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Nestin negatively regulates postsynaptic differentiation of the neuromuscular synapse

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Abstract

Positive and negative regulation of neurotransmitter receptor aggregation on the postsynaptic membrane is a critical event during synapse formation. Acetylcholine (ACh) and agrin are two opposing signals that regulate ACh receptor (AChR) clustering during neuromuscular junction (NMJ) development. ACh induces dispersion of AChR clusters that are not stabilized by agrin via a cyclin-dependent kinase 5 (Cdk5)-mediated mechanism, but regulation of Cdk5 activation is poorly understood. Here we show that the intermediate filament protein nestin physically interacts with Cdk5 and is required for ACh-induced association of p35, the co-activator of Cdk5, with the muscle membrane. Blockade of nestin-dependent signaling inhibits ACh-induced Cdk5 activation and the dispersion of AChR clusters in cultured myotubes. Similar to the effects of Cdk5 gene inactivation, knockdown of nestin in agrin-deficient embryos significantly restores AChR clusters. These results suggest that nestin is required for ACh-induced, Cdk5-dependent dispersion of AChR clusters during NMJ development.

Introduction

Synapses are the cellular basis of neural connectivity and therefore are fundamental to nervous system function. A cardinal feature of the chemical synapse is the presence of a postsynaptic apparatus containing high concentrations of neurotransmitter receptors closely associated with numerous extracellular, transmembrane, and cytoplasmic scaffolding and signaling components. One of the most prominently studied synapses is the neuromuscular junction (NMJ), a tripartite apparatus comprised of precisely aligned presynaptic motor

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Author contributions

J.Y. designed and performed majority of experiments, conducted data analysis, and wrote the manuscript; B.D. conducted the transgenic mouse experiment; F.d.W. conducted the transgenic mouse experiment and assisted in data interpretation; T.W.G. assisted in data interpretation and wrote the manuscript; J.E.E. provided constructs and assisted in data interpretation; K-F.L. supervised the project, designed experiments, and wrote the manuscript.

Competing financial interests

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nerve terminals, postsynaptic skeletal muscle-derived acetylcholine receptor (AChR) clusters, and perisynaptic Schwann cells1, 2. Previous studies have demonstrated that postsynaptic differentiation at the NMJ is initiated autonomously (pre-patterned) within the muscle, while the nerve and/or Schwann cells provide both positive and negative signals that sculpt presynaptic and postsynaptic patterning and differentiation3–6. Positive signals promote the clustering of postsynaptic AChR clusters and position these clusters in close proximity to specialized, presynaptic nerve terminals, whereas negative signals act to disperse AChR clusters not apposed to presynaptic nerve terminals (a.k.a. aneural clusters). Recent data have shown that agrin and acetylcholine (ACh) are key positive and negative signals, respectively, during the initial stages of NMJ formation. ACh disperses AChR clusters that are not stabilized by agrin in part by modulating cyclin-dependent kinase 5 (Cdk5)-mediated cascades within the muscle5, 7. Cdk5 activation requires its co-activator p35, which is targeted to the membrane8. Although the activation of Cdk5 in muscle by nerve-derived ACh is required for Ach-induced dispersion of AChR clusters in agrin mutants5, the molecular mechanism by which ACh regulates muscle Cdk5 activity is unknown.

Several lines of evidence led us to investigate the role of the intermediate filament protein nestin in NMJ development. First, although nestin is expressed in mitotically active cells in the mammalian nervous system and is widely used as a progenitor/stem cell marker for neurons and glia9, 10, nestin mRNA is highly enriched in subsynaptic nuclei at the adult NMJs11, 12, and nestin protein is co-localized with AChR clusters13, 14. Second, the unique structure of nestin suggests that it may link the cytoskeleton to postsynaptic AChR clusters. Although both nestin and other intermediate filament members possess an N-terminal α–helical rod domain and a C-terminal non-α-helical tail domain, which permit the formation of intermediate filament heteropolymers with vimentin and other intermediate filaments, only nestin contains a long C-terminal tail domain with sequences that can bind microtubules or microfilaments15. Regulation of microtubules and microfilaments plays an important role in AChR cluster formation and stability16–22. Finally, in addition to their structural role23, the intermediate filament serves as scaffolds for numerous signaling molecules and frequently are themselves the targets of kinases or phosphatases. Consistent with an active role for intermediate filament in cell signaling, phosphorylation and dephosphorylation of intermediate filament regulates their subcellular localization and association with signaling molecules24–27. Interestingly, nestin is phosphorylated by Cdk5 at multiple sites, including Thr316 and Thr149528, but it is not clear if nestin modulates kinase activation or if phosphorylation of nestin plays a role in development of the nervous system, including the NMJ.

Here we provide *in vivo* and *in vitro* evidence that nestin is required for Cdk5-dependent, ACh-induced dispersion of AChR clusters on muscle. Nestin increases muscle Cdk5 activity by elevating the levels of the Cdk5 co-activator p35 in the muscle membrane in response to ACh stimulation. Both knockdown of nestin and over-expression of a nestin phosphorylation mutant at Thr316 reduce ACh-induced Cdk5 activation and dispersion of AChR clusters. Finally, reducing nestin expression in agrin null mutant mice prevents the dispersal of numerous AChR clusters that occurs in these mice. Thus, our studies suggest

that nestin promotes dispersion of AChR clusters that are not stabilized by agrin during NMJ development by regulating Cdk5 activation.

Results

Membrane-associated Cdk5 activation requires nestin

Nestin is mainly expressed in muscle and co-localized with AChR clusters at the adult NMJ13, 14, but it is not clear whether nestin and AChR are also co-localized at the embryonic NMJ. To investigate this possibility, embryonic day 18.5 (E18.5) hind limb muscle sections were stained with an anti-nestin antibody and Cy3-conjugated α-BTX. As shown in Figure 1a, nestin was co-localized with AChR clusters at the NMJ in E18.5 wild type muscle.

As Cdk5 is activated by ACh stimulation in myotubes5, we sought to determine whether Cdk5 phosphorylates nestin in cultured C2C12 myotubes upon ACh stimulation. When stimulated by the ACh agonist, carbachol (CCh), nestin was phosphorylated as revealed by an anti-phospho-nestin antibody (Fig. 1b). This effect was inhibited by the Cdk5 inhibitor Roscovitine. These results suggest that ACh induces the phosphorylation of nestin by Cdk5 in myotubes.

Recent data suggest that intermediate filaments may function as a scaffold or provide a platform for targeting and activation of signaling molecules in different cellular compartments29–32. A couple of lines of evidence led us to hypothesize that nestin regulates Cdk5 activation at the postsynaptic membrane of the NMJ. First, the direct action of ACh is restricted to the muscle membrane. Second, p35, which contains an N-terminal myristoylation signal motif, segregates with the plasma membrane fraction33. Finally, recruitment of p35 to the membrane enhances its downstream signaling34. To test this hypothesis, we performed nestin RNAi (RNA-interference) experiments in order to examine if nestin affects the levels of Cdk5 and p35 associated with the membrane fraction of cultured C2C12 myotubes (Fig. 2). As shown in Figure 2a, lentiviruses expressing short hairpin RNAi sequences (shRNA) against nestin markedly reduce nestin protein. As controls, we also generated lentiviruses expressing shRNA against scrambled shRNA and control shRNA against caspase-3 (Fig. 2a). Upon CCh stimulation, Cdk5 protein levels did not change in either the total lysate or membrane fraction (Fig. 2b). However, CCh induced an increase in both total and membrane-associated Cdk5 activity. Treatment with CCh also resulted in a clear increase in membrane-associated p35 protein. Next, we sought to determine whether nestin is required for the CCh-induced elevation of p35 levels in the muscle membrane. When infected with nestin RNAi-expressing but not control viruses, C2C12 myotubes displayed a significant reduction in the level of nestin expression, but not expression of erbB2 or the AChR α1 subunit (Fig. 2a and Supplementary Fig. 1). The CCh-induced increase of membrane-associated p35 and Cdk5 activity was largely abolished in nestin-RNAi-expressing myotubes (Fig. 2b, c), suggesting that nestin is required for CCh-induced association of p35 with the muscle membrane and, hence, Cdk5 activation. The elevated association may result from increased recruitment of p35 to the membrane and/or maintenance of the association of p35 with the plasma membrane, thereby leading to increased formation of the enzymatically active Cdk5/p35 complex.

To test whether the activation of membrane-associated Cdk5 requires the recruitment of p35 to the cell membrane, we used a mutant form of p35 (p35 G2A) that cannot be myristolated and thus recruited to the membrane33, 35. When p35 G2A was overexpressed in myotubes expressing nestin RNAi, we failed to observe either recruitment of p35 to the membrane fraction or activation of membrane-associated Cdk5 (Fig. 2b, c). However, total Cdk5 activity was significantly upregulated in nestin RNAi-expressing, p35 G2A-overexpressing myotubes (Fig. 2b, c).

Nestin-dependent AChR cluster dispersion induced by ACh

Our data show that nestin is required for the increase of Cdk5 activity induced by ACh. Because Cdk5 activity is required for the dispersal of AChR clusters caused by ACh, we tested whether the clustering of postsynaptic AChRs was affected by silencing nestin expression in C2C12 myotubes. We measured both the ability of agrin to induce the aggregation of AChR and the stability of these agrin-induced AChR clusters in response to ACh stimulation. There was no difference between nestin-RNAi-infected myotubes and control scramble-RNAi-infected myotubes in agrin-induced AChR clustering (Fig. 3a, b). In contrast to the dispersal of AChR clusters caused by CCh in scrambled-RNAi-infected myotubes (Fig. 3a, b), nestin-RNAi-infected myotubes showed resistance to the AChR-dispersing effect of CCh (Fig. 3a, b). The quantification of the number of AChR clusters under these conditions reveals that this blockade effect is significant but partial (Fig. 3b). Thus, the silencing of nestin expression does not affect the aggregation of AChRs but blocks the ACh-induced dispersal of AChR clusters in myotubes.

We next wanted to test if experimentally elevating Cdk5 activity by introducing an exogenous construct into nestin RNAi-expressing myotubes would restore CCh-induced dispersion of AChR clusters. In order to test this possibility, we took advantage of the p35 G2A mutant, which robustly upregulates Cdk5 activity even in the absence of nestin (Fig. 2b, c). Although nestin RNAi prevented the dispersion of AChR clusters by CCh, as shown above, co-expression of p35 G2A and nestin RNAi re-instated the dispersion and thus over-rode the dispersal-blocking effects of nestin RNAi (Supplementary Fig. 2). Because this increase of Cdk5 activity is sufficient to disperse AChR clusters in the absence of nestin, these results support the idea that nestin mediates the CCh-induced dispersion of AChR clusters through a Cdk5-dependent pathway.

Nestin T316A mutant blocks AChR cluster dispersion

Phosphorylation regulates the structure and function of intermediate filaments36–38. Phosphorylation of substrates may in turn regulate kinase activity as shown by the unfolded protein response kinase PERK39. We decided to further investigate whether phosphorylation of nestin regulates ACh-induced Cdk5 activity and AChR dispersion. Although multiple amino acids in nestin are phosphorylated by Cdk5, Thr-316 is the major Cdk5 phosphorylation site that has been previously characterized.

We generated wild-type (WT) and Thr316Ala (T316A) point mutant nestin:GFP fusion constructs, as GFP fusion does not interfere with the function of nestin31. To test whether the phospho-nestin mutant T316A affects the phosphorylation of endogenous nestin by

Cdk5, we overexpressed these nestin-GFP constructs with or without the T316A mutation in cultured myotubes. Endogenous nestin was phosphorylated upon CCh stimulation when nestin-WT-GFP constructs were transfected (Supplementary Fig. 3, right lanes), an effect that was blocked by co-administration with the Cdk5 inhibitor Roscovitine. However, when nestin-T316A-GFP was introduced, the phosphorylation of endogenous nestin was blocked (Supplementary Fig. 3, left lanes).

Upon confirming that phospho-mutant nestin can block the phosphorylation of endogenous nestin, we used these constructs to test whether blockade of nestin phosphorylation would affect AChR clustering. C2C12 myotubes transfected with nestin-WT-GFP displayed clustering of AChRs in response to agrin treatment and exhibited the dispersal of these AChRs upon treatment with ACh (Fig. 4a, right panels). In contrast, while myotubes transfected with nestin-T316A-GFP exhibited normal agrin-induced AChR clustering, they failed to display CCh-induced dispersal of AChR clusters (Fig. 4a, left panels). Quantitative analysis revealed that the blockade of ACh-induced dispersion of AChR clusters in nestin-T316A-GFP-transfected myotubes was significant but partial (Fig. 4b). Together with the finding that nestin is required for the activation of Cdk5 (Fig. 2), these data raise the possibility that the phospho-nestin mutant may block Cdk5 activation.

To investigate how the manipulation of nestin causes it to interact with Cdk5 and interfere with Cdk5 activity, we tested whether phospho-nestin mutant T316A and Cdk5 interact physically in cultured myotubes. We were able to pull down Cdk5 with an anti-GFP antibody when Cdk5 and p35 were co-expressed with nestin-WT-GFP (Fig. 5a, lanes 3 and 4, top blot). However, when co-expressed with nestin-T316A-GFP, significantly more Cdk5 was precipitated with anti-GFP antibodies (Fig. 5a, lane 4 vs. 3, middle blot). These data indicate that impaired phosphorylation of nestin by Cdk5 leads to the enhanced association of nestin-T316A with Cdk5/p35. Additionally, we observed that the overexpression of nestin-T316A-GFP blocked Cdk5 activity (Fig. 5a, lanes 4 vs. 3, bottom blot), suggesting that the enhanced association of nestin-T316A-GFP with Cdk5/p35 impairs the activity of Cdk5. To corroborate this idea, 293T cells were transfected with Cdk5 and p35. Total lysate was incubated with GST-WT-nestin or GST-T316A-nestin and then pulled down using glutathione-conjugated agarose beads. The supernatant was then subjected to a routine Cdk5 activity assay and the glutathione-conjugated agarose beads were treated with sample buffer and subjected to Western blot analysis. As shown in Figure 5b (top panel), Cdk5 activation was markedly reduced when the lysate was pre-incubated with GST-T316A-nestin, and the amounts of Cdk5 and p35 pulled down were significantly increased by GST-T316A nestin (Fig. 5b, second and third blots). These results support the idea that the phospho-nestin T316A functions as a dominant negative mutant with respect to Cdk5 activation.

Reducing nestin decreases AChR dispersion in agrin mutants

To investigate the role of nestin *in vivo*, we used a lentivirus-based nestin RNAi expressing vector to generate transgenic mice that express RNAi against nestin (Supplementary Fig. 4). Several lines of nestin knockdown mice were established and characterized. The expression level of nestin in muscles from nestin RNAi-expressing mice was determined. Nestin expression in both the diaphragm and leg muscle at E18.5 was significantly lower than that

in wild type mice (Fig. 6a), indicating effective knockdown of nestin expression in muscle during embryonic development. Similarly, whereas wild type mice exhibited postsynaptic localization of nestin immunoreactivity at the NMJ, as determined by AChR cluster co-localization, this immunoreactivity was abolished in muscles from nestin-RNAi expressing mice (Fig. 6b). Similar results were observed in three independent lines of transgenic mice. No gross abnormality was observed in nestin knockdown mice, consistent with nestin null mutants generated via the gene-targeting technology by Nagy and colleagues (Mohseni *et al.* submitted).

To investigate whether nestin also affects the dispersion of AChR clusters *in vivo*, we generated nestin knockdown mice. We found that the number of AChR clusters in E18.5 nestin knockdown mice is slightly higher than that in wild type controls (Table 1). No aneural AChR clusters were observed in E18.5 nestin knockdown mutants (Fig. 7, top right). Next, we determined whether nestin is required for the dispersion of AChR clusters in agrin mutants. In contrast to agrin single mutants, E18.5 agrin mutants that also express nestin RNAi display numerous AChR clusters (Fig. 7, bottom right). The number (Table 1) and area (Supplementary Table 1) of AChR clusters of nestin RNAi/agrin mutants were significantly higher than those of agrin single mutants (Fig. 7, bottom left), but lower than those of wild type controls (Fig. 7, top left). When E15.5 and E16.5 embryos were examined, similar results were obtained (Table 1 and Supplementary Table 1). Consistent with our results, Mohseni *et al.* (submitted) show that AChR clustering is significantly maintained in nestin/agrin double null mutants.

We further determined whether the presynaptic differentiation of motor axon terminals, which is also completely absent in agrin mutants, was restored in nestin RNAi/agrin mutants. In contrast to the rescue of presynaptic differentiation observed in Cdk5/agrin double mutants, presynaptic differentiation was not observed in nestin RNAi/agrin mutants (Fig. 7, bottom right, inset). The results raise the possibility that a nestin-independent mechanism regulates presynaptic differentiation. Together, these results indicate that blocking nestin expression diminishes the ACh-induced dispersion of AChR clusters in agrin mutants and identify nestin as a critical component of this Cdk5-mediated negative regulatory pathway of AChR clustering.

Discussion

The ubiquitous expression of nestin throughout the nervous system suggests that it may play an important physiological role in this system, yet to date such a role has remained elusive. Using biochemical and genetic approaches, our current studies demonstrate that nestin is critically important for dispersing AChR clusters via increasing membrane-association of the Cdk5 co-activator p35, and, hence, Cdk5 activation during NMJ development.

Based on our results, we propose a working model on how the subcellular location of nestin and phosphorylation of nestin by Cdk5 contributes to a sustained activation of Cdk5 (Supplementary Fig. 5). Initially, there is a constant association between unphosphorylated nestin and Cdk5 at the postsynaptic membrane. However, this Cdk5 is largely inactive because there is very little p35 associated. Upon the simulation of ACh, cytoplasmic p35 is

recruited by unphosphorylated nestin to the synaptic membrane at the NMJ, forming an active tertiary nestin-Cdk5-p35 complex. The binding of p35 to Cdk5 activates Cdk5, which in turn phosphorylates nestin. Phosphorylated nestin becomes unstable, dissociates from the Cdk5/p35 complex and the intermediate filament network. The release and subsequent degradation of phospho-nestin terminates the association between active Cdk5 and p35, thereby increasing the free pool of Cdk5 and p35 for a sustained activation. Two lines of evidence support this idea. First, we observed that there was a stronger association between a phospho-mutant form of nestin and the Cdk5/p35 complex, suggesting that the release of nestin from Cdk5 requires the phosphorylation of nestin. This finding is consistent with an earlier report showing that the phosphorylation of Thr-316, which is adjacent to the α-helical rod domain of nestin, leads to the dissociation of nestin from the heterogeneous intermediate filament complex and the eventual disassembly of the nestin filament network28. Second, the enhanced association of an exogenous phospho-mutant nestin with Cdk5 was also correlated with impaired Cdk5 activity and decreased phosphorylation of endogenous nestin. A similar regulation between kinase and substrate has been reported for the unfolded protein response kinase PERK39.

It is worth noting that ACh may prolong Cdk5 activation by regulating the processing of p35 to p25, a constitutive activator of Cdk533. For example, Chen *et al.* showed that ACh induced p25 production by Ca^{++}-dependent calpain activation after a longer treatment of ACh agonist (> 6 hrs)40. However, we were not able to detect changes of p25 in our study. This discrepancy could be due to different culture systems or treatment regimes. Alternatively, given the longer time course of ACh-induced p25 generation in myotubes reported by Chen *at al.*, we cannot completely rule out the possibility that calpain-dependent p25 generation acts in parallel in a longer timeframe to nestin-dependent activation of Cdk5/p35.

Interestingly, in contrast to the results from E16.5 rat *ex vivo* sternomastoid muscle cultures by Bloch41, Lin *et al.* showed that ACh enhances stability of AChR clusters in E14.5 mouse diaphragm muscle preparation42. These results suggest a model wherein ACh is a stabilization enhancing factor for synaptic AChR clusters that are previously stabilized by agrin-dependent signaling. Our results, however, cannot distinguish between synaptic vs. non-synaptic clusters as any negative effects of nestin are likely antagonized by agrin at synaptic sites. Nevertheless, our results strongly support the interpretation that nestin is required for ACh-induced, Cdk5-dependent dispersion of AChR clusters that are not stabilized by agrin. It will be of interest in the future to elucidate mechanisms by which agrin-mediated signaling influences the opposing effects of ACh. Furthermore, although motor axons are the likely source of ACh, we cannot rule out that ACh may be also released locally by muscle as previous studies show that an ACh-like compound is synthesized and released by myotubes43.

Because ectopic clusters were not observed in nestin knockdown mutants and because Cdk5 deletion and nestin knockdown only partially restore AChR clusters in agrin mutants, it is likely that additional dispersing factors or signaling pathways play a role in dispersing AChR clusters. For example, phosphatase Shp-2 has been shown to negatively regulate AChR clustering in cultures44. Although no ectopic clusters were observed in Shp-2

mutants45, it will be of interest to determine whether the deletion of Shp-2 in agrin mutants also restores AChR clustering.

Additionally, whereas both Cdk5/agrin and ChAT/agrin mutants exhibit a clear rescue of presynaptic differentiation5, there is a lack of presynaptic rescue in nestin RNAi/agrin mutants. As ACh has recently been demonstrated to inhibit presynaptic differentiation through a non-postsynaptic mechanism46, it is possible that Cdk5 but not nestin regulates the release of ACh, thereby providing an explanation to the discrepancy. Alternatively, the effects of nestin may be restricted to postsynaptic differentiation, and other molecules regulating the effects of Cdk5 in the muscle may exert retrograde effects on presynaptic differentiation.

In summary, our data are among the first to characterize a requirement for nestin during neural development. These data support the significance of *in vitro* findings showing that nestin is a Cdk5 target and expand the role of nestin beyond the realm of cellular integrity and into that of cell signaling.

Methods

RNAi lentivirus construction

The lentivirus construct that overexpresses RNAi against nestin was generated as previously described47. The RNAi sequence against nestin was previously shown to effectively knock down nestin expression in neural stem cells ST15A31. This nestin knockdown construct was further cloned into the pDest-CMV-GFP by LR clonase reaction transfer (Invitrogen, CA). pDest-CMV-GFP is a lentiviral vector developed in Dr. Inder Verma's lab at the Salk Institute for introducing RNAi constructs into various mammalian cells47.

Generation of Nestin RNAi transgenic mice

We used the pDest-CMV-GFP lentiviral vector to generate mice overexpressing RNAi against nestin47, 48. Lentiviral particles were infected into 8-cell stage embryos, which were implanted into pseudo-pregnant females and allowed to develop full term. Several lines of mice survived until adulthood and exhibited no significant differences in weight and gross behavior as compared to wild type mice. The use of animals complies with the guidelines of the Animal Care and Use Committee of the Salk Institute.

DNA constructs and protein purification

Construction of nestin-WT-GFP was described previously31. A T316A point mutant of nestin was made by site-directed mutagenesis using Phusion® Site-Directed Mutagenesis Kit (New England Biolabs). GST-nestin constructs were generated using the GST Gene Fusion System into pGEX Vectors (GE Healthcare). GST-nestin proteins were purified using glutathione-conjugated agarose (Invitrogen).

Cell cultures and AChR cluster quantification

C2C12 myoblasts were cultured in DMEM with high glucose and 20% fetal bovine serum (FBS). When confluent, the culture medium was changed to differentiation medium, which

was DMEM with high glucose and 2% normal horse serum (NHS). Myotubes usually were formed after 48 hrs in differentiation medium. Myotubes were then subject to 1 ng/ml agrin treatment overnight, before further treatment. When the Cdk5 inhibitor Roscovitine was used, the culture was pretreated with 50 μm Roscovitine for 2 hrs before the drug treatment. The quantification of AChR clustering was performed as previously described5. Briefly, the number and the area of AChR clusters were measured in ImageJ. The length of the myotube was defined as the longest axis of the myotube in the particular field. The area of individual AChR cluster was measured as positive area above a set threshold of α-BTX signal in a particular field. Only the AChR clusters of 1 μm or longer at its longest axis were measured.

Cdk5 activity assay

A Cdk5 activity assay based on H1 Histone phosphorylation was carried out as previously described5. To test the effect of phospho-mutant nestin on Cdk5 assay in vitro, GST-nestin purified by glutathione agarose was pre-incubated with lysate from 293 cells transfected with Cdk5 and p35 for 1 hr at 4°C. At the end of the incubation, the mixes were centrifuged and the supernatant was used for the Cdk5 activity assay.

Immunocytochemistry

Diaphragm muscles were fixed in 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.3) overnight at 4°C, rinsed briefly with phosphate-buffered saline (PBS, pH 7.3), incubated in 0.1 M glycine in PBS for 1 h, rinsed briefly with PBS and then washed with 0.5% Triton X-100 in PBS. The muscles were blocked in dilution buffer (500 mM NaCl, 0.01 M phosphate buffer, 3% bovine serum albumin (BSA), 5% goat serum and 0.01% thimerosal) overnight at 4°C, and then incubated with primary rabbit antibodies against neurofilament-150 (Chemicon) or synaptophysin (DAKO) in dilution buffer overnight at 4°C. After being washed three times for 1 h each in 0.5% Triton X-100 in PBS, the muscles were incubated with fluorescein-conjugated goat anti-rabbit IgG (1:600; Cappel) and Cy3-α BTX (Molecular Probes) overnight at 4°C. The muscles were then washed three times for 1 hr each with 0.5% Triton X-100 in PBS and once with PBS and flat-mounted in Vectorshield solution (Vector). The quantification of AChR cluster number and cluster area was performed as previously described5.

Western blotting

Antibody against Phospho-Thr316 in nestin was generated as described previously 49. Anti-Cdk5 (C-8) and p35 (N-20) antibodies were from Santa Cruz, CA. Anti-V5 antibodies were from Invitrogen, CA. For co-immunopricipitation, total cell lysate was incubated with primary antibody on a rocking platform at 4°C for 2 hrs and then pulled down using protein A/G plus-agarose beads (Santa Cruz, CA). Washed agarose beads were boiled in the presence of loading dye and subjected to for SDS-PAGE and Western blotting analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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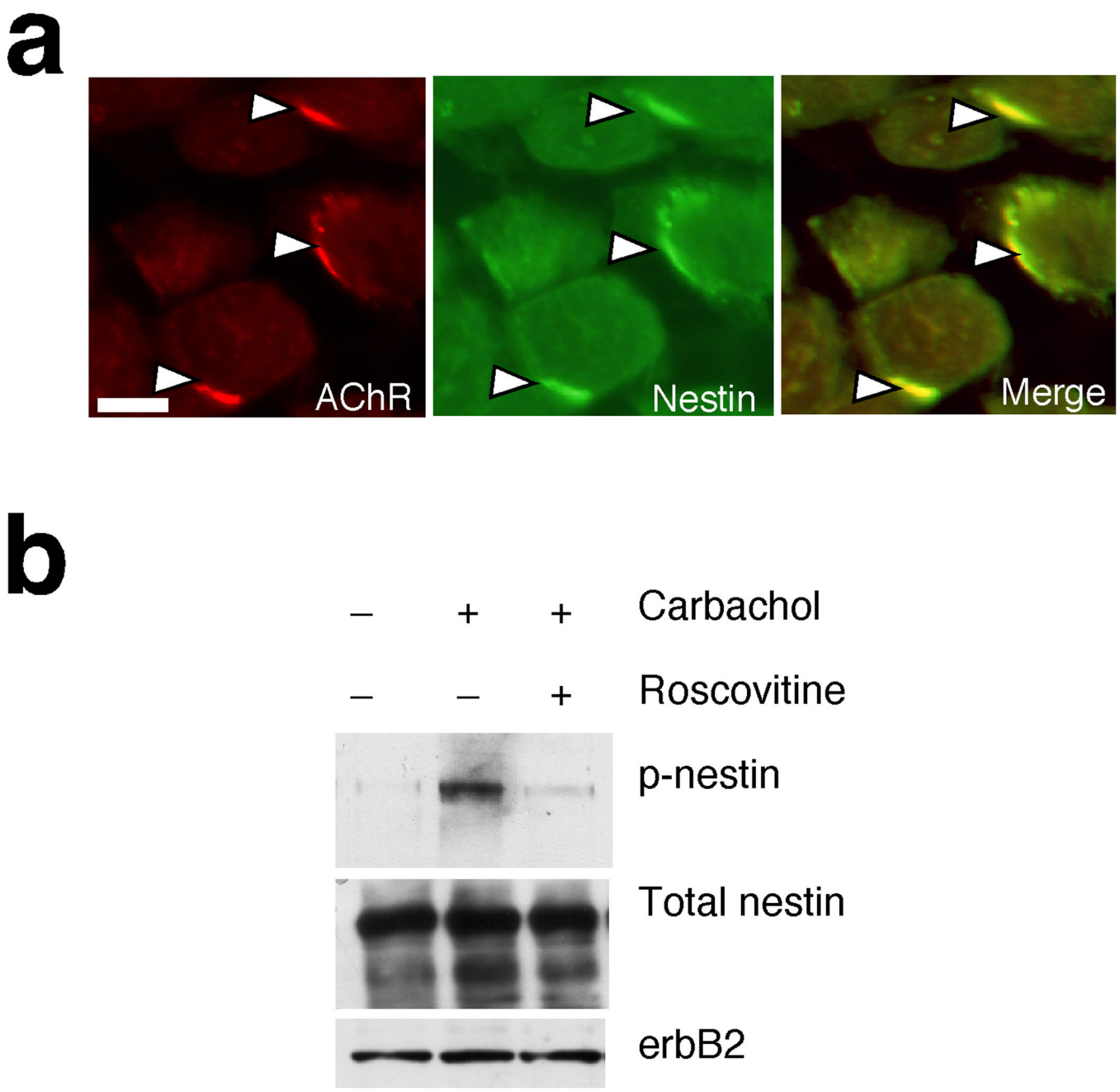

Figure 1. Colocalization of nestin and AChR clusters and phosphorylation of nestin upon acetylcholine stimulation

a. E18.5 leg muscle cross-sections were stained with anti-nestin antibody (green, middle and right panels) and Cy3-labeled α-bungarotoxin (α-BTX; red, left and right panels) for AChR clusters. Nestin was co-localized with AChR clusters (white arrowheads). Scale bar: 10 μm. **b.** C2C12 myotubes stimulated overnight with the cholinergic agonist carbachol (CCh) exhibited a robust phosphorylation of nestin on Thr-316 that was blocked by the Cdk5 inhibitor roscovitine. In contrast, total nestin and control erbB2 protein levels were not affected by these treatments.

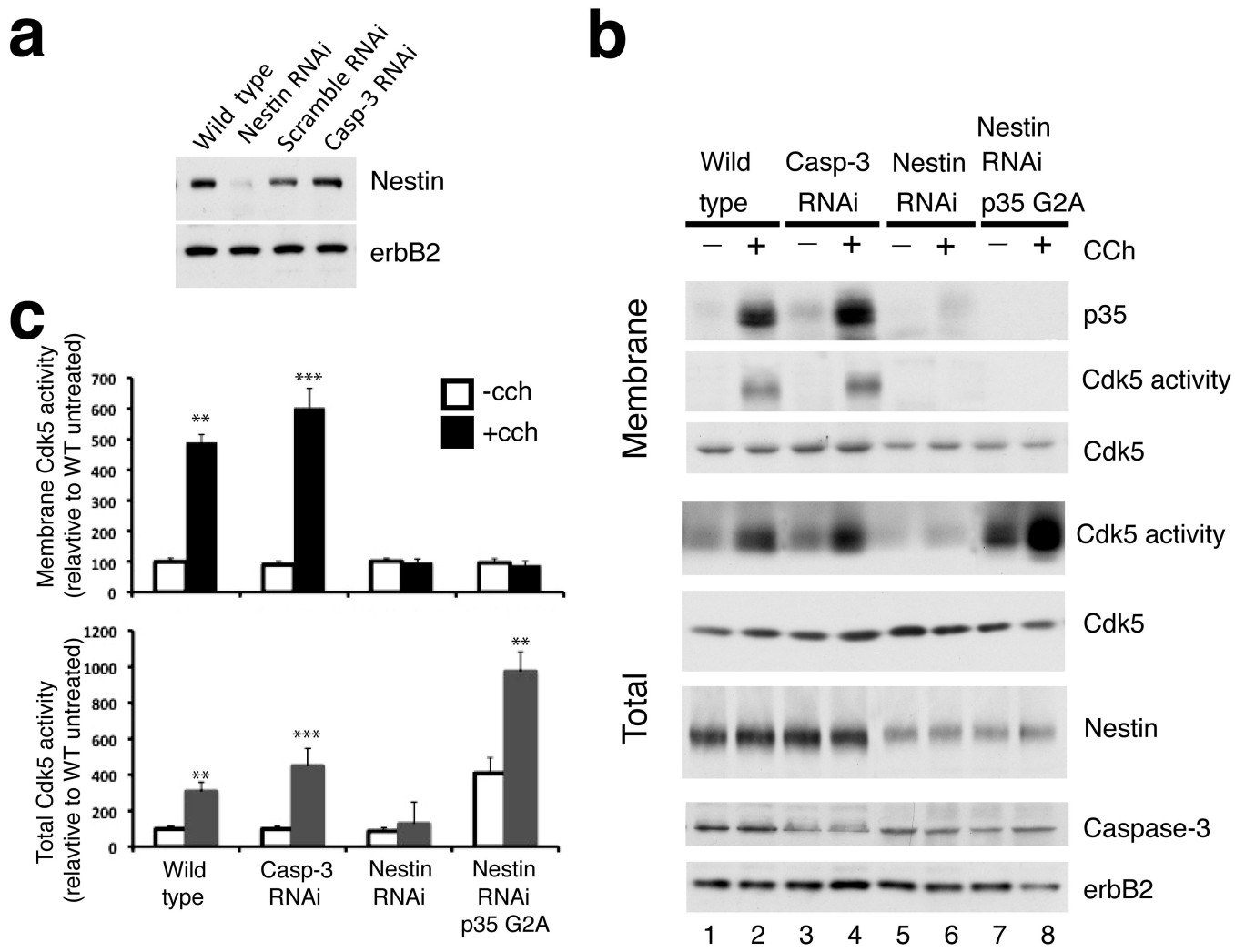

Figure 2. Nestin is required for the recruitment of p35 to the muscle membrane to activate Cdk5

a. C2C12 myotubes were either uninfected (wild tye) or infected with lentiviruses expressing nestin RNAi (LV-nestin RNAi), scrambled RNAi. Total lysate was immunoblotted with anti-nestin or anti-erbB2 antibodies. Significantly lower levels of nestin were observed in the cells infected with LV-nestin RNAi than in either control lysate. **b.** Whereas wild type myotubes showed a robust increase in membrane-associated p35 levels (lanes 1,2) and Cdk5 activity (lanes 1,2) when treated with CCh, LV-nestin RNAi-infected myotubes exhibited markedly less (p35) or a complete absence (Cdk5 activity) of these membrane-associated increases (lanes 5,6 vs. 1,2). When RNAi against caspase-3 was use, the recruitment of p35 to membrane and the membrane-associated Cdk5 activation were not affected (lanes 3,4 vs. 1,2). Overexpression of the p35 G2A mutant in LV-nestin RNAi-infected myotubes had no effect on p35 recruitment and Cdk5 activation (lanes 7,8 vs. 5,6). CCh treatment caused a corresponding activation of Cdk5 in total lysate extracted from wild type myotubes but not from LV-nestin RNAi-infected myotubes (lanes 1,2 vs. 5,6). No change was observed between any conditions in the overall levels of Cdk5 protein in either the membrane or total lysate fractions. Nestin protein levels were reduced accordingly in

LV-nestin RNAi-infected. ErbB2 expression in myotubes was not affected. **c.** Quantification of Cdk5 activity in membrane and in total lysates, normalized to Cdk5 protein. Cdk5 activity of wild type myotubes without any treatment was set as 100. Data are expressed as mean ±SEM. ** $p<0.01$, *** $p<0.001$, n=3.

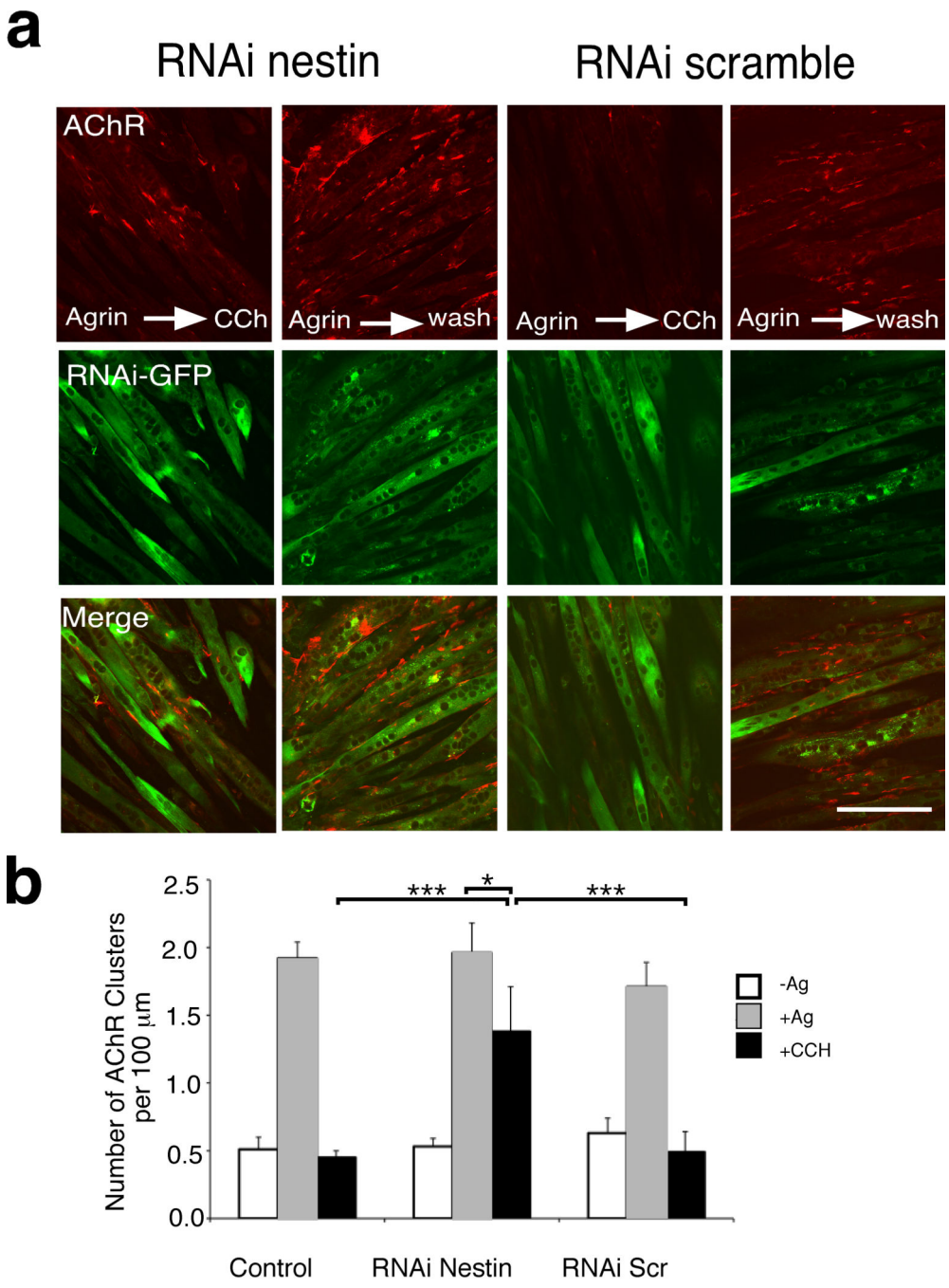

Figure 3. ACh-induced dispersion of AChR clusters was blocked when nestin was knocked down

C2C12 myotubes were infected with RNAi against nestin (**a**, left two columns) or a control scrambled sequence (**a**, right two columns). The control RNAi had no effect on either the clustering of AChRs induced by agrin treatment (Agrin → wash) or the dispersion of AChR by the ACh agonist carbachol (CCh) in myotubes (Agrin → CCh). Nestin knockdown did not affect the agrin-induced clustering of AChRs (Agrin → wash). However, the ACh-induced dispersion of AChR was blocked when nestin was knocked down (Agrin → CCh). Number of AChR clusters per 100 μm of myotubes was quantified in **b**. Scale bar: 10 μm.

Open bars, untreated myotubes; grey bars, agrin-treated myotubes (imaged 16 hrs after washing out agrin with fresh media); solid bars, CCh-treated myotubes (imaged 16 hrs after washing out agrin and treated with media containing CCh). Data are expressed as mean ±SEM (n=6). *, $p<0.05$; ***, $p<0.005$.

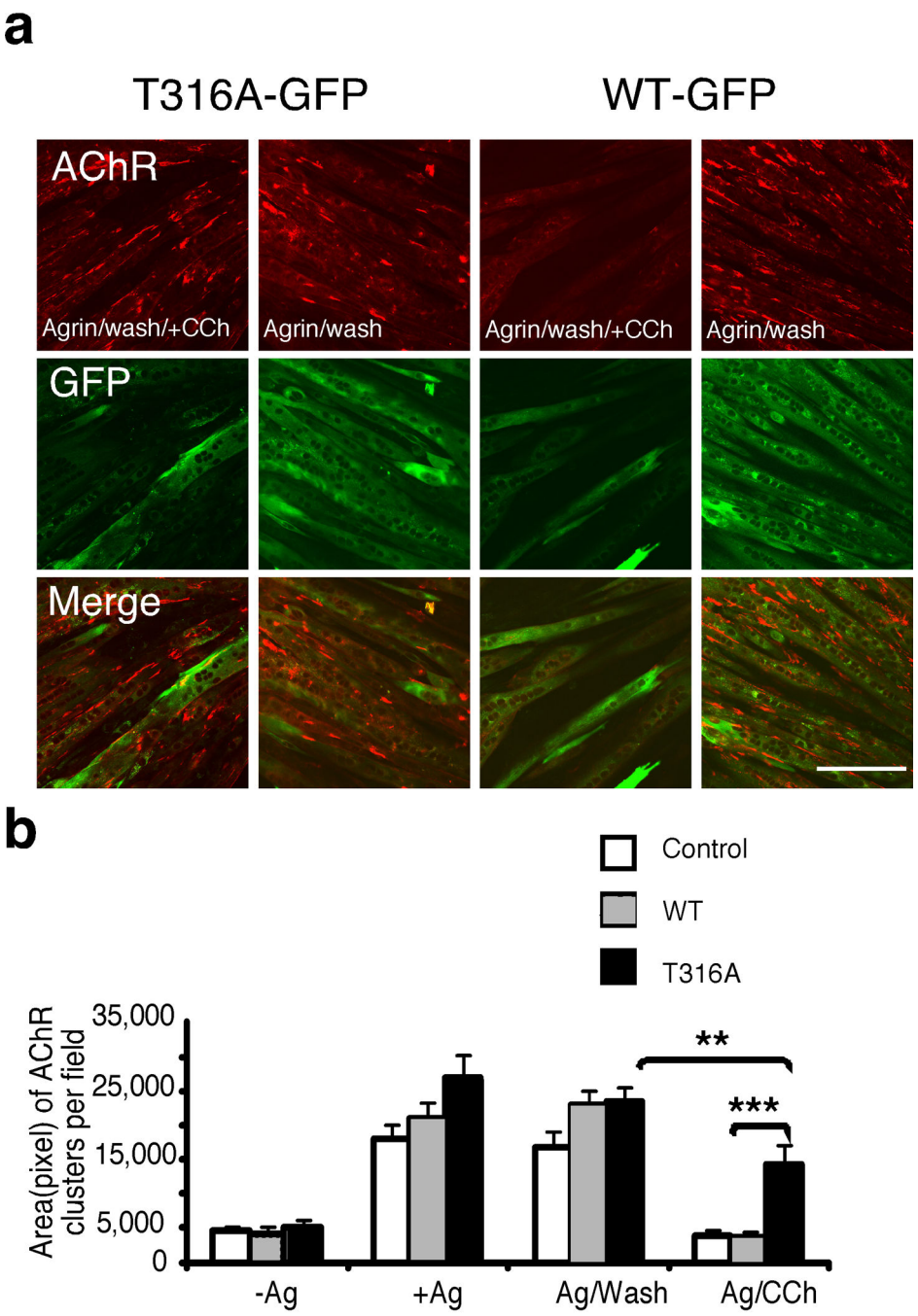

Figure 4. Phosphorylation of nestin by Cdk5 is critical for the dispersion of AChRs in myotubes

C2C12 myotubes were transfected with nestin-T316A-GFP or nestin-WT-GFP constructs and examined for the clustering of AChRs labeled by Cy3-conjugated-α-BTX and for RNA transduction by GFP-immunostaining (green in middle and bottom rows in **a**). Agrin-induced aggregation of AChRs was not affected in either nestin-WT-GFP or nestin-T316A-GFP transfected myotubes. However, similar to the effects of nestin RNAi, CCh-induced dispersion of AChR clusters was blocked in nestin-T316A-GFP but not nestin-WT-GFP transfected myotubes (**a**). Scale bar: 10 μm. This effect is quantified in **b**. Open bars, wild

type, untransfected myotubes; grey bars, nestin-WT-GFP overexpressing myotubes; solid bars, nestin-T316A-GFP overexpressing myotubes. Data are expressed as mean±SEM. ** $p<0.01$; *** $p<0.005$, n=6.

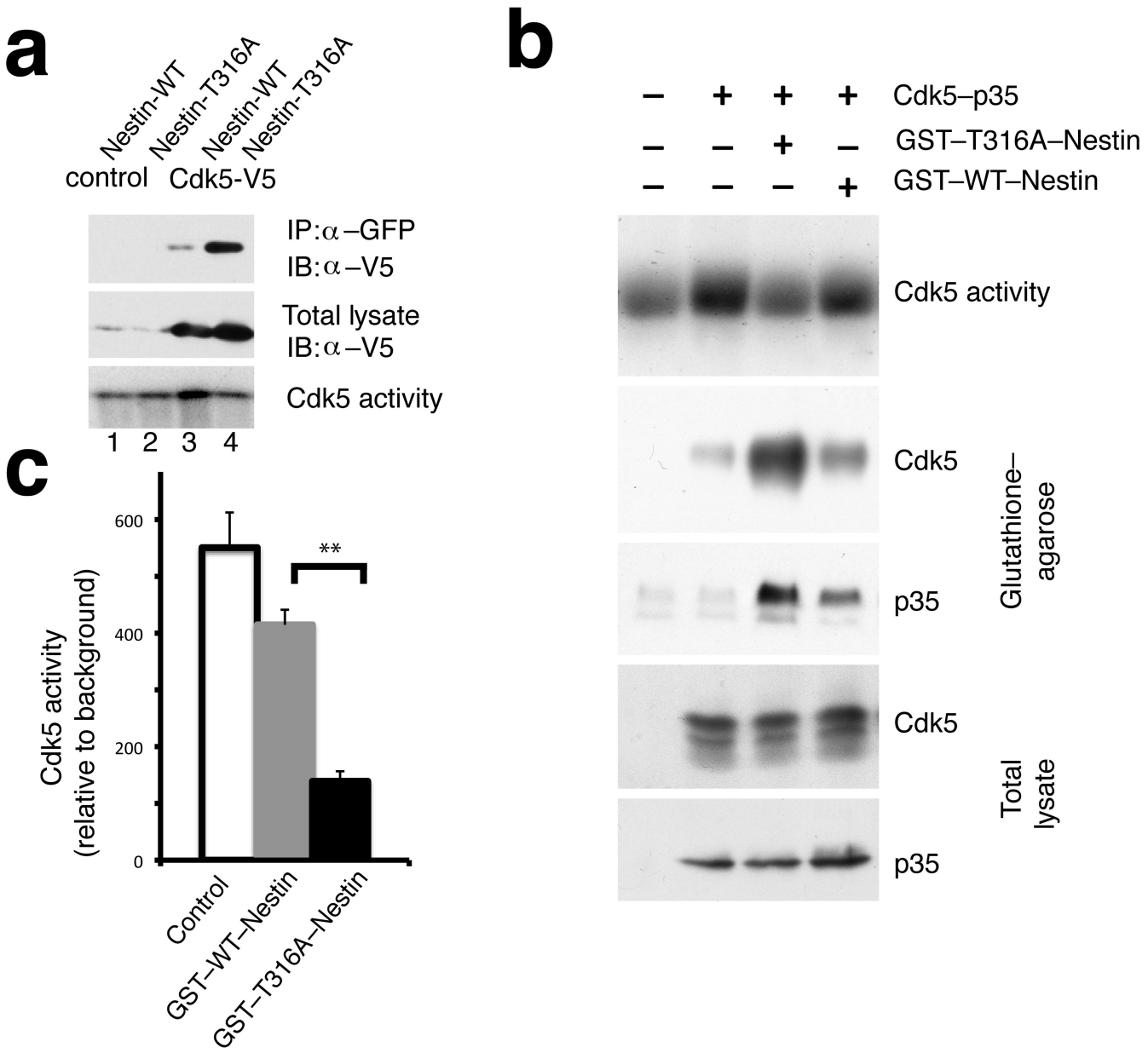

Figure 5. The T316A nestin mutant preferentially binds Cdk5 and inhibits Cdk5 activation

a. Nestin-WT-GFP or Nestin-T316A-GFP constructs were transfected alone (control, lanes 1 and 2) or co-transfected with a V5-tagged Cdk5 construct into C2C12 myotubes (Cdk5-V5, lanes 3 and 4). The lysates were immunoprecipitated with anti-GFP antibodies and immunoblotted with anti-V5 antibodies (top blot). Total lysates were also immunoblotted with anti-V5 antibodies as a positive control (middle blot), or with were assayed for Cdk5 activity (bottom blot). In co-transfected myotubes, significantly higher levels of Cdk5 were pulled down with mutant vs. WT nestin (top blot, lane 4 vs. lane 3), yet Cdk5 activity was markedly reduced (bottom blot, lane 4 vs. lane 3). **b**. Total lysates from cells transfected with Cdk5 and p35 were incubated with GST-WT-nestin or GST-T316A-nestin and pulled down with glutathione beads. Similar to results in myotubes, higher levels of Cdk5 were

found associated with GST-T136A-nestin-containing than with GST-WT-containing beads (second and third blots, third vs. fourth lane), but lower levels of Cdk5 activity were observed in supernatants taken from samples incubated with mutant GST-T316A-nestin as compared to those incubated with GST-WT-nestin (first blot, third vs. fourth lane). **c**. Quantification of Cdk5 activity in top panel **b**, normalized to the background (left-most lane in top panel **b**). Data are expressed as mean±SEM. ** $p<0.01$, $n=3$.

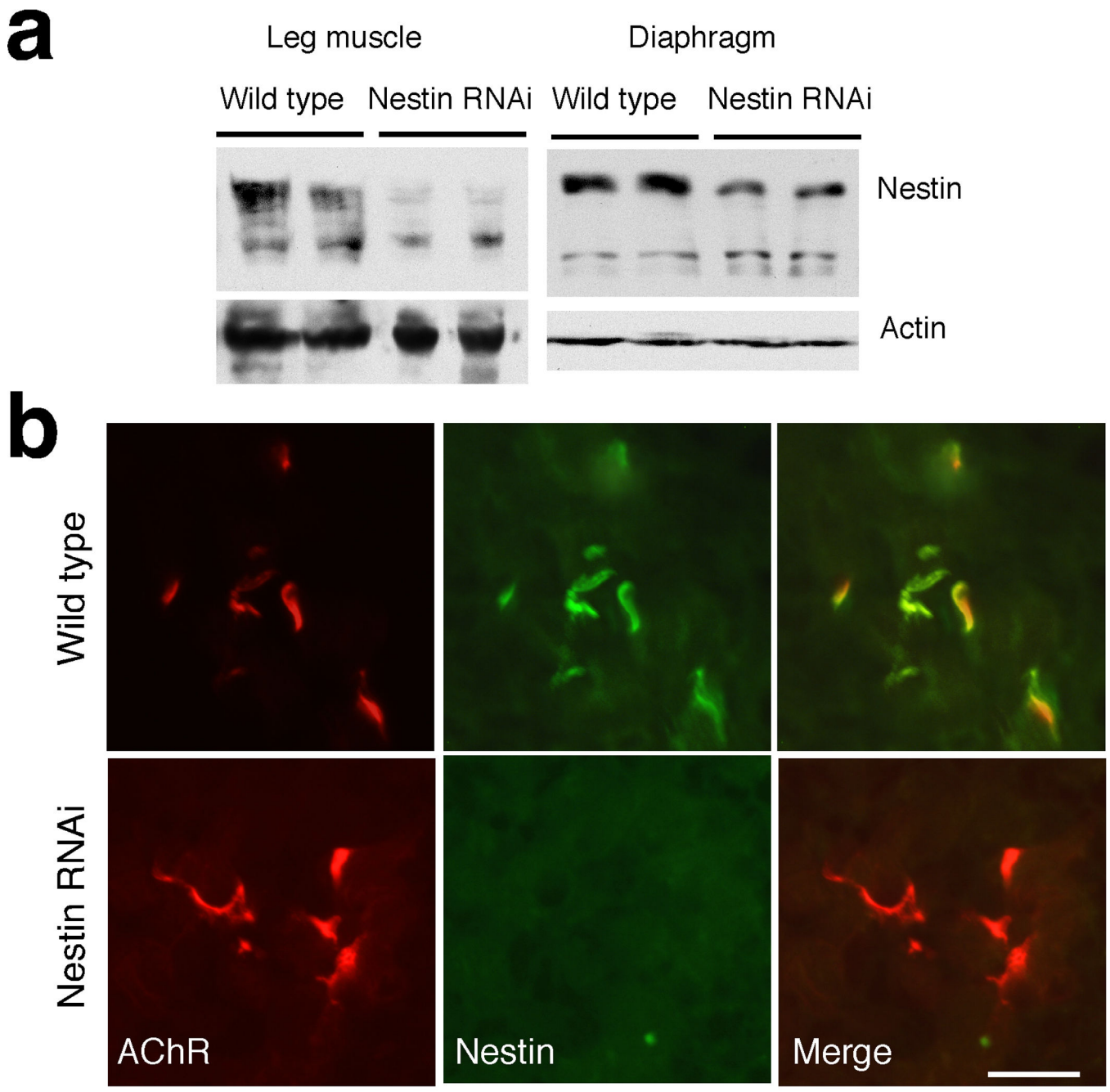

Figure 6. Knockdown of nestin expression in muscle

a. Significantly less nestin immunoreactivity was observed in muscle samples from embryos expressing RNAi against nestin (left two lanes in each top blot) than from WT embryos (right two lanes in each top blot). **b**. E18.5 hindlimb muscle sections were stained with anti-nestin antibody (green, middle and right panels) and Cy3-α-BTX (red, left and right panels) for AChR clusters. In WT muscle, nestin was co-localized with AChR clusters. In nestin RNAi-expressing hindlimb muscle, there was no detectable nestin staining.

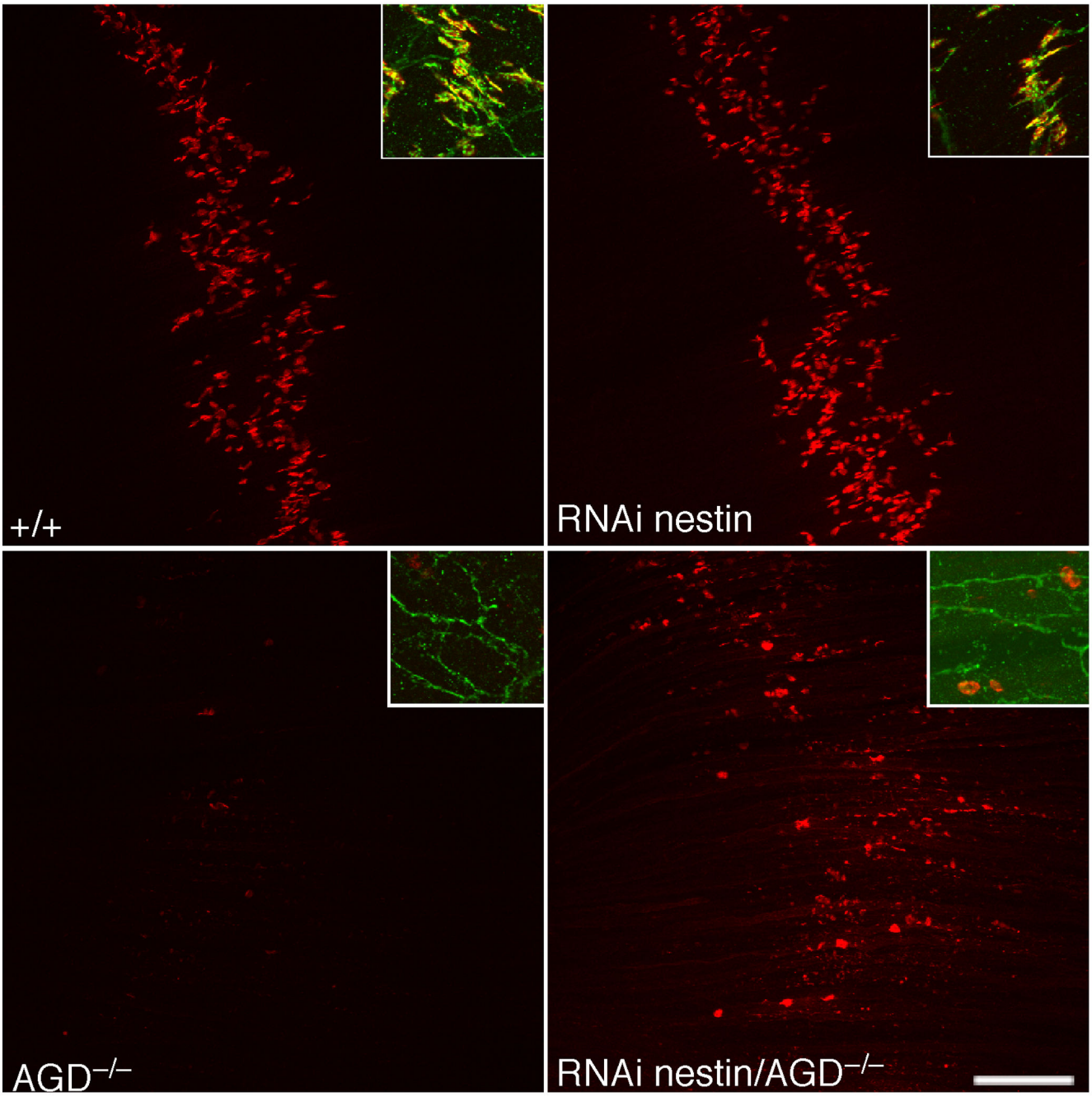

Figure 7. Knockdown of nestin expression in vivo rescues the postsynaptic AChR clusters in agrin-null mice

Diaphragm muscle from E18.5 wild type (+/+) (top left) and nestin RNAi-expressing (top right) embryos showed a centrally restricted band of innervated AChR clusters by Cy3-α–BTX staining (red). In agrin-null mutant ($AGD^{-/-}$) embryos (bottom left), there were no observable AChR clusters on the diaphragm at E18.5 due to the dispersing effect of ACh released from motor nerve terminals. In nestin RNAi-expressing/AGD double mutant embryos (bottom right), there were significantly more AChR clusters along the central band as compared to that of AGD single mutant embryos. In contrast to the restoration of AChR

clusters, there was no rescue of presynaptic specialization observed in nestin RNAi expressing/AGD double mutant embryos, as determined by double staining of AChR clusters with Cy3-αBTX (red) and of motor nerve terminals with synaptophysin antibodies (red and green, respectively, insets). Scale bar, 100 μm.

Table 1

AChR cluster number/field on E15.5, E16.5 and E18.5 diaphragms

Genotypes	E15.5	E16.5	E18.5
Wild Type	65.4±10.3	146.5±31.3	296.2±8.2
Nestin RNAi	95.4±23.3ND	206.5±41.8ND	338.3±12.1^{*a}
AGD$^{-/-}$	21.3±3.2	22.5±1.9	26.7±9.8
Nestin RNAi/AGD$^{-/-}$	38.6±9.1^{**b}	99.3±10.8^{***b}	111.9±10.1^{***b}

The numbers of AChR clusters are counted in a matching area of the dorsal quarter of right diaphragm muscle from embryos. Values are expressed as mean±SEM.

* $p<0.05$;

** $p<0.01$;

*** $p<0.005$;

n=7. ND, no significant difference.

^{a}Compared with Wild Type

^{b}Compared with AGD$^{-/-}$