Cellular/Molecular

Nuclear Factor κB Controls Acetylcholine Receptor Clustering at the Neuromuscular Junction

Jia Wang,* Xiu-Qing Fu,* Wen-Liang Lei, Tong Wang, Ai-Li Sheng, and Zhen-Ge Luo

Institute of Neuroscience and State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

At the vertebrate neuromuscular junction (NMJ), acetylcholine receptor (AChR) clustering is stimulated by motor neuron-derived glycoprotein Agrin and requires a number of intracellular signal or structural proteins, including AChR-associated scaffold protein Rapsyn. Here, we report a role of nuclear factor κB (NF-κB), a well known transcription factor involved in a variety of immune responses, in regulating AChR clustering at the NMJ. We found that downregulating the expression of RelA/p65 subunit of NF-κB or inhibiting NF-κB activity by overexpression of mutated form of IκB (inhibitor κB), which is resistant to proteolytic degradation and thus constitutively keeps NF-κB inactive in the cytoplasma, impeded the formation of AChR clusters in cultured C2C12 muscle cells stimulated by Agrin. In contrast, overexpression of RelA/p65 promoted AChR clustering. Furthermore, we investigated the mechanism by which NF-κB regulates AChR clustering. Interestingly, we found that downregulating the expression of RelA/p65 caused a marked reduction in the protein and mRNA level of Rapsyn and upregulation of RelA/p65 enhanced Rapsyn promoter activity. Mutation of NF-κB binding site on Rapsyn promoter prevented responsiveness to RelA/p65 regulation. Moreover, forced expression of Rapsyn in RelA/p65 downregulated muscle cells partially rescued AChR clusters, suggesting that NF-κB regulates AChR clustering, at least partially through the transcriptional regulation of Rapsyn. In line with this notion, genetic ablation of RelA/p65 selectively in the skeletal muscle caused a reduction of AChR density at the NMJ and a decrease in the level of Rapsyn. Thus, NF-κB signaling controls AChR clustering through transcriptional regulation of synaptic protein Rapsyn.

Introduction

The transcription factor nuclear factor κB (NF-κB) is best known for its roles in inflammation and immune responses (Chen and Greene, 2004; Lin and Karin, 2007). NF-κB is composed of heterodimers or homodimers of NF-κB subunits, RelA (p65), RelB, c-Rel, p50, and p52, among which the most prominent dimer is that of RelA/p65 and p50, with RelA/p65 containing transactivation domains (Hayden and Ghosh, 2004; Karin, 2006). The activity of NF-κB is tightly controlled by inhibitor κB (IκB), which keeps NF-κB inactive in the cytoplasm (Albensi and Mattson, 2000). Phosphorylation by IKK (IκB kinase) causes proteolytic degradation of IκB, resulting in NF-κB translocation to the nucleus where it binds to the κB consensus sequence within target gene promoters and regulates gene transcription (DiDonato et al., 1997; Zandi et al., 1998; Albensi and Mattson, 2000). A growing body of evidence has suggested roles of NF-κB proteins in nervous system, including neural development, neurodegeneration, synaptic plasticity, and behavior (Mattson et al., 2000; Meffert et al., 2003; Meffert and Baltimore, 2005; Mattson and Meffert, 2006; Lubin and Sweatt, 2007). Here, we have investigated the role and mechanism of NF-κB signaling in the postsynaptic organization of vertebrate NMJ.

At the vertebrate NMJ, clustering of acetylcholine receptor (AChR) at postsynaptic skeletal muscle surface is stimulated by Agrin, a motor neuron-derived glycoprotein, through activation of muscle-specific tyrosine kinase (MuSK) (Valenzuela et al., 1995; DeChiara et al., 1996; Glass et al., 1996; Sanes and Lichtman, 1999, 2001) and the coreceptor LRP4 (Kim et al., 2008; Zhang et al., 2008). A number of intracellular proteins regulating this process have been identified (Wu et al., 2010). Among them, AChR-associated protein Rapsyn plays a pivotal role in AChR cluster formation and stabilization (Phillips et al., 1991a,b; Gautam et al., 1995; Apel et al., 1997). Genetic ablation of Rapsyn prevents AChR clustering and NMJ formation (Gautam et al., 1995; Lin et al., 2001), and overexpression of Rapsyn is sufficient to induce AChR clustering in heterologous cells (Qu et al., 1996; Apel et al., 1997). Recent studies have shown that Agrin induces, whereas ACh disperses, AChR clusters during NMJ synaptogenesis (Lin et al., 2005; Misgeld et al., 2005), and this counteractive interaction is mediated by Rapsyn, which stabilizes AChR clusters by inhibiting Calpain, a calcium-dependent protease involved in ACh-induced dispersion of AChR clusters (Chen et al., 2007).

Received April 26, 2010; revised June 29, 2010; accepted July 2, 2010.

This work was supported by National Natural Science Foundation of China Grants 30721004 and 30825013, National Basic Research Program Grants 2006CB806600 and 2011CB809002, Key State Research Program of China Grant 2006CB943900, Chinese Academy of Sciences Grant KSCX2-YW-R-102, and Program of Shanghai Subject Chief Scientist Grant 08XD14050. We thank Dr. C. Wang at Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, for the IκB^{SR} and RelA/p65 constructs, Dr. Y. Z. Wang at Institute of Neuroscience, Chinese Academy of Sciences, for the RelA/p65 construct, and Dr. M. C. Hung at M. D. Anderson Cancer Center, University of Texas, for the κB-luc construct. We are grateful to Dr. L. Mei for valuable reagents, Dr. R. M. Schmid for RelA$^{floxed/floxed}$ mouse line, and Dr. P. Noakes for Rapsyn$^{+/-}$ mouse line. The Institute of Neuroscience Imaging Facility provided technical assistance for microscope analysis.

*J.W. and X.-Q.F. contributed equally to this work.

Correspondence should be addressed to Dr. Zhen-Ge Luo, Institute of Neuroscience, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China. E-mail: zgluo@ion.ac.cn.

DOI:10.1523/JNEUROSCI.2118-10.2010

 0270-6474/10/3011104-10$15.00/0

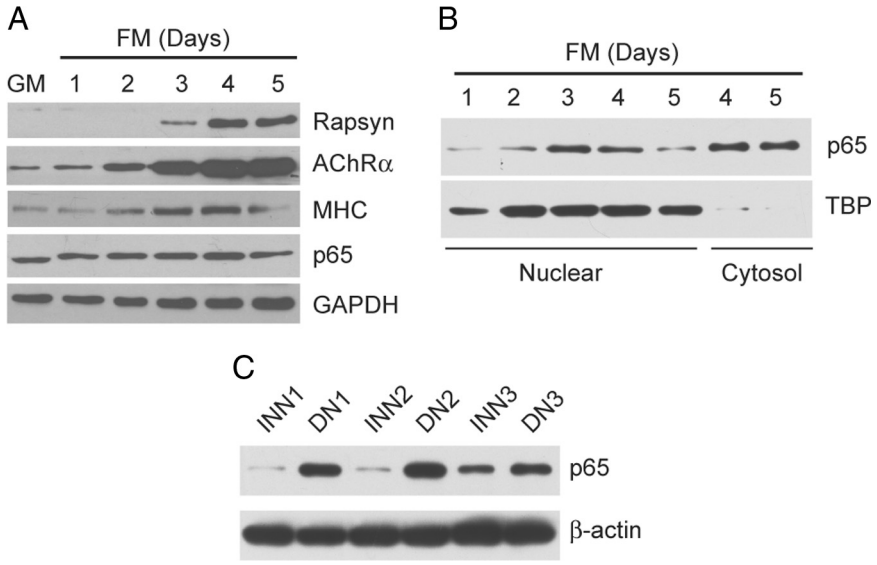

Figure 1. RelA/p65 is expressed in skeletal muscle cells. ***A***, ***B***, C2C12 myoblasts in growth media (GM) or myotubes in differentiation/fusion media (FM) for different days were lysed or subjected to nuclear purification. Cell lysates (***A***) or nuclear extracts (***B***) were subjected to immunoblotting (IB) with indicated antibodies. TBP was used as nuclear loading control. ***C***, Two weeks after denervation, soleus muscle was separated and homogenates (20 μg of proteins) were subjected to IB with indicated antibodies. Muscles without denervation were used as controls. Three mice were analyzed. DN, Denervated; INN, innervated.

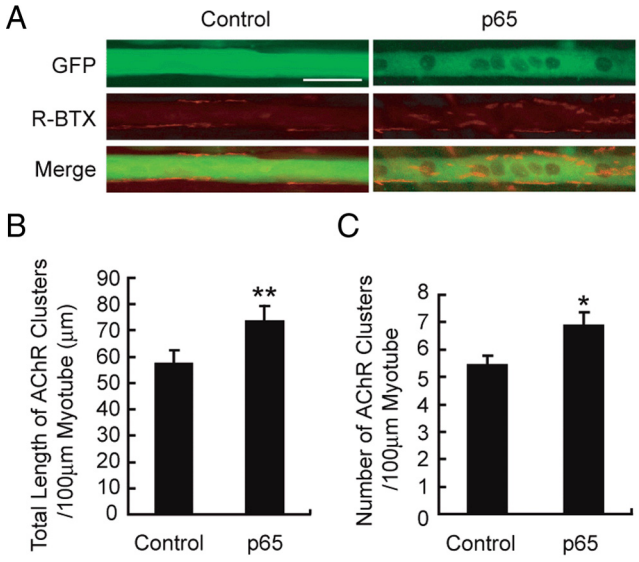

Figure 2. Upregulation of RelA/p65 promotes AChR clustering. ***A***, C2C12 myoblasts were transfected with vectors encoding GFP-p65 or GFP, and the fully differentiated myotubes were treated with Agrin (5 ng/ml) for 6 h to induce the formation of AChR clusters. Transfected myotubes were then stained with R-BTX. Scale bar, 50 μm. ***B***, ***C***, Quantification of total AChR cluster length (***B***) and numbers (***C***) in 100 μm of transfected myotubes marked with GFP ($n = 119$ for control, $n = 50$ for p65). Data are shown as mean ± SEM from three experiments. *$p < 0.05$, **$p < 0.01$, one-way ANOVA with Student's *t* test.

The importance of Rapsyn at the NMJ has prompted us to investigate the mechanisms governing Rapsyn expression.

Here, we have investigated the function of NF-κB in the assembly of the postsynaptic apparatus at the vertebrate NMJ. Upregulation of NF-κB promoted, whereas downregulation or inhibition of NF-κB attenuated, AChR clustering in cultured skeletal muscle cells. Mechanistic studies showed that RelA/p65 subunit of NF-κB is essential for the expression of Rapsyn. In line with these findings, elimination of RelA/p65 in the skeletal muscle caused a decrease of AChR density at the NMJ. Thus, NF-κB signaling plays an important role in AChR clustering by transcriptional regulation of synaptic protein Rapsyn.

Materials and Methods

Reagents. Antibodies against Rapsyn and MuSK were described in our previous studies (Luo et al., 2002; Chen et al., 2007). Other antibodies were from the following companies: Millipore Bioscience Research Reagents (β-actin), Invitrogen [green fluorescent protein (GFP)], Clontech (AChRα), Santa Cruz (NF-κB p65, NF-κB p50, rabbit IgG), Abcam [TATA binding protein (TBP)], and Sigma-Aldrich [myosin heavy chain (MHC)]. Tetramethyl-rhodamine-α-bangarotoxin (R-BTX) was purchased from Invitrogen, and recombinant rat C-terminal Agrin was from R&D Systems.

Constructs. The construct harboring a fusion of the NF-κB p65 subunit with GFP (GFP-p65) was provided by Dr. Yi-Zheng Wang (Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China) (Tao et al., 2006). Stabilized IκB ($I\kappa B^{SR}$) was provided by Dr. Chen Wang (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) (Sun et al., 2007). Reporter construct of κB, κB-Luc, was provided by Dr. Mien-Chie Hung (M. D. Anderson Cancer Center, University of Texas, Houston, TX) (Deng et al., 2002). The reporter construct that contains the 5605 nt 5′-flanking region of Rapsyn gene and the downstream cDNA encoding firefly luciferase (R5k-Luc) was described in our previous study (Wang et al., 2008). Vector-based p65 small interference RNAs (siRNAs) were encoded by pSUPER vectors, with the following sequences: #366, 5′-GAAGCGAGACCTGGAGCAA-3′; #1410, 5′-CTCAGAGTTTCAGCAG-CTC-3′. The siRNA that does not target any known mammalian gene (5′-TTCTCCGAACGTGTCACGT-3′) was cloned into pSUPER as a control.

Cell culture, transfection, and Western blotting. C2C12 myoblasts were maintained in DMEM with 20% fetal bovine serum [growth media (GM)]. Fusion of myoblasts into myotubes was induced by switching to the differentiation/fusion media (FM) (DMEM supplemented with 3% horse serum). C2C12 myoblasts in 50% confluence were transfected with Lipofectamine 2000 (Invitrogen), or FuGene HD (Roche Applied Science), following the manufacturer's instructions, and then switched to FM. Whole-cell lysates in the Nonidet P-40 buffer containing protease inhibitor mixture (Calbiochem) or nuclear extracts were prepared, followed by SDS-PAGE and Western blot with indicated antibodies.

C2C12 stable cell line. Stable C2C12 clones expressing RelA/p65 siRNA (siRNA#366, siRNA#1410) or control siRNA were generated by transfection with pSUPER vectors encoding individual siRNA, respectively. Transfections were performed with Cell Line Nucleofector Kit V (Amaxa) according to the manufacturer's protocol, and G418-resistant clones were collected after 2 weeks in growth media.

Luciferase assay. C2C12 myoblasts were cotransfected with 0.2 μg of GFP-p65 or GFP, and 0.1 μg of R5k-Luc reporter plasmids in 24-well plate. After differentiation, luciferase activity in myotube lysates was measured using the dual luciferase kit (Promega). The relative luciferase activity of the reporter over cotransfected *Renilla* luciferase encoded by pRL-TK was normalized, with values at control group set as 1. The ratio of R5k-Luc to pRL-TK was 10:1.

Quantitative reverse transcription-PCR. Total RNA was isolated from C2C12 myotubes using Trizol reagent (Invitrogen). Reverse transcription was performed by using oligo-dT primers according to the manufacturer's protocol. Quantitative reverse transcription-PCR was performed on Mx3000P real-time PCR instrument (Stratagene) with a SYBR Premix Ex Taq kit (Takara). The housekeeping gene GAPDH was

used as the reference for quantification. Primer sequences were as follows: Rapsyn, 5′-ATATCGGGCCATGAGCCAGTAC-3′ (forward) and 5′-TCACAACACTCCATGGCACTGC-3′ (reverse); GAPDH, 5′-TGAAGCAGGCATCTGAGGG-3′ (forward) and 5′-CGAAGGTGGAAGAGTGGGAG-3′ (reverse).

Electrophoretic mobility shift assay. For electrophoretic mobility shift assay (EMSA) analysis, nuclear proteins of C2C12 myotubes were extracted by using a modified procedure described previously (Deryckere and Gannon, 1994). Briefly, myotubes were washed for three times using 1× PBS and lysed with low-salt solution (0.6% NP-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM PMSF, and 1:200 protease inhibitor mixture set III), followed by centrifugation at 2000 rpm for 30 s. The supernatant was incubated for 20 min on ice and then centrifuged for 5 min at 13,000 rpm. The pelleted nuclei components were resuspended in a high-salt solution (25% glycerol, 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, and 1:200 protease inhibitor mixture set III), and incubated on ice for 20 min. After brief centrifugation, insoluble cellular debris was discarded, and the supernatant was aliquoted, stored at −80°C until use. The probes used in EMSA were as follows: NF-κB-A, 5′-TCCAAGTGGGTAATTCCATGAAC-3′; NF-κB-B, 5′-GCTAGACAGGTGAAATCCCTTCT-3′; NF-κB-C, 5′-GATGACCTGGAACTTTCTATGTA-3′; NF-κB-D, 5′-AATGGGGTGGAATGTTCCATTAG-3′; MHC (positive control), 5′-CAGGGCTGGGGATTCCCCATCTCCACAGTTTCACTTC-3′; mutated NF-κB, 5′-AGTTGAGGCGACTTTCCCAGGC-3′. These probes were labeled with biotin using 3′-end DNA labeling kit (Pierce) and incubated with 10 μg of nuclear protein at room temperature for 20 min using the LightShift Chemiluminescent EMSA kit (Pierce). The DNA–protein complex was separated on a non-denatured 6% PAGE and visualized by chemiluminescent nucleic acid detection module kit (Pierce). For supershift assay, antibodies against p65, p50, or control rabbit IgG were incubated with the mixture of nuclear extracts and the probes for 20 min before non-denatured PAGE.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) analysis was performed following the manufacturer's instruction (Millipore) with minor modifications. Briefly, fully differentiated C2C12 myotubes (cultured on 10 cm dishes) were fixed with 1% formaldehyde for 10 min, and then 2.5 M glycine to stop fixation. Nuclear fractions were collected and lysed in 350 μl of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1). After extensive sonication, 150 μl of supernatant was mixed with 1350 μl of ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1), followed by immunoprecipitation with indicated antibodies. The precipitated complexes were washed and eluted as instructed, and coupled DNA was extracted for PCR analysis. For the PCR analysis of *Rapsyn* 5′ flanking region (−696 to −496 nt), the primers were as follows: 5′-GGCTGAGACAGGTAGATCTCTC-3′ (forward) and 5′-AAGCCAGGGTAGCCTCAAACTC-3′ (reverse).

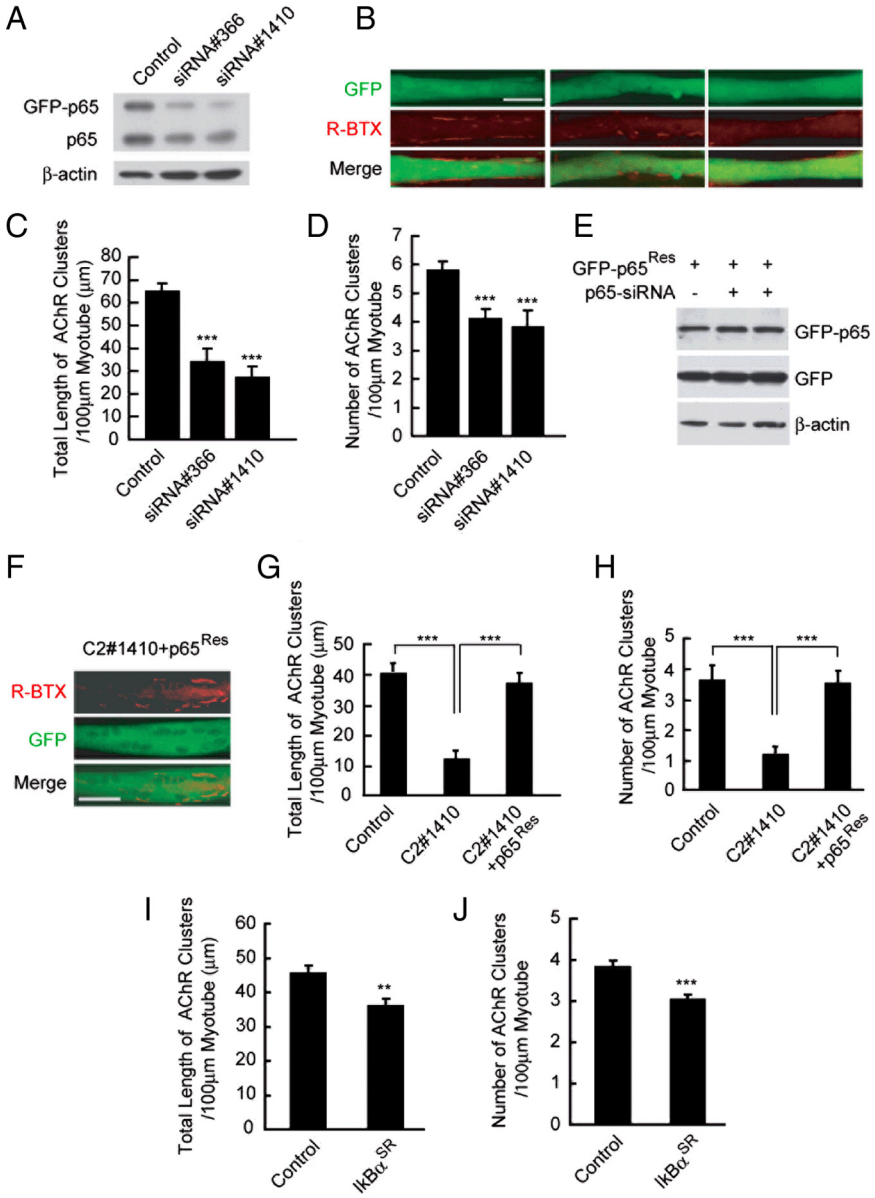

Figure 3. Downregulation or inhibition of NF-κB signaling inhibits AChR clustering. ***A***, HEK-293 cells were cotransfected with GFP-p65 and pSUPER-nonsilencing or pSUPER-p65-siRNA. Cell lysates were subjected to immunoblotting (IB) with antibodies against p65 or β-actin. ***B***, C2C12 myoblasts were transfected with control (pSUPER-nonsilencing) or pSUPER-p65-siRNA (siRNA#366 or siRNA#1410) plasmids. Fully differentiated myotubes were treated with Agrin to induce the formation of AChR clusters and stained with R-BTX. ***C***, ***D***, Quantification of total length (***C***) and number (***D***) of AChR clusters ($n = 69$ for control; $n = 45$ for siRNA#366; $n = 25$ for siRNA#1410). Data are shown as mean ± SEM from three experiments. ***E***, HEK293 cells were cotransfected with GFP-$p65^{Res}$ and pSUPER (lane 1) or pSUPER-p65-siRNA (lanes 2, 3). Cell lysates were subjected to IB with anti-GFP antibody to determine levels of GFP-p65 and pSUPER-encoded GFP, using β-actin as loading control. ***F***, Stable C2C12 cell lines expressing p65-siRNA were transfected with GFP-$p65^{Res}$, and fully differentiated myotubes were treated with Agrin (5 ng/ml) for 6 h. AChR clusters were marked by R-BTX. ***G***, ***H***, Total length (***G***) and number (***H***) of AChR clusters in 100 μm myotubes are shown in the histograms ($n = 50$ for control; $n = 50$ for C2#1410; $n = 54$ for C2#1410 with $p65^{Res}$). ***I***, ***J***, Total cluster length (***I***) and number (***J***) of AChR clusters in 100 μm myotubes transfected with control vector or plasmid encoding $I\kappa B\alpha^{SR}$ are shown in the histograms ($n = 62$ for control; $n = 69$ for $I\kappa B\alpha^{SR}$). $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA with Turkey's honestly significant difference *post hoc* tests. Scale bar, 50 μm.

Mice. $RelA^{floxed/floxed}$ mice with *loxP* sites flanking exons 7 and 10 of the RelA/p65 gene (Algül et al., 2007) were crossed to HSA-Cre mice (Brennan and Hardeman, 1993; Miniou et al., 1999; Luo et al., 2003), generating mutant mice with muscle-specific deletion of RelA/p65. The conditional knock-out (CKO) mice survived until adulthood without apparent behavioral abnormalities. Rapsyn heterozygous mice ($Rapsyn^{+/-}$) were kindly provided by Peter Noakes (University of Queensland, Brisbane, Queensland, Australia). All experimental

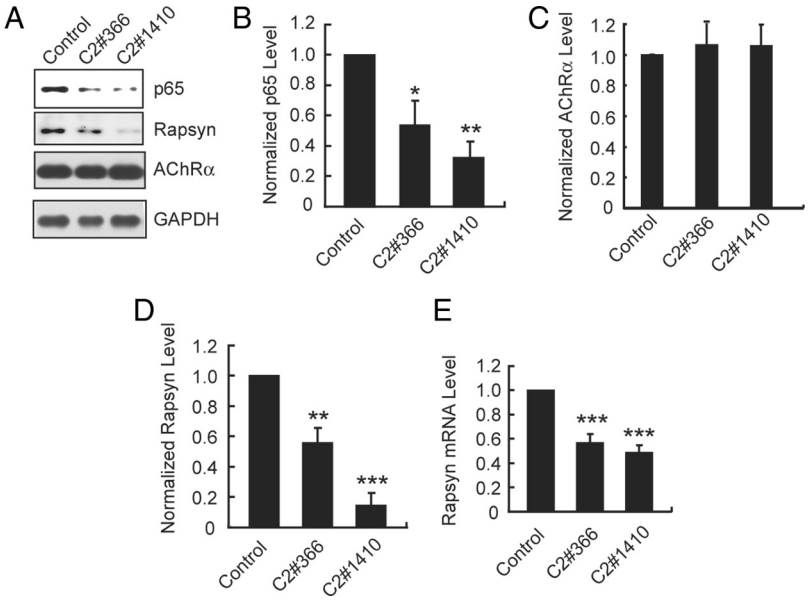

Figure 4. RelA/p65 promotes *Rapsyn* gene expression. ***A***, Cell lysates of stable C2C12 cell lines with RelA/p65 knockdown were subjected to IB with indicated antibodies. ***B–D***, Quantification of RelA/p65 (***B***), AChRα (***C***), or Rapsyn (***D***) protein levels from three independent experiments. ***E***, mRNA level of Rapsyn was determined by quantitative-PCR analysis. Values of control cells were normalized as 1.0. Shown are mean ± SEM from at least three experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student's *t* test.

protocols followed institutional guidelines for animal care and administration.

Immunohistochemistry and AChR cluster assays. Cultured muscle cells were stained with indicated antibodies after fixation in 4% paraformaldehyde for 30 min, followed by incubation with secondary antibody or together with R-BTX to label AChR clusters (Luo et al., 2003). Images were collected with confocal microscope. The total length of all AChR clusters or number of clusters in 100 μm myotube was quantitatively analyzed following the method described previously (Chen et al., 2007). For whole-mount staining of diaphragm, soleus, or sternomastoid muscles, staining procedure was followed as described in the previous study (Luo et al., 2003), with anti-synaptophysin antibody labeling synaptic nerve terminals. Given that the most ventral side of the diaphragm has little anatomical variation across mice, AChR clusters in this region were analyzed.

Results

Expression of RelA/p65 in developing skeletal muscle or cultured muscle cells

First, we determined the localization of RelA/p65 in teased soleus muscles from adult mice. Immunohistochemistry analysis showed that RelA/p65 distributed surrounding postsynaptic AChR patches, which were positively stained with R-BTX (supplemental Fig. S1*A*,*B*, available at www.jneurosci.org as supplemental material). This pattern is similar to that seen for the *Drosophila* homolog of NF-κB, Dorsal, which has been shown to distribute surrounding glutamate receptor (GluT) clusters at the NMJ (Heckscher et al., 2007). In addition, RelA/p65 was also found in other cell types, such as Schwann cells around the NMJ (supplemental Fig. S1*B*, available at www.jneurosci.org as supplemental material).

Given the presence of RelA/p65 in multiple cell types (e.g., muscle cells and perisynaptic Schwann cells) (supplemental Fig. S1*A*,*B*, available at www.jneurosci.org as supplemental material), we determined the expression and localization of RelA/p65 in cultured muscle cells. C2C12 myoblasts were kept in GM and then switched to FM for different days to induce myotube fusion. The differentiation status of C2C12 muscle cells was evaluated with the expression of MHC, a marker for mature muscle cell (Fig. 1*A*). Of note, the expression of AChRα and Rapsyn was progressively induced in differentiating muscle cells (Fig. 1*A*). Interestingly, although the level of RelA/p65 remained constant in muscle cells at different developmental stages (Fig. 1*A*), it was found to be accumulated in nuclear fractions of differentiating myotubes, with the peak appearing on day 3 in fusion media (Fig. 1*B*). This nuclear localization pattern of RelA/p65 is coincident with the expression pattern of Rapsyn and AChRα (Fig. 1*A*). Given the role of Agrin in AChR clustering, we determined whether RelA/p65 expression changes on exposure of myotubes to Agrin. We found that muscle cells treated with Agrin exhibited no changes in the protein level (supplemental Fig. S2, available at www.jneurosci.org as supplemental material) or nuclear localization of RelA/p65 (data not shown). It is known that denervation of skeletal muscles causes upregulation of several postsynaptic proteins to compensate the loss of presynaptic input (Evans et al., 1987; Goldman and Staple, 1989; Luo et al., 2002). Interestingly, marked increase in the level of RelA/p65 was observed in denervated (DN) muscles, compared with that of innervated (INN) muscles, whereas β-actin remained unchanged (Fig. 1*C*), suggesting the regulation of RelA/p65 by electrical muscle activity. These results prompted us to investigate the role of RelA/p65 in developing muscle cells, in particular the process of postsynaptic differentiation at the NMJ.

Role of p65 in AChR clustering in cultured muscle cells

To investigate the role of RelA/p65 in postsynaptic differentiation, we first determined the gain-of-function effect of NF-κB by overexpressing GFP-p65 in cultured C2C12 muscle cells. The transfected myotubes were treated with Agrin (5 ng/ml) for 6 h to induce the formation of AChR clusters (Fig. 2*A*). Interestingly, overexpression of GFP-p65 resulted in marked increase in the formation of AChR clusters, compared with control cells transfected with vehicle GFP vector (control). The effect on AChR cluster formation was reflected from increased total length (Fig. 2*B*) and number (Fig. 2*C*) of AChR clusters in p65-transfected muscle cells.

Next, we determined the loss-of-function effect of NF-κB by downregulating the endogenous RelA/p65 with the siRNA approach. We designed two siRNAs, siRNA#366 and siRNA#1410, against mouse p65, both of which could suppress the expression of exogenous and endogenous p65 efficiently in human embryonic kidney 293 (HEK-293) cells (Fig. 3*A*). Transfection of C2C12 muscle cells with plasmids encoding these siRNAs resulted in marked reduction in the formation of AChR clusters, compared with cultures transfected with control (nonsilencing) siRNA (Fig. 3*B–D*). As shown in Figure 3, *C* and *D*, both total length and number of AChR clusters were decreased in RelA/p65 knockdown cells. Thus, p65 plays an important role in AChR cluster formation.

RelA/p65 has been shown to function as a negative regulator of MyoD-induced myogenesis or differentiation (Guttridge et al.,

1999, 2000; Bakkar et al., 2008). It is thus conceivable that RelA/p65 downregulation may affect myotube formation and thus inhibit AChR clustering. To determine the effect of RelA/p65 downregulation on muscle differentiation, we established stable cell lines of C2C12 with p65 knockdown, C2#366 and C2#1410 (for details, see Materials and Methods), and the efficiency of knockdown was confirmed by Western blot analysis (Fig. 4*A*,*B*). We found that C2#366 and C2#1410 cells were normally differentiated to form mature myotubes, which were positively stained with MHC, after 4–5 d cultured in FM, although this process was slightly delayed than that of control cells (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). Similar to that seen in transient-transfected muscle cells by p65-siRNAs, Agrin-induced AChR clustering was decreased in RelA/p65 knockdown stable cell lines (Figs. 3*F–H*, 6). To eliminate off-target effect of p65-siRNA, we generated a siRNA-resistant construct of p65, GFP-p65^{Res} (Fig. 3*E*). Expression of p65^{Res} in p65 knockdown muscle cells exhibited a rescue effect on either total length (Fig. 3*G*) or number of AChR clusters (Fig. 3*H*). Thus, the effect of p65 downregulation on AChR clustering is specific.

Under resting conditions, NF-κB subunits (e.g., RelA/p65 or p50) are usually located in the cytoplasm to form inactive complexes with inhibitory molecule IκB (Karin et al., 2004). After release from IκB, NF-κB dimmers can translocate from the cytoplasm into the nucleus and switch on or off target gene expression. To further examine the role of NF-κB signaling in AChR clustering, we took advantage of IκB^{SR}, the mutated form of IκB, which contains serine-to-alanine mutations at positions 32 and 36, and thus renders IκB to be resistant to proteasome-mediated degradation and constitutively keeps NF-κB in the cytoplasm and inhibits NF-κB activity (Diao et al., 2005; Sun et al., 2007). We found that transfection with IκB^{SR} caused a marked reduction in the total length and number of AChR clusters (Fig. 3*I*,*J*). This result is in line with the notion that NF-κB signaling is essential for AChR cluster formation.

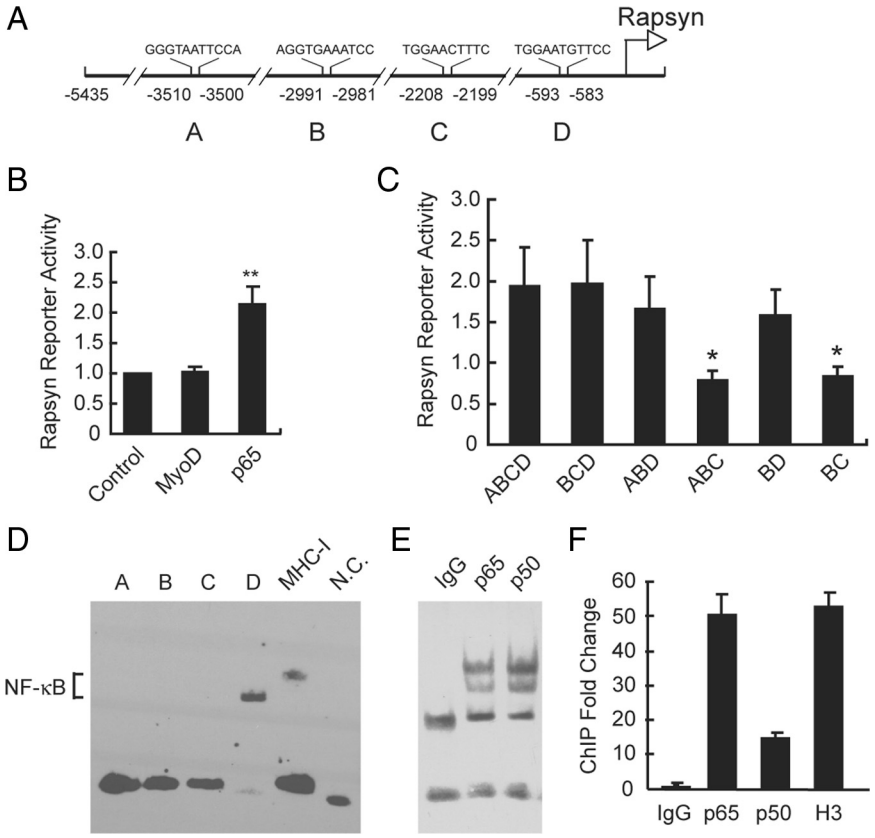

Figure 5. RelA/p65 promotes *Rapsyn* transcription. ***A***, Schematic diagram showing *Rapsyn* reporter construct R5k-Luc that contains the 5′-flanking region of *Rapsyn* gene and the downstream cDNA encoding firefly luciferase. Four putative NF-κB binding sites (A–D) were indicated in the R5K promoter (A, −3510 to −3500 nt; B, −2991 to −2981 nt; C, −2208 to −2199 nt; D, −593 to −583 nt). ***B***, C2C12 myotubes were cotransfected with R5k-luc and GFP-p65, MyoD, or control plasmid. Firefly and *Renilla* luciferase activity was assayed as described in Materials and Methods. *$p < 0.05$, **$p < 0.01$, Student's *t* test. ***C***, R5K-Luc (marked as ABCD) or the constructs with the deletion of indicted NF-κB binding sites (mutations lacking site A, C, D, AC, or AD were labeled as BCD, ABD, ABC, BD, or BC, respectively) were cotransfected with GFP-p65 or vehicle plasmid. The data shown are the ratio of relative reporter activity between p65-transfected and control cells. All the experiments were repeated at least six times. *$p <$ 0.05, one-way ANOVA with Turkey's honestly significant difference *post hoc* tests. ***D***, Nuclear extracts were prepared from C2C12 myotubes, and EMSA was performed with biotin-labeled probes corresponding to putative NF-κB binding sites. The MHC-I probe containing a bona fide NF-κB binding site was taken as a positive control and the mutated NF-κB binding site was taken as negative control (N.C.). ***E***, Supershift EMSA was performed with myotube nuclear extracts preincubated with normal IgG or specific antibodies against RelA/p65 or p50 subunits. ***F***, Nuclear extracts from C2C12 myotubes were incubated with normal IgG or antibodies against RelA/p65, p50, or acetylated histone H3; quantitative PCR analysis was conducted with coimmunoprecipitated DNA using *Rapsyn* promoter primers covering the region containing site D. Error bars indicate SEM.

p65 is essential for *Rapsyn* gene expression

Next, we investigated the mechanisms by which NF-κB regulates AChR clustering. Interestingly, we found that the protein level of Rapsyn showed a marked reduction in p65 knockdown cells (C2#366 and C2#1410), compared with control cells (Fig. 4*A*,*D*). However, p65 knockdown had no effect on the level of AChRα (Fig. 4*A*,*C*). Thus, RelA/p65 may regulate Rapsyn expression. Given that activated RelA/p65 is capable of regulating target gene transcription, we further examined the mRNA level of Rapsyn in p65 knockdown cells. Quantitative PCR showed that the level of Rapsyn mRNA was dramatically decreased in p65 knockdown cells (Fig. 4*E*). Thus, RelA/p65 regulates Rapsyn gene transcription.

p65 promotes Rapsyn transcription

We further examined whether NF-κB signaling affects transcription of Rapsyn by using promoter reporter assays. NF-κB dimers have been proposed to bind to consensus sequence GGGRNNYYCC of the target gene promoter (R is purine, Y is pyrimidine, and N is any base) (Miyamoto and Verma, 1995). We analyzed the sequence of the Rapsyn 5′-flanking region between −5435 and +170, and identified four putative NF-κB consensus binding sites (Fig. 5*A*, sites A–D). The reporter construct R5k-Luc that contains the 5605 nt 5′-flanking region of *Rapsyn* gene and the downstream cDNA encoding firefly luciferase has been demonstrated in our previous study (Wang et al., 2008). The relative luciferase activity of the reporter over cotransfected *Renilla* luciferase encoded by pRL-TK was measured in C2C12 myotubes. We found that cotransfection with RelA/p65 caused a marked increase in the promoter activity of R5k-Luc, compared with con-

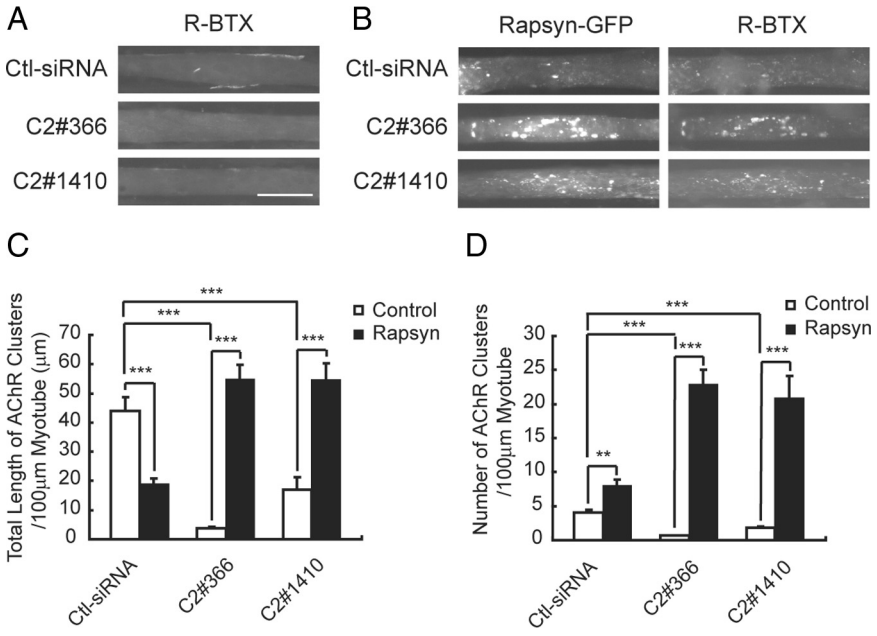

Figure 6. Forced expression of Rapsyn prevents the failure in AChR clustering caused by RelA/p65 downregulation. Stable C2C12 cell lines with RelA/p65 knockdown (C2#366 or C2#1410) were transfected with empty vector or that encoding Rapsyn-GFP. Fully differentiated myotubes were treated with Agrin for 6 h. AChR clusters were labeled with R-BTX. Shown in ***A*** and ***B*** are images from representative experiments. Scale bar, 50 μm. ***C***, ***D***, Quantification of AChR cluster length (***C***) and number (***D***) in 100 μm myotubes ($n = 50$ for Ctl-siRNA, $n = 87$ for C2#366, $n = 77$ for C2#1410 in control group; $n = 26$ for Ctl-siRNA, $n = 33$ for C2#366, $n = 17$ for C2#1410 in Rapsyn-GFP group). Data are shown as means ± SEM from at least three experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA with Turkey's honestly significant difference *post hoc* tests.

trol cells cotransfected with vehicle plasmid (Fig. 5*B*). Given that Rapsyn promoter contains several E-box consensus sequence CANNTG, which shall be responsive to a variety of myogenic determination factors, including MyoD, myogenin, Myf5, and Myf6 (Ohno et al., 2003) and the known regulation of other NMJ proteins, such as AChR and MuSK, by MyoD (Cheng et al., 1992; Prody and Merlie, 1992; Kim et al., 2003), we determined whether Rapsyn is regulated by MyoD. We found that indeed MyoD had no effect on the promoter activity of R5k-Luc (Fig. 5*B*). Together, these results suggest that RelA/p65 promotes *Rapsyn* gene expression.

To determine which NF-κB-binding motifs on *Rapsyn* promoter are involved in regulating the *Rapsyn* promoter activity, we generated site-specific mutated forms of *Rapsyn* reporter constructs. As shown in Figure 5*C*, myotubes transfected with mutated *Rapsyn* reporter constructs lacking site D (ABC or BC) exhibited a loss of responsiveness to RelA/p65 upregulation, and the constructs containing intact site D (ABCD, BCD, ABD, or BD) retained the responsiveness to RelA/p65 upregulation. These results suggest that site D is essential for RelA/p65-dependent *Rapsyn* gene expression. In accordance with this notion, only biotin-labeled probes containing site D showed a band shift in an EMSA, when mixed with nuclear extracts prepared from C2C12 myotubes (Fig. 5*D*). As the positive control, the probe derived from the major histocompatability complex class I (MHC-I) also exhibited a band shift in the EMSA (Fig. 5*D*). In contrast, mutated NF-κB probe [negative control (N.C.)] exhibited no band shift (Fig. 5*D*). This result suggests that protein complexes from nuclear extracts of cultured myotubes bind to the consensus DNA sequence for NF-κB in *Rapsyn* promoter region.

To determine which NF-κB subunits are responsible for the observed band shift, we performed a supershift assay by adding antibodies against RelA/p65 or p50, two major subunits of the NF-κB family, when performing EMSA analysis using site D probe. We found that incubation with both p65 and p50 antibodies resulted in supershifted band, indicating that RelA/p65 or p50 binds to site D, directly or indirectly (Fig. 5*E*). In line with this notion, ChIP assay also showed a remarkable association between RelA/p65 and the specific Rapsyn promoter region containing site D element (Fig. 5*F*). Although both p65 and p50 showed association with Rapsyn promoter in the ChIP assay, p65 seemed to have higher binding affinity than p50 in binding with Rapsyn promoter (Fig. 5*F*). These observations have provided direct evidence that NF-κB regulates *Rapsyn* gene expression.

p65 regulation of *Rapsyn* is required for AChR clustering

In light of the regulation by RelA/p65 of AChR clustering and Rapsyn expression, we hypothesized that p65 might regulate AChR clustering by modulating Rapsyn expression. To test this hypothesis, stable RelA/p65 knockdown C2C12 muscle cells were transfected with exogenous Rapsyn driven by a promoter that does not respond to NF-κB regulation. We found that overexpression of Rapsyn-GFP in these RelA/p65 knockdown cells prevented the failure in the formation of AChR clusters caused by the loss of RelA/p65, as shown by the rescue of total length and number of AChR clusters (Fig. 6*C*,*D*). Of note, overexpression of Rapsyn-GFP induced formation of many small AChR clusters (Fig. 6*B*), as reflected from increased number of AChR clusters, compared with control cells without RelA/p65 downregulation (Fig. 6*D*). Previous studies have shown that proper ratio between AChR and Rapsyn in muscle cells is important for AChR cluster formation (LaRochelle and Froehner, 1986; Yoshihara and Hall, 1993; Han et al., 1999). Both decreased and increased expression of Rapsyn have been shown to inhibit AChR clustering in myotubes (Yoshihara and Hall, 1993; Han et al., 1999). In agreement with these observations, we found that overexpression of Rapsyn in control cells caused a decrease in the length of AChR clusters (Fig. 6*C*), and an increase in the number of small clusters (Fig. 6*B*,*C*). The decrease in the length of clusters caused by Rapsyn overexpression was not observed in C2#366 and C2#1410 muscle cells (Fig. 6*C*), probably because of appropriate levels of reconstituted Rapsyn. Thus, strict regulation of Rapsyn expression is essential for the formation of normal AChR clusters, and RelA/p65 plays an important role in governing Rapsyn expression.

Role of p65 in NMJ development *in vivo*

Finally, to evaluate the role of RelA/p65 in neuromuscular junction development *in vivo*, we used the *loxP*/*Cre* approach to selectively eliminate the *RelA/p65* gene in skeletal muscle. Gene encoding for the Cre recombinase was under the control of human skeletal actin promoter (HSA) (Brennan and Hardeman, 1993; Miniou et al., 1999). We crossed the $RelA^{floxed/floxed}$ mice with *loxP* sites flanking exons 7 and 10 of the RelA/p65 gene (Algül et al., 2007) to HSA-Cre mice, and thus obtained the mutant mice with muscle-specific deletion of RelA/p65. The mutant

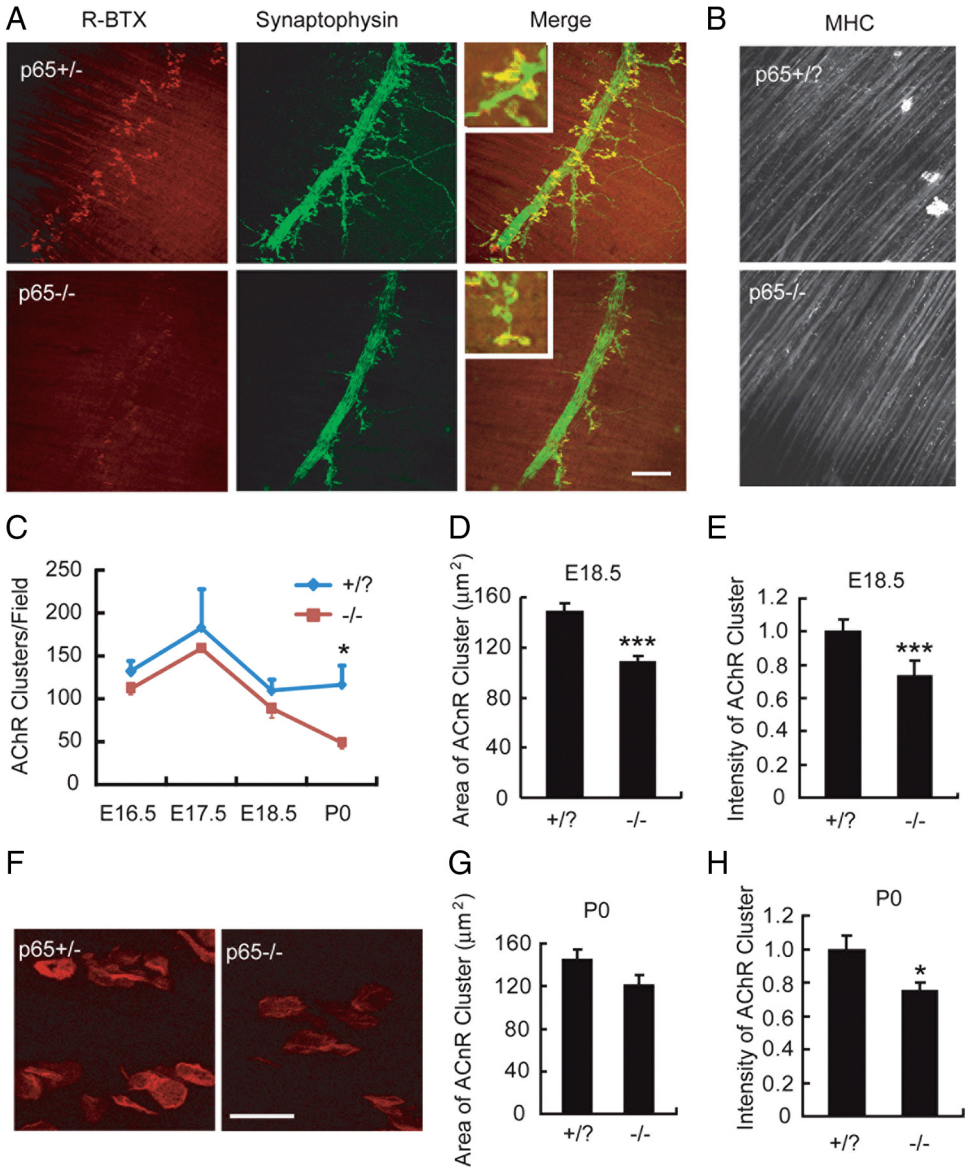

Figure 7. Aberrant AChR clustering in RelA/p65 mutant mice at early stages. ***A***, ***B***, Diaphragm muscles from littermate $p65^{+/-}$ or $p65^{-/-}$ mice were stained with antibody against synaptophysin (green) to mark synaptic nerve branches, MHC to mark muscle fibers, and R-BTX to label AChRs. Shown are representative images from the ventral quadrant of the right side hemidiaphragm of heterozygous ($p65^{+/-}$, top panels) or homozygous p65 knock-out mice ($p65^{-/-}$, bottom panels) at P0. Scale bar, 100 μm. ***C***, Numbers of AChR clusters in diaphragms at indicated stages were measured ($n = 8$ for E16.5 +/?, $n = 4$ for E16.5 −/−; $n = 12$ for E18.5 +/?, $n = 6$ for E18.5 −/−; $n = 6$ for P0 +/?, $n = 6$ for P0 −/−). ***D***, ***E***, Average area (in square micrometers) (***D***) or intensity (***E***) of individual AChR clusters from diaphragms of wild-type or mutant mice at E18.5 ($n = 56$ for +/?; $n = 79$ for −/−). ***F***, Representative images of AChR clusters in +/− or −/− mice at P0. Scale bar, 20 μm. ***G***, ***H***, Average area (in square micrometers) (***G***) or intensity (***H***) of individual AChR clusters at P0 ($n = 18$ for +/?; $n = 14$ for −/−). Data are shown as means ± SEM. *$p < 0.05$, ***$p < 0.001$, Student's *t* test.

mice exhibited normal muscle differentiation (Fig. 7*B*). However, AChR clusters appeared abnormal in the diaphragms of these mutant mice, in particular in the late developmental stages (Fig. 7*A*), as shown by the analysis of number, size, and intensity of AChR clusters (Fig. 7*C–H*). We found there were fewer AChR clusters in the diaphragms of RelA/p65 mutant mice, compared with littermate controls at P0 (Fig. 7*A*,*C*,*F*). However, no difference was observed for the number of clusters at embryonic stages [embryonic day 16.5 (E16.5) to E18.5] (Fig. 7*C*), probably because of the weak expression of Cre driven by HSA promoter in the skeletal muscle at early developmental stages (Brennan and Hardeman, 1993; Luo et al., 2003; Chen et al., 2007). Interestingly, diaphragms of mutant mice exhibited smaller AChR clusters at E18.5, as reflected from the decreased area of individual clusters (Fig. 7*D*). Furthermore, the intensity of AChR clusters was lower in mutant mice than in littermate control mice at E18.5 (Fig. 7*E*) or postnatal day 0 (P0) (Fig. 7*F*,*H*). Mild but not significant decrease in the area of individual AChR clusters was found in P0 mutant mice (Fig. 7*G*). Given that Rel/p65 CKO mice survived to adulthood, we examined the AChR clusters at later stages. By the end of the second postnatal week (P15), most of the AChR patches at mouse NMJs have attained the pretzel-like adult-form structures (Marques et al., 2000). We found that RelA/p65 mutant mice at P30 exhibited a marked decrease in the area occupied by AChR patches, compared with wild-type control mice (Fig. 8*A*,*B*). Of note, the AChR patches in mutant mice were usually fragmented (Fig. 8*A*) Together, all these results indicate that NF-κB regulates AChR clustering *in vivo*.

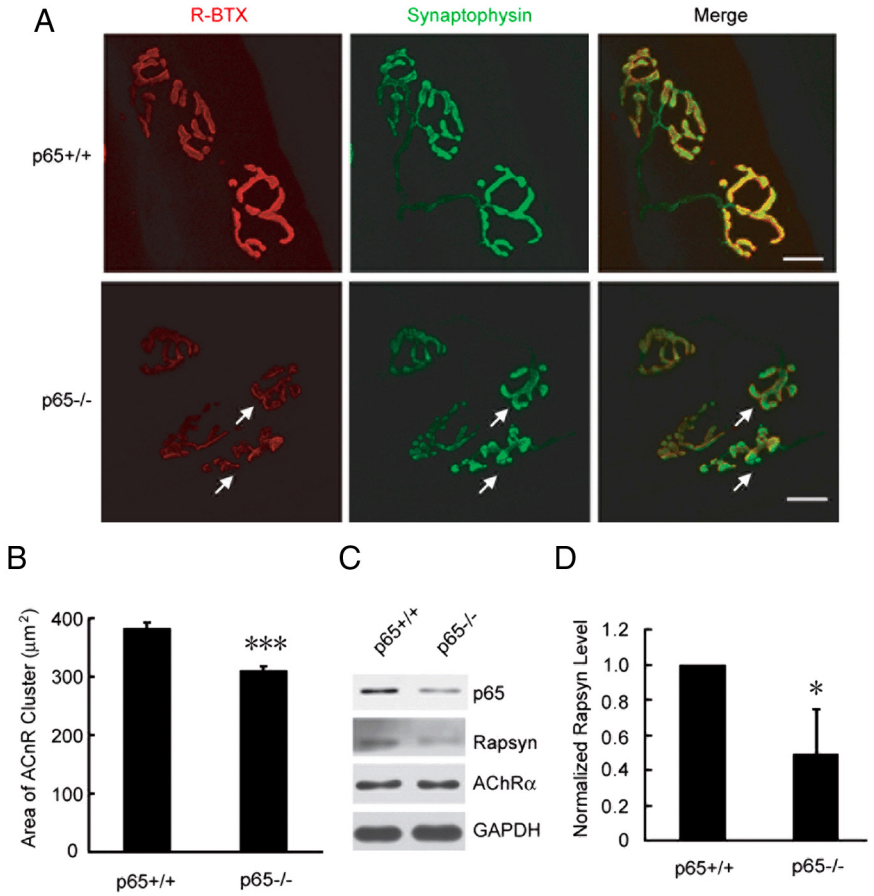

Figure 8. Aberrant AChR clustering in adult RelA/p65 mutant mice. ***A***, Sternomastoid muscles from P30 mice (+/+, $p65^{floxed/?}$; −/−, $p65^{floxed/floxed}$;HSA^{cre}) were stained with R-BTX and anti-Synaptophysin antibody. Shown are representative images. Scale bar, 20 μm. ***B***, Quantification for the average area of individual NMJs ($n = 76$ for +/+; $n = 72$ for −/−). ***$p < 0.001$, Student's *t* test. ***C***, Homogenates of soleus muscles isolated from RelA/p65 knock-out mice or littermate wild-type mice at P0 were probed with indicated antibodies. ***D***, Quantification for Rapsyn levels from three experiments. *$p < 0.05$, ***$p < 0.001$, Student's *t* test. Error bars indicate SEM.

In line with the notion that NF-κB regulates Rapsyn expression in cultured muscle cells, we found Rapsyn level was decreased in the muscle of RelA/p65 mutant mice compared with littermate control wild-type mice (Fig. 8*C*,*D*). The level of Rapsyn was reduced by ~50% in RelA/$p65^{+/-}$ mice. Indeed, $Rapsyn^{+/-}$ mice, which showed ~50% reduction in Rapsyn levels (supplemental Fig. S4*A*, available at www.jneurosci.org as supplemental material), usually exhibited fragmented AChR clusters (supplemental Fig. S4*B*, available at www.jneurosci.org as supplemental material). The area occupied by each NMJ was smaller in $Rapsyn^{+/-}$ mice than in littermate control mice ($Rapsyn^{+/+}$) (supplemental Fig. S4*C*, available at www.jneurosci.org as supplemental material). These defects phenocopied that of RelA/$p65^{+/-}$ mice. Thus, both *in vitro* and *in vivo* evidence support the notion that NF-κB signaling plays an important role in the formation or stabilization of AChR clusters, by regulating the expression of synaptic gene *Rapsyn*.

Discussion

NF-κB system proteins have been implicated in neurite differentiation, neuronal survival, synaptic plasticity and memory, and neuronal disorders (Schmidt-Ullrich et al., 1996; O'Neill and Kaltschmidt, 1997; Schneider et al., 1999; Mattson et al., 2000; Mattson and Camandola, 2001; Meffert et al., 2003; Gutierrez et al., 2005; Meffert and Baltimore, 2005; Mattson and Meffert, 2006; Lubin and Sweatt, 2007). In this study, we demonstrate an important role of NF-κB in AChR clustering at the vertebrate NMJ, extending the reported role of *Drosophila* homolog of NF-κB, Dorsal, in specifying GluR density during NMJ development (Heckscher et al., 2007). Furthermore, we found that RelA/p65 subunit of NF-κB controls AChR clustering via transcriptional regulation of synaptic protein Rapsyn, providing a mechanism and specific target of NF-κB action.

At the vertebrate NMJ, AChR clustering underneath nerve terminals are stimulated by Agrin/MuSK signaling and involves a number of intracellular proteins (Sanes and Lichtman, 1999, 2001), most of which exert clustering function via transmitting signals from surface, scaffolding receptors with cytoskeleton components, or regulating cytoskeleton organization directly (Weston et al., 2000; Luo et al., 2002, 2003; Finn et al., 2003; Wang et al., 2003; Okada et al., 2006; Linnoila et al., 2008). In this study, we have identified a role of NF-κB in regulating AChR clustering, based on evidence obtained in cultured muscle cells and *in vivo*. First, in C2C12 myotubes, downregulating the expression of RelA/p65 subunit of NF-κB by siRNA caused a reduction in the formation of AChR clusters induced by Agrin (Fig. 3*A–H*). In contrast, upregulation of NF-κB by overexpression of RelA/p65 increased AChR clusters (Fig. 2). Second, inhibiting NF-κB activity by overexpressing $I\kappa B^{SR}$, the mutated form of IκB that is believed to be resistant to proteolytic degradation, and thus constitutively renders NF-κB in an inactive state, exhibited a similar effect with that of NF-κB siRNA. Third, the mutant mice with muscle-specific knock-out of RelA/p65 exhibited a remarkable reduction in the intensity or size of AChR clusters, although the expression level of AChR was not changed (Figs. 7, 8). This effect was significant at late embryonic (E18.5), perinatal (P0), or adult stage (P30), rather than earlier stages (E16.5 and E17.5), probably because the expression of gene driven by HSA promoter progressively increases during muscle development (Luo et al., 2003; Chen et al., 2007). Nevertheless, we conclude that NF-κB plays a pivotal role in AChR cluster formation or stabilization.

At the *Drosophila* NMJ, NF-κB and IκB homologs Dorsal and Cactus distribute surrounding postsynaptic GluR clusters, where Dorsal controls GluR density presumably via posttranscriptional mechanisms (Heckscher et al., 2007). Interestingly, NF-κB subunit RelA/p65 was found to be enriched at the regions around AChR clusters at the mouse NMJ (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). In differentiating C2C12 skeletal muscle cells, the nuclear localization pattern of RelA/p65 coincides with that of Rapsyn expression (Fig. 1*B*). Furthermore, there are several NF-κB consensus sequences in the *Rapsyn* promoter (Fig. 5*A*, motifs A to D). EMSA analysis shows that both RelA/p65 and p50 subunits of NF-κB can bind to motif

D. In agreement with this notion, upregulation of RelA/p65 increased activity of *Rapsyn* promoter with site D sequence intact, but had no effect on *Rapsyn* promoter with site D deleted (Fig. 5*C*). In support of the essential role of NF-κB in regulating Rapsyn expression, a remarkable reduction in the level of Rapsyn was observed in C2C12 muscle cells with RelA/p65 downregulated (Fig. 4) or in muscle tissues derived from RelA/p65 knockout mice (Fig. 8*C*,*D*). Finally, the loss of AChR clusters in RelA/p65 downregulated muscle cells was partially prevented by overexpressing Rapsyn (Fig. 6). All these results support the conclusion that NF-κB controls AChR clustering, at least partially, through the transcriptional regulation of synaptic protein Rapsyn. It remains possible that NF-κB also regulates other synaptic proteins at the NMJ.

How might NF-κB be regulated during NMJ development? One of the most potent activators of NF-κB is tumor necrosis factor-α (TNFα) (Baker and Reddy, 1998; Mattson et al., 2000). Interestingly, TNFα activation of NF-κB has been implicated in mechanisms of muscle wasting (Guttridge et al., 2000), and denervation- or tumor-induced muscle loss (Cai et al., 2004). Thus, TNFα signaling may be involved in synaptogenesis at the NMJ. Importantly, glia-derived TNFα has been shown to play important role in synaptic scaling (Stellwagen and Malenka, 2006) and modulate synaptic plasticity by controlling membrane insertion of NMDA receptors (Wheeler et al., 2009). In addition to TNFα, other NF-κB activators identified in nervous system include nerve growth factors, oxyradicals, amyloid β-peptide, and neurotransmitter glutamate (Mattson et al., 2000; Mattson and Camandola, 2001; Gutierrez et al., 2005; Gallagher et al., 2007; Gavaldà et al., 2009). Previous studies have observed increased nerve sprouting or branching in Rapsyn mutant mice (Gautam et al., 1995; Banks et al., 2001), but this phenotype was not found in RelA/p65 mutant mice. We believe that, as a transcription factor, RelA/P65 may also regulate expression of other genes, in addition to *Rapsyn*. Furthermore, genetic ablation of p65 was selectively in skeletal muscle cells, rather than globally. Muscle-specific knock-down of RelA/p65 inhibits AChR clustering and correspondingly causes matching of nerve terminals with AChR clusters.

Synaptic transcription has been proposed to play important role in regulating synaptogenesis at the vertebrate NMJ (Schaeffer et al., 2001; Burden, 2002). In addition to inducing AChR cluster formation, Agrin/MuSK signaling has also been shown to play important roles in regulating synaptic AChR expression (Yang et al., 2001; Lacazette et al., 2003; Jaworski and Burden, 2006). Although bath application of Agrin had no apparent effect on RelA/p65 expression (supplemental Fig. S2, available at www.jneurosci.org as supplemental material), we could not exclude the possible regulation of RelA/p65 by synaptic Agrin that attached to the synaptic basal lamina. It would be of interest to identify the mechanism that underlies NF-κB regulation at the NMJ. Our demonstration that NF-κB regulates Rapsyn expression, together with the other reports of δ- or β-catenin regulation of Rapsyn expression at the NMJ (Rodova et al., 2004; Wang et al., 2008), suggests the intriguing mechanism by which transcription factors influence synaptogenesis.

References

Albensi BC, Mattson MP (2000) Evidence for the involvement of TNF and NF-kappaB in hippocampal synaptic plasticity. Synapse 35:151–159.

Algül H, Treiber M, Lesina M, Nakhai H, Saur D, Geisler F, Pfeifer A, Paxian S, Schmid RM (2007) Pancreas-specific RelA/p65 truncation increases susceptibility of acini to inflammation-associated cell death following cerulein pancreatitis. J Clin Invest 117:1490–1501.

Apel ED, Glass DJ, Moscoso LM, Yancopoulos GD, Sanes JR (1997) Rapsyn is required for MuSK signaling and recruits synaptic components to a MuSK-containing scaffold. Neuron 18:623–635.

Baker SJ, Reddy EP (1998) Modulation of life and death by the TNF receptor superfamily. Oncogene 17:3261–3270.

Bakkar N, Wang J, Ladner KJ, Wang H, Dahlman JM, Carathers M, Acharyya S, Rudnicki MA, Hollenbach AD, Guttridge DC (2008) IKK/NF-kappaB regulates skeletal myogenesis via a signaling switch to inhibit differentiation and promote mitochondrial biogenesis. J Cell Biol 180:787–802.

Banks GB, Chau TN, Bartlett SE, Noakes PG (2001) Promotion of motoneuron survival and branching in rapsyn-deficient mice. J Comp Neurol 429:156–165.

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

Burden SJ (2002) Building the vertebrate neuromuscular synapse. J Neurobiol 53:501–511.

Cai D, Frantz JD, Tawa NE Jr, Melendez PA, Oh BC, Lidov HG, Hasselgren PO, Frontera WR, Lee J, Glass DJ, Shoelson SE (2004) IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. Cell 119:285–298.

Chen F, Qian L, Yang ZH, Huang Y, Ngo ST, Ruan NJ, Wang J, Schneider C, Noakes PG, Ding YQ, Mei L, Luo ZG (2007) Rapsyn interaction with calpain stabilizes AChR clusters at the neuromuscular junction. Neuron 55:247–260.

Chen LF, Greene WC (2004) Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol 5:392–401.

Cheng TC, Hanley TA, Mudd J, Merlie JP, Olson EN (1992) Mapping of myogenin transcription during embryogenesis using transgenes linked to the myogenin control region. J Cell Biol 119:1649–1656.

DeChiara TM, Bowen DC, Valenzuela DM, Simmons MV, Poueymirou WT, Thomas S, Kinetz E, Compton DL, Rojas E, Park JS, Smith C, DiStefano PS, Glass DJ, Burden SJ, Yancopoulos GD (1996) The receptor tyrosine kinase MuSK is required for neuromuscular junction formation in vivo. Cell 85:501–512.

Deng J, Miller SA, Wang HY, Xia W, Wen Y, Zhou BP, Li Y, Lin SY, Hung MC (2002) beta-Catenin interacts with and inhibits NF-kappa B in human colon and breast cancer. Cancer Cell 2:323–334.

Deryckere F, Gannon F (1994) A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. Biotechniques 16:405.

Diao L, Zhang B, Fan J, Gao X, Sun S, Yang K, Xin D, Jin N, Geng Y, Wang C (2005) Herpes virus proteins ICP0 and BICP0 can activate NF-kappaB by catalyzing IkappaBalpha ubiquitination. Cell Signal 17:217–229.

DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. Nature 388:548–554.

Evans S, Goldman D, Heinemann S, Patrick J (1987) Muscle acetylcholine receptor biosynthesis. Regulation by transcript availability. J Biol Chem 262:4911–4916.

Finn AJ, Feng G, Pendergast AM (2003) Postsynaptic requirement for Abl kinases in assembly of the neuromuscular junction. Nat Neurosci 6:717–723.

Gallagher D, Gutierrez H, Gavalda N, O'Keeffe G, Hay R, Davies AM (2007) Nuclear factor-κB activation via tyrosine phosphorylation of inhibitor κB-α is crucial for ciliary neurotrophic factor-promoted neurite growth from developing neurons. J Neurosci 27:9664–9669.

Gautam M, Noakes PG, Mudd J, Nichol M, Chu GC, Sanes JR, Merlie JP (1995) Failure of postsynaptic specialization to develop at neuromuscular junctions of rapsyn-deficient mice. Nature 377:232–236.

Gavaldà N, Gutierrez H, Davies AM (2009) Developmental switch in NF-kappaB signalling required for neurite growth. Development 136:3405–3412.

Glass DJ, Bowen DC, Stitt TN, Radziejewski C, Bruno J, Ryan TE, Gies DR, Shah S, Mattsson K, Burden SJ, DiStefano PS, Valenzuela DM, DeChiara TM, Yancopoulos GD (1996) Agrin acts via a MuSK receptor complex. Cell 85:513–523.

Goldman D, Staple J (1989) Spatial and temporal expression of acetylcholine receptor RNAs in innervated and denervated rat soleus muscle. Neuron 3:219–228.

Gutierrez H, Hale VA, Dolcet X, Davies A (2005) NF-kappaB signalling regulates the growth of neural processes in the developing PNS and CNS. Development 132:1713–1726.

Guttridge DC, Albanese C, Reuther JY, Pestell RG, Baldwin AS Jr (1999) NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol 19:5785–5799.

Guttridge DC, Mayo MW, Madrid LV, Wang CY, Baldwin AS Jr (2000)

NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. Science 289:2363–2366.
Han H, Noakes PG, Phillips WD (1999) Overexpression of rapsyn inhibits agrin-induced acetylcholine receptor clustering in muscle cells. J Neurocytol 28:763–775.
Hayden MS, Ghosh S (2004) Signaling to NF-kappaB. Genes Dev 18:2195–2224.
Heckscher ES, Fetter RD, Marek KW, Albin SD, Davis GW (2007) NF-kappaB, IkappaB, and IRAK control glutamate receptor density at the *Drosophila* NMJ. Neuron 55:859–873.
Jaworski A, Burden SJ (2006) Neuromuscular synapse formation in mice lacking motor neuron- and skeletal muscle-derived Neuregulin-1. J Neurosci 26:655–661.
Karin M (2006) Nuclear factor-kappaB in cancer development and progression. Nature 441:431–436.
Karin M, Yamamoto Y, Wang QM (2004) The IKK NF-kappa B system: a treasure trove for drug development. Nat Rev Drug Discov 3:17–26.
Kim CH, Xiong WC, Mei L (2003) Regulation of MuSK expression by a novel signaling pathway. J Biol Chem 278:38522–38527.
Kim N, Stiegler AL, Cameron TO, Hallock PT, Gomez AM, Huang JH, Hubbard SR, Dustin ML, Burden SJ (2008) Lrp4 is a receptor for Agrin and forms a complex with MuSK. Cell 135:334–342.
Lacazette E, Le Calvez S, Gajendran N, Brenner HR (2003) A novel pathway for MuSK to induce key genes in neuromuscular synapse formation. J Cell Biol 161:727–736.
LaRochelle WJ, Froehner SC (1986) Determination of the tissue distributions and relative concentrations of the postsynaptic 43-kDa protein and the acetylcholine receptor in Torpedo. J Biol Chem 261:5270–5274.
Lin W, Burgess RW, Dominguez B, Pfaff SL, Sanes JR, Lee KF (2001) Distinct roles of nerve and muscle in postsynaptic differentiation of the neuromuscular synapse. Nature 410:1057–1064.
Lin W, Dominguez B, Yang J, Aryal P, Brandon EP, Gage FH, Lee KF (2005) Neurotransmitter acetylcholine negatively regulates neuromuscular synapse formation by a Cdk5-dependent mechanism. Neuron 46:569–579.
Lin WW, Karin M (2007) A cytokine-mediated link between innate immunity, inflammation, and cancer. J Clin Invest 117:1175–1183.
Linnoila J, Wang Y, Yao Y, Wang ZZ (2008) A mammalian homolog of *Drosophila* tumorous imaginal discs, Tid1, mediates agrin signaling at the neuromuscular junction. Neuron 60:625–641.
Lubin FD, Sweatt JD (2007) The IkappaB kinase regulates chromatin structure during reconsolidation of conditioned fear memories. Neuron 55:942–957.
Luo ZG, Wang Q, Zhou JZ, Wang J, Luo Z, Liu M, He X, Wynshaw-Boris A, Xiong WC, Lu B, Mei L (2002) Regulation of AChR clustering by Dishevelled interacting with MuSK and PAK1. Neuron 35:489–505.
Luo ZG, Je HS, Wang Q, Yang F, Dobbins GC, Yang ZH, Xiong WC, Lu B, Mei L (2003) Implication of geranylgeranyltransferase I in synapse formation. Neuron 40:703–717.
Marques MJ, Conchello JA, Lichtman JW (2000) From plaque to pretzel: fold formation and acetylcholine receptor loss at the developing neuromuscular junction. J Neurosci 20:3663–3675.
Mattson MP, Camandola S (2001) NF-kappaB in neuronal plasticity and neurodegenerative disorders. J Clin Invest 107:247–254.
Mattson MP, Meffert MK (2006) Roles for NF-kappaB in nerve cell survival, plasticity, and disease. Cell Death Differ 13:852–860.
Mattson MP, Culmsee C, Yu Z, Camandola S (2000) Roles of nuclear factor kappaB in neuronal survival and plasticity. J Neurochem 74:443–456.
Meffert MK, Baltimore D (2005) Physiological functions for brain NF-kappaB. Trends Neurosci 28:37–43.
Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS, Baltimore D (2003) NF-kappa B functions in synaptic signaling and behavior. Nat Neurosci 6:1072–1078.
Miniou P, Tiziano D, Frugier T, Roblot N, Le Meur M, Melki J (1999) Gene targeting restricted to mouse striated muscle lineage. Nucleic Acids Res 27:e27.
Misgeld T, Kummer TT, Lichtman JW, Sanes JR (2005) Agrin promotes synaptic differentiation by counteracting an inhibitory effect of neurotransmitter. Proc Natl Acad Sci U S A 102:11088–11093.
Miyamoto S, Verma IM (1995) Rel/NF-kappa B/I kappa B story. Adv Cancer Res 66:255–292.
Ohno K, Sadeh M, Blatt I, Brengman JM, Engel AG (2003) E-box mutations in the RAPSN promoter region in eight cases with congenital myasthenic syndrome. Hum Mol Genet 12:739–748.
Okada K, Inoue A, Okada M, Murata Y, Kakuta S, Jigami T, Kubo S, Shiraishi H, Eguchi K, Motomura M, Akiyama T, Iwakura Y, Higuchi O, Yamanashi Y (2006) The muscle protein Dok-7 is essential for neuromuscular synaptogenesis. Science 312:1802–1805.
O'Neill LA, Kaltschmidt C (1997) NF-kappa B: a crucial transcription factor for glial and neuronal cell function. Trends Neurosci 20:252–258.
Phillips WD, Kopta C, Blount P, Gardner PD, Steinbach JH, Merlie JP (1991a) ACh receptor-rich membrane domains organized in fibroblasts by recombinant 43-kildalton protein. Science 251:568–570.
Phillips WD, Maimone MM, Merlie JP (1991b) Mutagenesis of the 43-kD postsynaptic protein defines domains involved in plasma membrane targeting and AChR clustering. J Cell Biol 115:1713–1723.
Prody CA, Merlie JP (1992) The 5′-flanking region of the mouse muscle nicotinic acetylcholine receptor beta subunit gene promotes expression in cultured muscle cells and is activated by MRF4, myogenin and myoD. Nucleic Acids Res 20:2367–2372.
Qu Z, Apel ED, Doherty CA, Hoffman PW, Huganir RL (1996) The synapse-associated protein rapsyn regulates tyrosine phosphorylation of proteins colocalized at nicotinic acetylcholine receptor clusters. Mol Cell Neurosci 8:171–184.
Rodova M, Kelly KF, VanSaun M, Daniel JM, Werle MJ (2004) Regulation of the rapsyn promoter by kaiso and delta-catenin. Mol Cell Biol 24:7188–7196.
Sanes JR, Lichtman JW (1999) Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 22:389–442.
Sanes JR, Lichtman JW (2001) Induction, assembly, maturation and maintenance of a postsynaptic apparatus. Nat Rev Neurosci 2:791–805.
Schaeffer L, de Kerchove d'Exaerde A, Changeux JP (2001) Targeting transcription to the neuromuscular synapse. Neuron 31:15–22.
Schmidt-Ullrich R, Mémet S, Lilienbaum A, Feuillard J, Raphaël M, Israel A (1996) NF-kappaB activity in transgenic mice: developmental regulation and tissue specificity. Development 122:2117–2128.
Schneider A, Martin-Villalba A, Weih F, Vogel J, Wirth T, Schwaninger M (1999) NF-kappaB is activated and promotes cell death in focal cerebral ischemia. Nat Med 5:554–559.
Stellwagen D, Malenka RC (2006) Synaptic scaling mediated by glial TNF-alpha. Nature 440:1054–1059.
Sun S, Tang Y, Lou X, Zhu L, Yang K, Zhang B, Shi H, Wang C (2007) UXT is a novel and essential cofactor in the NF-kappaB transcriptional enhanceosome. J Cell Biol 178:231–244.
Tao Y, Yan D, Yang Q, Zeng R, Wang Y (2006) Low K^+ promotes NF-kappaB/DNA binding in neuronal apoptosis induced by K^+ loss. Mol Cell Biol 26:1038–1050.
Valenzuela DM, Stitt TN, DiStefano PS, Rojas E, Mattsson K, Compton DL, Nuñez L, Park JS, Stark JL, Gies DR (1995) Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury. Neuron 15:573–584.
Wang J, Jing Z, Zhang L, Zhou G, Braun J, Yao Y, Wang ZZ (2003) Regulation of acetylcholine receptor clustering by the tumor suppressor APC. Nat Neurosci 6:1017–1018.
Wang J, Ruan NJ, Qian L, Lei WL, Chen F, Luo ZG (2008) Wnt/beta-catenin signaling suppresses Rapsyn expression and inhibits acetylcholine receptor clustering at the neuromuscular junction. J Biol Chem 283:21668–21675.
Weston C, Yee B, Hod E, Prives J (2000) Agrin-induced acetylcholine receptor clustering is mediated by the small guanosine triphosphatases Rac and Cdc42. J Cell Biol 150:205–212.
Wheeler D, Knapp E, Bandaru VV, Wang Y, Knorr D, Poirier C, Mattson MP, Geiger JD, Haughey NJ (2009) Tumor necrosis factor-alpha-induced neutral sphingomyelinase-2 modulates synaptic plasticity by controlling the membrane insertion of NMDA receptors. J Neurochem 109:1237–1249.
Wu H, Xiong WC, Mei L (2010) To build a synapse: signaling pathways in neuromuscular junction assembly. Development 137:1017–1033.
Yang X, Arber S, William C, Li L, Tanabe Y, Jessell TM, Birchmeier C, Burden SJ (2001) Patterning of muscle acetylcholine receptor gene expression in the absence of motor innervation. Neuron 30:399–410.
Yoshihara CM, Hall ZW (1993) Increased expression of the 43-kD protein disrupts acetylcholine receptor clustering in myotubes. J Cell Biol 122: 169–179.
Zandi E, Chen Y, Karin M (1998) Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. Science 281:1360–1363.
Zhang B, Luo S, Wang Q, Suzuki T, Xiong WC, Mei L (2008) LRP4 serves as a coreceptor of agrin. Neuron 60:285–297.