

# Specific Agrin Isoforms Induce cAMP Response Element Binding Protein Phosphorylation in Hippocampal Neurons

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The synaptic basal lamina protein agrin is essential for the formation of neuromuscular junctions. Agrin mediates the postsynaptic clustering of acetylcholine receptors and regulates transcription in muscles. Agrin expression is not restricted to motor neurons but can be demonstrated throughout the CNS. The functional significance of agrin expression in neurons other than motor neurons is unknown. To test whether agrin triggers responses in neurons that lead to the activation of transcription factors, we have analyzed phosphorylation of the transcriptional regulatory site serine 133 of the transcription factor CREB (cAMP response element binding protein) in primary hippocampal neurons. Our results indicate that the neuronal (Ag4,8), but not the non-neuronal (Ag0,0), isoform of agrin

induces CREB phosphorylation in hippocampal neurons. The kinetics of agrin- and BDNF-induced CREB phosphorylation are similar: peak levels are reached in minutes and are strongly reduced 2 hr later. Neuronal responses to agrin require extracellular calcium, and, in contrast to tyrosine kinase inhibitors, the specific inhibition of protein kinase A (PKA) does not affect agrin-evoked CREB phosphorylation. Our results show that hippocampal neurons specifically respond to neuronal agrin in a  $Ca^{2+}$ -dependent manner and via the activation of tyrosine kinases.

**Key words:** agrin isoforms; CREB phosphorylation; hippocampal neurons; PKA; receptor tyrosine kinases; synapses; calcium

Many functional properties of synaptic transmission depend on the precise localization and stoichiometry of specialized molecules present on pre- and postsynaptic membranes. The high concentration of neurotransmitter receptors at postsynaptic sites is a general feature of synapses. The search for molecules able to regulate synaptic differentiation led to the discovery of agrin, an extracellular molecule capable of inducing the aggregation of acetylcholine receptors (AChR) on the surface of myotubes (McMahan, 1990; Burden, 1998). A critical role for agrin in the formation of neuromuscular junctions (NMJ) has been demonstrated *in vitro* and *in vivo*. Blocking the activity of neuronal agrin with specific antibodies in motor neuron/myotube cocultures prevents AChR aggregation at contact sites (Reist et al., 1992). Moreover, homologous recombination experiments revealed that agrin expression is essential for embryonic development and that NMJ do not form in homozygous agrin-deficient embryos (Gautam et al., 1996).

Agrin mediates AChR clustering in part via the activation of the receptor tyrosine kinase muscle-specific kinase (MuSK) (Jennings et al., 1993; Glass et al., 1996). MuSK phosphorylation in myotubes occurs rapidly after agrin addition, NMJ formation is similarly affected in MuSK-null mice and in agrin-deficient mice, and agrin does not induce AChR clustering in MuSK-deficient myotubes (DeChiara et al., 1996; Fuhrer et al., 1997; Hopf and Hoch, 1998).

The AChR-aggregating activity of agrin is regulated by alter-

native splicing. Splicing at one particular location of the agrin mRNA, referred to as the Z position, produces isoforms that differ by ~1000-fold in their ability to cluster AChR on myotubes *in vitro* (Ferns et al., 1992, 1993; Ruegg et al., 1992; Rupp et al., 1992). The alternative splicing of agrin mRNA is regulated during development and is tissue-specific (Hoch et al., 1993; O'Connor et al., 1994; Ma et al., 1995; Stone and Nikolics, 1995; N. Cohen et al., 1997). Highly active agrin isoforms are found exclusively in neurons, whereas non-neuronal tissues, including muscle, express isoforms with low AChR-aggregating activity (Hoch et al., 1993; Ma et al., 1993; O'Connor et al., 1994; Stone and Nikolics, 1995; N. Cohen et al., 1997). Besides motor neurons, agrin is expressed in many different types of neurons throughout the CNS, including CA1 and CA3 pyramidal cells in the hippocampus and granule cells in the dentate gyrus, where agrin expression is regulated during development and by neuronal activity (O'Connor et al., 1995; N. Cohen et al., 1997). Despite its widespread expression in the nervous system, the role of agrin in neurons remains undetermined. Preliminary anatomical examination of agrin isoform-deficient mice does not reveal any gross abnormalities, suggesting that defective agrin expression does not compromise early aspects of neural development such as neurogenesis and axon guidance (Gautam et al., 1996). Although NMJ are absent in agrin-mutated mice, it is not known whether agrin mediates synapse formation in the CNS. Along with its effect in synapse formation, it has been proposed that agrin may function as a neural adhesion molecule and as a stop signal for sensory and motor neurons (Campagna et al., 1995; Chang et al., 1997; Martin and Sanes, 1997).

Recently, it has been shown that agrin induces specific changes of gene expression in muscle. Muscle and neuronal agrin increase the expression of utrophin in cultured myotubes, and agrin regulates the expression of AChR subunits *in vivo* (Jones et al., 1996;

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I. Cohen et al., 1997; Meier et al., 1997, 1998; Gramolini et al., 1998). Defective transcription in muscle fibers also has been observed in agrin-deficient mice (Gautam et al., 1996). To determine whether agrin isoforms induce specific responses in neurons that lead to the activation of transcription factors, we have analyzed the phosphorylation of the cAMP response element binding protein (CREB) in primary hippocampal neurons. CREB is a transcription factor that regulates gene expression in many cell types, including neurons (Ginty, 1997; Tamai et al., 1997). Phosphorylation of CREB at serine 133 is required for CREB-mediated transcription (Ginty et al., 1993; Montminy, 1997). CREB can be phosphorylated via the activation of many kinases, including RSK2, a member of the pp90(RSK) family, protein kinase A (PKA), and  $\text{Ca}^{2+}$ -calmodulin-dependent kinase IV (CaMK IV) (Sheng et al., 1991; Ginty et al., 1994; Xing et al., 1996; Montminy, 1997). Therefore, CREB phosphorylation is considered an integrative component of the cellular response underlying a variety of physiological processes (Tamai et al., 1997). In hippocampal neurons, for example, CREB phosphorylation is induced in response to trophic factors and by patterns of activity that lead to changes of synaptic efficacy (Bito et al., 1996; Deisseroth et al., 1996; Martin and Kandel, 1996). Our data show that specific agrin isoforms are able to induce CREB phosphorylation in hippocampal neurons, that this response requires extracellular calcium, and that it is mediated by tyrosine kinases.

## MATERIALS AND METHODS

**Primary cell cultures.** Four to five hippocampi of 2- to 3-d-old Sprague Dawley rats were dissected rapidly (<40 min). CA1–CA3 regions were isolated, sliced into four to five small pieces each, washed once in Hank's buffer, and incubated for 3–5 min at room temperature with trypsin (3 mg/ml) (Sigma, St. Louis, MO) and DNase (0.6 mg/ml) (Sigma) in digestion solution [containing (in mM) 137 NaCl, 5 KCl, 7  $\text{Na}_2\text{HPO}_4$ , 25 HEPES, and 4  $\text{NaHCO}_3$ , pH 7.4]. Tissues were dissociated by gentle pipetting in Hank's buffer supplemented with 0.3% BSA, 12 mM  $\text{MgSO}_4$ , and 0.6 mg/ml DNase. Approximately 350,000 hippocampal neurons in 1 ml of Neurobasal medium (Life Technologies, Grand Island, NY) were cultured per well in 12-well plates precoated with poly-D-lysine. Arabino cytokine (5  $\mu\text{M}$ ; Sigma) was added on the second day of culture.

**Stimulation protocols.** Agrin-containing supernatant (conditioned media) were prepared as described (Ferns et al., 1993). The amounts of agrin isoforms in conditioned medium were normalized by Western blotting to standardized agrin quantities. Both the Ag0,0-conditioned and Ag4,8-conditioned media were used at 1:300 dilution, corresponding to ~50 pM agrin. Control medium from untransfected cells also was used at the same dilution. Ag4,8-conditioned medium (1:300) was tested for AChR clustering activity by using C2 myotubes as described (Ferns et al., 1993). Brain-derived neurotrophic factor (BDNF; kindly provided by Dr. David Ginty, Johns Hopkins University, Baltimore MD) was used at 40 ng/ml. Conditioned medium containing the 50 pM Ag4,0 isoform was tested also. Various conditioned media and BDNF were added to hippocampal cultures for 10 min. All pharmacological agents were added to the medium 20–30 min before stimulation. Cell morphology was evaluated after treatments; no alterations in processes and/or cell bodies were observed. TTX, CNQX, genistein, herbimycin A, forskolin, and Rp-8-cAMP were purchased from Calbiochem (San Diego, CA); MK801 was purchased from RBI (Natick, MA).

**Immunostaining.** At 10 min after the stimulation the cells were fixed in 4% paraformaldehyde for 30 min and processed for immunocytochemistry according to the ABC method, as previously described (Ji and Rupp, 1997). Briefly, cultures were incubated with anti-pCREB (Polyclonal antibody, 1:2000; Upstate Biotechnology, Lake Placid, NY) at 4°C overnight. Then the cells were incubated with biotinylated secondary antibody and subsequently with ABC complex. The reaction product finally was visualized with DAB/hydrogen peroxide in acetate buffer containing ammonium nickel sulfate. Synaptophysin staining occurred as follows: cells were fixed in 4% paraformaldehyde/4% sucrose for 30 min at 4°C. Fixed cells were permeabilized in 1% BSA/0.4% saponin (Sigma) for 1 hr at 4°C. Synaptophysin-specific monoclonal antibody SY38

(Boehringer Mannheim, Indianapolis, IN) and FITC-conjugated goat anti-mouse antibodies (Jackson Laboratories, Bar Harbor, ME) were used as suggested by the manufacturers.

**Western blot.** Hippocampal cultures were lysed after treatment in 100  $\mu\text{l}$  of boiling lysis buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 10% 2-mercaptoethanol, and 0.1% bromophenol blue) per well. Lysate (20  $\mu\text{l}$ ) was loaded onto SDS-PAGE gradient gel (4–15%, Bio-Rad Laboratories, Melville, NY) and transferred onto nitrocellulose filters. Filters were incubated with anti-pCREB antibodies (1:3000; Upstate Biotechnology) overnight at 4°C. pCREB-immunoreactive bands were visualized via the ECL detection kit (Amersham, Arlington Heights, IL) and exposed onto X-ray films (XAR-5, Kodak, Rochester, NY) for 5–20 min. Subsequently, the blots were submerged in stripping solution (62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol), incubated at 50°C for 30 min, and probed with anti-CREB antiserum (1:2000; New England Biolabs, Beverly, MA) to detect the total level of CREB expression.

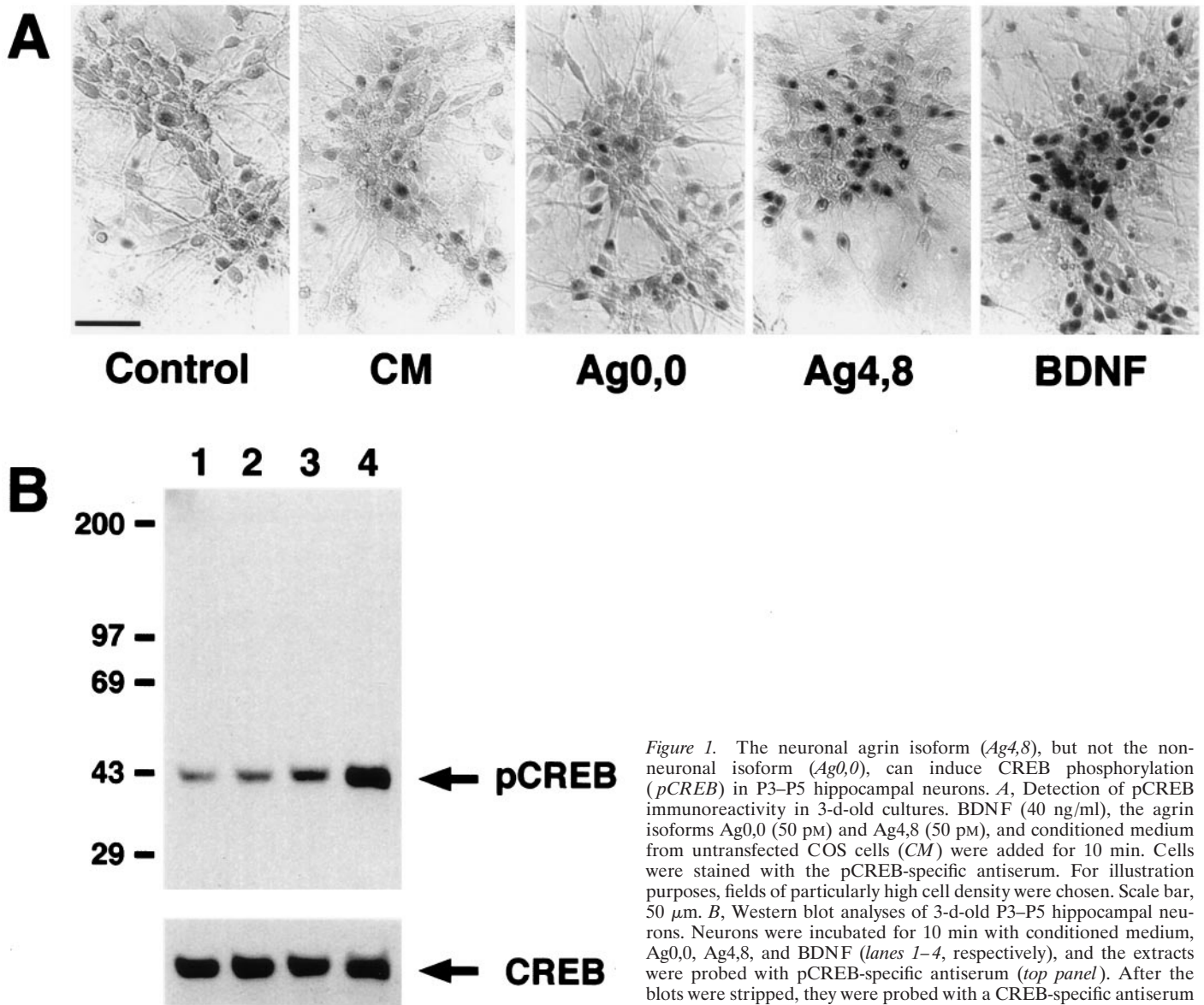
**Quantification.** pCREB-positive neurons were counted in each well. Counting was performed by a person unaware of the treatment protocol. Neurons with distinct nuclear staining, which were obviously different from the background, were counted as pCREB-positive. Five optic fields per 2.5 cm diameter well (one at the center and four at ~0.5 cm from the edge of the well) were selected for counting at 20 $\times$  magnification. The total number of neurons ranged from 70 to 230 per optic field. The percentage of pCREB-immunoreactive nuclei per optic field was calculated. The percentages of five fields were averaged as a mean, and four to six experiments in duplicate were included in each treatment. The data were assessed by an ANOVA test. The criterion for statistical significance was  $p < 0.05$ .

## RESULTS

### Agrin induces CREB phosphorylation in hippocampal neurons

The 3-d-old primary neurons obtained from postnatal day 3 (P3) rat hippocampi were exposed to conditioned media containing an equal amount (~50 pM) of neuronal (Ag4,8) and non-neuronal (Ag0,0) agrin isoforms. CREB phosphorylation at serine 133 (pCREB) was detected by using a specific polyclonal serum that recognizes CREB phosphorylated, but not CREB unphosphorylated (Ginty et al., 1993). pCREB immunoreactivity was analyzed by Western blotting with protein extracts prepared from hippocampal neurons incubated with agrin isoforms and compared with the response elicited by BDNF (40 ng/ml), a strong inducer of pCREB in neurons (Montminy, 1997). Exposure to Ag4,8 and BDNF, but not to Ag0,0 or conditioned medium from untransfected COS cells, induced an increase in CREB phosphorylation (Fig. 1*A,B*). To quantify this response more precisely, we determined the number of pCREB-immunoreactive cells. A significant increase in the percentage of cell nuclei immunoreactive for pCREB, as compared with conditioned medium, was detected in cells exposed to Ag4,8 and BDNF, but not to Ag0,0 (82%  $p < 0.01$ , 159%  $p < 0.01$ , and -1.4%  $p > 0.05$ , respectively) (Fig. 2*B*). The pCREB response to both Ag4,8 and BDNF is transient, reaches its maximum within 10 min, and has decreased significantly at 2 hr (Fig. 2*C*). A larger number of hippocampal neurons respond to BDNF than to the Ag4,8 isoform.

Hippocampal neurons express many types of agrin isoforms, and secreted full-length agrin preferentially associates with the plasma membrane (Campanelli et al., 1991; O'Connor et al., 1995; N. Cohen et al., 1997). In motor neurons, agrin is secreted at axon terminals where it is incorporated into the synaptic basal lamina (Magill-Solc and McMahan, 1990; Reist et al., 1992). In fully differentiated NMJ both  $\alpha$ -dystroglycan, the major agrin-binding protein in muscle, and MuSK, a signaling component of the agrin receptor, are concentrated at synaptic sites (Hall and Sanes, 1993). This suggests the possibility that synapse formation may lead to a synaptic concentration of agrin, agrin-binding



**Figure 1.** The neuronal agrin isoform (*Ag4,8*), but not the non-neuronal isoform (*Ag0,0*), can induce CREB phosphorylation (*pCREB*) in P3–P5 hippocampal neurons. **A**, Detection of *pCREB* immunoreactivity in 3-d-old cultures. BDNF (40 ng/ml), the agrin isoforms *Ag0,0* (50 pM) and *Ag4,8* (50 pM), and conditioned medium from untransfected COS cells (*CM*) were added for 10 min. Cells were stained with the *pCREB*-specific antiserum. For illustration purposes, fields of particularly high cell density were chosen. Scale bar, 50  $\mu$ m. **B**, Western blot analyses of 3-d-old P3–P5 hippocampal neurons. Neurons were incubated for 10 min with conditioned medium, *Ag0,0*, *Ag4,8*, and BDNF (*lanes 1–4*, respectively), and the extracts were probed with *pCREB*-specific antiserum (*top panel*). After the blots were stripped, they were probed with a CREB-specific antiserum to assess the total amount of CREB expression (*bottom panel*).

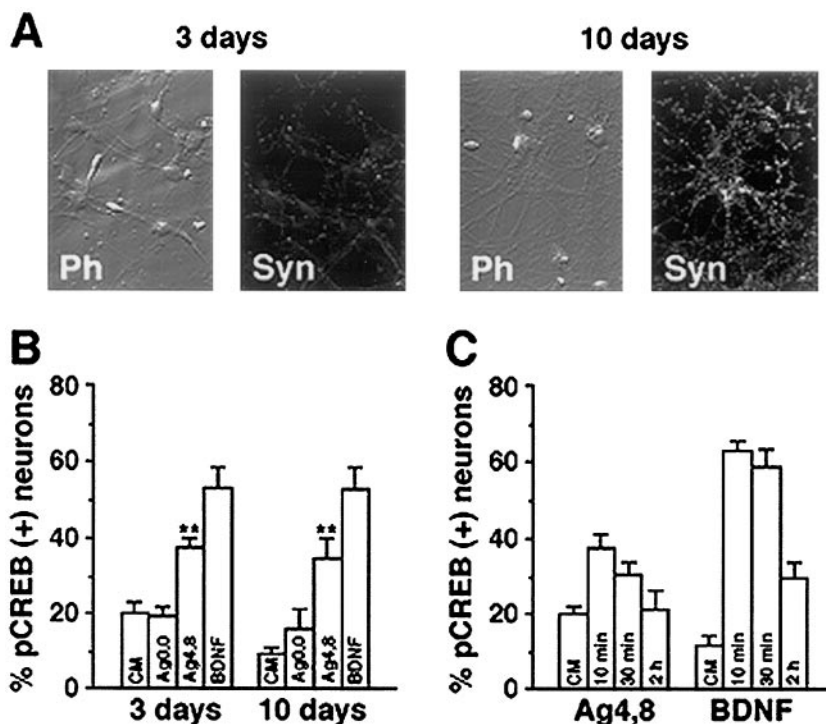
proteins, and putative neuronal agrin receptors. To determine whether synapse formation affects the neuronal response to agrin in hippocampal neurons, we analyzed *pCREB* induction in 10-d-old cultures. The formation of functional synapses in these cultures occurs after 6–7 d of plating (Banker and Goslin, 1988; Fletcher et al., 1994). Because of the different cellular densities used in these studies, we performed immunocytochemistry to determine the pattern of expression of the synaptic vesicle protein synaptophysin in 3- and 10-d-old cultures. The appearance of punctate synaptophysin immunoreactivity is used routinely as an indicator of synapse formation. Synaptophysin immunoreactivity in 3-d-old cultures predominantly is localized diffusely to the soma and throughout processes. A few immunoreactive puncta also can be detected. In contrast, in 10-d-old cultures the staining profile is different. The characteristic punctate pattern of synaptophysin immunoreactivity is evident on the soma and on neuritic processes (Fig. 2*A*). Similarly to neurons cultured for 3 d in which functional synaptic contacts are absent, a robust *pCREB* induction is observed with BDNF and *Ag4,8*, but not with *Ag0,0* and

control conditioned medium (Fig. 2*B*). The magnitude and the kinetics of the *Ag4,8*-induced and BDNF-induced CREB phosphorylation are similar among 3- and 10-d-old cultured neurons (data not shown). The 50 pM *Ag4,0* isoform did not induce significant *pCREB* both in 3- and 10-d-old neurons (1%  $p > 0.05$  and 0.9%  $p > 0.05$ , respectively, as compared with control conditioned medium;  $n = 3$ ). These data show that hippocampal neurons selectively respond to the same highly active agrin isoforms as muscle fibers and that synapse formation does not affect the neuronal responses to agrin.

#### Agrin-induced CREB phosphorylation depends on extracellular calcium

Having established the ability of the *Ag4,8* isoform to induce *pCREB* specifically in hippocampal neurons, we next examined the signaling pathways involved and whether this response is regulated similarly to the AChR clustering mediated by agrin at NMJ. The agrin-induced clustering of AChR on muscle fibers is dependent on extracellular  $Ca^{2+}$  (Henderson et al., 1984; Wal-





**Figure 2.** *A*, Profile of synaptophysin immunoreactivity in 3- and 10-d-old cultures. A shift from diffuse to punctate staining is observed between these two time points, indicating the absence and presence of synapses, respectively. *Ph*, Phase contrast, *Syn*, synaptophysin. *B*, Quantification of agrin-induced pCREB in 3- and 10-d-old cultures. The agrin isoform Ag4,8 and BDNF, but not Ag0,0, induce an increase in the percentage of pCREB-positive neurons both in 3- and 10-d-old cultures (\*\* $p < 0.01$  as compared with control medium, ANOVA;  $n = 4$ ). *C*, Agrin induces a transient increase in pCREB-immunoreactive cells. Incubation with Ag4,8 or BDNF was analyzed at 0, 10, 30, and 120 min, respectively. Both the Ag4,8 and BDNF responses reach a maximum within 10 min and are reduced within 2 hr. Despite the difference in magnitude, the BDNF- and Ag4,8-induced CREB phosphorylation essentially follows similar kinetics. *CM*, Control medium.

lace, 1988). To test the role of extracellular  $Ca^{2+}$  for the agrin-induced induction of pCREB, we treated 3-d-old hippocampal neurons with the calcium chelator EGTA (4 mM) for 20 min before agrin treatment. EGTA pretreatment completely blocked pCREB induction by Ag4,8 but did not affect the BDNF response (Fig. 3*A,B*). It has been shown previously that extracellular  $Ca^{2+}$  is not required for BDNF-induced CREB phosphorylation (Finkbeiner et al., 1997). Extracellular  $Ca^{2+}$  may be required for agrin binding to its receptor or may regulate CREB phosphorylation as a consequence of opening of  $Ca^{2+}$ -permeable ion channels after depolarization (Ginty, 1997). Agrin may cause depolarization by mediating an acute release of glutamate in the media. To distinguish among these possibilities, we stimulated 3-d-old hippocampal neurons with Ag4,8 in the presence of the  $Na^+$  channel blocker tetrodotoxin (TTX; 10  $\mu M$ ) or MK801 (10  $\mu M$ ) and CNQX (10  $\mu M$ ), which are specific antagonists of the NMDA and AMPA subfamilies of glutamate receptors, respectively. The presence of TTX or MK801 and CNQX in the medium partially reduces (21 and 23%, respectively) agrin-induced pCREB (Fig. 3*C*). In all conditions, however, agrin did produce a significant increase of pCREB-positive cells (38 and 43%, respectively). The magnitude of the agrin response was comparable when depolarization was prevented or glutamate receptors were blocked. The response to BDNF was not influenced by TTX or antagonists of glutamate receptors (Fig. 3*C*). These results demonstrate that the neuronal response to agrin requires extracellular  $Ca^{2+}$  and that a component of this response is partly attributable to the stimulation of glutamate release or increased receptor sensitization (see below).

#### Agrin does not activate the PKA signaling pathway

CREB phosphorylation can occur via the activation of cAMP-dependent PKA after the induction of adenylyl cyclase activity and the subsequent rise of intracellular cAMP (Montminy, 1997). To test whether agrin activates this signaling pathway, we used the cyclic nucleotide analog Rp-8-CPT-cAMP, a specific PKA

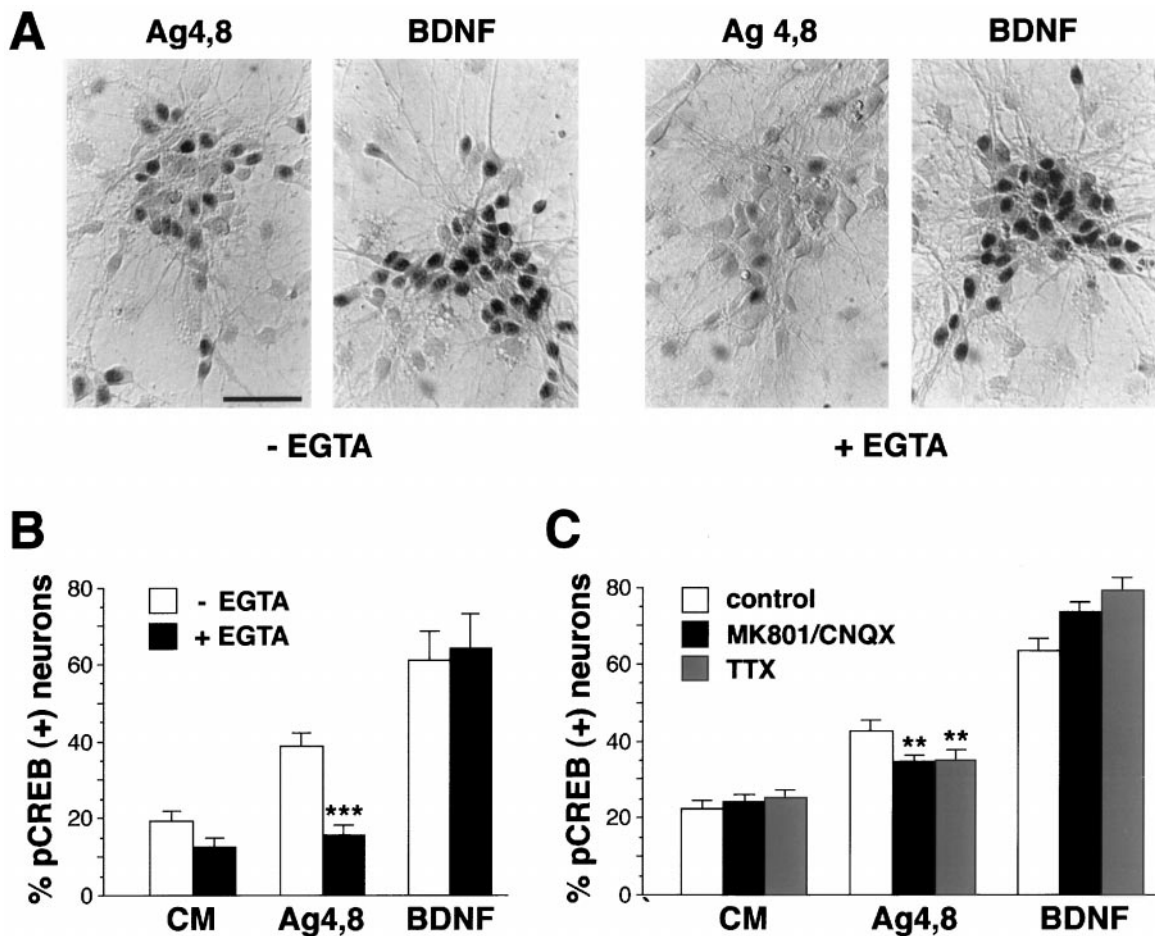
inhibitor, to test the involvement of PKA (Fig. 4). The efficiency of Rp-8-CPT-cAMP inhibition in 3-d-old hippocampal neurons was tested with forskolin, a PKA activator. As described previously, the application of forskolin (75  $\mu M$ ) induces CREB phosphorylation (Gonzalez and Montminy, 1989). This response is inhibited significantly (95%) by Rp-8-CPT-cAMP (50  $\mu M$ ). In contrast, the Ag 4,8-induced and BDNF-induced responses are not affected significantly by Rp-8-CPT-cAMP, suggesting that agrin-induced pCREB in hippocampal neurons does not result from the activation of PKA.

#### Agrin-induced CREB phosphorylation in neurons is regulated by tyrosine kinases

Activation of the receptor tyrosine kinases is required to induce AChR clustering at NMJ (Peng et al., 1993; Wallace, 1994; DeChiara et al., 1996; Ferns et al., 1996; Glass et al., 1996, 1997; Hopf and Hoch, 1998). To investigate the dependency of the agrin-induced phosphorylation of CREB on the activity of tyrosine kinases, we incubated 3-d-old hippocampal neurons with the specific protein tyrosine kinase inhibitors genistein and herbimycin A. Pretreatment (20 min) with either genistein (100  $\mu M$ ) or herbimycin A (1.5  $\mu M$ ) resulted in an inhibition of the Ag4,8 and BDNF-induced pCREB (Fig. 5). Genistein treatments equally affected the agrin- and the BDNF-induced responses by 71 and 69% inhibition, respectively. Herbimycin A was more effective in suppressing the agrin-induced pCREB (92%) than the BDNF-induced one (55%). No morphological changes could be observed in treated cells, suggesting that short-time exposure (30 min) with these inhibitors did not affect the hippocampal neurons adversely.

#### DISCUSSION

Agrin induces a response in hippocampal neurons that leads to the phosphorylation of the transcription factor CREB. Several parameters tested in this study indicate that the neuronal re-

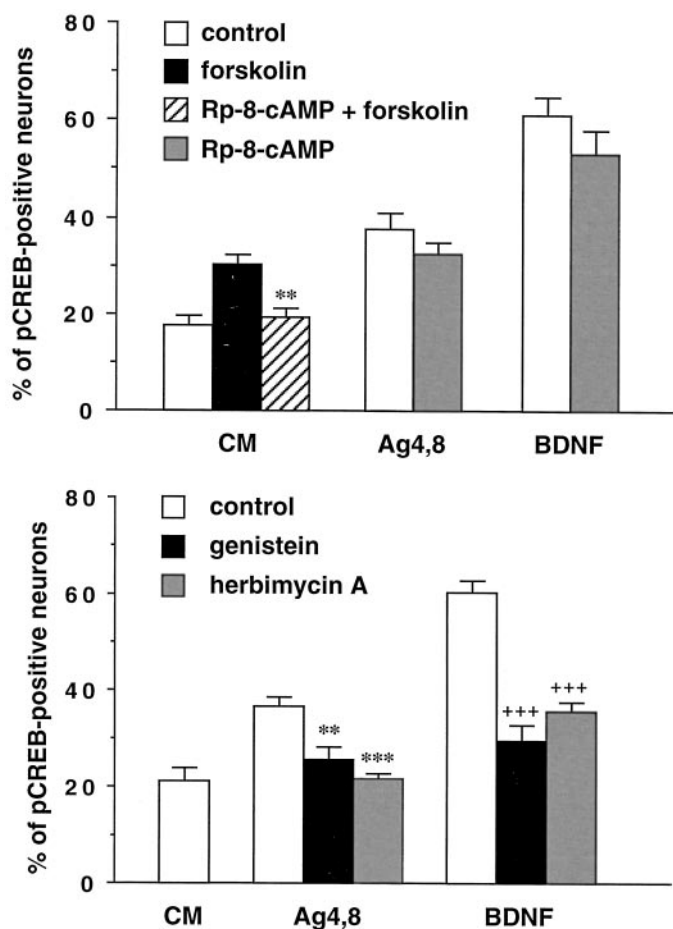


**Figure 3.** Agrin-induced pCREB in hippocampal neurons depends on extracellular  $Ca^{2+}$ . *A, B*, Agrin-induced pCREB is inhibited significantly by EGTA (4 mM). \*\*\* $p < 0.001$ , ANOVA ( $n = 4$ ), as compared with agrin response without EGTA. The BDNF-induced pCREB is not affected. *C*, Preventing depolarization and blocking glutamate receptors partially reduce agrin responses in hippocampal neurons. The pCREB-positive neurons induced by agrin in the presence of TTX or a combination of the NMDA receptor antagonist dizocilpine maleate (*MK-801*) (10  $\mu$ M) and the non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (*CNQX*) (10  $\mu$ M) are reduced by 21% ( $p < 0.05$ , ANOVA;  $n = 5$ ) and 23% ( $p < 0.05$ , ANOVA;  $n = 4$ ), respectively, when compared with the agrin response. However, a comparison between responses to agrin and conditioned medium in the presence of TTX or glutamate receptor antagonists still shows a significant increase in pCREB-positive cells of 38% (\*\* $p < 0.01$ , ANOVA;  $n = 5$ ) and 48% (\*\* $p < 0.01$ , ANOVA;  $n = 4$ ), respectively. Scale bar, 50  $\mu$ m.

response to agrin and the agrin-induced aggregation of the AChR at NMJ are regulated similarly. In particular, only the agrin isoforms with the highest AChR clustering activity (Ag4,8), but not the ones with low activity (Ag0,0), induce pCREB in hippocampal neurons. Another similarity is the role that tyrosine kinases play in mediating agrin responses in muscle and neurons. The role of the receptor tyrosine kinase MuSK in myotubes is well defined (Peng et al., 1993; Wallace, 1994; DeChiara et al., 1996; Ferns et al., 1996; Glass et al., 1996, 1997; Hopf and Hoch, 1998). One of the early events in AChR clustering is the binding of agrin to MuSK, which becomes phosphorylated at tyrosine residues within minutes of agrin addition (Glass et al., 1996). Other phosphorylation events downstream of the initial activation of MuSK also occur. Tyrosine phosphorylation of the  $\beta$ -subunit of the AChR is concomitant to AChR clustering, but it does not seem to be necessary for this process (Wallace et al., 1991; Swope et al., 1995; Ferns et al., 1996). We have demonstrated that, as in myotubes, the activation of tyrosine kinases is an essential step in the signaling cascade activated by agrin in neurons.

There is a similar requirement for extracellular  $Ca^{2+}$  for agrin

function in neurons and muscle. Extracellular  $Ca^{2+}$  is required for both agrin-induced CREB phosphorylation in hippocampal neurons and for the agrin-induced aggregation of AChR on myotubes. There are two possible explanations for the  $Ca^{2+}$  dependence in muscle.  $Ca^{2+}$  may influence the binding of agrin to its receptor; it has been reported that in the absence of  $Ca^{2+}$  agrin does not bind to the myotube surface (Nastuk et al., 1991; Ma et al., 1993; Gee et al., 1994). Alternatively, engagement of the agrin receptor may trigger a signaling cascade that leads to  $Ca^{2+}$  influx. Because the addition of neither the AChR antagonist  $\alpha$ -bungarotoxin nor the  $Na^+$  channel blocker tetrodotoxin affects agrin-induced AChR aggregation, neither AChR activation nor  $Na^+$  channel-dependent depolarization must be required for  $Ca^{2+}$  influx into muscle fibers (Anderson et al., 1977; Godfrey et al., 1988). A role for a transient rise of intracellular  $Ca^{2+}$  concentration has been proposed recently for agrin-induced AChR clustering (Mook-Jung and Gordon, 1995; Megeath and Fallon, 1998). However, the precise source responsible for these intracellular calcium transients remains undetermined. There are other possible roles for  $Ca^{2+}$  in the neural



**Figure 4. Top.** Agrin does not activate the PKA signaling pathway. The specific PKA inhibitor Rp-8-CPT-cAMP ( $50 \mu\text{M}$ ) does not affect the agrin- and the BDNF-induced pCREB. However, Rp-8-CPT-cAMP greatly inhibits (95%) pCREB induced by the PKA activator forskolin ( $75 \mu\text{M}$ );  $**p < 0.01$ , ANOVA;  $n = 5$ .

**Figure 5. Bottom.** Agrin-induced pCREB is inhibited significantly by tyrosine kinase inhibitors. The protein tyrosine kinase inhibitors genistein ( $100 \mu\text{M}$ ) and herbimycin A ( $1.5 \mu\text{M}$ ) greatly reduce the agrin- and the BDNF-induced CREB phosphorylation in day 3 hippocampal neurons.  $**p < 0.01$ ,  $***p < 0.001$ , ANOVA;  $n = 4$ , as compared with agrin induction.  $+++p < 0.001$ , ANOVA;  $n = 4$ , as compared with BDNF.

response to agrin. Again, a  $\text{Ca}^{2+}$  requirement for agrin binding to its receptor(s) or voltage-gated  $\text{Ca}^{2+}$  channels activation may be involved. In neurons, however, the addition of TTX or glutamate receptor antagonists reduces the magnitude of the agrin response even before the establishment of functional synapses. This suggests that a component of the neuronal response to agrin is attributable to acute release of glutamate and/or increased glutamate responsivity. Both cases would result in  $\text{Na}^+$  channel-dependent depolarization, which would activate voltage-gated  $\text{Ca}^{2+}$  channels. How could agrin stimulate neurotransmitter release? It has been reported that agrin promotes the differentiation of presynaptic specializations in motor neurons (Campagna et al., 1997). It is possible that an acute exposure to agrin may affect the proteins involved in synaptic vesicle release, thus favoring the fusion of vesicles that are docked either along, or in the proximity of, the plasma membrane of growth cones. Alternatively, agrin

may change the cellular responses to glutamate. This may occur via sensitization of glutamate receptors, possibly via post-translational modifications, or via stimulation of receptor insertion in the plasma membrane, as proposed for silent synapses (Malenka and Nicoll, 1997).

Our results show that the neuronal response to agrin is unaffected by synapse formation. Both the background levels and the magnitude of agrin-induced CREB phosphorylation are comparable in neurons before or after functional synapses are formed. This suggests that endogenous agrin does not saturate agrin receptors and that desensitization of the receptors occurs. Neuronal agrin receptors may aggregate partially at synaptic sites, or they may not aggregate at all. This also may explain the persistent neuronal response to exogenously applied agrin. Alternatively, agrin expression may decrease after synapse formation to levels that are no longer able to trigger a response. This hypothesis is consistent with results showing that agrin expression decreases at developmental times concomitant with synapse formation *in vivo* (Rupp et al., 1991; Thomas et al., 1993; Stone and Nikolics, 1995; N. Cohen et al., 1997; Li et al., 1997) and that a similar regulation of agrin expression is observed *in vitro* (Li et al., 1997). Last, agrin may not be required for synapse formation in hippocampal neurons but may function as an adhesion molecule. This hypothesis is supported by data showing that sensory and motor neurons adhere to agrin and that, in muscle, integrins also can function as an additional agrin receptor (Chang et al., 1997; Martin and Sanes, 1997). Whether agrin can induce the activation of receptors for adhesion molecules in hippocampal neurons has not yet been determined.

Finally, it has been shown that CREB phosphorylation in hippocampal neurons plays an important role in the regulation of gene expression, including the  $\text{Ca}^{2+}$ -dependent activation of transcription, which is thought to underlie long-term changes of synapse strength (Deisseroth et al., 1996; Ginty, 1997). Our data, along with results showing that agrin expression in neurons is regulated by synapse formation and by electrical activity (O'Connor et al., 1995; N. Cohen et al., 1997; Li et al., 1997), are consistent with the hypothesis that agrin may play a role in the regulation of synaptic efficacy. Taken together, our data support the hypothesis that hippocampal neurons and muscle fibers express agrin receptor(s) with the same specificity for agrin isoforms and that the receptor expressed in neurons is likely to be a novel member of the receptor tyrosine kinase family, because MuSK expression is confined to muscle and spleen (Jennings et al., 1993; Glass et al., 1996).

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