Cdk5 is involved in neuregulin-induced AChR expression at the neuromuscular junction

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Here we describe an important involvement of Cdk5/p35 in regulating the gene expression of acetylcholine receptor (AChR) at the neuromuscular synapse. Cdk5 and p35 were prominently expressed in embryonic muscle, and concentrated at the neuromuscular junction in adulthood. Neuregulin increased the p35-associated Cdk5 kinase activity in the membrane fraction of cultured C2C12 myotubes. Co-immunoprecipitation studies revealed the association between Cdk5, p35 and ErbB receptors in muscle and cultured myotubes. Inhibition of Cdk5 activity not only blocked the NRG-induced AChR transcription, but also attenuated ErbB activation in cultured myotubes. In light of our finding that overexpression of p35 alone led to an increase in AChR promoter activity in muscle, Cdk5 activation is sufficient to mediate the up-regulation of AChR gene expression. Taken together, these results reveal the unexpected involvement of Cdk5/p35 in neuregulin signaling at the neuromuscular synapse.

Cyclin-dependent kinase 5 (Cdk5), a member of a large family of protein kinases, is a serine/threonine kinase originally identified as the catalytic subunit of a brain cdc2-like kinase and shown to be homologous to other cyclin-dependent kinases[1]. Unlike other members of the family, Cdk5 exhibits distinct functions that are not related to cell cycle control. The activity of Cdk5 requires association with its neuronal-specific activators, p35 or its isoform p39. Thus, whereas Cdk5 is expressed ubiquitously in mammalian tissues, its kinase activity is most abundant in the CNS[2–4]. Cdk5 and p35 gene targeting studies suggest that Cdk5 activity is critical in neuronal development, including axon outgrowth, axon guidance and neuronal migration[5–7]. Consistent with the proposed roles of Cdk5 in the nervous system, several of its identified substrates are cytoskeletal proteins essential in neuronal cytoarchitecture, such as neurofilament and tau[1,8]. The prominent expression of Cdk5 and its activators in the limbic system, such as in the hippocampus, suggests that one of the functions of Cdk5 may be related to neuronal plasticity such as neuronal sprouting and synapse formation in the brain[9]. In addition to the CNS, Cdk5 activity is also detected in muscle cells during differentiation and has been suggested to be essential for myogenesis[10]. Indeed, expression of Cdk5 dominant negative mutant in *Xenopus* embryo has been reported to disrupt somitic muscle development[11]. The potential function of Cdk5 and its activators at the neuromuscular synapse, however, has not been explored.

During the formation of synapses, pre- and postsynaptic cells undergo complex modification to form specialized apparatus in order to accommodate the rapid and precise neurotransmission. The synaptic transmission at the neuromuscular junction (NMJ) is ensured by the high concentration of acetylcholine receptors (AChR) at the postsynaptic muscle membrane. The transcription of AChR occurs at the subsynaptic nuclei and is controlled by nerve-derived factors such as neuregulin-1 (NRG-1). NRG-1 (from this point forward, NRG, also known as acetylcholine receptor inducing activity) is a member of a family of growth factors that also include glial growth factor, heregulin and neu differentiation factor. (For review, see refs. 12 and 13.) At the NMJ, NRG is responsible for the induction of synapse-specific gene expression, such as AChR subunits, sodium channels, utrophin and various components of the G protein signaling cascade[14–18].

The actions of NRG on target muscle cells are mediated by ErbB receptors, which belong to the epidermal growth factor receptor related family of tyrosine kinases. NRG and the receptors are localized and enriched at the neuromuscular endplates[19]. Binding of NRG to ErbB3 and ErbB4 initiates the homo- or heterodimerization of the receptors and subsequent tyrosine phosphorylation. Whereas all three ErbB receptors are tyrosine phosphorylated in response to NRG, ErbB3 does not display the tyrosine kinase activity. Activation of downstream signaling cascades, involving molecules such as Ras, Raf, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and PI3 kinase, results in the transcription of AChR subunits[20–22].

In the present study, the potential involvement of Cdk5 in synapse formation was explored using the NMJ as a model system. We report here that Cdk5 and p35 are prominently expressed in muscle during embryonic stages, and localized to the NMJ in adult muscle. The p35-associated Cdk5 kinase activity in the membrane fraction of cultured C2C12 myotubes can be increased by

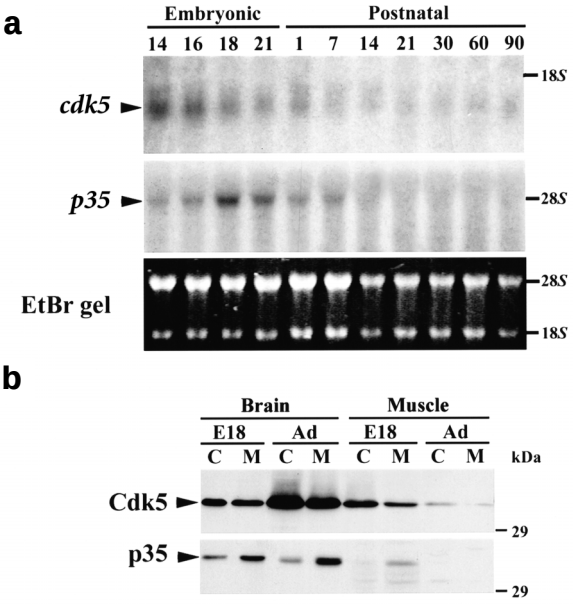

Fig. 1. Cdk5 and p35 were prominently expressed in muscle during embryonic development. (**a**) Northern blot analysis of the mRNA expression of *cdk5* (top, 10 μg total RNA per lane) and *p35* in muscle (middle, 20 μg total RNA per lane) during development. Bottom, ethidium bromide (EtBr)-stained gel indicated equal loading of RNA samples. (**b**) Western blot analysis of the expression of Cdk5 and p35 in membrane (M) and cytosolic fractions (C) of brain (20 μg protein per lane) and muscle (80 μg protein per lane). E18, embryonic day 18; Ad, adult.

NRG. In addition, our findings reveal the unexpected involvement of Cdk5/p35 in the NRG signaling pathway. The association of the ErbB receptors and Cdk5/p35 suggests the existence of a functional signaling complex at the NMJ, where many other postsynaptic molecules are also localized. Taken together, these results identify Cdk5/p35 as key players in NRG signaling at the neuromuscular synapse. It would be of interest to examine whether a similar functional role for Cdk5/p35 exists at the CNS synapse.

Results

As a first step to explore potential functions of Cdk5 and p35 at the neuromuscular synapse, the developmental profiles of their gene expression in rat muscle were examined by northern blot analysis (Fig. 1a). The level of *cdk5* transcript in muscle was abundant during embryonic day 14 to 16 (E14–16); the expression then decreased during postnatal stages. The transcript of *p35* was also detected in rat muscle during embryonic development; its expression peaked at embryonic stage E18 (Fig. 1a).

To compare the subcellular localization of Cdk5 and p35 in brain and muscle, western blot analysis was done using membrane and cytosolic fractions. Our findings showed that the expression of Cdk5 and p35 was less abundant in muscle than in brain (Fig. 1b). Moreover, unlike the expression profile in brain, Cdk5 and p35 were more prominently expressed during embryonic stages in muscle when compared to adult muscle. Whereas p35 protein was mainly concentrated in the embryonic muscle membrane fractions, Cdk5 could be detected in both cytosolic and membrane fractions. The localization of Cdk5 and p35 in adult muscle was examined by immunohistochemical analysis. Our results demonstrated that Cdk5 as well as p35 were concentrated at the adult NMJ, localized with AChR (Fig. 2a). To confirm the expression of Cdk5 on the postsynaptic specializations, similar analysis was done with denervated muscle. The axon terminal normally degenerates after denervation by day 4, as demonstrated by the disappearance of the expression of a presynaptic marker, synapsin I (Fig. 2b). However, the concentration of p35 staining remained at the NMJ 10 days after nerve cut, albeit with reduced intensity (Fig. 2b). A similar result was obtained with Cdk5 (data not shown). Our data suggests that in addition to their expression in axons, both p35

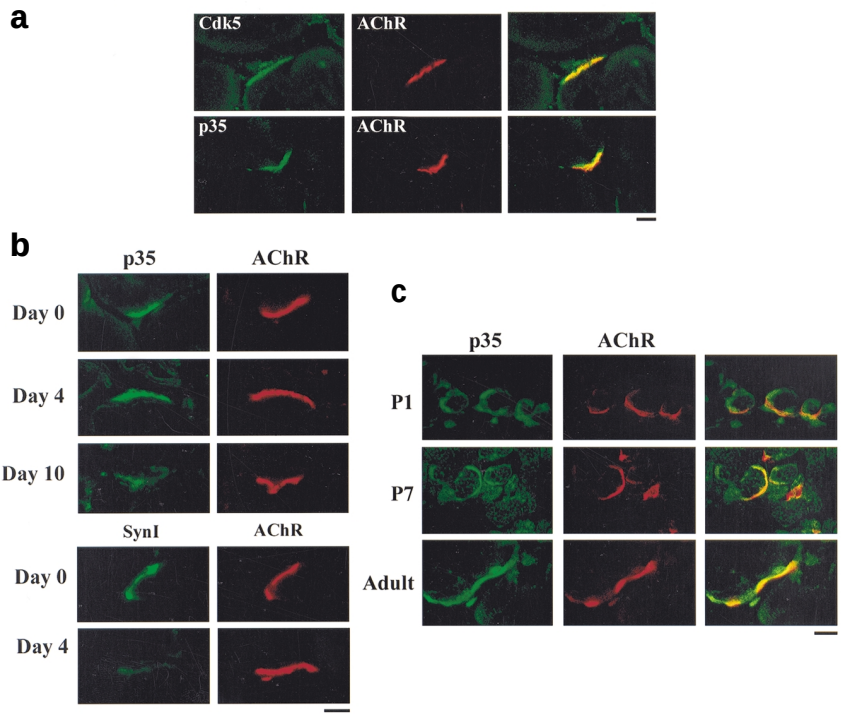

Fig. 2. Localization of Cdk5 and p35 in muscle during development and after nerve injury. (**a**) Immunohistochemical analysis revealed the colocalization of Cdk5 and p35 (left) with AChR (middle) at the adult rat NMJ. Right, superimposed images with the overlapping region indicated in yellow. The specific staining could no longer be observed when the antibodies were preincubated with the peptides used to raise the antibodies (data not shown). (**b**) Localization of p35 after denervation. Double immunostaining of p35 and AChR was done on muscle sections from rat gastrocnemius muscle at day 0, day 4 or day 10 after nerve cut. p35 staining remained at the NMJ 10 days after nerve cut (top), whereas the staining of synapsin I (SynI) disappeared at day 4 after denervation (bottom). (**c**) Developmental profile of p35 localization in muscle. Double immunostaining was done on muscle sections of various developmental stages (P1, P7 and adult). Left, p35; middle, AChR; right, superimposed images with the overlapping region indicated in yellow. Scale bar, 10 μm.

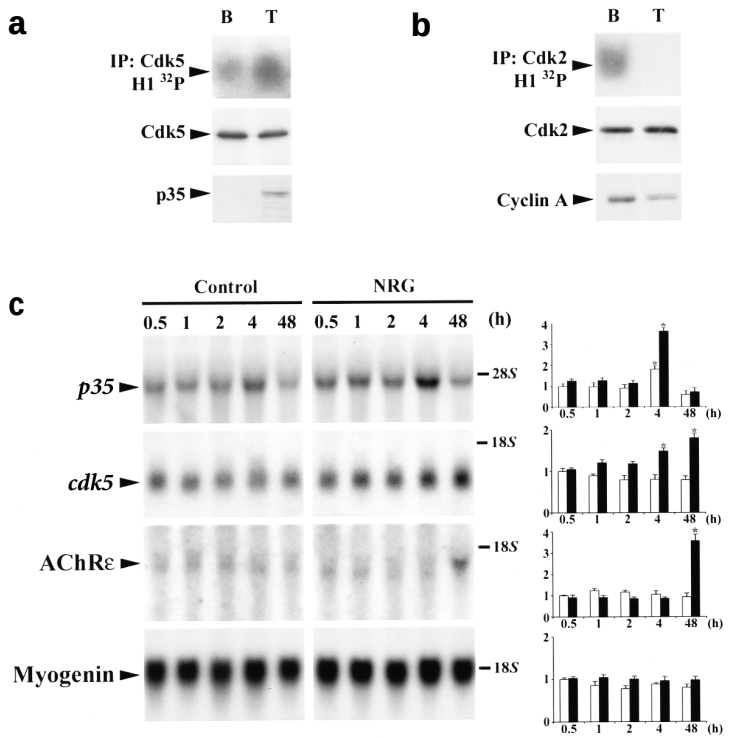

Fig. 3. Regulation of expression of Cdk5 and p35 in NRG-treated myotubes. (**a**) Top, Cdk5 kinase activity in C2C12 myoblasts (B) and myotubes (T); middle, total Cdk5 protein; bottom,total p35 protein. (**b**) Top, Cdk2 kinase activity in C2C12 myoblasts (B) and myotubes (T); middle , total Cdk2 protein; bottom, total Cyclin A protein, (**c**) NRG increased the mRNA expression of *cdk5* and *p35* in C2C12 cultured myotubes that had been differentiated for 3 days. Total RNA was collected from C2C12 myotubes without (control, white bars) or with NRG treatment (NRG, black bars) for various time periods (0.5–48 h) as indicated. Left, northern blot analysis of *p35*, *cdk5*, AChRε and myogenin; right, quantitation of results. Arbitrary units were used for the *y*-axis to indicate the relative intensity. Each data point represented the mean ± s.e.m. of one representative experiment; $n = 3$; $^*p < 0.005$. Similar results on the temporal profile of the changes in mRNA levels were obtained in six independent experiments.

and Cdk5 were concentrated at synaptic sites in adult muscle fibers. To examine the cellular localization of p35 during development, immunohistochemical analysis was done on muscle sections. Whereas p35 staining was observed at the developing NMJ (postnatal day 1; P1), extra-synaptic expression could also be detected at the circumference of muscle fibers (Fig. 2c). By P7, p35 staining became more concentrated at the synaptic sites, and colocalized with AChR in adult muscle.

Cultured C2C12 myotubes were used as a model system to investigate the potential functional roles of Cdk5 and p35 in postsynaptic specializations. Cdk5 kinase activity could be detected in C2C12 myoblasts and was upregulated in myotubes (Fig. 3a). Unlike Cdk5, the activity of Cdk2 in C2C12 myotubes diminished to an undetectable level (Fig. 3b). We examined whether mRNA expression of p35 and Cdk5 in cultured myotubes could be regulated by NRG, a nerve-derived factor that is responsible for the induction of synapse-specific genes. Treatment of C2C12 myotubes with NRG resulted in an increase in *p35* and *cdk5* transcripts (Fig. 3c). The induction of acetylcholine receptor epsilon subunit (AChRε) observed after 48 hours of NRG treatment served as positive control. The expression of myogenin was not induced by NRG (Fig. 3c), suggesting that the observed up-regulation of the expression of *p35* and *cdk5* was specific and not due to changes in the differentiation status of myotubes.

In addition to the induction in the gene expression of *p35*, the ability of NRG to increase Cdk5 kinase activity in C2C12 myotubes was examined. Upon treatment with NRG, Cdk5-associated histone H1 kinase activity increased approximately twofold by one hour (Fig. 4a). To confirm whether the effect of NRG on Cdk5 kinase activity was specific, we examined the ability of NRG to stimulate the activity of another serine-threonine kinase, protein kinase A (PKA). Unlike forskolin, which increased PKA activity fourfold in C2C12 myotubes, NRG did not affect PKA activity (Fig. 4b). The distribution of Cdk5 activity in NRG-treated C2C12 myotubes was examined using membrane and cytosolic fractions. The increased Cdk5 activity observed after one hour of NRG treatment was associated with p35 in the membrane fraction (Fig. 4c). A time-course study of the p35-associated Cdk5 activity demonstrated that the maxi-

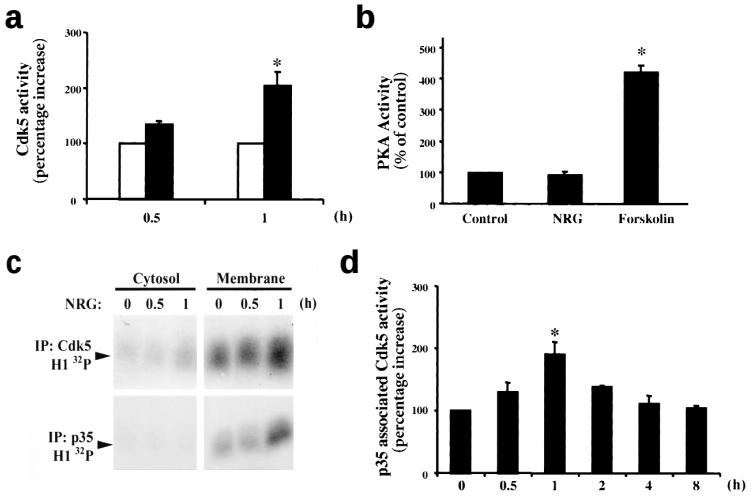

Fig. 4. NRG increased Cdk5 kinase activity associated with membrane fractions of C2C12 myotubes. (**a**) C2C12 myotubes were treated without (control, white bars) or with NRG treatment (NRG, black bars) for 0.5 or 1 h as indicated. Cdk5 kinase activity was assessed as described in Methods, using histone H1 peptide as a substrate. Each data point represented the mean ± s.e.m. of one representative experiment; $n = 3$. Similar results were obtained in five independent experiments. Cdk5 kinase activity was consistently higher in NRG-treated C2C12 myotubes ($^*p < 0.005$). (**b**) C2C12 myotubes were treated with NRG for 1 h, and PKA activity was measured as described in Methods. Treatment with forskolin served as the positive control. (**c**) C2C12 myotubes were treated with NRG for 0.5 or 1 h. Cdk5- or p35-associated kinase activity was measured in cytosolic or membrane fractions as described in Methods. (**d**) C2C12 myotubes were treated with NRG for 0.5 to 8 h. The p35-associated kinase activity was measured in membrane fraction as described in Methods.

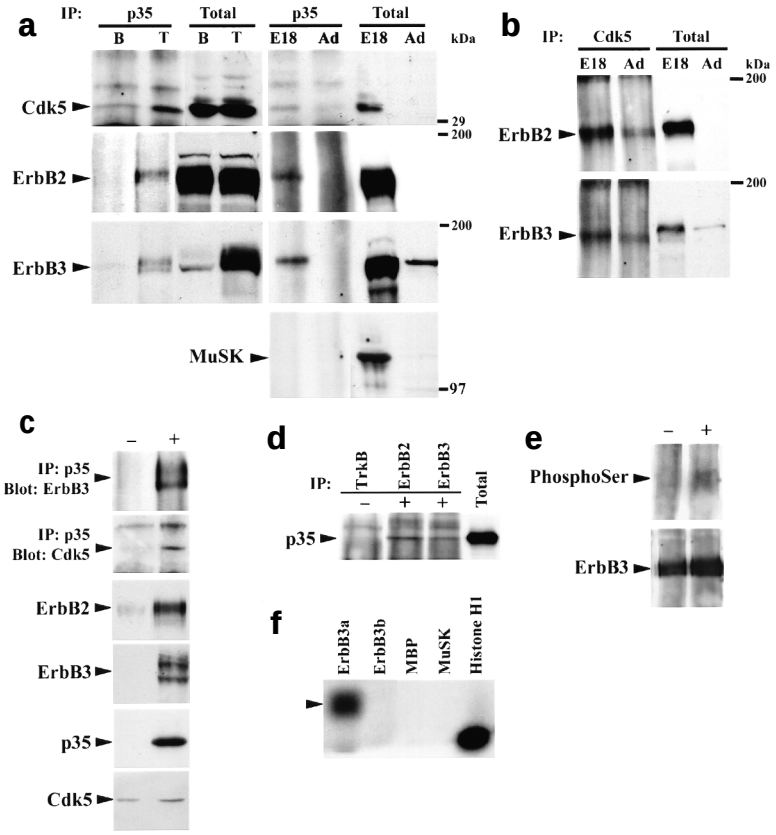

Fig. 5. Association of Cdk5 and p35 with ErbB receptors and serine/threonine phosphorylation of ErbB3. (**a**) Co-immunoprecipitation of p35 with Cdk5, ErbB2 and ErbB3 in cultured myotubes and muscle. Cell lysates were immunoprecipitated with p35 antibody and immunoblotted with antibodies specific for Cdk5, ErbB2 or ErbB3. Undifferentiated myoblasts, B; differentiated myotubes, T; embryonic day 18 muscle, E18; adult muscle, Ad. Total protein expression of Cdk5, ErbB2 and ErbB3 is also shown. When cell lysates were immunoprecipitated with p35 antibody and immunoblotted with MuSK antibody, no association was observed. (**b**) Co-immunoprecipitation of Cdk5 with ErbB2 and ErbB3 in muscle. Cell lysates were immunoprecipitated with Cdk5 antibody and immunoblotted with antibodies specific for ErbB2 or ErbB3. (**c**) COS-7 cells were transfected with vector (–) or with p35, ErbB2 and ErbB3 (+) as described in Methods. Cell lysates were immunoprecipitated with p35 antibody and immunoblotted with ErbB3 or Cdk5 antibody. Lower panels, total protein for ErbB2, ErbB3, p35 and Cdk5. (**d**) COS-7 cells were transfected with p35, ErbB2 and ErbB3 (+), and lysates were immunoprecipitated with ErbB2 or ErbB3 antibodies followed by immunoblotting with p35 antibody. A parallel control experiment was done by transfecting COS-7 cells with p35 and TrkB (–), followed by immunoprecipitating the cell lysates with TrkB antibody and immunoblotting with p35 antibody. (**e**) ErbB3 immunoprecipitated from C2C12 myotubes could be phosphorylated by reconstituted Cdk5/p25 on serine residues. The *in vitro* phosphorylation assay was done as described in Methods. Without active Cdk5 (–); with active Cdk5 (+). (**f**) *In vitro* phosphorylation of two ErbB3 peptides (ErbB3a and ErbB3b), as well as peptides of MBP, MuSK and histone H1.

mal increase was observed at one hour after NRG treatment (Fig. 4d). By 48 hours, p35-associated Cdk5 activity returned to below basal level (data not shown). The maximal induction of p35 mRNA level was not observed until four hours after the addition of NRG (Fig. 3c); thus, the rapid increase in p35-associated Cdk5 activity upon NRG treatment is unlikely to be accounted for by an increase in p35 transcription.

Because the activity of Cdk5 requires the association with its activators, we examined whether the interaction between Cdk5 and p35 could be detected in myotubes. Association between p35 and Cdk5 was demonstrated in cultured myotubes as well as muscle by immunoprecipitation studies (Fig. 5a). C2C12 cells express ErbB2 and ErbB3 and very little ErbB4, if any. Whereas an abundant level of ErbB2 expression was detected in both myoblasts and myotubes, prominent expression of ErbB3 was only detected in myotubes (Fig. 5a). Complex formation between p35, ErbB2 and ErbB3 could be demonstrated in cultured myotubes (Fig. 5a). Furthermore, similar association between p35 and ErbB receptors, as well as between Cdk5 and ErbB receptors, was also demonstrated in embryonic muscle (E18; Fig. 5a and b). On the other hand, muscle specific kinase (MuSK, another receptor tyrosine kinase localized at the NMJ) could not be detected in the complex (Fig. 5a), thus verifying the specificity of the association observed. To confirm the association of p35 and ErbB receptors, the cDNA constructs encoding p35, ErbB2 and ErbB3 were transfected into COS-7 cells, and cell lysates were immunoprecipitated with p35 antibody followed by immunoblotting with ErbB3 or Cdk5 antibody. We observed an association between p35 and ErbB3, as well as between p35 and Cdk5 (Fig. 5c). Similar results were obtained using the ErbB2 and ErbB3 antibodies for immunoprecipitation and p35 antibody for immunoblotting (Fig. 5d). Taken together, our findings on the association of Cdk5, p35, ErbB2 and ErbB3 suggest the existence of a complex involving these postsynaptic molecules in muscle.

Analysis of the sequences encoding rat ErbB3 identifies the consensus sequence motif -S/T-P-X-K/R- for Cdk5 phosphorylation at several positions. Our studies on the *in vitro* phosphorylation assay demonstrated that ErbB3 immunoprecipitated from C2C12 myotubes could be phosphorylated by active Cdk5 on serine residues (Fig. 5e). We investigated the ability of active Cdk5 to phosphorylate two peptides of ErbB3 corresponding to the potential sites for Cdk5 serine/threonine phosphorylation. We found that ErbB3a (TPIK; amino acid positions 871–874) could be phosphorylated on threonine by active Cdk5 *in vitro* (Fig. 5f), whereas phosphorylation of ErbB3b (SPPR; amino acids 1204–1207) on serine could be detected upon longer exposure of the autoradiogram (data not shown). On the other hand, two peptides derived from myelin basic protein (MBP) or muscle specific kinase (MuSK), which were not phosphorylated by active Cdk5, served as negative control (Fig. 5f).

To investigate whether NRG-mediated AChR transcription depends on Cdk5 kinase activity, we first examined the effect of roscovitine, a specific Cdk5 kinase inhibitor[23]. Pretreatment of C2C12 myotubes with roscovitine before the addition of NRG was able to attenuate the NRG-mediated induction of AChRε as well as acetylcholine receptor alpha subunit (AChRα) mRNA (Fig. 6a). Similarly, in primary chick muscle culture, roscovitine was able to inhibit the NRG-induced AChRα transcription (Fig. 6b). We examined the ability of the Cdk5 inhibitor to block ERK activation, which is required for NRG-

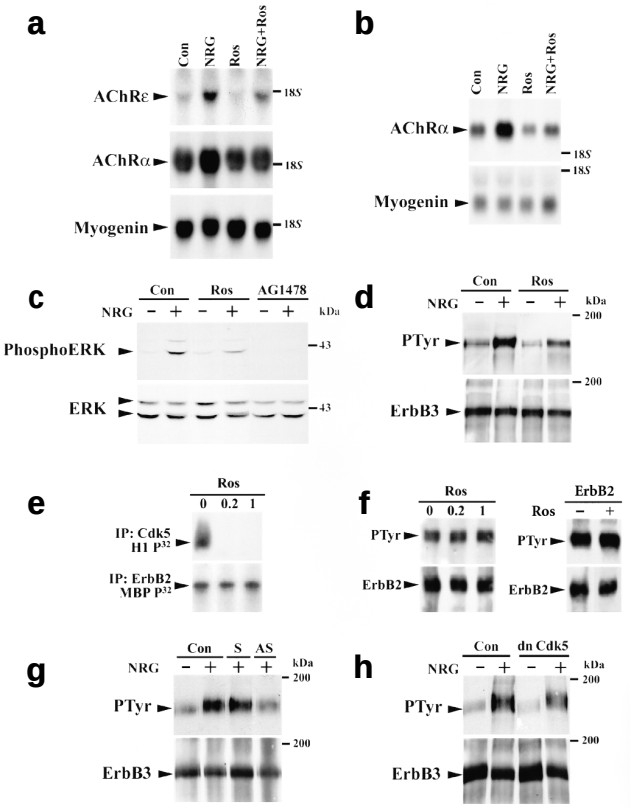

Fig. 6. Inhibition of Cdk5 activity attenuated the NRG-induced AChR transcription and ErbB3 phosphorylation in cultured myotubes. (**a**) Northern blot analysis of AChRε, AChRα and myogenin in NRG-treated C2C12 myotubes in the presence or absence of roscovitine (Ros, 10 μM). Con, control. (**b**) Northern blot analysis of AChRα and myogenin in primary chick muscle culture treated with NRG in the presence or absence of Ros. (**c**) Western blot analysis of phosphoERK (top) and total ERK (bottom) in NRG-treated C2C12 myotubes in the presence or absence of Ros. Pretreatment of myotube with AG1478 served as control to show the complete inhibition of NRG-stimulated ERK phosphorylation. (**d**) Western blot analysis of tyrosine phosphorylated ErbB3 and total ErbB3 (bottom) in NRG-treated C2C12 myotubes in the presence or absence of Ros. (**e**) *In vitro* Cdk5 kinase activity (histone H1 as substrate) and ErbB2 kinase activity (MBP as substrate) was examined in the absence or presence of Ros (0.2 and 1 μM). (**f**) *In vitro* phosphorylation of ErbB2 immunoprecipitated from C2C12 myotubes, and *in vivo* autophosphorylation of ErbB2 overexpressed in COS-7 cells were examined in the absence or presence of Ros. (**g**) Antisense (AS) or sense (S) oligonucleotides specific for Cdk5 were added to C2C12 myotubes as described in Methods. Without NRG (–); with NRG (+). Top, tyrosine phosphorylation of ErbB3; bottom, total ErbB3 protein. (**h**) Constructs encoding vector alone (Con) or Cdk5 dominant negative (dnCdk5) were transiently transfected into C2C12 cells as described in Methods. Top, tyrosine phosphorylation of ErbB3; bottom, ErbB3 protein.

induced expression of AChR subunits in muscle cultures[20,22]. Treatment of C2C12 myotubes with roscovitine (10 μM) inhibited phosphorylation of ERK (Fig. 6c). Tryphostin AG1478 (a potent and selective inhibitor of ErbB protein kinase; 1 nM), which suppressed NRG-induced ERK activation, served as control. Roscovitine also attenuated the NRG-stimulated ErbB3 (Fig. 6d) and ErbB2 (data not shown) phosphorylation in a similar manner. To rule out the possibility that roscovitine might directly inhibit ErbB2, the ability of roscovitine to directly inhibit kinase activity and phosphorylation of ErbB2 was examined. Whereas roscovitine completely inhibited Cdk5 kinase activity *in vitro*, it did not affect ErbB2 kinase activity (Fig. 6e) or phosphorylation of ErbB2 *in vitro* (Fig. 6f). Similarly, roscovitine did not inhibit autophosphorylation of ErbB receptors in COS-7 cells transfected with ErbB2 (Fig. 6f) or ErbB2/3 (data not shown). These data indicated that the inhibitory effect of roscovitine is specific for Cdk5.

To provide additional evidence that Cdk5 activity was involved in NRG-induced activation of ErbB receptors, we analyzed the consequences of Cdk5 suppression by antisense oligonucleotide treatment or by transfection of dominant negative Cdk5 construct. Treatment of C2C12 myotubes with Cdk5 antisense oligonucleotides inhibited the NRG-induced tyrosine phosphorylation of ErbB3 (Fig. 6g), whereas control cultures treated with the same concentration of the corresponding sense nucleotides were not affected. A similar inhibitory effect on NRG-induced ErbB3 tyrosine phosphorylation was observed when dominant negative Cdk5 construct was transiently transfected into C2C12 cells (Fig. 6h).

To examine the functional consequence of overexpressing p35 on the induction of AChRε mRNA expression, C2C12 cells were co-transfected with p35 and AChRε promoter-luciferase construct. A significant increase (~2-fold) in luciferase expression in C2C12 myotubes was observed following p35 overexpression (Fig. 7a), comparable to that observed in the control myotubes after NRG treatment[24]. However, p35 overexpression did not regulate the promoter of acetylcholinesterase (Fig. 7a). Overexpression of p35 in the transfected C2C12 cells was confirmed by western blot analysis (Fig. 7b), with most of the expressed p35 protein confined to the membrane fraction (data not shown). To verify that Cdk5 kinase activity contributes to the regulation of AChRε *in vivo*, the expression constructs encoding p35 and AChRε promoter-luciferase reporter were directly co-injected into mouse tibialis anterior muscle as described in the Methods. We found that overexpression of p35 *in vivo* led to an approximately threefold increase in the promoter activity of AChRε (Fig. 7c).

Discussion

In this study, we describe an important functional involvement of Cdk5 in the NRG signaling pathway that regulates AChR gene expression at the NMJ. The prominent expression of Cdk5 and p35 in embryonic muscle, together with their localization at the adult NMJ, suggest that they have potential functions at the synapse. The association of Cdk5, p35 and ErbB receptors can be demonstrated in cultured myotubes as well as in muscle. Inhibition of Cdk5 activity attenuates NRG-induced AChR gene expression and ErbB3 tyrosine phosphorylation in C2C12 myotubes. In light of our finding that overexpression of p35 alone leads to an increase in AChRε promoter activity in cultured myotubes as well as *in vivo*, Cdk5 activation is sufficient to mediate the up-regulation of AChR gene expression. The unexpected involvement of Cdk5 and its activators described in this study suggests that they are important in NRG signaling at the neuromuscular synapse.

Cdk5 activity depends on its association with activators such as p35; however, not much is known regarding the mechanisms that regulate Cdk5/p35 kinase activity. Whereas a recent report has provided convincing evidence for the ability of c-Abl, a non-receptor tyrosine kinase, to phosphorylate Cdk5 and enhance Cdk5/p35 kinase activity in the brain, the identity of the extracellular signal(s) involved remains to be determined[25]. Our finding of the ability of NRG to regulate the gene expression of

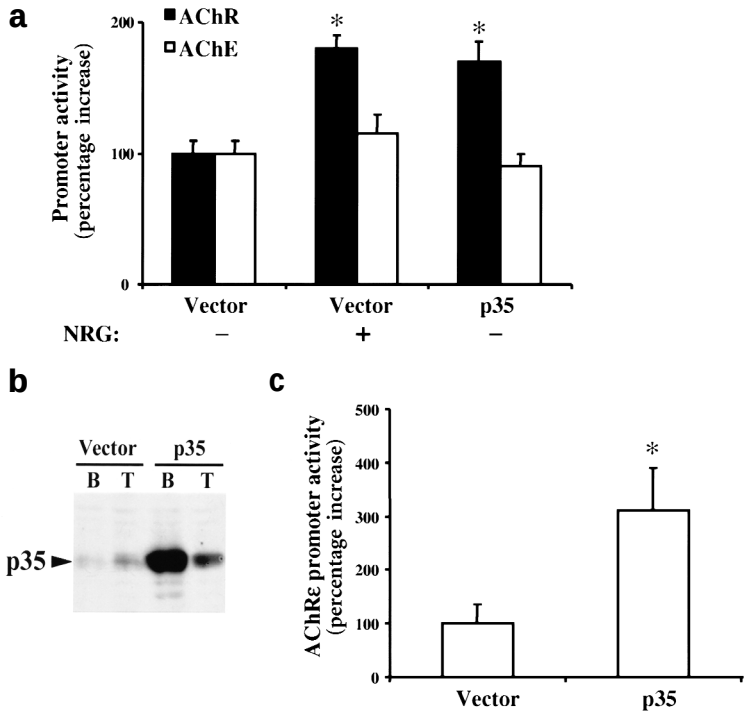

Fig. 7. Overexpression of p35 increased the expression of AChRε transgene both *in vitro* and *in vivo*. (**a**) Control C2C12 myoblasts were transfected with AChRε416-pGL3, βgal-pCMV and p35-pMT21 (p35) or pMT21 (vector) as described in Methods. The AChE promoter was used to examine whether the effect of p35 overexpression was specific for AChR. NRG was added to one set of the vector transfected cells to serve as positive controls (+). Each data point represents the mean ± s.e.m.; $n = 6$. AChRε promoter activity in p35 overexpressing C2C12 cells was significantly different from vector-transfected cells (*$p < 0.005$). The results have been confirmed in six independent experiments. (**b**) Western blot analysis confirmed the overexpression of p35 in C2C12 myoblasts transfected with p35 expression construct. Myoblast, B; myotube, T. (**c**) p35 increased the ε-transgene expression *in vivo*. Tibialis anterior muscles of 3-week-old mice were injected with AChRε416-pGL3, βgal-pCMV and p35-pMT21 or pMT21. Each data point represents the mean ± s.e.m.; $n = 8$. AChRε promoter activity in p35-injected mice was significantly different from mice injected with vector alone (*$p < 0.005$).

both p35 as well as Cdk5 activity in cultured myotubes is an important demonstration of how growth factors can influence Cdk5 activity in muscle. However, whether the regulation of Cdk5 is mediated by nerve-derived NRG or by NRG originated from muscle remains to be determined. Like p35, the expression of its isoform p39 is developmentally regulated and localized to the adult NMJ (unpublished observations). It remains to be determined whether the activity of Cdk5 is regulated by p35, p39 or a novel isoform(s) in muscle, and whether these activators subserve distinct functions during different developmental stages. Studies with p35 and p39 null mutant mice should shed light on the overlapping and distinct roles of these activators at the neuromuscular synapse.

NRG activates multiple signaling cascades that converge to regulate the gene expression of AChR subunits. Upon NRG-induced activation of ErbB receptors, recruitment of adapter proteins including Grb2 and Shc initiates the Ras/Raf/MAP kinase cascade that involves the activation of both ERKs and JNK[20,21]. Ras activates the ERKs pathway and Rac, a member of Rho subfamily of GTP-binding protein, activates the JNK pathway[26]. The ability of the Cdk5 inhibitor to attenuate the NRG-induced AChR expression as well as phosphorylation of ErbB and ERKs suggests that Cdk5 may regulate the NRG signaling cascade by modulating the activation of ErbB receptors. However, it remains possible that Cdk5 also regulates other downstream signaling molecules, such as JNK. Potential interactions of Cdk5 with known signaling molecules activated by NRG need to be further investigated.

Serine/threonine kinases are involved in regulating the phosphorylation and activity of receptor tyrosine kinases[27,28]. In the search for potential Cdk5 substrates in muscle, we have identified several postsynaptic proteins that contain the phosphorylation consensus sequence motif -S/T-P-X-K/R-, such as ErbB2 and ErbB3. We report here that phosphorylation of ErbB3 on serine and threonine residues by active Cdk5 could be demonstrated using *in vitro* phosphorylation assays. Our data is consistent with the possibility that phosphorylation of these serine/threonine sites by Cdk5/p35 may regulate the activity of ErbB receptors. The association of p35, Cdk5, ErbB2 and ErbB3 demonstrated in this study suggests the existence of a signaling complex involving Cdk5/p35 in muscle. The ability of Cdk5 to regulate the phosphorylation of cytoskeletal proteins[29] raises the possibility that Cdk5 activity is involved in the molecular organization of various postsynaptic proteins at the NMJ. More intriguing, however, is the possibility that such complex formation may be mediated by regulating the phosphorylation of the repertoire of Cdk5 substrates that are highly concentrated at the neuromuscular synapse. Similar complexes involving Cdk5 and its activators may well exist in the CNS to mediate the NRG-regulated gene expression of various receptors, such as NMDA receptor subunit, GABA receptor subunit as well as nicotinic AChR[30–32]. In light of the recent report on the association between ErbB4 and PSD-95, a protein enriched in the postsynaptic density and important for recruiting various components of the postsynaptic scaffold[33–35], it would be of interest to examine whether PSD-95 also interacts with Cdk5/p35 at the CNS synapse.

Methods

Chemicals and antibodies. Recombinant NRGβ1 was purified as previously described[17]. Antibodies specific for p35 (c-19), Cdk2 (M2), cyclin A (H-432), Cdk5 (c-8 and DC-17), ErbB2 (c-18) and ErbB3 (c-17) were purchased from Santa Cruz Biotechnology (Santa Cruz, California). ERKs and phospho-ERKs antibodies were purchased from New England Biolabs, Beverly, Massachussetts). The p35 monoclonal antibody (4E3) was a gift from L.H. Tsai (Department of Pathology, Harvard Medical School). Phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology (Lake Placid, New York), phosphoserine antibody from Zymed (San Francisco, California) and synapsin I antibody from Molecular Probes (Eugene, Oregon). Roscovitine and AG1478 were purchased from Calbiochem (San Diego, California).

Cell culture. Mouse C2C12 cells were normally maintained and differentiated as previously described[36]. Myotubes were cultured for at least three days before RNA extraction, phosphorylation and kinase assays. Primary chick muscle cultures were prepared from the hind-leg muscles of E13 chicks[36,37]. Both C2C12 and chick myotubes were treated with NRG (3 nM) for various time periods as indicated. The inhibitors, such as roscovitine (10 μM) and AG1478 (1 nM), were added to the myotube culture four hours before NRG treatment.

RNA extraction and northern blot analysis. Total RNAs of C2C12 cells and rat tissues were prepared by guanidinium thiocyanate extraction and lithium chloride/urea extraction, respectively[17]. Northern blot analysis was done as previously described[38]. The cDNA probes used included a C-terminal fragment (580 bp) of human *cdk5* and N-terminal fragment (570 bp) of bovine *p35*.

Protein extraction and western blot analysis. Brain and muscle tissues were homogenized in lysis buffer (20 mM Tris, pH 7.6, 150 mM NaCl and 1 mM EDTA) with protease inhibitors. After centrifuging, the supernatant was saved as cytosolic fractions, and the insoluble pellet was re-extracted with the homogenization buffer supplemented with 0.25% NP40; the supernatant was saved as membrane fractions. For co-immunoprecipitation studies, 1.5 mg protein of membrane fraction was incubated with the corresponding antibody (2 μg) at 4°C overnight and then incubated with 40 μl of protein-G sepharose at 4°C for 1 h. The samples were washed and resuspended in 2× sample buffer. Western blot analysis was done as previously described[36].

Kinase assay. For Cdk5 kinase assay, the immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer. The *in vitro* kinase reaction was then done at 30°C for 30 min with kinase buffer containing 100 μM histone H1 peptide and 100 μM [γ-^{32}P] ATP as described[39]. Protein kinase A activity in C2C12 myotubes after 60 min treatment with NRG was measured using Peptag assay (Promega, Madison, Wisconsin). For assaying the *in vitro* kinase activity of ErbB2, ErbB2 immunoprecipitated from C2C12 myotubes was incubated with roscovitine for 30 min in 30 μl of kinase buffer at 30°C, in the presence of 250 μM [γ-^{32}P] ATP and 2 μg of myelin basic protein (MBP) as substrate.

In vitro phosphorylation assay. For the *in vitro* phosphoserine assay, ErbB3 immunoprecipitated from C2C12 myotubes was incubated with reconstituted Cdk5/p25 for 30 min at 30°C in 50 μl of kinase buffer, in the presence of 100 μM cold ATP. The phosphorylated protein was separated on a 6% SDS-PAGE and then blotted with a phosphoserine antibody. To assay the ability of different peptides to be phosphorylated by active Cdk5, two peptides of ErbB3 containing the potential phosphorylation sites were synthesized (Research Genetics, Huntsville, Alabama): SEAKTPIKWMAL (amino acids 871–874) and KRRGSPPRPPRP (1204–1207), denoted as ErbB3a and ErbB3b, respectively. Histone H1 peptide (PKTPKKAKKL, amino acids 9–18) served as the positive control. Two other peptides that are not potential Cdk5 substrates, muscle specific kinase (MuSK, SELLLDRLHPNPMYQ, amino acids 540–554) and MBP (QKRPSQRSKYL, amino acids 4–14) were used as negative controls. Peptide phosphorylating activity of the reconstituted Cdk5/p25 was measured. For the *in vitro* phosphorylation assay of ErbB receptors, ErbB2 immunoprecipitated from C2C12 myotubes was incubated with roscovitine for 30 min in 30 μl of kinase buffer in the presence of 250 μM cold ATP. The phosphorylated protein was separated on a 6% SDS-PAGE and then blotted with phosphotyrosine antibody or ErbB2 antibody.

Immunohistochemical analysis. The rat muscle sections (10 μm) were collected from various developmental stages and after nerve injury as previously described[40] and fixed with 2% paraformaldehyde/5% sucrose in PBS for 15 min at room temperature, washed and permeabilized with 0.4% Triton X-100. Double staining was performed as previously described[40]. The sections were then washed and analyzed using a Bio-Rad confocal scanning microscope.

Cdk5 antisense oligonucleotides. Antisense phosphorothioate oligonucleotide (S-modified) encoding the inverse complement of nucleotides +49/+63 (5′-TTCTCGTATTTCTGC-3′) of rat Cdk5 was used in this study[41]. The antisense and its corresponding sense oligonucleotides were purchased from Genset Oligos (La Jolla, California). C2C12 myotubes were treated with the oligonucleotides as previously described[42]. C2C12 cells were differentiated for 48 h before the addition of various concentrations (70–200 μM) of the oligonucleotides. Maximal inhibitory effect was observed at an oligonucleotide concentration of 140 μM.

Transfection studies and AChRε promoter–reporter gene constructs. COS-7 cells were transfected with the ErbB2, ErbB3 and p35 expression constructs using lipofectamine plus (GIBCO-BRL, Rockville, Maryland). Cells were collected 48 h after transfection to assay for the association of these proteins by immunoprecipitation. Similar transient transfection into C2C12 cells was also done using a dominant negative Cdk5 construct (where amino acid Asp 144 was changed to Asn 144)[43]. The transfection efficiency for C2C12 cells was estimated to be ~30%.

A fragment of AChRε promoter (416-nucleotide of the N-terminus)[44] was inserted upstream of a luciferase reporter vector, pGL3 (Promega). This construct [ε416-pGL3 (1 μg)] was transfected into C2C12 cells together with p35 cDNA expression construct subcloned in pMT21 (4 μg) and βgal-pCMV (0.5 μg) using calcium phosphate method. Twenty-four hours after transfection, C2C12 myoblasts were induced to differentiate by switching to 2% HS, incubated for 2 days, followed by luciferase and β-galactosidase assays. In addition to using the AChRε promoter, similar studies were done using the promoter for acetylcholinesterase (AChE). The luciferase assay was done using a kit purchased from Promega and the β-galactosidase activity was measured using a luminescent assay (Luminescent β-gal enzyme kit; Clontech, Palo Alto, California). Luciferase activity was normalized against β-galactosidase activity to correct for the variations in transfection efficiency.

For *in vivo* transfection studies, 10 μg of ε416-pGL3, 1 μg of βgal-pCMV and 40 μg of p35 cDNA construct or vector alone (pMT21), were injected into the tibialis anterior muscles of 3-week-old mice[44]. The injection volume was 10 μl, and the muscle was removed and homogenized in reporter lysis buffer (2 ml, Promega) one week after injection. The cell lysates were centrifuged and the supernatants were collected for luciferase and β-galactosidase assay.

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