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Rapsyn Interaction with Calpain Stabilizes AChR Clusters at the Neuromuscular Junction

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SUMMARY

Agrin induces, whereas acetylcholine (ACh) disperses, ACh receptor (AChR) clusters during neuromuscular synaptogenesis. Such counteractive interaction leads to eventual dispersal of nonsynaptic AChR-rich sites and formation of receptor clusters at the postjunctional membrane. However, the underlying mechanisms are not well understood. Here we show that calpain, a calcium-dependent protease, is activated by the cholinergic stimulation and is required for induced dispersion of AChR clusters. Interestingly, the AChR-associated protein rapsyn interacted with calpain in an agrin-dependent manner, and this interaction inhibited the protease activity of calpain. Disrupting the endogenous rapsyn/calpain interaction enhanced CCh-induced dispersion of AChR clusters. Moreover, the loss of AChR clusters in agrin mutant mice was partially rescued by the inhibition of calpain via overexpressing calpastatin, an endogenous calpain inhibitor, or injecting calpeptin, a cell-permeable calpain inhibitor. These results demonstrate that calpain participates in ACh-induced dispersion of AChR clusters, and rapsyn stabilizes AChR clusters by suppressing calpain activity.

INTRODUCTION

Neuromuscular synapses are formed between motor neurons and skeletal muscle fibers. AChRs are concentrated at the postsynaptic membrane, which guarantees efficient and accurate neurotransmission. AChR clustering is a dynamic process, whereby nascent clusters underneath nerve terminals are stabilized by agrin, a motor-neuron-derived glycoprotein, and the AChR-associated protein rapsyn (McMahan, 1990; Sanes and Lichtman, 1999, 2001). Meanwhile, motor neurons release negative signals to disperse noninnervated clusters and refine clusters at the synapses. Genetic studies suggest that ACh may serve as a negative signal. AChR clusters are larger in mice deficient in choline acetyltransferase (ChAT), the key enzyme for ACh synthesis, and numerous AChR clusters are maintained in ChAT and agrin double mutant mice (Lin et al., 2005; Misgeld et al., 2002, 2005), whereas few AChR clusters are present in the muscle of agrin single mutant mice (Burgess et al., 1999; Gautam et al., 1996; Lin et al., 2001; Yang et al., 2001). The interplay between positive and negative signals is necessary for precise matching of nerve terminals to individual postsynaptic apparatuses (Sanes and Lichtman, 1999, 2001).

Recent evidence suggests that Cdk5, a cytoplasmic serine/threonine kinase, may be an effector in dispersing or refining AChR clusters. ACh activates Cdk5, and inhibition of Cdk5 by genetic ablation or pharmacological inhibition increases AChR cluster size in cultured muscle fibers and in mutant mice (Fu et al., 2005; Lin et al., 2005). The dispersing activity of ACh can be counteracted by agrin (Lin et al., 2005; Misgeld et al., 2005), presumably via the activation of MuSK, a muscle-specific tyrosine kinase receptor concentrated in the postsynaptic region of neuromuscular junctions (Glass et al., 1996). Thus, the overall inhibitory effect by ACh and Cdk5 is reversed locally by agrin signaling so that AChR clusters are stabilized at the neuromuscular junction (NMJ). Rapsyn is believed to be required for agrin-induced AChR clustering and stabilization (Apel et al., 1997; Froehner, 1991; Fuhrer et al., 1999; Gautam et al., 1996; Phillips et al., 1997). However, the effectors of rapsyn are not well defined. Here we provide evidence that rapsyn acts by inhibiting calpain to regulate AChR cluster stabilization at the NMJ.

Calpains are a family of calcium-activated intracellular cysteine proteases, which are ubiquitously expressed in various mammalian cells (Goll et al., 2003). In the brain, calpains are involved in many physiological events including LTP (Carafoli and Molinari, 1998; Oliver et al., 1989; Staubli et al., 1988) or neurotoxic insults ranging from

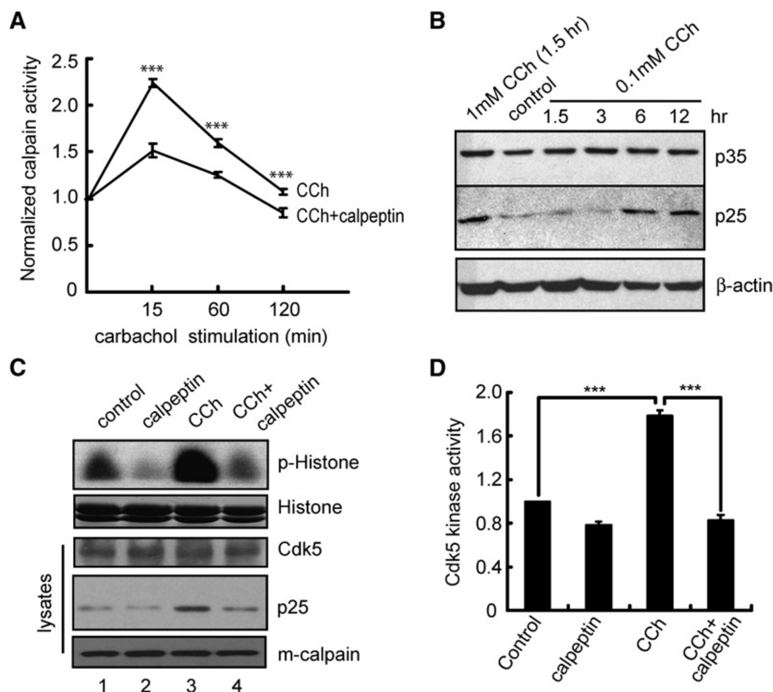

Figure 1. Activation of Calpain by Cholinergic Agonist

(A) Activation of calpain by stimulation with carbachol (CCh). C2C12 myotubes were treated with CCh (0.1 mM), without or with 20 μM calpeptin for the indicated times. The activity of calpain in cell lysates was measured using Suc-Leu-Tyr-AMC as the substrate. The value of control cells was taken as 1.0. Data are shown as mean ± SEM (n = 3). ***$p < 0.001$, CCh versus CCh + calpeptin at each time point.
(B) Accumulation of p25 in CCh-treated muscle cells. C2C12 myotubes were treated without (control) or with 0.1 mM or 1 mM CCh for the indicated times. Lysates (30 μg of protein) were subjected to immunoblotting with antibodies against p35 or β-actin.
(C and D) Dependence of CCh-induced Cdk5 activation on calpain activity. C2C12 myotubes were treated with or without calpeptin (20 μM) for 2 hr prior to incubation with CCh (0.1 mM) for additional 5 hr. The activity of Cdk5 purified by immunoprecipitation was assayed in vitro using histone H1 as substrate in the presence of [γ-^{32}P]-ATP. Normalized Cdk5 activity was summarized in (D), where Cdk5 activity of control cells was taken as 1.0. Data are shown as mean ± SEM (n = 3; ***$p < 0.001$, Student's t test).

ischemia to Alzheimer's disease (Lee et al., 2000; Nixon, 2000; Patrick et al., 1999). A number of proteins have been identified as calpain substrates from various tissues (Goll et al., 2003). Calpain cleavage of p35, a regulatory partner of Cdk5, generates p25, which causes hyperactivation of Cdk5 (Ahlijanian et al., 2000; Patrick et al., 1999; Patzke et al., 2003). Transient expression of p25 in the hippocampus enhances LTP and facilitates hippocampus-dependent memory, whereas prolonged p25 expression results in loss of synapses and death of neurons (Fischer et al., 2005).

In the present study, we demonstrate a role of calpain in regulating postsynaptic differentiation at the NMJ. The cholinergic stimulation increased calpain activity, and this in turn resulted in an accumulation of p25, a potent activator of Cdk5. Inhibition of calpain attenuated cholinergic agonist-induced dispersal of AChR clusters in cultured muscle cells and prevented the loss of AChR clusters in agrin mutant mice. We also investigated regulatory mechanisms of calpain activity and found that rapsyn interacted with calpain and inhibited the cleavage activity of calpain. Together these studies identify a critical signaling mechanism by which ACh destabilizes AChR clusters and reveal a function of rapsyn in regulating AChR clustering and NMJ formation.

RESULTS

Activation of Calpain by the Cholinergic Agonist Carbachol

The activity of calpain is known to be regulated by calcium (Carafoli and Molinari, 1998; Glading et al., 2002; Goll et al., 2003). It has been shown that cholinergic stimulation or depolarization of muscle cells causes calcium elevation (Allard et al., 1996; Cherednichenko et al., 2004). We investigated whether cholinergic stimulation regulates calpain activity in muscle cells. As shown in Figure 1A, treatment with carbachol (CCh), a nonhydrolyzable cholinergic agonist that triggers Ca^{2+} transients in muscle cells (data not shown), caused an increase of calpain activity in C2C12 myotubes; the activity peaked 15 min after CCh stimulation and declined thereafter. The CCh-induced activation of calpain was attenuated when muscle cells were pretreated with calpeptin, a cell-permeable calpain inhibitor. These results demonstrate that cholinergic stimulation increases calpain activity in muscle cells. Cdk5 in muscle cells can be activated by ACh (Lin et al., 2005), although exact mechanisms remain unclear. p35 is a regulatory partner of Cdk5 and a specific substrate of calpain (Kusakawa et al., 2000; Lee et al., 2000; Nath et al., 2000). Proteolytic cleavage of p35 by calpain generates p25, which causes hyperactivation of Cdk5 (Fischer et al., 2005; Kusakawa et al., 2000; Lee et al., 2000; Patrick et al., 1999). We investigated whether CCh regulates p25 production. As shown in Figure 1B, the amount of p25 increased in C2C12 myotubes treated with CCh. By contrast, the level of p35 appeared to be constant, probably due to rapid turnover rates and the addition of newly synthesized p35 (O'Hare et al., 2005; Patrick et al., 1998). The CCh-induced p25 accumulation was suppressed in muscle cells that were pretreated with calpeptin (Figure 1C, lane 3 and 4), suggesting the specific role of calpain in p25 production. To investigate the function of calpain-mediated p25 production, we evaluated the consequence of

calpain inhibition on Cdk5 kinase activity. CCh treatment induced a robust increase in [^{32}P]-labeled histone H1, indicative of Cdk5 activation, consistent with a previous study (Lin et al., 2005) (Figures 1C and 1D). Interestingly, CCh-induced Cdk5 activation was inhibited by calpeptin (Figures 1C and 1D), suggesting a role of calpain in cholinergic agonist-induced Cdk5 activation.

Increased AChR Cluster Stability by Calpain Inhibition

Next we investigated whether calpain was involved in cholinergic agonist-induced dispersion of AChR clusters. C2C12 myotubes were first treated with agrin to induce AChR clusters, and then switched to agrin-free medium in the presence of CCh with or without calpeptin. CCh treatment promoted disassembly of AChR clusters by reducing both total length and number of clusters (Figures 2A–2C). This result is in agreement with the earlier reports that CCh destabilizes AChR clusters (Lin et al., 2005; Misgeld et al., 2005). The reduction in AChR clusters by CCh was ameliorated in calpeptin-treated cells (Figures 2B and 2C), suggesting that calpain may participate in "cholinergic" agonist-induced dispersal of AChR clusters.

To further examine the role of calpain in regulating AChR clusters, we suppressed calpain expression by small interference RNA (siRNA). Because m- but not μ-calpain is the dominant form expressed in C2C12 cells and in skeletal muscles (data not shown), we screened several siRNA sequences and identified calpain-siRNA (CP-siRNA) that could suppress the expression of endogenous m-calpain effectively (Figure 2D). Similar to the treatment with calpeptin, transfection with calpain-siRNA prevented the dispersion of AChR clusters by CCh (Figures 2E and 2F). These results support the notion that calpain is involved in "cholinergic" stimulation-induced dispersal of AChR clusters.

Rapsyn Interaction with Calpain

Interestingly, when using rapsyn as bait to search for binding proteins in yeast two-hybrid screens, we identified the large subunit of m-calpain. m-Calpain is composed of two subunits: a distinct 80 kDa large subunit, and a small subunit (28 kDa) that is shared with μ-calpain (Carafoli and Molinari, 1998; Goll et al., 2003; Sorimachi et al., 1997). The large subunit contains four domains: a short prodomain (domain I) in the N-terminus, a catalytic protease domain (domain II), a regulatory domain (domain III), and a calcium-binding domain (domain IV) containing EF hand motifs (Glading et al., 2002; Goll et al., 2003). The original clone isolated from yeast contained 266 residues (aa 435–700) covering a partial domain III and domain IV, suggesting that the binding region resides in domains III and IV (Figure 3B). To map the domain necessary for the interaction, Myc-tagged domains III (Myc-DIII) or IV (Myc-DIV) was coexpressed with HA-rapsyn in HEK293 cells. Immunoprecipitation (IP) of rapsyn resulted in co-IP of m-calpain domain III, but not domain IV, indicating that rapsyn is associated with m-calpain via domain III, but not domain IV (Figure 3C).

Rapsyn contains seven tetratricopeptide repeat (TPR) domains mediating self-association (Ramarao et al., 2001; Ramarao and Cohen, 1998), one coiled-coil domain binding to the AChR (Bartoli et al., 2001; Ramarao et al., 2001), and a RING domain interacting with β-dystroglycan (Bartoli et al., 2001) (Figure 3A). To determine which region in rapsyn interacts with calpain, a series of constructs encoding different rapsyn fragments were expressed in HEK293 cells. Cell lysates were incubated with His-m-calpain immobilized on beads. Constructs that contained the TPR domains (the full-length rapsyn and rapsyn$_{1–320}$) associated with His-m-calpain (Figure 3D). However, the coiled-coil domain and the RING domain were barely detectable in the precipitates. These results indicate that the TPR domains in rapsyn mediate interaction with calpain. Similar results were obtained in co-IP experiments (Figure 3E). The interaction and domain mapping results are summarized in Figure 3B. The rapsyn-calpain interaction did not appear to be regulated by calcium, since the association was unchanged by the addition or depletion of calcium (Figure 3F).

Inhibition of Calpain Activity by Rapsyn

The catalytic activity of calpain can be regulated through the interaction of domain III with calcium, phospholipids, and/or regulatory proteins (Benetti et al., 2001; Glading et al., 2002; Tompa et al., 2001). In light of its interaction through domain III with rapsyn, we reasoned that the latter might regulate calpain's activity. To test this possibility, calpain activity in mouse brain lysates was assayed in vitro, with p25 production as readout, in the absence or presence of GST-rapsyn. Calpain was activated by calcium, which promotes the cleavage of p35 to p25 (Figure 4A, lane 2) (Ahlijanian et al., 2000; Patrick et al., 1999; Patzke et al., 2003). This proteolytic activity was inhibited by calpeptin, indicating the specificity (Figure 4A, lane 4). Remarkably, GST-rapsyn, but not GST, inhibited p35 cleavage and p25 production (Figure 4A, lane 3), suggesting that rapsyn might inhibit calpain activity. To examine whether rapsyn regulates calpain activity in cells, p35-Myc was cotransfected with rapsyn-EGFP or EGFP in HEK293 cells (Figure 4B). Transfected cells were treated with ionomycin, a calcium ionophore that causes calcium influx to activate calpain (Croall, 1989). Treatment with ionomycin resulted in the p35 cleavage and p25 production (Figure 4B, lane 2). However, ionomycin-induced p35 cleavage and p25 production were inhibited in cells expressing rapsyn (Figure 4B, lane 1). To further evaluate the role of rapsyn in regulating calpain activity in muscle cells, we explored the consequence of suppressing rapsyn expression. Two rapsyn-siRNAs (#233 and #270) were generated and transfected into HEK293 cells. siRNA-233, but not control siRNA, suppressed exogenous expressed rapsyn in HEK293 cells and endogenous rapsyn in C2C12 muscle cells (Figures 4C and 4G). However, the effect of siRNA-270 was minimal, if any (Figure 4C), and was thus not used in the following experiments. Transfection of muscle cells with siRNA-233 had no effect on

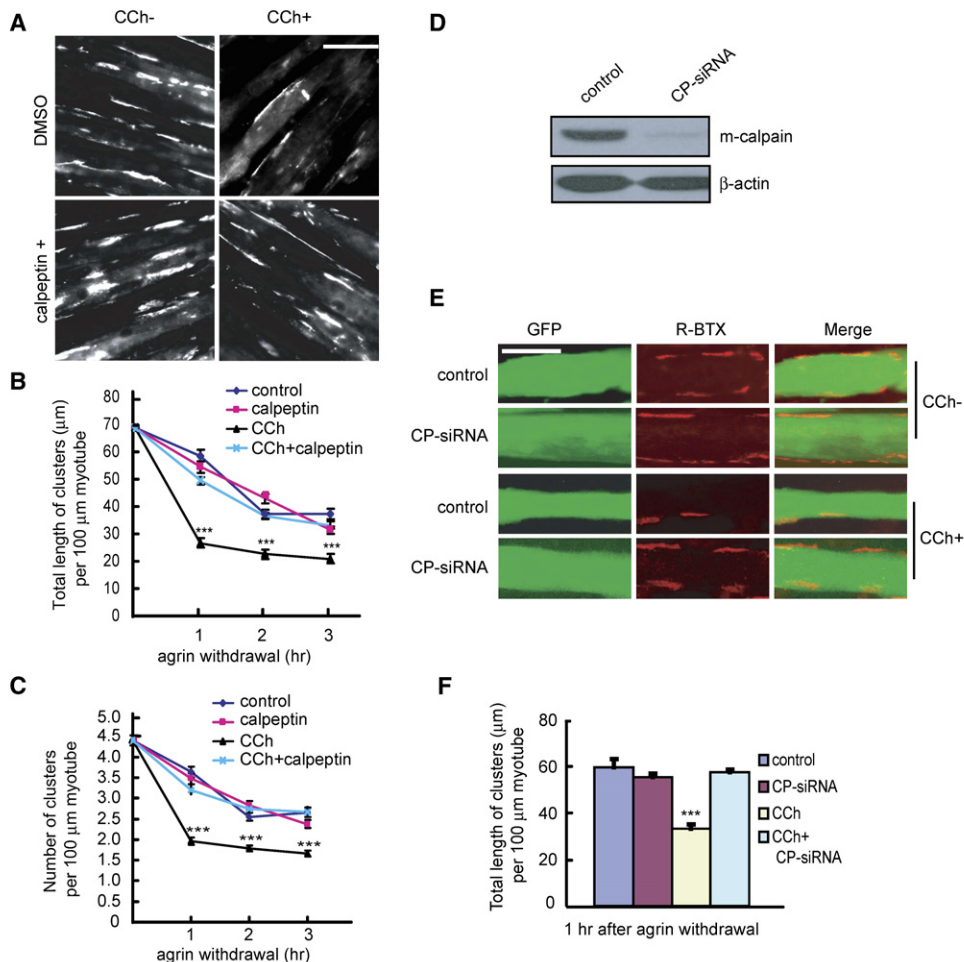

Figure 2. Cholinergic Agonist-Induced Dispersion of AChR Clusters Is Attenuated by Calpain Inhibition or Downregulation

(A) Increased stability of AChR clusters in calpeptin-treated muscle cells. C2C12 myotubes were stimulated with agrin (10 ng/ml, 12 hr) to induce AChR clusters and switched to agrin-free medium containing 0.1 mM CCh, either alone or together with calpeptin (20 μM), and incubated for 1–3 hr. AChR clusters were visualized by R-BTX. Shown are images from a representative experiment that was repeated three times with similar results. Scale bar, 50 μm.

(B and C) Total length (B) and number of clusters bigger than 5 μm in length (C) per 100 μm myotube were quantitatively analyzed. The value from DMSO-treated cells after agrin withdrawal was used as control. Data are shown as mean ± SEM from at least three experiments (***$p < 0.001$ in comparison to control, Student's t test). The following number of myotubes was scored: 226 for control, 198 for calpeptin, 278 for CCh, and 233 for CCh + calpeptin.

(D) Downregulation of m-calpain expression in muscle cells by siRNA. C2C12 cells transfected with parental plasmid pSUPER-EGFP or that encoding siRNA of m-calpain (CP-siRNA) were lysed, and resulting lysates were probed for m-calpain levels with β-actin as control.

(E) Increased stability of AChR clusters in muscle cells transfected with CP-siRNA. C2C12 myoblasts transfected with control or CP-siRNA plasmids were induced for differentiation 12 hr after transfection. After treatment with agrin for 12 hr, myotubes were switched to agrin-free medium without or with CCh (0.1 mM) and incubated for 1 hr. AChR clusters were visualized by R-BTX. Coexpressed GFP was used to show cell morphology.

(F) Total length of AChR clusters per 100 μm transfected myotube. Data are presented as mean ± SEM of three independent experiments. At least 100 myotubes were analyzed for each condition. ***$p < 0.001$, in comparison to control cells after agrin withdrawal.

calpain expression, but elevated p25 levels in control and CCh-treated myotubes (Figure 4D). These results indicate that suppression of rapsyn expression increases calpain activity, in line with the notion that rapsyn inhibits calpain activity in vitro.

Inhibition of Calpain by Agrin in Muscle Cells

Since rapsyn is required for AChR clustering (Apel et al., 1997; Froehner, 1991; Fuhrer et al., 1999; Gautam et al., 1995; Phillips et al., 1997), we asked whether agrin regulates the interaction between rapsyn and calpain. First, we investigated whether rapsyn and calpain interact in vivo, which was analyzed by IP experiments in control and agrin-stimulated C2C12 myotubes (Figure 4E). IP of rapsyn resulted in co-IP of calpain (Figure 4E, lane 3). By contrast, calpain was not detectable in precipitates with a control antibody (Flag) or in a reaction when the antibodies were omitted (No Ab) (Figure 4E, lane 1 and 2),

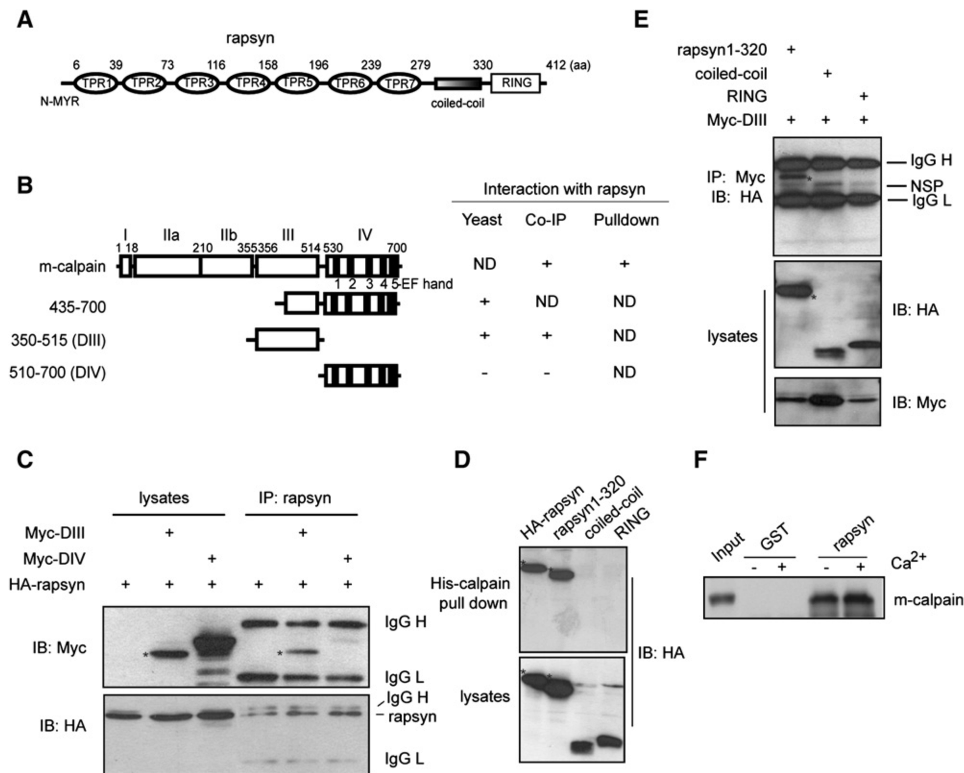

Figure 3. Rapsyn Interacts with Calpain
(A) Schematic structure of rapsyn domains.
(B) Identification of domains in calpain necessary for interaction with rapsyn. Data were summarized from yeast two-hybrid, coimmunoprecipitation (CoIP), and pulldown assays. +, positive interaction; −, no interaction; ND, not detected.
(C–E) Calpain domain III associates with rapsyn TPR domains. HEK293 cells coexpressing HA-rapsyn proteins and/or Myc-tagged calpain domains were lysed. Resulting lysates were subjected to IP with antibodies against rapsyn (C) or Myc (E) and subsequent immunoblotting (IB) with indicated antibodies. IgG H, IgG heavy chain; IgG L, IgG light chain; NSP, nonspecific protein. The asterisks indicate positive interacting bands. (D) The lysates of HEK293 cells transfected with HA-tagged rapsyn were incubated with His-tagged calpain large subunit immobilized on Ni-NTA metal affinity resin. Bound proteins were probed with anti-HA antibody.
(F) The rapsyn-calpain interaction is not regulated by calcium. C2C12 cells were lysed in the modified RIPA buffer containing 5 mM EGTA or 5 mM Ca^{2+}, respectively. Lysates were incubated with GST-rapsyn or GST immobilized on glutathione-sepharose beads. Bound proteins were subjected to IB with anti-calpain antibody.

indicating the specificity of the interaction. The interaction between rapsyn and calpain was increased by 2.5-fold in agrin-treated C2C12 myotubes (Figure 4E, histogram). Quantitative analysis showed that in the absence of agrin, ~12% of calpain in C2C12 myotubes interacted with rapsyn, whereas the amount of rapsyn-interacting calpain increased to ~30% in response to agrin stimulation (see Figure 4 legend for detailed calculation). Since rapsyn is associated with AChR and this association is increased upon agrin stimulation (Moransard et al., 2003), we examined whether calpain is in the complex of AChR. As shown in Figure 4F, IP with antibody against AChR alpha subunit (AChRα) resulted in co-IP of m-calpain, as well as rapsyn. The association of AChR with m-calpain or rapsyn was increased upon agrin stimulation (Figure 4F, lanes 4 and 6). These associations were specific, since IP with control antibody did not show any positive bands (Figure 4F, lanes 3 and 5). In line with the notion that m-calpain associates with rapsyn and AChRs, m-calpain was found to be colocalized with AChRs at the NMJ (Figure S1 in the Supplemental Data available with this article online).

The observation that agrin increased the rapsyn-calpain interaction raised the possibility that agrin might regulate calpain's activity in muscle cells. We found that CCh-induced accumulation of p25 was decreased when the cells were pretreated with agrin (Figure 4G, lanes 1–3), suggesting an inhibitory role of agrin in calpain activity. The inhibitory effect of agrin requires rapsyn, since downregulation of rapsyn in muscle cells prevented agrin from suppressing CCh-induced p25 elevation (Figure 4G, lane 4). In addition, CCh-induced increase of calpain activity was also suppressed by agrin (Figure 4H). Taken together, these results suggest that agrin may inhibit calpain activity by increasing the rapsyn-calpain interaction. To further explore the regulation of calpain in vivo, we compared the calpain activity between synaptic and nonsynaptic

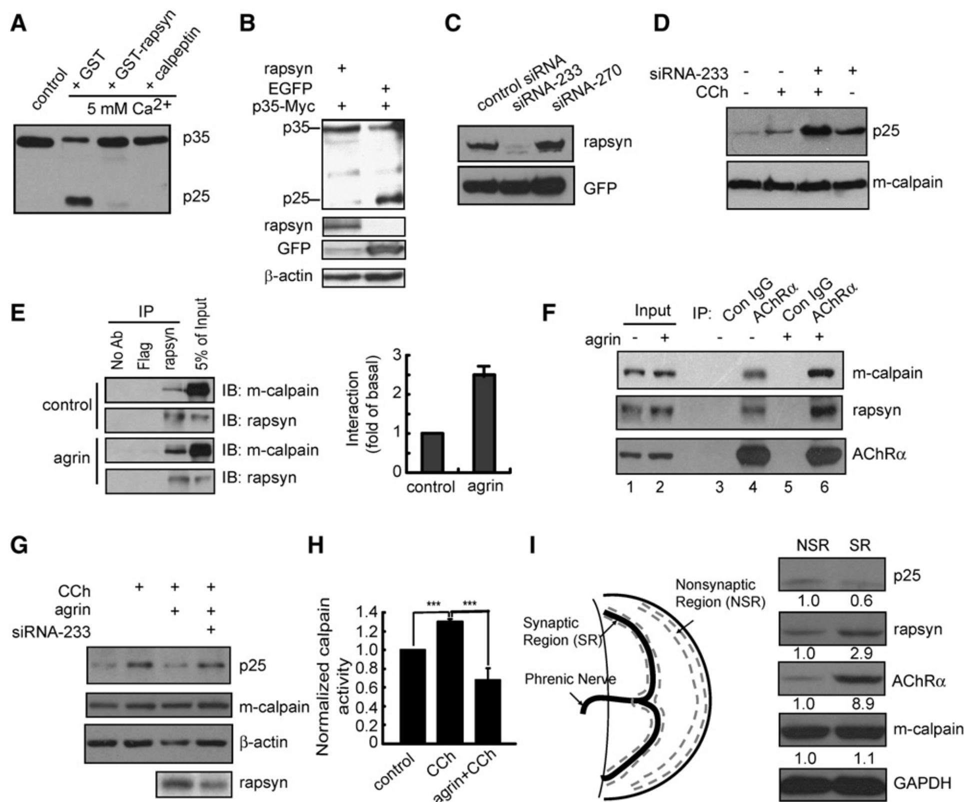

Figure 4. Regulation of Calpain Activity by Rapsyn and Agrin

(A) Inhibition of brain calpain activity by rapsyn. Rat brain lysates were incubated without (control) or with 5 mM Ca^{2+}. The calpain activity in the lysates was assayed by measuring the level of p25, which was revealed by IB. In some experiments, lysates were incubated with calpeptin (20 μM), GST (2 μg), or GST-rapsyn (2 μg).

(B) Rapsyn inhibits calpain activity in cells. HEK293 cells transfected with p35-myc and rapsyn-EGFP or EGFP were treated with ionomycin for 1 hr. The cell lysates were probed with indicated antibodies.

(C) Suppression of rapsyn expression by siRNA. HEK293 cells were transfected with HA-rapsyn and EGFP, together with control or rapsyn-siRNA233 or -270. Lysates were probed with antibodies against HA or GFP.

(D) Increase in p25 production in C2C12 cells transfected with rapsyn-siRNA-233. C2C12 myotubes were transfected with control siRNA or siRNA-233. Differentiated myotubes were treated with CCh (0.1 mM, 5 hr), and the level of p25 or m-calpain was probed.

(E) Increased interaction of rapsyn with calpain in agrin-stimulated muscle cells. C2C12 myotubes were treated with or without agrin (10 ng/ml, 8 hr). Lysates were subjected to IP with monoclone anti-rapsyn or anti-Flag (control) antibodies. Resulting immunocomplexes were probed with antibodies against m-calpain or rapsyn. Five percent of inputs were loaded to quantitate the efficacy of IP. Since only ~1% or 2.5% of calpain was coprecipitated with rapsyn in the absence or presence of agrin, respectively, and ~8% rapsyn was IP-ed, the total amount of m-calpain that was associated with rapsyn should be ~12-fold (100/8) of that seen in the blots, i.e., ~12% (~1% times 12) and ~30% (2.5% times 12), before and after agrin stimulation. Quantitative analysis of data is shown in the histogram. The amount of rapsyn-associated m-calpain without agrin treatment was taken as 1.0. Data are shown as mean ± SEM (n = 3).

(F) Increased association of calpain with the AChR/rapsyn complex. C2C12 myotubes were treated as in (E), and resulting lysates were IP-ed with antibody against AChRα. AChR-associated m-calpain or rapsyn was probed.

(G) Reduced p25 accumulation in agrin-stimulated muscle cells. C2C12 myoblasts were transfected with siRNA-233 (lane 4) or control siRNA (lane 1–3), then induced for differentiation 12 hr after transfection. Resulting myotubes were treated with or without agrin (10 ng/ml, overnight) prior to CCh exposure (0.1 mM, 5 hr). Cell lysates were subjected to IB with indicated antibodies.

(H) Inhibition of CCh-induced calpain activity by agrin treatment. C2C12 myotubes were pretreated without or with agrin (10 ng/ml, 15 min) before exposure to CCh (0.1 mM, 30 min). Calpain activity was determined using Suc-Leu-Tyr-AMC as the substrate. Data are shown as mean ± SEM (n = 3; ***$p < 0.001$, Student's t test).

(I) Comparison of p25 levels in synaptic and nonsynaptic regions. Different parts of diaphragm (SR, synaptic region covered by phrenic nerve; NSR, marginal region of muscle distant from nerve branches) were dissected out. Same amount of homogenates (100 μg protein) from NSR or SR was subjected to IB. The relative level of indicated proteins was marked underneath each blot by taking the value of NSR as 1.0. The level of p25 reflects relative activity of calpain in different regions of the muscle.

regions (Figure 4I, left) in the skeletal muscle by examining levels of p25 and calpain in these regions. As shown in Figure 4I, the level of rapsyn or AChRα is several-fold higher in synaptic region (SR) than in nonsynaptic region (NSR). However, the level of m-calpain appeared to be similar between NSR and SR regions. μ-calpain was barely

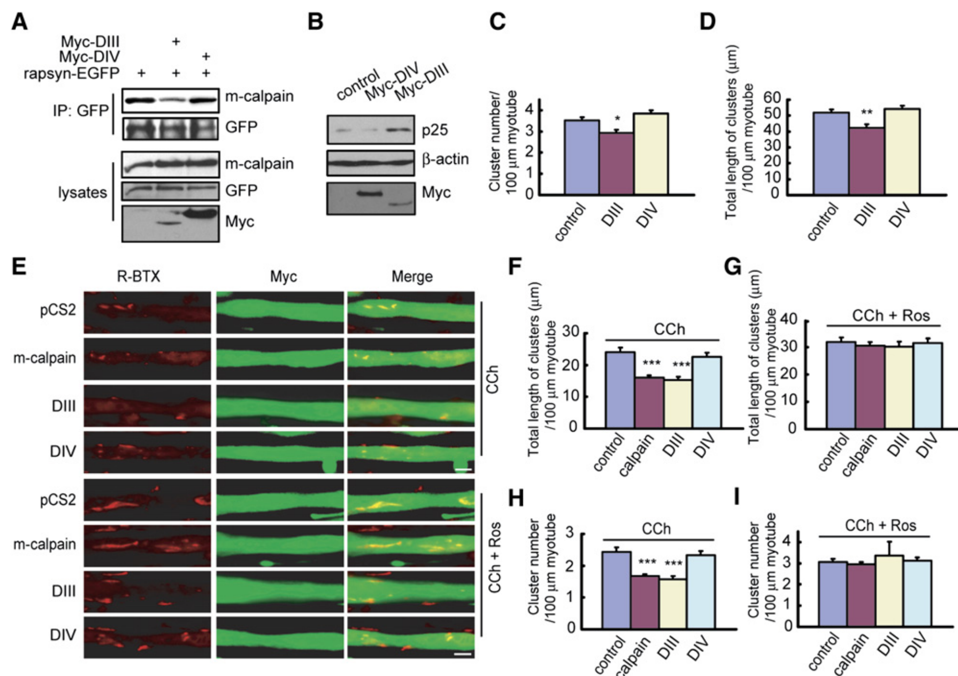

Figure 5. Disruption of the Rapsyn-Calpain Interaction Decreases AChR Cluster Stability
(A) Disruption of the rapsyn-calpain interaction by calpain domain III. C2C12 myoblasts were transfected with rapsyn-EGFP, without or with Myc-DIII or DIV. Cell lysates of transfected myotubes were subjected to IP with antibodies against GFP, followed by IB with indicated antibodies.
(B) Overexpression of calpain domain III elevates CCh-induced p25 accumulation. Transfected C2C12 myotubes were treated with CCh (0.1 mM, 5 hr). Amounts of transfected calpain fragments or endogenous p25 were probed.
(C and D) The effects of calpain mutants on agrin-induced AChR clustering. C2C12 myoblasts were transfected with the empty vector (pCS2 + MT) or that encoding DIII, or -DIV. Resulting myotubes were stimulated with agrin (10 ng/ml, 12 hr) to induce AChR clusters. The number and total length of AChR clusters per 100 μm myotube were shown in (C) and (D), respectively. Data are shown as mean ± SEM from three experiments (*p < 0.05, **p < 0.01, Student's t test). The following numbers of transfected-myotubes were analyzed: 77 for pCS2, 83 for DIII, and 116 for DIV.
(E) AChR cluster stability in muscle cells expressing calpain proteins in the absence or presence of roscovitine (Ros). C2C12 cells were transfected with pCS2 + MT or calpain constructs, and the transfected myotubes were treated with agrin as in (C) and (D), followed by exposure to agrin-free medium for 2 hr in the presence of CCh (0.1 mM) (top four sets in [E]) or of CCh (0.1 mM) plus Ros (10 μM) (bottom four sets in [E]). The myotubes from these two treatments that have been stained with R-BTX or anti-Myc antibodies (red and green fluorescence, respectively). Scale bar, 25 μm.
(F–I) Quantitative analysis of total length (F and G) and number (H and I) of AChR clusters per 100 μm Myc-positive myotube. Data are shown as mean ± SEM from three experiments (***p < 0.001 in comparison with control, Student's t test). The following numbers of transfected-myotubes were scored: 90 for pCS2 + MT (control), 147 for calpain, 145 for DIII, 104 for DIV.

detectable in the diaphragm muscle (data not shown). Interestingly, the level of p25 in SR was only ~60% of that in NSR (Figure 4I, right), suggesting that calpain is locally inhibited at the NMJ, probably by agrin and rapsyn.

Disruption of the Rapsyn-Calpain Interaction Increases Calpain Activity and Promotes CCh-Induced Dispersion of AChR Clusters

To evaluate the role of the rapsyn-calpain interaction in AChR clustering, we sought to disrupt such interaction in muscle cells. Sufficient to bind to rapsyn, domain III (DIII) may disrupt the rapsyn interaction with endogenous calpain, and thus release its inhibitory effect. As shown in Figure 5A, the amount of m-calpain coprecipitated with rapsyn was decreased in muscle cells expressing Myc-DIII. As expected, expression of Myc-DIII in muscle cells resulted in an increase of CCh-induced p25 production (Figure 5B, lane 3, top panel). By contrast, Myc-DIV, which did not inhibit the rapsyn-calpain interaction (Figure 5A), had no effect on p25 production (Figure 5B). To determine whether the rapsyn-calpain interaction regulates AChR clustering, wild-type or mutated forms of calpain were overexpressed in C2C12 muscle cells. Transfected myotubes, identified by anti-Myc antibody, were scored for AChR clusters induced by agrin. The number and total length of AChR clusters were significantly decreased in C2C12 myotubes expressing Myc-DIII, but not Myc-DIV (Figures 5C and 5D), suggesting a regulatory role of calpain in agrin-induced formation of AChR clusters.

To investigate the effects of calpain on cluster stability, transfected myotubes were first stimulated with agrin and switched to agrin-free medium containing CCh to destabilize AChR clusters. As shown in Figures 5F and 5H, the CCh-induced declustering of AChR clusters was facilitated in muscle cells expressing m-calpain or DIII, but not DIV, suggesting that the disruption of the rapsyn-calpain

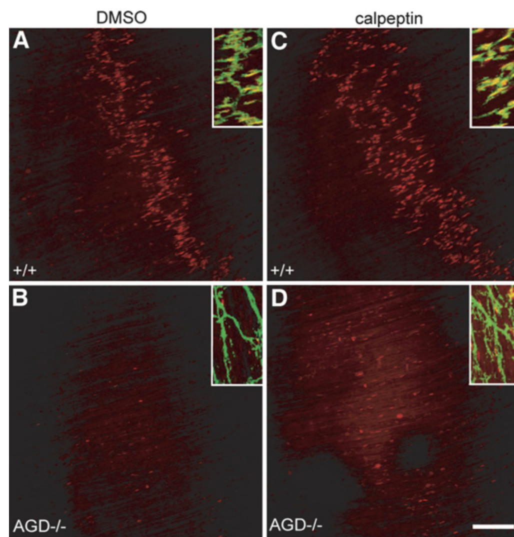

Figure 6. Maintenance of AChR Clusters in Agrin Mutant Mice by Injecting Calpain Inhibitor
Calpeptin (15 mg/kg) or vehicle DMSO was injected i.p. into pregnant mice (E13.5) daily for 3 days. Diaphragm muscles of agrin mutant mice ($AGD^{-/-}$, [B] and [D]) or littermate control mice (+/+, [A] and [C]) at E16.5 were dissected out and whole-mount stained with antibody against synaptophysin to label presynaptic vesicles (green in insets), along with R-BTX (red). AChR clusters in the most ventral region of the left hemidiaphragm were analyzed. Scale bar, 100 μm.

interaction facilitated the dispersing effect of cholinergic stimulation. Representative myotube images showing this are seen in Figure 5E (top four sets). The reduction of AChR cluster stability in calpain-transfected cells is in line with the notion that calpain gain of function disturbs AChR clusters. Remarkably, the effects of calpain or DIII on CCh-induced declustering were abolished by treatment with roscovitine (Ros), an inhibitor of Cdk5 (Figures 5G and 5I). Representative myotube images showing this are seen in Figure 5E (bottom four sets). Taken together, these results suggest that the interaction of rapsyn with calpain regulates AChR clustering by inhibiting Cdk5.

Calpain Inhibition Maintains AChR Clusters in Agrin Mutant Mice

Having shown that calpain inhibition stabilized AChR clusters in cultured muscle cells (Figure 2), we next investigated the effect of calpain inhibition on synaptogenesis in vivo (for the effect of calpeptin on the formation of AChR clusters in cultured muscle cells, see the Supplemental Results and Figure S2). Because inhibition of ChAT or Cdk5 by genetic ablation or pharmacological inhibitors (for Cdk5) has been shown to maintain AChR clusters in agrin mutant mice (Lin et al., 2005; Misgeld et al., 2005), we speculated that calpain inhibition should have a similar effect if calpain functions in the same pathway. To test this possibility, calpeptin was injected into pregnant mice daily from E13.5 for 3 days. E16.5 diaphragm muscles of agrin mutants ($AGD^{-/-}$) (Burgess et al., 1999) or littermate wild-

Table 1. Analysis of AChR Clusters in E16.5 Control and Mutant Mice

Genotype	Average Area of Clusters	Number of Clusters/Field
Control (+/+)	34.0 ± 3.8 (149)	363 ± 22 (3)
$AGD^{-/-}$	12.4 ± 2.7 (28)	23 ± 3 (3)
Control (DMSO)	35.4 ± 5.0 (100)	369 ± 8 (3)
Control (calpeptin)	38.4 ± 5.0 (80)	456 ± 9[a] (3)
$AGD^{-/-}$ (DMSO)	9.0 ± 1.6 (66)	36 ± 9 (3)
$AGD^{-/-}$ (calpeptin)	17.7 ± 4.4[b] (61)	96 ± 13[b] (3)
CS Tg	34.6 ± 3.4 (119)	461 ± 23[c] (3)
CS Tg;$AGD^{-/-}$	23.8 ± 3.3[d] (64)	105 ± 13[e] (3)

Average area (μm^2) of individual AChR clusters and number of AChR clusters per field. Data are presented as mean ± SEM; numbers in brackets indicate numbers of AChR clusters (middle lane) measured or animals (right lane) evaluated.
[a] $p < 0.01$, compared with control (DMSO).
[b] $p < 0.05$, compared with $AGD^{-/-}$ (DMSO).
[c] $p < 0.01$, compared with control (+/+).
[d] $p < 0.05$, compared with $AGD^{-/-}$.
[e] $p < 0.001$, compared with $AGD^{-/-}$.

type controls were dissected out and stained for AChR clusters. As shown in Figure 6B, few AChR clusters were present in the muscle of $AGD^{-/-}$ mice injected with vehicle DMSO, compared to control (Figure 6A). However, numerous AChR clusters were observed when AGD mutant mice were treated with calpeptin (Figure 6D). Some of these clusters were not apposed by nerve terminals positive for synaptophysin, a marker for presynaptic differentiation (Figure 6D, inset; Figure S3, arrows). Quantitatively, both average area of individual clusters and the number of clusters were significantly increased in calpeptin-treated $AGD^{-/-}$ mice than that observed in DMSO-treated $AGD^{-/-}$ mice (Table 1 and Figure S3B). The rescue effect of calpeptin was apparent in both the most ventral (Figure 6D) and dorsal regions (Figure S3) of diaphragm muscles from $AGD^{-/-}$ mice. Control littermates treated with calpeptin also exhibited an increase in the number of AChR clusters compared to DMSO-treated control mice (Figures 6A and 6C, Figure S4, and Table 1). Interestingly, the majority of AChR clusters of control mice either injected with DMSO or calpeptin were colocalized with nerve terminals stained positive for synaptophysin (Figures 6A and 6C, yellow in insets, and Figure S4), probably due to the coincidence of post- and presynaptic differentiation. These results suggest that inhibition of calpain is important for the stabilization of AChR clusters. AChR clusters can be formed independent of innervation (Lin et al., 2001; Yang et al., 2001). The formation of these aneural AChR clusters requires rapsyn and MuSK, but not agrin (Lin et al., 2001; Yang et al., 2001). To explore whether calpain inhibition has any effect on this prepatterned AChR clustering, we injected calpeptin daily into pregnant mice from E10.5 for 4 days, and AChR clusters

were analyzed at E14.5, the time when numerous aneural AChR clusters in *AGD*$^{-/-}$ diaphragm muscles are visible. We found that treatment with calpeptin resulted in a slight increase in the proportion of large-sized clusters and the formation of wider endplate in *AGD*$^{-/-}$ mice. However, these changes were not significant (Figure S5).

Overexpression of Calpastatin Maintains AChR Clusters in Agrin Mutants

Since pharmacological inhibitors, including calpeptin, may have potential side effects, we took advantage of calpastatin, an endogenous protein inhibitor for calpain (Goll et al., 2003), to further determine whether calpain acts to disperse AChR clusters in vivo. Transgenic mice were generated by overexpressing calpastatin under the control of the human skeletal actin (HSA) promoter (Figure 7A), which has been shown to drive muscle-specific expression (Brennan and Hardeman, 1993; Luo et al., 2003). Western blot showed that calpastatin was expressed at a remarkable high level in skeletal muscles of the transgenic mice (CS Tg) compared with wild-type control mice (Figure 7B). Low expression of calpastatin was detectable in other tissues, and this might be endogenous proteins (Figure 7C). The expression level of calpastatin in the muscle of CS Tg mice was low at E14.5 and detectable at E16.5 and gradually increased thereafter (Figure 7B). This expression pattern of HSA-driven transgene is in agreement with the promoter activity during development (Brennan and Hardeman, 1993). The multiple bands in CS Tg might be different types of calpastatin resulting from both alternative splicing and proteolytic processing (Takano et al., 1993). We found that the level of p25, an indicator for calpain activity, in the skeletal muscle of CS Tg mice was lower than that of control mice (Figure 7D, top panel). By contrast, overexpression of calpastatin had no effect on levels of other synaptic proteins including rapsyn, MuSK, AChRα, and m-calpain (Figure 7D). The relative activity of calpain was quantitatively estimated by dividing the p25 level with the m-calpain level. We found a significant decrease in the calpain activity in CS Tg mice (Figure 7E).

Next we analyzed NMJ development of control or agrin-deficient mice with calpastatin transgene. In agreement with the observation that injection with calpeptin caused an increase in the number of clusters in wild-type mice, AChR clustering was also increased in CS Tg mice compared with control littermates (Figures 7F and 7H and Table 1). Importantly, numerous AChR clusters remained in *AGD*$^{-/-}$*;CS Tg* mice (Figure 7I), compared to control *AGD*$^{-/-}$ (Figure 7G). Quantitatively, both number and size of AChR clusters were significantly higher in *AGD*$^{-/-}$*;CS Tg* mice when compared with AGD mutants (Figures 7J and 7K and Table 1). This difference was remarkable not only at various embryonic stages, i.e., E16.5 and E17.5 (Figures 7J and 7K; Figure S6), but also in different parts of diaphragms, ranging from the most ventral to most dorsal regions (for the analysis of ventral regions, see Figures 7F–7K and Table 1; for the analysis of dorsal regions, see Figure S7). As shown in Figures 7F–7K and Figure S6, fewer AChR hotspots remained in *AGD*$^{-/-}$ mice at E17.5 compared with E16.5; overexpression of calpastatin markedly increased the AChR clusters at E17.5 as well as E16.5. However, the rescue effect at E17.5 appeared to be not more profound than that at E16.5 (see Figures 7J and 7K, compare the fold increase of cluster numbers and area in *AGD*$^{-/-}$*;CS Tg* mice to that of *AGD*$^{-/-}$ mice at E16.5 and E17.5, for numbers: 4.5- ± 0.6-fold at E16.5 versus 4.1- ± 1.1-fold at E17.5, for area: 1.9- ± 0.2-fold at E16.5 versus 2.0- ± 0.4-fold at E17.5), although the expression of calpastatin in CS Tg mice gradually increased during development (Figure 7B). It is possible that there are other dispersing factors that act to disassemble AChR clusters independent of calpain. Unlike AGD/Cdk5 or AGD/ChAT double mutants, where presynaptic differentiation was apparent compared to AGD single mutant mice (Lin et al., 2005), most AChR clusters in *AGD*$^{-/-}$*;CS Tg* mice were not apposed by presynaptic nerve terminals (see inset in Figure 7I, and Figure S6, arrows). Thus calpain inhibition in the skeletal muscle increased AChR clusters in agrin mutants, but had no effect on presynaptic differentiation.

DISCUSSION

The present study uncovers a role of calpain, a family of calcium-dependent protease, in regulating postsynaptic differentiation at the NMJ. Cholinergic activation stimulates calpain, whose inhibition stabilizes AChR clusters in cultured muscle cells and in agrin mutant mice. Interestingly, rapsyn interacts with calpain and inhibits calpain activity. Blockade of the rapsyn-calpain interaction enhances CCh-induced AChR cluster dispersal. Furthermore, agrin increases rapsyn interaction with calpain and inhibits CCh-induced calpain activation. One substrate of calpain may be p35, whose cleavage by calpain generates p25, a potent activator of Cdk5 (Ahlijanian et al., 2000; Patrick et al., 1999; Patzke et al., 2003). Cdk5 inhibition counteracts the effects of calpain on AChR cluster dispersal. Together, these results support a model depicted in Figure 8 supporting the following hypothesis: AChR clusters are dispersed in muscle cells by electric activity stimulated by cholinergic inputs; agrin stimulates the interaction of rapsyn, which is spatially localized in synapses, with calpain to inhibit its enzyme activity to allow for formation of new AChR clusters and/or maintenance of existing AChR clusters.

Activation of AChRs leads to calcium influx via the ligand-gated ion channel (i.e., AChRs) or indirectly via sodium influx-stimulated calcium from intracellular stores (Allard et al., 1996; Cherednichenko et al., 2004). However, the role of calcium in AChR clustering is complex (Bloch, 1983; Megeath and Fallon, 1998; Tseng et al., 2003). How calcium acts to alter the stability of AChR clusters is yet to be investigated. Calpain is a family of proteases that are activated by calcium. Our findings in this paper suggest a role of calpain in regulating AChR clusters.

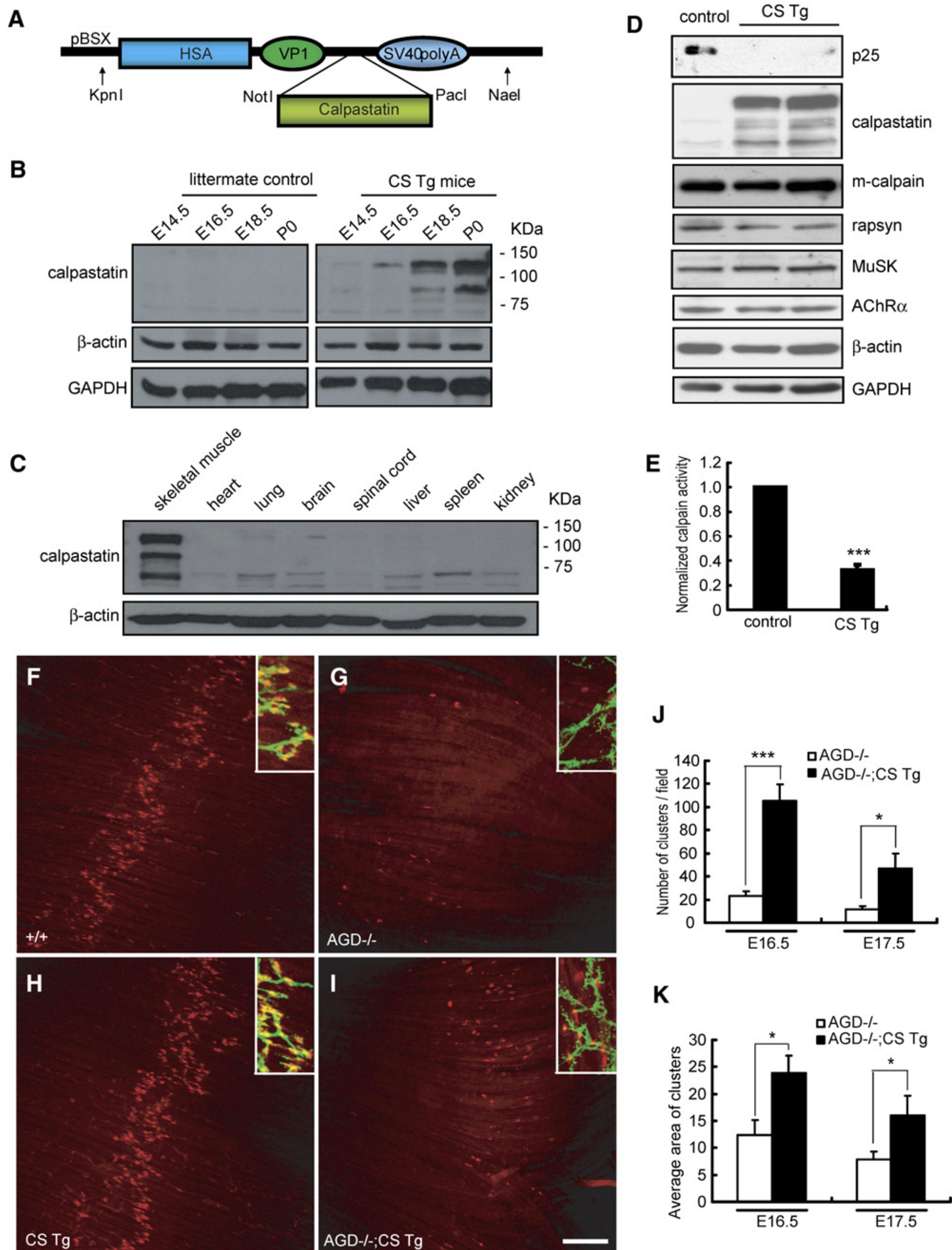

Figure 7. The Loss of AChR Clusters in Agrin Mutant Mice Is Prevented by Overexpressing Calpastatin

(A) Construct for the generation of calpastatin transgenic mice. HSA, human skeletal actin promoter.

(B) Developmental expression of calpastatin in the skeletal muscle of transgenic mice. Homogenates of the skeletal muscle from calpastatin transgene (CS Tg) or littermate wild-type control mice at different developmental stages, embryonic day 14.5 (E14.5) to postnatal day 0 (P0), were probed for expression of calpastatin or loading controls.

(C) Muscle-specific expression of calpastatin transgene. Same amounts of proteins from various tissues were probed for the level of calpastatin, using β-actin as control.

(D) Decreased p25 levels in CS Tg mice. Same amounts of proteins isolated from skeletal muscles of CS Tg or control mice at E18.5 were probed for the level of p25 and other indicated proteins.

(E) Decreased calpain activity in CS Tg mice. The level of p25 normalized with m-calpain reflects relative activity of calpain from mice skeletal muscles. The calpain activity of control mice was taken as 1.0. Data are shown as mean ± SEM (n = 4 for control and n = 7 for CS Tg; ***$p < 0.001$; Student's t test).

(F–I) Representative images of AChR clusters and nerve terminals from the most ventral sides of mice diaphragm muscles. E16.5 diaphragm muscles from control (+/+) (F), $AGD^{-/-}$ (G), CS Tg (H), and $AGD^{-/-}$;*CS Tg* (I) mice were double stained with R-BTX (red) and anti-synaptophysin antibody (green). Scale bar, 100 μm.

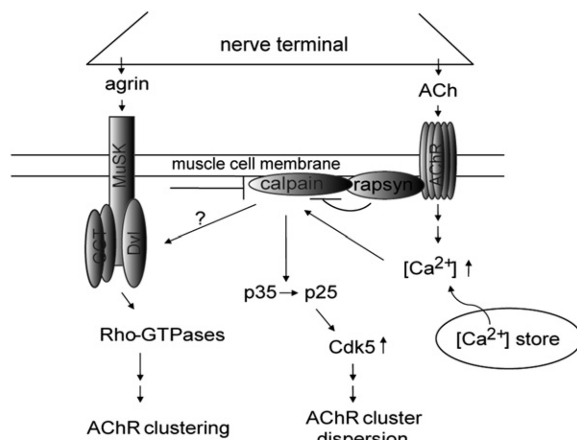

Figure 8. The Working Hypothesis
ACh activates Cdk5 in a manner dependent on calpain. Activated Cdk5 disperse AChR clusters. Agrin increases the interaction of rapsyn with calpain and thus inhibits calpain activity to stabilize AChR clusters at synapses.

By using a specific substrate or probing the levels of p25 to monitor calpain activity, we show that CCh causes calpain activation (Figure 1). Furthermore, inhibition of calpain stabilizes AChR clusters in vitro (Kim and Nelson, 2000) (Figure 2) and in vivo (Figures 6 and 7), whereas overexpression of calpain promotes cluster dispersal (Figure 5). These results indicate a negative role of calpain in AChR clustering. One possible target of calpain is Cdk5, which is activated by CCh in cultured muscle cells and whose ablation leads to enlarged AChR clusters (Fu et al., 2005; Lin et al., 2005). This notion is supported by the following observations. First, CCh-induced Cdk5 activation was dependent on calpain. Second, CCh treatment caused the accumulation of p25, a potent activator of Cdk5. Third, overexpression of calpain promoted CCh-induced AChR cluster dispersion, which was ameliorated by inhibition of Cdk5. In addition to p25, calpain may also have other substrates. Several molecules have been identified to function downstream of agrin/MuSK signaling to induce AChR clustering (Luo et al., 2002, 2003; Okada et al., 2006; Wang et al., 2003; Weston et al., 2000). It is possible that calpain may also cleave other substrates to destabilize AChR clusters. We note that Cdk5 activation happens shortly after "cholinergic" stimulation (Lin et al., 2005) (data not shown), whereas the production of p25 happens slowly (Figure 1). This suggests that other events, such as phosphorylation modifications (Hallows et al., 2003; Sharma et al., 1999), may also contribute to Cdk5 activation.

Intracellular calcium elevation, elicited by synaptic activity, stimulates calpain, which has been implicated in regulating the integrity and localization of synaptic proteins, and synaptic plasticity (Lee et al., 2000; Lynch and Seubert, 1989; Patrick et al., 1999; Siman and Noszek, 1988). Calpain activity is regulated by multiple mechanisms including phosphorylation, autolysis, and interaction with phospholipids (Carafoli and Molinari, 1998; Glading et al., 2002). In addition, calpain activity is also regulated by interacting proteins, mainly calpastatin (Benetti et al., 2001; Carafoli and Molinari, 1998; Glading et al., 2002). Here we identified rapsyn as a regulator for calpain. Rapsyn is a peripheral membrane protein that is essential for AChR clustering and neuromuscular junction formation (Gautam et al., 1995; Lin et al., 2001; Phillips et al., 1991a, 1991b; Qu et al., 1996). This protein is thought to function downstream of the agrin/MuSK signaling, leading to AChR clustering (Apel et al., 1997). The present study reveals an unexpected mechanism by which rapsyn regulates AChR clusters. Rapsyn interacts with calpain and inhibits calpain activity in vitro and in vivo. The region in calpain for rapsyn interaction is domain III, a region that is known to regulate calpain activity (Hosfield et al., 2001; Rizo and Sudhof, 1998; Tompa et al., 2001). Disruption of the rapsyn-calpain interaction activates calpain and promotes AChR cluster dispersal. Remarkably, p25 levels, which reflect calpain activity, at the synaptic region are lower than that of nonsynaptic region (Figure 4I), supporting the conclusion that calpain is spatially inhibited at the NMJ, most probably by agrin and rapsyn. In agreement with this notion, inhibition of calpain partially prevented the loss of AChR clusters in agrin mutant mice (Figures 6 and 7). Recently it has been shown that inhibition or ablation of Cdk5 or ablation of ChAT had a similar rescue effect (Lin et al., 2005; Misgeld et al., 2005). In addition to the rescue phenotypes, AGD/ChAT double mutants had increased presynaptic differentiation, and all AChR clusters were opposed by synaptic nerve terminals in AGD/ChAT or *AGD/Cdk5*$^{-/-}$ mice (Lin et al., 2005; Misgeld et al., 2005). However, this phenotype was not observed in *AGD*$^{-/-}$*;CS Tg* mice, probably due to the muscle-specific expression of calpastatin. It is possible that the presynaptic phenotypes of ChAT or Cdk5 mutant mice were caused by the ablation of these genes in motor neurons.

Taken together, the inhibitory effect of calpain, activated by cholinergic activation, could be reversed by agrin, which inhibits calpain by increasing its interaction with rapsyn. This study provides insight into the role of rapsyn in synapse formation and reveals a mechanism of the agrin pathway. It will shed light on the regulatory mechanism for calpain in CNS synaptogenesis.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies

Double-stranded oligonucleotides targeting against rapsyn or m-calpain were synthesized by Shanghai GenePharma Co.LTD. The siRNA sequence was designed against a region starting from 233 or 270 nt of mouse rapsyn cDNA. The sequence of #233 is 5′-ATGCTGACTTCC

(J and K) Quantitation of AChR clusters. The average area of individual clusters and number of clusters in the same field of the most ventral sides of left diaphragm muscles from E16.5 or E17.5 mice are shown in (J) and (K), respectively. Data are presented as mean ± SEM (n = 3; *p < 0.05, ***p < 0.001, Student's t test). The following numbers of clusters were analyzed for average area: 40 for *AGD*$^{-/-}$ and 50 for *AGD*$^{-/-}$*;CS Tg* (E17.5).

TGCTCGAAA-3′ (forward) and 5′-TTTCGAGCAGGAAGTCAGCAT-3′ (reverse). The sequence of #270 is 5′-GCGCAGCAATGAGAAGCT ATG-3′ (forward) and 5′-CATAGCTTCTCATTGCTGCGC-3′ (reverse). The sequence of m-calpain siRNA is 5′-GGATGGCGATTTCTGC ATC-3′ (forward) and 5′-GATGCAGAAATCGCCATCC-3′ (reverse). The control (nonsilencing) siRNA was purchased from QIAGEN-Xeragon, and it does not target any known mammalian gene (the targeted sequence is 5′-AATTCTCCGAACGTGTCACGT-3′). Rabbit antisera were generated against the GST-rapsyn C terminus (aa 354–412) or m-calpain domain IV (aa 510–700). Monoclonal anti-rapsyn was generously provided by Stan Frohner. Other antibodies were from Chemicon (β-actin), Santa Cruz (CDK5, p35, GFP), BD/Clontech (β-catenin, Myc), and Sigma (HA, MHC). The calpain inhibitor calpeptin was from Calbiochem, R-BTX was from Molecule Probes, agrin was from R&D, glutathione-sepharose was from Amersham Pharmacia, and protein A or protein G agarose was from Roche.

Constructs

Rapsyn was subcloned into pEGFP-N1 in frame with EGFP. Different domains of rapsyn were inserted into pKH3 with 3xHA epitopes at the N-terminus: TPRs (aa 1–320), coiled-coil (aa 290–360), and RING-H2 (aa 354–412). m-Calpain mutants (domain III, aa 350–515; domain IV, aa 510–700) were subcloned into pCS2 + MT in frame with 6xMyc epitopes at the N terminus, or into pGEX-2T to produce GST-fusion proteins. HA-calpain was described previously (Benetti et al., 2001). p35 and Cdk5 cDNA were obtained with reverse transcription-PCR using rat brain total RNA as template and subcloned into pCS2 + MT and pKH3, respectively.

Cell Culture, Transfection, and Biochemical Characterization

HEK293 cells and C2C12 muscle cells were cultured as described (Luo et al., 2002). Cells were transfected by the standard calcium phosphate method or with FuGENE6 (Roche) following the manufacturer's instruction. Cell lysates were prepared in the modified RIPA buffer including protease inhibitors (Luo et al., 2003). For pull-down assay, cell lysates (0.5–1.0 mg protein/ml) were incubated with 5 μg GST-fusion protein immobilized on sepharose beads for 4 hr at 4°C. Bound proteins were resolved by SDS-PAGE. For immunoprecipitation, lysates (0.3–0.5 mg of protein in a final volume of 1 ml) were incubated with respective antibodies (2.5–3 μg) and subsequently with 40 μl protein A or protein G agarose beads. Immunoprecipitated proteins were resolved by SDS-PAGE and subsequent immunoblotting. All experiments were repeated at least three times.

Calpain Activity Assay

Calpain activity was measured using Suc-Leu-Tyr-7-amino-4-methylcoumarin (Suc-Leu-Tyr-AMC) as the substrate (Chen et al., 2001). C2C12 myotubes were treated with DMSO or 0.1 mM CCh, without or with 20 μM calpeptin for indicated time. Cell lysates (100 μg of protein) were added to 100 μl reaction buffer (145 mM NaCl, 100 mM Tris-HCl [pH 7.3]) containing 80 μM Suc-Leu-Tyr-AMC and reacted for 30 min at room temperature. The amount of AMC release resulting from calpain cleavage was determined by measuring the value of fluorescence (excitation at 360 nm and emission at 460 nm) using a GENios fluorescence system. All measurements were performed in triplicate.

Generation of Transgenic Mice Overexpressing Calpastatin in the Skeletal Muscle

The human calpastatin cDNA was inserted between the NotI and PacI site of plasmid pBSX-HSAvpA downstream of the HSA promoter/VP1 intron. The HSA-calpastatin fragment was excised using KpnI and NaeI, gel purified, and microinjected into C57BL/6JxSJL fertilized eggs, which were then reimplanted into pseudopregnant recipient mice. Primer 1 covers the initial methionine: 5′-ATAAGAATGCGG CCGCATGAATCCCACAGAAACCAAGGC-3′; primer 2 covers the stop codon: 5′-CCTTAATTAACTAGTCATCTTTTGGCTTGGAAGTTT-3′. Calpastatin transgenic mice (CS Tg) were genotyped by PCR analysis of mice tail DNA. The transgenic mice were fertile and did not show gross anatomic defects within 6 months of observation after birth. Morphologically, muscle fibers revealed grossly normal architecture similar to that of control mice (data not shown). To determine transgene expression at the protein level, skeletal muscle and other tissues were sliced and sonicated in extraction buffer (modified RIPA containing 1 mM DTT and protease inhibitors) and then subjected to ultracentrifugation (150,000 g). Aliquots of supernatants were subjected to Western blotting using anti-calpastatin antibody. CS Tg mice were crossed with agrin mutant mice ($AGD^{-/-}$) to generate the mice with agrin deficiency and calpastatin overexpression.

Immunohistochemistry

Frozen sections of muscle or cells in culture were stained with indicated antibodies, washed, and incubated with FITC-conjugated goat anti-rabbit or anti-mouse antibody, and together with tetramethylrhodamine-α-bangarotoxin (R-BTX) to label the AChR (Luo et al., 2003). Images were collected with a Zeiss confocal microscope using a 40× objective. The total length of all AChR clusters or number of clusters bigger than 5 μm in 100 μm myotubes were quantitatively analyzed. For diaphragm samples, staining procedure was followed as shown in the previous study (Luo et al., 2003). Synaptic nerve terminals were marked by staining with anti-synaptophysin antibody. AChR clusters labeled by R-BTX at the most ventral left side of the diaphragm that has little anatomical variation across mice were quantitatively analyzed. In some cases, AChR clusters at dorsal regions were also analyzed.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/55/2/247/DC1/.

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