine SM. Sleuthing molecular targets for neurological diseases at the neuromuscular junction. Nat. Rev. Neurosci. 2003; 4:339–352. [PubMed: 12728262]

Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J. Biol. Chem. 2000; 275:8945–8951. [PubMed: 10722742]

Ferns MJ, Campanelli JT, Hoch W, Scheller RH, Hall Z. The ability of agrin to cluster AChRs depends on alternative splicing and on cell surface proteoglycans. Neuron. 1993; 11:491–502. [PubMed: 8398142]

Fertuck HC, Salpeter MM. Quantitation of junctional and extra-junctional acetylcholine receptors by electron microscope autoradiography after 125I-alpha-bungarotoxin binding at mouse neuromuscular junctions. J. Cell Biol. 1976; 69:144–158. [PubMed: 1254640]

Froehner SC, Gulbrandsen V, Hyman C, Jeng AY, Neubig RR, Cohen JB. Immunofluorescence localization at the mammalian neuro-muscular junction of the Mr 43,000 protein of Torpedo postsynaptic membranes. Proc. Natl. Acad. Sci. USA. 1981; 78:5230–5234. [PubMed: 7029531]

Froehner SC, Luetje CW, Scotland PB, Patrick J. The post-synaptic 43K protein clusters muscle nicotinic acetylcholine receptors in Xenopus oocytes. Neuron. 1990; 5:403–410. [PubMed: 1698395]

Gan-Erdene T, Nagamalleswari K, Yin L, Wu K, Pan ZQ, Wilkinson KD. Identification and characterization of DEN1, a deneddylase of the ULP family. J. Biol. Chem. 2003; 278:28892–28900. [PubMed: 12759362]

Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC, Sanes JR, Merlie JP. Failure of postsynaptic specialization to develop at neuro-muscular junctions of rapsyn-deficient mice. Nature. 1995; 377:232–236. [PubMed: 7675108]

Gautam M, Noakes PG, Moscoso L, Rupp F, Scheller RH, Merlie JP, Sanes JR. Defective neuromuscular synaptogenesis in agrin-deficient mutant mice. Cell. 1996; 85:525–535. [PubMed: 8653788]

Gillespie SK, Balasubramanian S, Fung ET, Huganir RL. Rapsyn clusters and activates the synapse-specific receptor tyrosine kinase MuSK. Neuron. 1996; 16:953–962. [PubMed: 8630253]

Gong L, Yeh ET. Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway. J. Biol. Chem. 1999; 274:12036–12042. [PubMed: 10207026]

Han H, Noakes PG, Phillips WD. Overexpression of rapsyn inhibits agrin-induced acetylcholine receptor clustering in muscle cells. J. Neurocytol. 1999; 28:763–775. [PubMed: 10859577]

Jacobson C, Côté PD, Rossi SG, Rotundo RL, Carbonetto S. The dystroglycan complex is necessary for stabilization of acetylcho-line receptor clusters at neuromuscular junctions and formation of the synaptic basement membrane. J. Cell Biol. 2001; 152:435–450. [PubMed: 11157973]

Kim N, Burden SJ. MuSK controls where motor axons grow and form synapses. Nat. Neurosci. 2008; 11:19–27. [PubMed: 18084289]

Kirsch J, Betz H. The postsynaptic localization of the glycine receptor-associated protein gephyrin is regulated by the cytoskeleton. J. Neurosci. 1995; 15:4148–4156. [PubMed: 7790902]

Komander D, Rape M. The ubiquitin code. Annu. Rev. Biochem. 2012; 81:203–229. [PubMed: 22524316]

LaRochelle WJ, Froehner SC. Determination of the tissue distributions and relative concentrations of the postsynaptic 43-kDa protein and the acetylcholine receptor in Torpedo. J. Biol. Chem. 1986; 261:5270–5274. [PubMed: 3514614]

Lee Y, Rudell J, Ferns M. Rapsyn interacts with the muscle acetylcholine receptor via alpha-helical domains in the alpha, beta, and epsilon subunit intracellular loops. Neuroscience. 2009; 163:222–232. [PubMed: 19482062]

Li XM, Dong XP, Luo SW, Zhang B, Lee DH, Ting AK, Neiswender H, Kim CH, Carpenter-Hyland E, Gao TM, et al. Retrograde regulation of motoneuron differentiation by muscle beta-catenin. Nat. Neurosci. 2008; 11:262–268. [PubMed: 18278041]

Liakopoulos D, Doenges G, Matuschewski K, Jentsch S. A novel protein modification pathway related to the ubiquitin system. EMBO J. 1998; 17:2208–2214. [PubMed: 9545234]

Lim LE, Campbell KP. The sarcoglycan complex in limb-girdle muscular dystrophy. Curr. Opin. Neurol. 1998; 11:443–452. [PubMed: 9847993]

Luo ZG, Je HS, Wang Q, Yang F, Dobbins GC, Yang ZH, Xiong WC, Lu B, Mei L. Implication of geranylgeranyltransferase I in synapse formation. Neuron. 2003; 40:703–717. [PubMed: 14622576]

Luo S, Zhang B, Dong XP, Tao Y, Ting A, Zhou Z, Meixiong J, Luo J, Chiu FC, Xiong WC, Mei L. HSP90 beta regulates rapsyn turnover and subsequent AChR cluster formation and maintenance. Neuron. 2008; 60:97–110. [PubMed: 18940591]

Maimone MM, Merlie JP. Interaction of the 43 kd postsynaptic protein with all subunits of the muscle nicotinic acetylcholine receptor. Neuron. 1993; 11:53–66. [PubMed: 8338668]

Matthews-Bellinger J, Salpeter MM. Distribution of acetylcho-line receptors at frog neuromuscular junctions with a discussion of some physiological implications. J. Physiol. 1978; 279:197–213. [PubMed: 307600]

Matthews-Bellinger JA, Salpeter MM. Fine structural distribution of acetylcholine receptors at developing mouse neuromuscular junctions. J. Neurosci. 1983; 3:644–657. [PubMed: 6827314]

McMahan UJ. The agrin hypothesis. Cold Spring Harb. Symp. Quant. Biol. 1990; 55:407–418. [PubMed: 1966767]

Mendoza HM, Shen LN, Botting C, Lewis A, Chen J, Ink B, Hay RT. NEDP1, a highly conserved cysteine protease that deNEDDylates Cullins. J. Biol. Chem. 2003; 278:25637–25643. [PubMed: 12730221]

Merlie JP, Sanes JR. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibres. Nature. 1985; 317:66–68. [PubMed: 3839905]

Muscat GE, Kedes L. Multiple 5′-flanking regions of the human alpha-skeletal actin gene synergistically modulate muscle-specific expression. Mol. Cell. Biol. 1987; 7:4089–4099. [PubMed: 2828926]

Neubig RR, Krodel EK, Boyd ND, Cohen JB. Acetylcholine and local anesthetic binding to Torpedo nicotinic postsynaptic membranes after removal of nonreceptor peptides. Proc. Natl. Acad. Sci. USA. 1979; 76:690–694. [PubMed: 34154]

Niethammer M, Valtschanoff JG, Kapoor TM, Allison DW, Weinberg RJ, Craig AM, Sheng M. CRIPT, a novel postsynaptic protein that binds to the third PDZ domain of PSD-95/SAP90. Neuron. 1998; 20:693–707. [PubMed: 9581762]

Nitkin RM, Smith MA, Magill C, Fallon JR, Yao YM, Wallace BG, McMahan UJ. Identification of agrin, a synaptic organizing protein from Torpedo electric organ. J. Cell Biol. 1987; 105:2471–2478. [PubMed: 2826489]

Noakes PG, Phillips WD, Hanley TA, Sanes JR, Merlie JP. 43K protein and acetylcholine receptors colocalize during the initial stages of neuromuscular synapse formation in vivo. Dev. Biol. 1993; 155:275–280. [PubMed: 8416841]

Ohno K, Engel AG, Shen XM, Selcen D, Brengman J, Harper CM, Tsujino A, Milone M. Rapsyn mutations in humans cause endplate acetylcholine-receptor deficiency and myasthenic syndrome. Am. J. Hum. Genet. 2002; 70:875–885. [PubMed: 11791205]

Okada K, Inoue A, Okada M, Murata Y, Kakuta S, Jigami T, Kubo S, Shiraishi H, Eguchi K, Motomura M, et al. The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science. 2006; 312:1802–1805. [PubMed: 16794080]

Phillips WD, Kopta C, Blount P, Gardner PD, Steinbach JH, Merlie JP. ACh receptor-rich membrane domains organized in fibro-blasts by recombinant 43-kildalton protein. Science. 1991; 251:568–570. [PubMed: 1703661]

Ramarao MK, Cohen JB. Mechanism of nicotinic acetylcholine receptor cluster formation by rapsyn. Proc. Natl. Acad. Sci. USA. 1998; 95:4007–4012. [PubMed: 9520483]

Ramarao MK, Bianchetta MJ, Lanken J, Cohen JB. Role of rapsyn tetratricopeptide repeat and coiled-coil domains in self-association and nicotinic acetylcholine receptor clustering. J. Biol. Chem. 2001; 276:7475–7483. [PubMed: 11087759]

Samuel MA, Valdez G, Tapia JC, Lichtman JW, Sanes JR. Agrin and synaptic laminin are required to maintain adult neuromuscular junctions. PLoS ONE. 2012; 7:e46663. [PubMed: 23056392]

Sanes JR, Lichtman JW. Development of the vertebrate neuro-muscular junction. Annu. Rev. Neurosci. 1999; 22:389–442. [PubMed: 10202544]

Sanes JR, Lichtman JW. Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat. Rev. Neurosci. 2001; 2:791–805. [PubMed: 11715056]

Sealock R, Wray BE, Froehner SC. Ultrastructural localization of the Mr 43,000 protein and the acetylcholine receptor in Torpedo post-synaptic membranes using monoclonal antibodies. J. Cell Biol. 1984; 98:2239–2244. [PubMed: 6725413]

Shen LN, Liu H, Dong C, Xirodimas D, Naismith JH, Hay RT. Structural basis of NEDD8 ubiquitin discrimination by the deNEDDylating enzyme NEDP1. EMBO J. 2005; 24:1341–1351. [PubMed: 15775960]

Sheng M, Hoogenraad CC. The postsynaptic architecture of excitatory synapses: a more quantitative view. Annu. Rev. Biochem. 2007; 76:823–847. [PubMed: 17243894]

Sheng M, Sala C. PDZ domains and the organization of supramolecular complexes. Annu. Rev. Neurosci. 2001; 24:1–29. [PubMed: 11283303]

Sobel A, Changeux JP. Purification and characterization of the cholinergic receptor protein in its membrane-bound and detergent-soluble forms from the electric organ of Torpedo marmorata. Biochem. Soc. Trans. 1977; 5:511–514. [PubMed: 902870]

Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP, Critchley S, et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature. 2009; 458:732–736. [PubMed: 19360080]

Thien CB, Walker F, Langdon WY. RING finger mutations that abolish c-Cbl-directed polyubiquitination and downregulation of the EGF receptor are insufficient for cell transformation. Mol. Cell. 2001; 7:355–365. [PubMed: 11239464]

Tintignac LA, Brenner HR, Rüegg MA. Mechanisms regulating neuromuscular junction development and function and causes of muscle wasting. Physiol. Rev. 2015; 95:809–852. [PubMed: 26109340]

Tyagarajan SK, Fritschy JM. Gephyrin: a master regulator of neuronal function? Nat. Rev. Neurosci. 2014; 15:141–156. [PubMed: 24552784]

van der Veen AG, Ploegh HL. Ubiquitin-like proteins. Annu. Rev. Biochem. 2012; 81:323–357. [PubMed: 22404627]

Vogt J, Harrison BJ, Spearman H, Cossins J, Vermeer S, ten Cate LN, Morgan NV, Beeson D, Maher ER. Mutation analysis of CHRNA1, CHRNB1, CHRND, and RAPSN genes in multiple pterygium syndrome/fetal akinesia patients. Am. J. Hum. Genet. 2008; 82:222–227. [PubMed: 18179903]

Waites CL, Craig AM, Garner CC. Mechanisms of vertebrate synaptogenesis. Annu. Rev. Neurosci. 2005; 28:251–274. [PubMed: 16022596]

Walker JH, Boustead CM, Witzemann V. The 43-K protein, v1, associated with acetylcholine receptor containing membrane fragments is an actin-binding protein. EMBO J. 1984; 3:2287–2290. [PubMed: 6389118]

Watson IR, Blanch A, Lin DC, Ohh M, Irwin MS. Mdm2-mediated NEDD8 modification of TAp73 regulates its transactivation function. J. Biol. Chem. 2006; 281:34096–34103. [PubMed: 16980297]

Weatherbee SD, Anderson KV, Niswander LA. LDL-receptor-related protein 4 is crucial for formation of the neuromuscular junction. Development. 2006; 133:4993–5000. [PubMed: 17119023]

Wu H, Xiong WC, Mei L. To build a synapse: signaling pathways in neuromuscular junction assembly. Development. 2010; 137:1017–1033. [PubMed: 20215342]

Wu H, Lu Y, Barik A, Joseph A, Taketo MM, Xiong WC, Mei L. β-Catenin gain of function in muscles impairs neuromuscular junction formation. Development. 2012a; 139:2392–2404. [PubMed: 22627288]

Wu H, Lu Y, Shen C, Patel N, Gan L, Xiong WC, Mei L. Distinct roles of muscle and motoneuron LRP4 in neuromuscular junction formation. Neuron. 2012b; 75:94–107. [PubMed: 22794264]

Wu H, Barik A, Lu Y, Shen C, Bowman A, Li L, Sathyamurthy A, Lin TW, Xiong WC, Mei L. Slit2 as a β-catenin/Ctnnb1-dependent retrograde signal for presynaptic differentiation. eLife. 2015; 4:e07266.

Yoshihara CM, Hall ZW. Increased expression of the 43-kD protein disrupts acetylcholine receptor clustering in myotubes. J. Cell Biol. 1993; 122:169–179. [PubMed: 7686162]

Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L. LRP4 serves as a coreceptor of agrin. Neuron. 2008; 60:285–297. [PubMed: 18957220]

Zong Y, Zhang B, Gu S, Lee K, Zhou J, Yao G, Figueiredo D, Perry K, Mei L, Jin R. Structural basis of agrin-LRP4-MuSK signaling. Genes Dev. 2012; 26:247–258. [PubMed: 22302937]

Zuo W, Huang F, Chiang YJ, Li M, Du J, Ding Y, Zhang T, Lee HW, Jeong LS, Chen Y, et al. c-Cbl-mediated neddylation antagonizes ubiquitination and degradation of the TGF-β type II receptor. Mol. Cell. 2013; 49:499–510. [PubMed: 23290524]

Highlights

- RING domain of rapsyn contains E3 ligase activity
- Rapsyn E3 ligase activity catalyzes neddylation as well as ubiquitination
- Agrin promotes AChR neddylation for cluster formation
- Rapsyn E3 ligase activity is required for AChR clustering and NMJ assembly

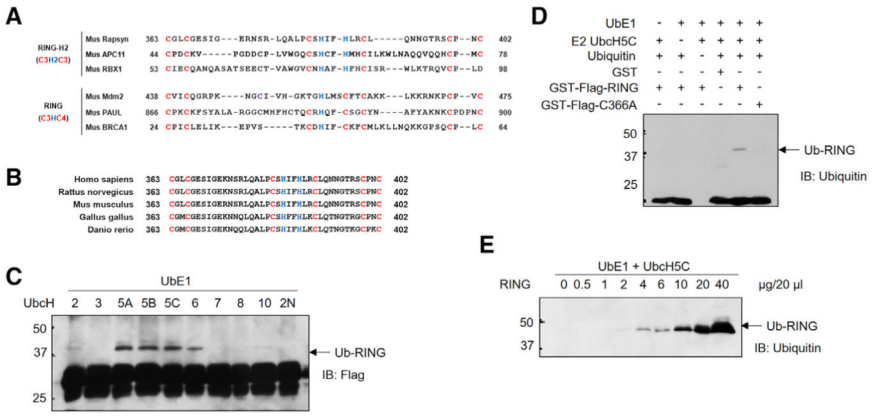

Figure 1. E3 Ligase Activity of the RING Domain of Rapsyn

(A) Rapsyn is a RING-H2-containing protein. Shown are amino sequences of RING-H2 proteins including APC11 and RBX1, and RING proteins including Mdm2, PAUL, and BRCA1. Conserved cysteines and histidines are indicated by red and blue, respectively.

(B) Conserved C3H2C3 motif in rapsyn of different species.

(C) E3 ligase activity in rapsyn RING domain. GST-Flag-RING was purified from bacteria by affinity chromatography and incubated with Ub, UbE1, and indicated E2 in in vitro ubiquitination assay. Ubiquitinated RING proteins were probed by anti-Flag antibody.

(D) Mutation of C366 in the rapsyn RING domain abolishes E3 ligase activity. E3 ligase activity was assayed as in (C), with indicated components.

(E) Dependence of E3 ligase activity on RING concentrations. E3 ligase activity was assayed as in (C), with increasing concentrations of RING domains.

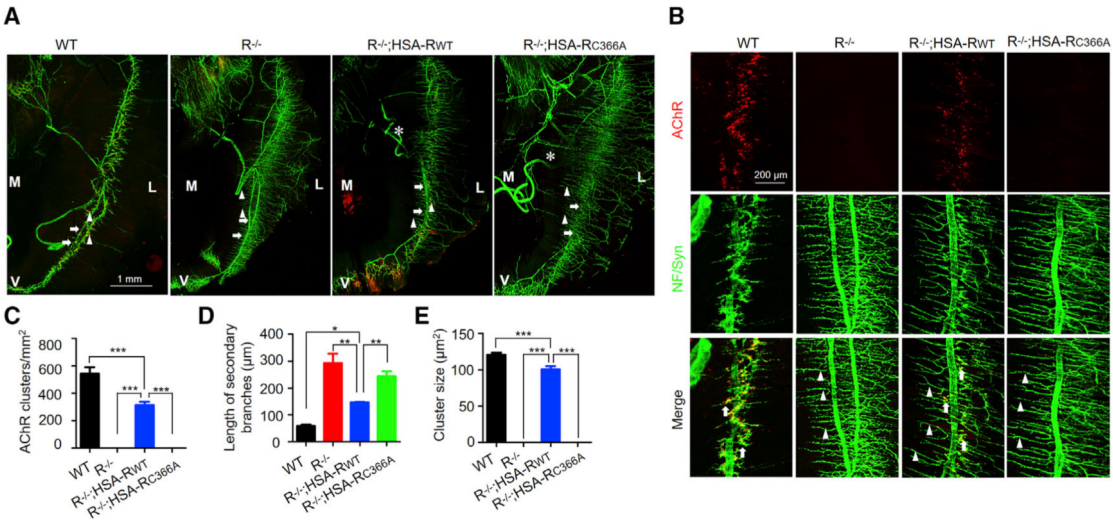

Figure 2. Inability of C366A Rapsyn to Rescue NMJ Deficits in Rapsyn Null Mutant Mice

(A and B) Diaphragms of P0 mice of indicated genotypes were stained whole mount with Alexa Fluor 594-BTX to label AChR clusters and with anti-NF/synaptophysin antibodies (green) to label axons and nerve terminals. Left ventral areas of diaphragms are shown. Arrows indicate primary branches, arrowheads indicate secondary branches, and asterisks indicate motor nerve. Images at (A) low and (B) high magnifications are shown.

(C–E) Quantitative analysis of (C) AChR clusters, (D) length of secondary nerve branches, and (E) cluster size in (B).

Data are shown as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001, one-way ANOVA. n = 3. L, lateral; M, medial.

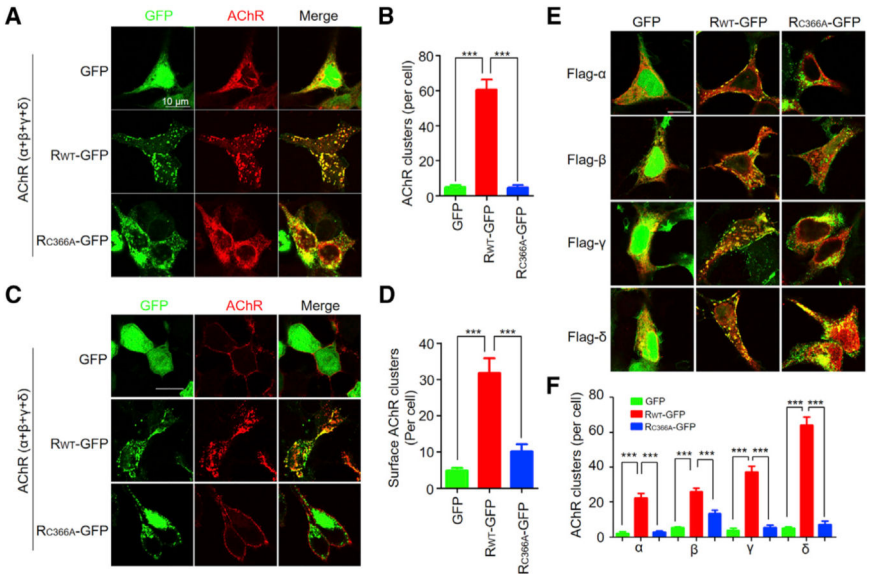

Figure 3. Requirement of E3 Ligase Activity for Rapsyn-Induced AChR Aggregation

(A) Fewer AChR aggregates in HEK293T cells expressing C366A mutant, compared with cells expressing WT rapsyn. HEK293T cells were transfected with indicated plasmids and, 48 hr later, were fixed and stained with Alexa Fluor 594-BTX to label AChR.

(B) Quantitative analysis of AChR aggregates.

(C) Reduced surface AChR aggregatesin HEK293T cells expressing C366A, compared with cells expressing WT rapsyn. Cells were transfected as in (A). After 48 hr, live, unfixed cells were stained with Alexa Fluor 594-BTX to label surface AChR.

(D) Quantitative analysis of data in (C).

(E) Fewer aggregates of AChR subunits in HEK293T cells expressing C366A rapsyn, compared with cells expressing WT. HEK293T cells were transfected with different individual AChR subunits and, 48 hr later, were fixed and stained with anti-Flag antibody to label perspective subunit.

(F) Quantitative analysis of data in (E).

Data were shown as mean ± SEM. ***p < 0.001, one-way ANOVA. n = 20 cells of three individual experiments.

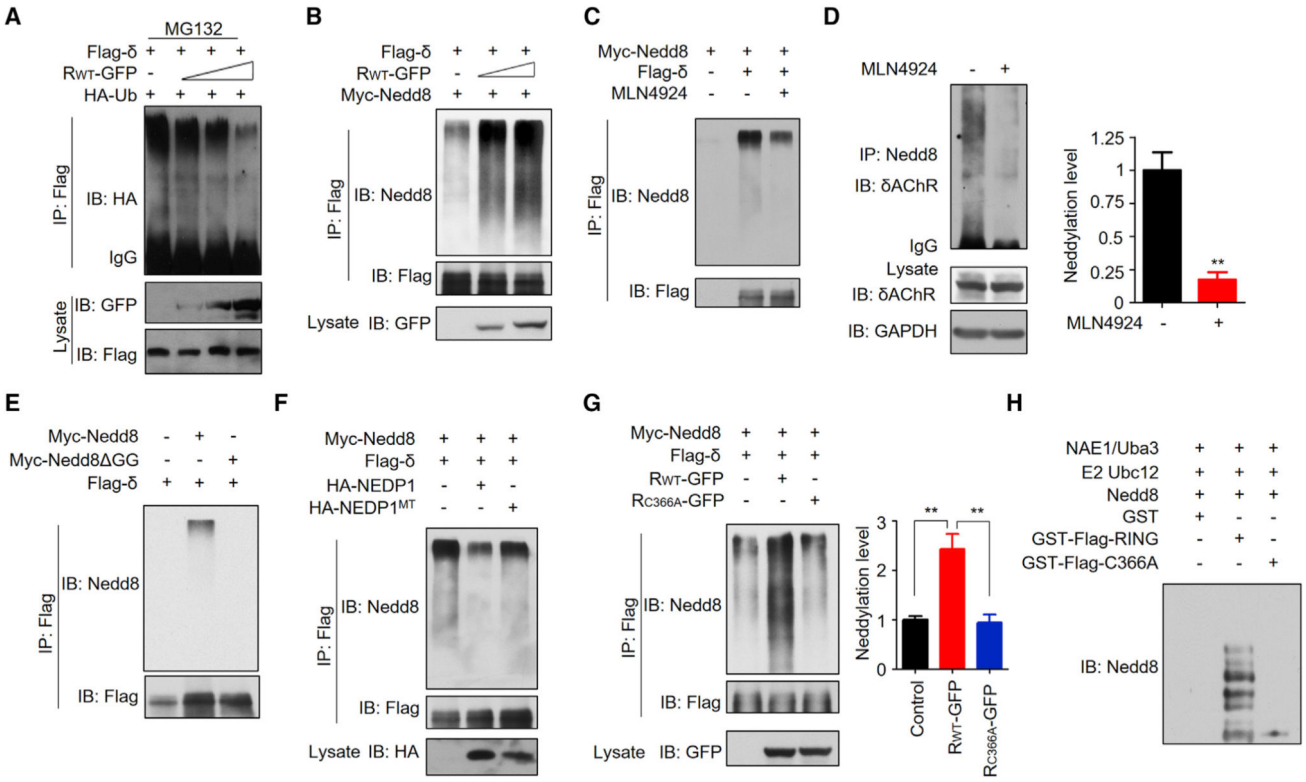

Figure 4. Neddylation of AChR by Rapsyn

(A) Reduced ubiquitin-conjugated δ subunit in rapsyn-expressing cells. HEK293T cells were transfected with Flag-δ and HA-Ub with or without GFP-fused WT rapsyn (R_{WT}-GFP). Ubiquitinated δ subunit was purified by IP with anti-Flag antibody and revealed by anti-HA antibody.

(B) Increased neddylated δ subunit in rapsyn-expressing cells. HEK293T cells were transfected with Flag-δ and Myc-Nedd8 with or without R_{WT}-GFP. Neddylated δ subunit was purified by IP with anti-Flag antibody and revealed by anti-Nedd8 antibody.

(C) Reduced neddylated δ subunit by MLN4924. HEK293T cells were transfected with Flag-δ and Myc-Nedd8 and treated with or without MLN4924. Neddylated δ subunit was revealed as in (B).

(D) Reduced neddylated δ subunit in MLN4924-treated C2C12 myotubes.

(E) Inability of Nedd8ΔGG mutant to be conjugated to δ subunit. HEK293T cells were transfected with Flag-δ with Myc-Nedd8 or Myc-Nedd8ΔGG. Neddylated δ subunit was revealed as in (B).

(F) δ subunit neddylation was inhibited by NEDP1. HEK293T cells were transfected with Flag-δ and Myc-Nedd8, without or with HA-tagged NEDP1 or NEDP1 mutant. Neddylated δ subunit was revealed as in (B).

(G) Dependence of δ subunit neddylation on E3 ligase activity of rapsyn. HEK293T cells were transfected with Flag-δ and Myc-Nedd8, without or with GFP-tagged WT or C366A rapsyn. Neddylated δ subunit was revealed as in (B).

(H) Neddylation E3 ligase activity in rapsyn RING domain. GST-Flag-RING and GST-Flag-C366A were purified from bacteria by affinity chromatography and incubated with Nedd8,

NAE1/Uba3, and Ubc12 in in vitro neddylation assay. Neddylated RING proteins were probed by anti-Nedd8 antibody.

Data are shown as mean ± SEM. (D) **p < 0.01, Student's t test; n = 4. (G) **p < 0.01, one-way ANOVA; n = 5.

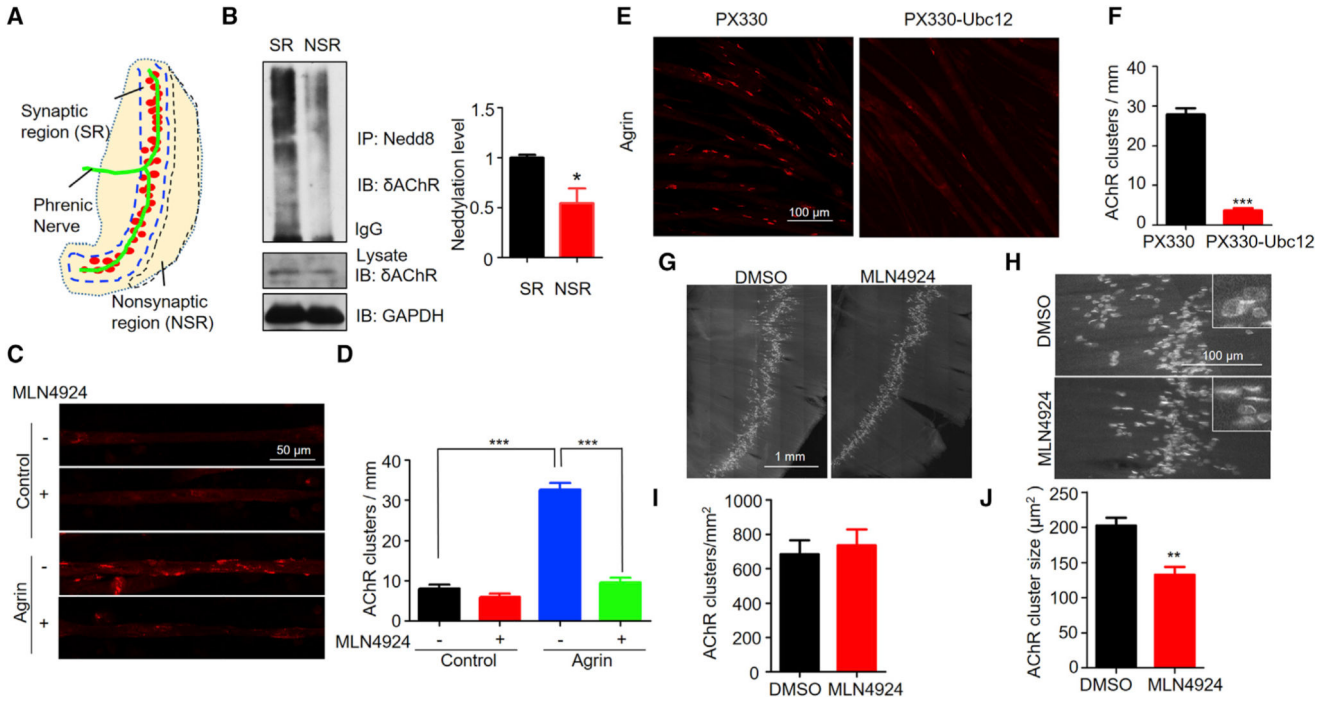

Figure 5. Attenuated AChR Clustering by Inhibiting Neddylation

(A) Diagram of synaptic region (SR) and nonsynaptic region (NSR) of a hemidiaphragm.

(B) Neddylated AChR δ subunit was enriched in the SR. Neddylated proteins were purified by immunoprecipitation and probed with anti-δ subunit antibody. Lysates were blotted for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as loading control (bottom).

(C) Inhibition of agrin-induced AChR clustering by MLN4924. C2C12 myotubes were treated with 100 ng/ml agrin and 20 μM MLN4924 for 12 hr, fixed, and stained with Alexa Fluor 594-BTX to label AChR clusters.

(D) Quantitative analysis of AChR clusters in (C).

(E) Inhibition of agrin-induced AChR clustering by Ubc12 knockdown. C2C12 myoblasts were transfected with PX330 (control) or PX330-Ubc12. Resulting myotubes were treated with 100 ng/ml agrin for 12 hr, fixed, and stained with Alexa Fluor 594-BTX to label AChR clusters.

(F) Quantitative analysis of AChR clusteres in (E).

(G and H) Reduced size of AChR clusters in mice treated with MLN4924. P0 pups were injected with 50 mmol MLN4924 once a day for 2 days. Diaphragms were stained whole mount with Alexa Fluor 594-BTX to label AChR clusters. Left ventral areas of diaphragms were shown at (G) low and (H) high magnification.

(I and J) Quantitative analysis of (I) AChR clusters and (J) cluster size in (H).

Data were shown as mean ± SEM. (B) *$p < 0.05$, Student's t test; n = 6. (D) ***$p < 0.001$, one-way ANOVA; n = 20 cells of three individual experiments. (F and J) **$p < 0.01$; ***$p < 0.001$, Student's t test; n = 20 cells of three individual experiments.

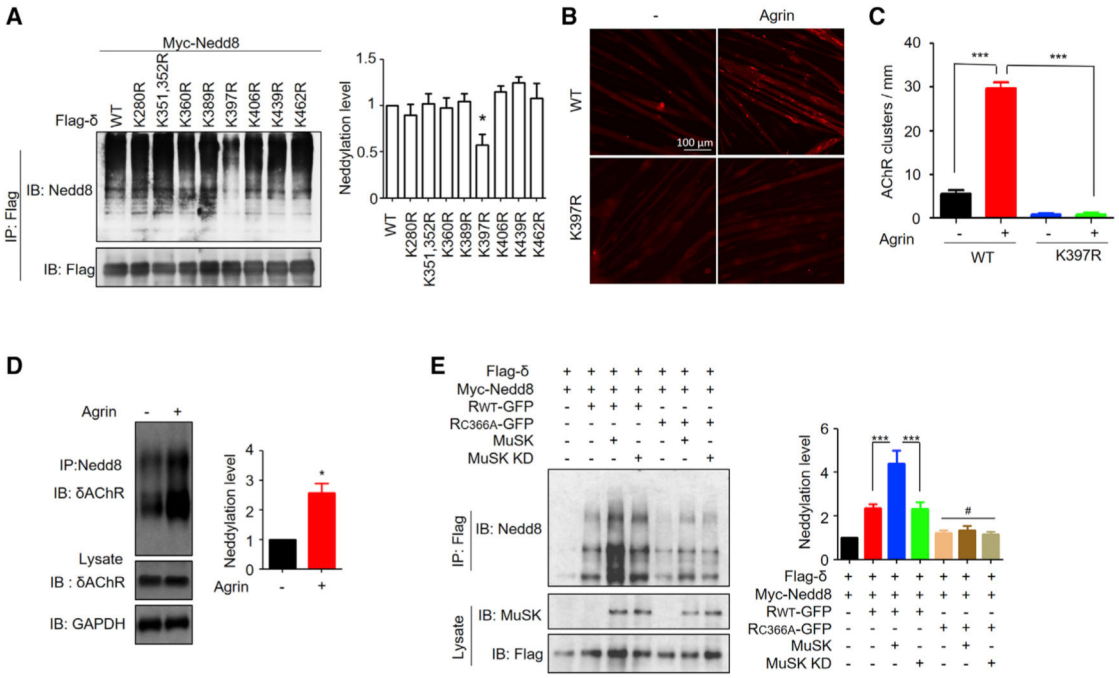

Figure 6. Requirement of δ-Subunit Neddylation for AChR Clustering and Regulation by Agrin

(A) K397 is a key site of neddylation in the δ subunit. HEK293T cells were transfected with Nedd8 and Flag-δ mutants. Neddylated δ subunit was purified by IP with anti-Flag antibody and revealed by anti-Nedd8 antibody.

(B) A few AChR clusters in K397R mutant myotubes. WT or K397R mutant myotubes were treated with 100 ng/ml agrin for 12 hr, fixed, and stained with Alexa Fluor 594-BTX to label AChR clusters.

(C) Quantitative analysis of AChR clusters in (B).

(D) Increased neddylation of the δ subunit by agrin. C2C12 myotubes were treated with or without agrin. Neddylated δ subunit was purified by anti-Nedd8 antibody and revealed by anti-δ subunit antibody.

(E) Regulation of the E3 ligase activity by MuSK. HEK293T cells were transfected with indicated plasmids. Neddylated δ subunit was purified by IP with anti-Flag antibody and revealed by anti-Nedd8 antibody.

Data are shown as mean ± SEM. (C) ***$p < 0.001$, one-way ANOVA; n = 20 cells of three individual experiments. (E) ***$p < 0.001$; $^{\#}p < 0.05$, one-way ANOVA; n = 4.

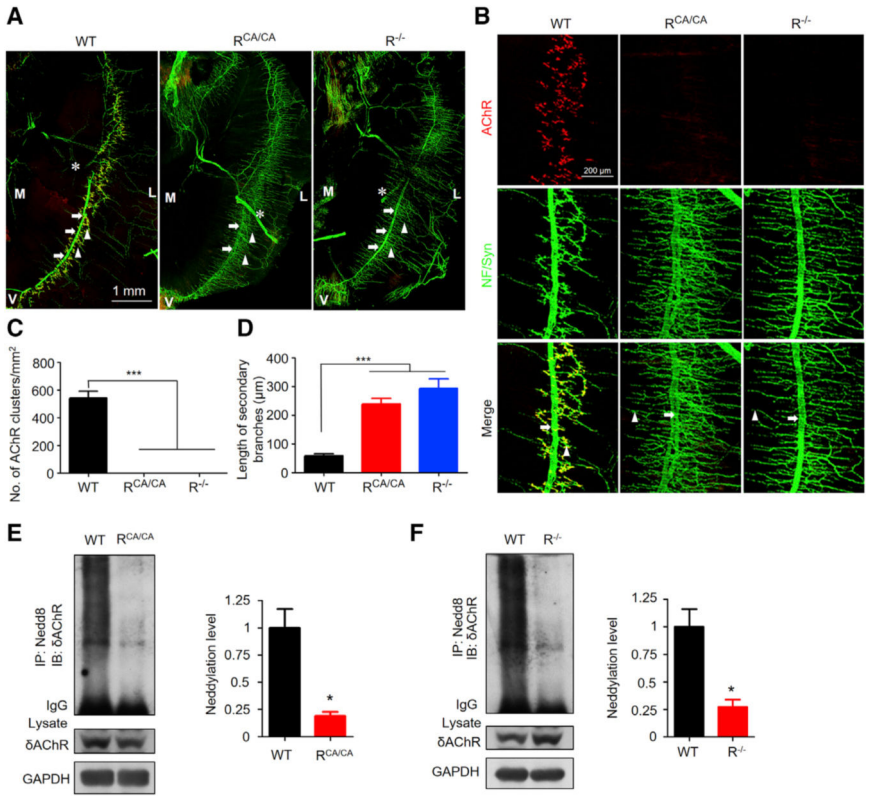

Figure 7. Aberrant NMJ Formation in Rapsyn C366A Mutant Mice

(A and B) Pre- and post-synaptic NMJ deficits in $R^{CA/CA}$ mice. Diaphragms of P0 mice of indicated genotypes were stained whole mount with Alexa Fluor 594-BTX (red) to label AChR clusters, and anti-NF/synaptophysin antibodies (green) to label axons and nerve terminals. Shown are left ventral areas of diaphragms at lower (A) and higher (B) magnifications. Arrows indicate primary branches, arrowheads indicate secondary branches, and asterisks indicate motor nerve.

(C and D) Quantitative analysis of (C) AChR clusters and (D) length of secondary branches of phrenic nerves.

(E and F) Reduced neddylated δ subunit in (E) $R^{CA/CA}$ and (F) rapsyn null muscles. Muscles of indicated genotypes were homogenized and subjected to immunoprecipitation with anit-Nedd8 antibody and western blotting with anti-δ subunit antibody. Lysates were blotted directly with antibodies against δ subunit and GAPDH (bottom panels) as control. Data were shown as mean ± SEM. (C and D) ***$p < 0.001$, one-way ANOVA; n = 4. (E and F) *$p < 0.05$, Student's t test; n = 4. L, lateral; M, medial.