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# $\alpha 3\text{Na}^+/\text{K}^+$ -ATPase Is a Neuronal Receptor for Agrin

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## SUMMARY

Agrin, through its interaction with the receptor tyrosine kinase MuSK, mediates accumulation of acetylcholine receptors (AChR) at the developing neuromuscular junction. Agrin has also been implicated in several functions in brain. However, the mechanism by which agrin exerts its effects in neural tissue is unknown. Here we present biochemical evidence that agrin binds to the  $\alpha 3$  subunit of the  $\text{Na}^+/\text{K}^+$ -ATPase (NKA) in CNS neurons. Colocalization with agrin binding sites at synapses supports the hypothesis that the  $\alpha 3\text{NKA}$  is a neuronal agrin receptor. Agrin inhibition of  $\alpha 3\text{NKA}$  activity results in membrane depolarization and increased action potential frequency in cortical neurons in culture and acute slice. An agrin fragment that acts as a competitive antagonist depresses action potential frequency, showing that endogenous agrin regulates native  $\alpha 3\text{NKA}$  function. These data demonstrate that, through its interaction with the  $\alpha 3\text{NKA}$ , agrin regulates activity-dependent processes in neurons, providing a molecular framework for agrin action in the CNS.

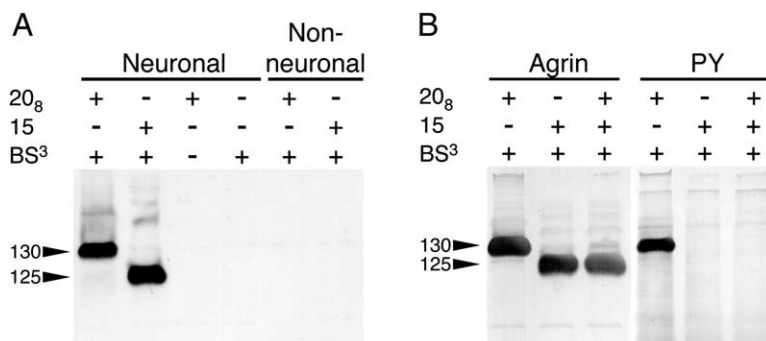
## INTRODUCTION

Agrin, a heparan sulfate proteoglycan, was originally isolated from the electric organs of marine rays based on its ability to induce the formation of high-density clusters of acetylcholine receptors (AChR) on the surface of cultured muscle cells (Nitkin et al., 1987). It is present at the earliest nerve-muscle contacts during development (Fallon et al., 1985) and, in mature muscles, is localized to the synaptic basal lamina that lies between the axon terminal and muscle fiber (Reist et al., 1987). Agrin is synthesized by motor neurons and antibodies against agrin block formation of motor neuron-induced clusters of AChR on cultured muscle cells (Reist et al., 1992). When expressed in muscle fibers in vivo, agrin induces formation of ectopic postsynap-

tic structures (Cohen et al., 1997a), whereas mutation of the *Agrn* gene blocks accumulation of AChR at developing neuromuscular junctions (Gautam et al., 1996). Thus, agrin is both sufficient and necessary for differentiation of the postsynaptic apparatus of the neuromuscular junction.

Much of what is known about agrin function at the neuromuscular junction has come from studies of the cell surface molecules with which it interacts. For example, a laminin binding domain at agrin's  $\text{NH}_2$  terminus anchors agrin to the basal lamina (Denzer et al., 1997), while binding to  $\alpha$ -dystroglycan provides a structural link to the muscle-fiber cytoskeleton that may stabilize the postsynaptic apparatus (Cote et al., 1999). It is the receptor tyrosine kinase MuSK, however, that is responsible for agrin-induced clustering of AChR. MuSK is concentrated in the postsynaptic muscle-fiber membrane (Valenzuela et al., 1995) and is rapidly phosphorylated in the presence of "active" agrin, which includes either one or two alternatively spliced exons at the z site ( $z^+$  agrin), but not the "inactive" isoform, which lacks a z site insert. Inhibition of MuSK phosphorylation blocks agrin-induced AChR clustering (Herbst and Burden, 2000), and mice lacking a functional *MuSK* gene display a phenotype similar to that of the agrin mutant (DeChiara et al., 1996).

Several lines of evidence suggest that agrin is also important for brain development. It is expressed by all populations of neurons in brain (O'Connor et al., 1994) and is concentrated at interneuronal synapses (Mann and Kröger, 1996; Hoover et al., 2003). Moreover, the highest levels of agrin expression in developing brain coincide with periods of synapse formation (Cohen et al., 1997b; Li et al., 1997). These observations suggest a function analogous to its role at the neuromuscular junction, and, consistent with this hypothesis, synapse formation between cultured hippocampal neurons is disrupted when either agrin expression or function is suppressed (Ferreira, 1999; Böse et al., 2000). However, the mechanism by which agrin exerts its effects in neurons must differ from that at the neuromuscular junction. First, MuSK expression is below detection in mammalian brain (Valenzuela et al., 1995). Second, mutation of the *Agrn* gene that blocks expression of  $z^+$  agrin and disrupts neuromuscular synapse formation has no effect on neuron-neuron synaptogenesis (Li et al., 1999; Serpinskaya et al., 1999).



**Figure 1. Agrin Binds to and Induces Tyrosine Phosphorylation of a ~110 kDa Protein on Neuron Surface Membranes**

(A) Immunoblot of cultured cortical cells cross-linked with BS<sup>3</sup> in the presence of either C-Ag20<sub>8</sub> or C-Ag15 probed with an antibody to a myc tag on the agrin fragments, showing agrin adducts of 130 and 125 kDa in neurons but not nonneuronal cells.

(B) Agrin adducts were immunoprecipitated with either an agrin antiserum (Agrin) or an anti-phosphotyrosine monoclonal antibody (PY) and then analyzed by immunoblotting for the myc tag. Crosslinking to C-Ag20<sub>8</sub> or C-Ag15 alone results in the appearance of the appropriately sized bands, but only the 125 kDa band was present when C-Ag20<sub>8</sub> was cross-linked in the presence of an excess of C-Ag15. Phosphorylation of the crosslinked complex is induced by C-Ag20<sub>8</sub> but not C-Ag15. Consistent with the competition studies, C-Ag20<sub>8</sub>-induced phosphorylation of the agrin adduct is blocked by C-Ag15.

However, agrin mutant neurons are resistant to excitotoxic injury, and heterozygous agrin mutant mice are less sensitive to systemic treatment with kainic acid (Hilgenberg et al., 2002), consistent with agrin regulating some aspect of neuronal activity.

Molecular identification of the receptor that mediates agrin's effects in neurons would greatly enhance our understanding of agrin function in the CNS. Evidence that such a receptor exists comes from biochemical studies showing that agrin induces expression of *c-fos* in cortical and other CNS neurons (Hilgenberg et al., 1999). Interestingly, signaling by the neuronal agrin receptor shares some similarity with agrin-induced AChR clustering in muscle, most notably its Ca<sup>2+</sup> dependence and sensitivity to inhibition of tyrosine kinase activity. However, in contrast to AChR clustering, where only z<sup>+</sup> agrin is active, z<sup>+</sup> and z<sup>-</sup> agrin isoforms are equally potent agonists of the neuronal agrin receptor (Hilgenberg et al., 1999; Hilgenberg and Smith, 2004), evidence that the neuronal receptor is distinct from MuSK. Activation of the agrin receptor leads to a rapid increase in intracellular Ca<sup>2+</sup>, which serves as the initiating event for many of agrin's effects on neurons (Hilgenberg and Smith, 2004). Consistent with a function regulating neuronal activity, recent studies using minimal fragments of agrin as affinity probes have shown that a receptor for agrin is concentrated at synaptic sites (Hoover et al., 2003). Here we show that this neuronal agrin receptor is a member of the Na<sup>+</sup>/K<sup>+</sup>-ATPase (NKA) family of ion pumps and that agrin binding inhibits pump activity, modulating membrane potential and action potential frequencies of cortical neurons in vitro and in vivo.

## RESULTS

### Agrin Binds the $\alpha$ 3NKA on Cortical Neurons

Biochemical and Ca<sup>2+</sup> imaging studies have provided evidence for an agrin receptor in cortical and other CNS

neurons, distinct from the MuSK/MASC receptor complex responsible for agrin signaling in skeletal muscle (Hilgenberg et al., 1999; Hilgenberg and Smith, 2004). Recently, we identified a minimal COOH-terminal region of agrin, C-Ag20, sufficient to activate the neuronal receptor and a smaller fragment, C-Ag15, which acts as an agrin antagonist (Hoover et al., 2003). As a first step toward identifying the binding site responsible for agrin activity in neurons, we used the membrane-impermeant bifunctional reagent bis[sulfosuccinimidyl] suberate (BS<sup>3</sup>) to chemically cross-link agrin fragments to components present on the surface of cells cultured from cerebral cortex. Cell membranes were then isolated and analyzed by immunoblotting with a monoclonal antibody (9E10.2, Invitrogen) against a COOH-terminal myc tag on the recombinant agrin fragments. Crosslinking C-Ag20<sub>8</sub> or C-Ag15 to cortical neurons resulted in the appearance of clear anti-myc immunoreactive bands with apparent molecular weights of ~130 and 125 kDa, respectively, much larger than the expected mass of the agrin fragments (Figure 1A). Agrin neither binds to nor activates nonneuronal cells (Hilgenberg et al., 1999; Hoover et al., 2003; Hilgenberg and Smith, 2004). Consistent with this observation, no specific labeling with the myc antibody was observed in blots of non-neuronal cells (Figure 1A). Similar results were obtained with a second crosslinking agent, dimethyl adipimidate (DMA), whose reactive groups are more closely spaced than BS<sup>3</sup> (8.6 Å versus 11.4 Å; data not shown). Taking into account the mass of the agrin fragments and assuming a 1:1 stoichiometry, the results suggest that agrin associates with a single class of sites, with an apparent molecular weight of ~110 kDa, present on neuron cell membranes.

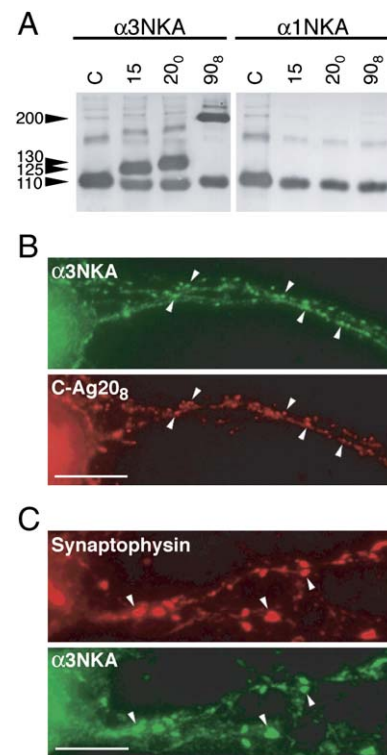
Ligand-induced phosphorylation is a common first step in membrane receptor activation, and inhibition of tyrosine kinase activity blocks agrin signaling in CNS neurons (Hilgenberg et al., 1999; Hilgenberg and Smith, 2004). To

determine whether agrin induces phosphorylation of the putative agrin receptor, membranes from neurons cross-linked to C-Ag20<sub>8</sub> or C-Ag15, either alone or in combination, were dissolved in a detergent-containing buffer, and aliquots were immunoprecipitated with either an agrin antiserum (Hoover et al., 2003) or anti-phosphotyrosine monoclonal antibody (mAb4G10; Upstate). Immunoprecipitated proteins were analyzed by immunoblotting for the COOH-terminal myc tag on the agrin fragments. In line with our initial results, anti-agrin immunoprecipitates probed for myc tagged C-Ag20<sub>8</sub> or C-Ag15 revealed two adducts of the expected molecular weight (Figure 1B). However, only the 130 kDa band crosslinked to C-Ag20<sub>8</sub> was phosphorylated. Even when used at a 10-fold higher concentration than C-Ag20<sub>8</sub>, C-Ag15 did not induce phosphorylation of the crosslinked complex. C-Ag15 was, however, an effective inhibitor of C-Ag20<sub>8</sub>, blocking both binding and phosphorylation by the larger agrin fragment (Figure 1B), consistent with its ability to antagonize agrin signaling.

The properties of the agrin adducts suggest that they represent a complex of an agrin fragment and a receptor that mediates responses to agrin in CNS neurons (Hilgenberg et al., 1999, 2002; Hoover et al., 2003; Hilgenberg and Smith, 2004). To determine the molecular identity of this putative agrin receptor, C-Ag20<sub>8</sub> adducts, crosslinked with either BS<sup>3</sup> or DMA, were affinity purified, and the identity of the component proteins was determined by mass spectrometry of their tryptic digests (Proteomic Research Services, Inc.). In addition to the expected peptide sequences for agrin, 4 to 12 peptides were present in each sample that matched the  $\alpha$ 3 subunit of the NKA. Similar results were also obtained when C-Ag90<sub>8</sub> (R&D Systems), a larger COOH-terminal fragment more commonly used in studies of agrin function, was used in place of C-Ag20<sub>8</sub>. Combined, the data for the three samples represented 17% coverage of the  $\alpha$ 3 subunit amino acid sequence overall.

To confirm the results of the mass spectrometry, we tested the ability of different NKA  $\alpha$  subunit antibodies to recognize the putative agrin- $\alpha$ 3NKA complex. When probed with an anti- $\alpha$ 3 subunit monoclonal antibody (XVIF9-G10; Novus Biologicals), immunoblots of cultured cortical neurons treated with BS<sup>3</sup> alone contained a major 110 kDa band corresponding to the  $\alpha$ 3 subunit (Figure 2A). Crosslinking in the presence of C-Ag15, C-Ag20<sub>8</sub>, or C-Ag90<sub>8</sub> resulted in  $\alpha$ 3-positive bands at 125, 130, and 200 kDa, respectively, consistent with agrin binding to the  $\alpha$ 3 subunit of the NKA. No molecular-weight shift was apparent when the same cell extracts were probed with an antibody to the closely related  $\alpha$ 1NKA (9A-5; Sigma), showing that agrin binding is specific for the  $\alpha$ 3 subunit (Figure 2A).

Previous studies have shown that the  $\alpha$ 3NKA is distributed in a nonuniform fashion over the soma and processes of cultured hippocampal neurons (Juhászova and Blaustein, 1997), reminiscent of the pattern of labeling observed using short agrin fragments as histochemical probes for



**Figure 2. Agrin Binds Specifically to the  $\alpha$ 3NKA on CNS Neurons**

(A) Blots of naive control cultured cortical neurons (C) and neurons crosslinked to the indicated agrin fragments (C-Ag15, C-Ag20<sub>8</sub>, and C-Ag90<sub>8</sub>) were probed with monoclonal antibodies against either the  $\alpha$ 3- or  $\alpha$ 1NKA. Only the  $\alpha$ 3NKA shows the predicted increases in molecular weight.

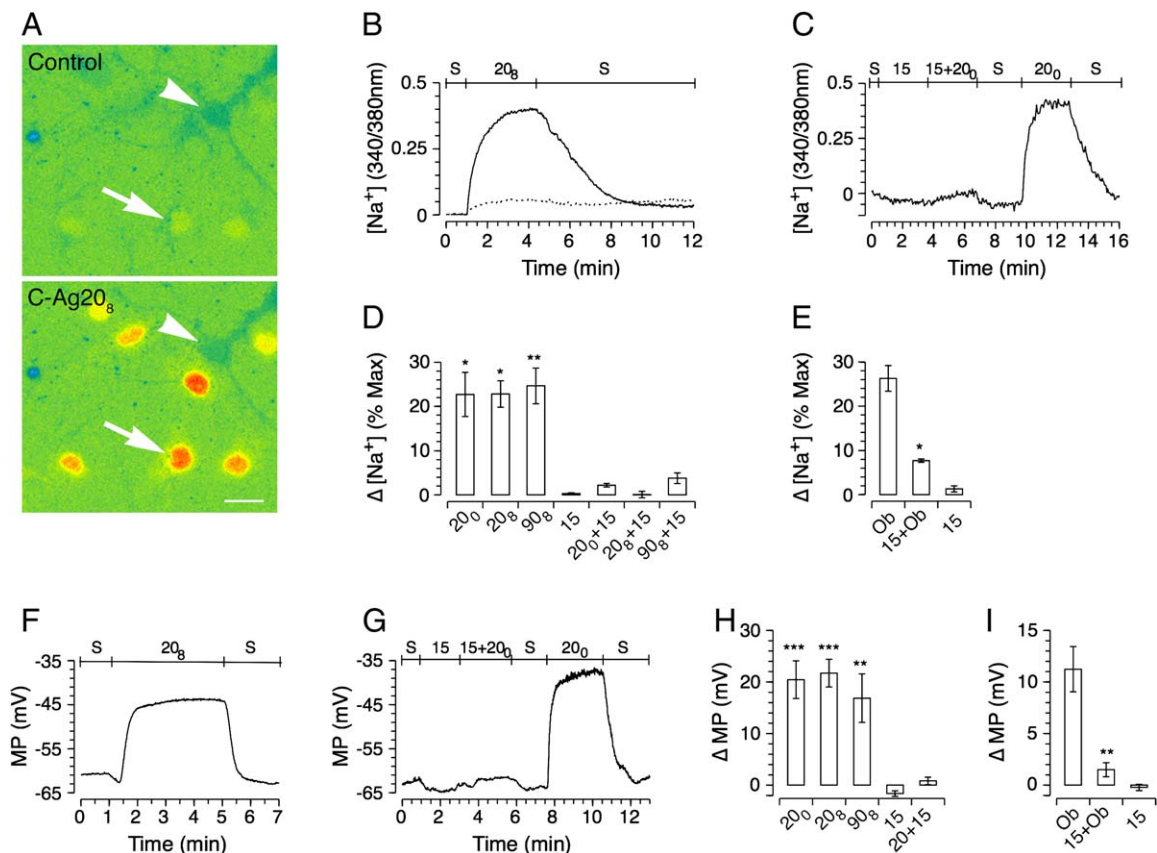
(B) Cortical neurons were double labeled for  $\alpha$ 3NKA and C-Ag20<sub>8</sub> binding sites. Consistent with the biochemical analysis, agrin binding sites and  $\alpha$ 3NKA are colocalized, appearing as small puncta distributed over the surface of the neuron soma and neurites.

(C) Double labeling with synaptophysin reveals  $\alpha$ 3NKA concentrated at synapses. Scale bars = 10  $\mu$ m.

agrin receptors on cultured cortical neurons (Hoover et al., 2003). Double labeling with the  $\alpha$ 3 subunit monoclonal antibody and C-Ag20<sub>8</sub> revealed extensive overlap between the  $\alpha$ 3NKA and agrin binding sites on cultured cortical neurons (Figure 2B). Consistent with our earlier studies (Hoover et al., 2003), double labeling for synaptophysin and the  $\alpha$ 3NKA shows agrin receptors diffusely distributed over the neuronal soma but concentrated at synapses (Figure 2C). Together with the results of the biochemical studies, these observations provide strong evidence that the  $\alpha$ 3NKA is a neuronal receptor for agrin.

#### Agrin Inhibits Activity of the $\alpha$ 3NKA

NKAs are heteromeric proteins composed of  $\alpha$  and  $\beta$  subunits. Multiple isoforms of each subunit are encoded by different genes that exhibit cell-specific patterns of expression. Expression of the  $\alpha$ 3 subunit in the CNS is neuron specific (Kaplan, 2002). Neurons but not nonneuronal



**Figure 3. Agrin Inhibits  $\alpha 3$ NKA Function**

(A) Pseudocolor images of cells loaded with the  $\text{Na}^+$ -sensitive dye SBFI-AM, before (Control) and 90 s after exposure to C-Ag20<sub>8</sub>.  $\text{Na}^+$  levels increase in the neurons (arrows), but not in nonneuronal cells (arrowheads), following agrin treatment. Scale bar = 20  $\mu\text{m}$ .  
 (B) Treatment with C-Ag20<sub>8</sub> triggers a rapid increase in neuronal intracellular  $\text{Na}^+$  (solid line, arrow in [A]) that returns to initial resting level upon being washed in normal saline solution (S). The small response in the nonneuronal cell (broken line, arrowhead in [A]) is due to fine neurites traversing the sampled region.  
 (C) Neuronal  $\text{Na}^+$  levels are unchanged following treatment with C-Ag15 alone, but C-Ag15 blocks the large increase induced by C-Ag20<sub>8</sub>.  
 (D) Treatment with a saturating concentration of either C-Ag20 isoform or C-Ag90<sub>8</sub> resulted in a significant increase in intracellular  $\text{Na}^+$  concentration, expressed as a percent of the maximal response to gramicidin, that could be blocked by coincubation with C-Ag15.  
 (E) The increase in neuronal  $\text{Na}^+$  levels induced by 3  $\mu\text{M}$  ouabain was significantly reduced by coincubation with C-Ag15.  
 (F) Whole-cell current-clamp record showing reversible membrane depolarization produced in a neuron by treatment with C-Ag20<sub>8</sub>.  
 (G) Treatment with C-Ag15 resulted in a small hyperpolarization and blocked the change in membrane potential induced by C-Ag20<sub>8</sub>.  
 (H) Treatment with "active" fragments of agrin causes membrane depolarization, whereas the membrane potentials of cells exposed to C-Ag20<sub>0</sub> or C-Ag20<sub>8</sub> in the presence of C-Ag15 were indistinguishable from their normal resting membrane potentials obtained before treatment (data for C-Ag20<sub>0</sub> and C-Ag20<sub>8</sub> were not different and have been pooled).  
 (I) Ouabain-induced neuron membrane depolarization is also blocked by C-Ag15. Bars show mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; paired Student's  $t$  test.

cells respond to treatment with agrin, suggesting a role for agrin in modulating the function of  $\alpha 3$  subunit-containing  $\text{Na}^+/\text{K}^+$  pumps. To test this hypothesis,  $\text{Na}^+$  imaging was used to determine the effect of agrin on cytoplasmic  $\text{Na}^+$  levels in cultured cortical cells.

Treatment with C-Ag20<sub>8</sub>, in the presence of tetrodotoxin (TTX; 1  $\mu\text{M}$ ), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10  $\mu\text{M}$ ), DL-2-amino-5-phosphonovaleric acid (APV; 50  $\mu\text{M}$ ), bicuculline methochloride (BMC; 10  $\mu\text{M}$ ), and d-tubocurarine (dTbC; 100  $\mu\text{M}$ ), to block action potentials and synaptic transmission caused a rapid increase in neuronal

cytoplasmic  $\text{Na}^+$ . The response to agrin was reversible and cell specific in that nonneuronal cells were unaffected by the treatment (Figures 3A and 3B). Consistent with its ability to bind but not activate the receptor, C-Ag15 alone had no significant effect on resting  $\text{Na}^+$  levels but blocked the increase induced by the larger agrin fragments (Figure 3C). Quantitative comparison of the effects of different agrin fragments showed that treatment with either of the alternatively spliced C-Ag20 isoforms or C-Ag90<sub>8</sub> resulted in a significant increase in neuronal intracellular  $\text{Na}^+$  (Figure 3D). In contrast, C-Ag15 alone had no effect on



resting  $\text{Na}^+$  levels but blocked the increase normally induced by each of the active agrin fragments.

Cardiac glycosides, such as ouabain, specifically inhibit NKA activity by binding to determinants present on the extracellular surface of the  $\alpha$  subunit (Kaplan, 2002). The short latency of the agrin response ( $t_{1/2}$  to peak =  $19.7 \pm 2.4$  s) suggested that agrin might be inhibiting the  $\alpha 3\text{NKA}$  in a similar manner. Supporting this hypothesis, treatment with a low concentration of ouabain to inhibit the high-affinity  $\alpha 3\text{NKA}$  resulted in an increase in neuronal  $\text{Na}^+$  levels similar to that seen following treatment with active agrin fragments (Figure 3E). Coincubation with C-Ag15 at the same concentration that blocked binding of the active agrin fragments also blocked the ouabain-induced increase in neuronal  $\text{Na}^+$ , providing strong evidence that agrin-induced inhibition of the  $\alpha 3\text{NKA}$  is mediated by binding directly to the  $\alpha 3$  subunit.

The NKA expels three intracellular  $\text{Na}^+$  ions for every two  $\text{K}^+$  ions taken up, directly affecting the membrane potential of all animal cells. In line with the results of the  $\text{Na}^+$  imaging, whole-cell current-clamp measurements showed that agrin treatment, in the presence of drugs to block action potentials and synaptic transmission, causes a rapid and reversible depolarization of cultured cortical neurons (Figures 3F and 3H). Moreover, C-Ag15 was also effective in blocking depolarization induced by the active agrin fragments (Figures 3G and 3H). Interestingly, treatment with C-Ag15 resulted in a small hyperpolarization ( $-1.0 \pm 0.4$  mV,  $p < 0.05$ ; Figures 3G and 3H), suggesting displacement of endogenous agrin. Consistent with the results of the  $\text{Na}^+$  imaging, ouabain-induced membrane depolarization was blocked by C-Ag15 (Figure 3I), confirming that agrin's effect on neuron membrane potential is mediated by its interaction with the  $\alpha 3\text{Na}^+/\text{K}^+$  pump.

#### Expression of the $\alpha 3$ Subunit of the NKA Is Sufficient for Agrin Binding and Agrin-Evoked Responses

Studies of MuSK, the receptor in skeletal muscle responsible for agrin-induced clustering of AChR, have shown that agrin-MuSK interaction requires a yet to be identified accessory component expressed only in muscle called MASC (Glass et al., 1996). To learn whether agrin interaction with the  $\alpha 3\text{NKA}$  might exhibit a similar dependency on cell context, we examined the properties of nonneuronal cells transiently transfected with pRc $\alpha 3$ , a plasmid expressing the rat  $\alpha 3$  subunit under control of the CMV promoter.

Immunostaining with an antibody to the  $\alpha 3\text{NKA}$  showed that nonneuronal cells transfected with pRc $\alpha 3$ , but not cells transfected with the enhanced green fluorescent protein marker plasmid pEGFP-C1 alone, expressed the  $\alpha 3$  subunit (data not shown). In line with these findings, agrin binding was only observed on the surface membranes of nonneuronal cells expressing pRc $\alpha 3$  (Figure 4A). Immunoblots of agrin fragments crosslinked to nonneuronal cells confirmed the interaction with the  $\alpha 3$  subunit expressed from the transfected plasmid (Figure 4B). Thus, expres-

sion of the  $\alpha 3$  subunit of the NKA is sufficient for agrin binding and is independent of cell context.

We next examined the physiological responses of nonneuronal cells transfected with pRc $\alpha 3$  to agrin. Treatment of nonneuronal cells expressing the  $\alpha 3$  subunit with either C-Ag20 isoform or C-Ag90<sub>8</sub> triggered a rapid increase in the concentration of intracellular  $\text{Na}^+$  ions that was not evident in normal cells or cells transfected with pEGFP-C1 alone (Figures 4C and 4D). Consistent with our earlier results,  $\text{Na}^+$  levels were unaffected by treatment with C-Ag15 alone, although C-Ag15 proved to be an effective antagonist for the active agrin fragments (Figure 4D).

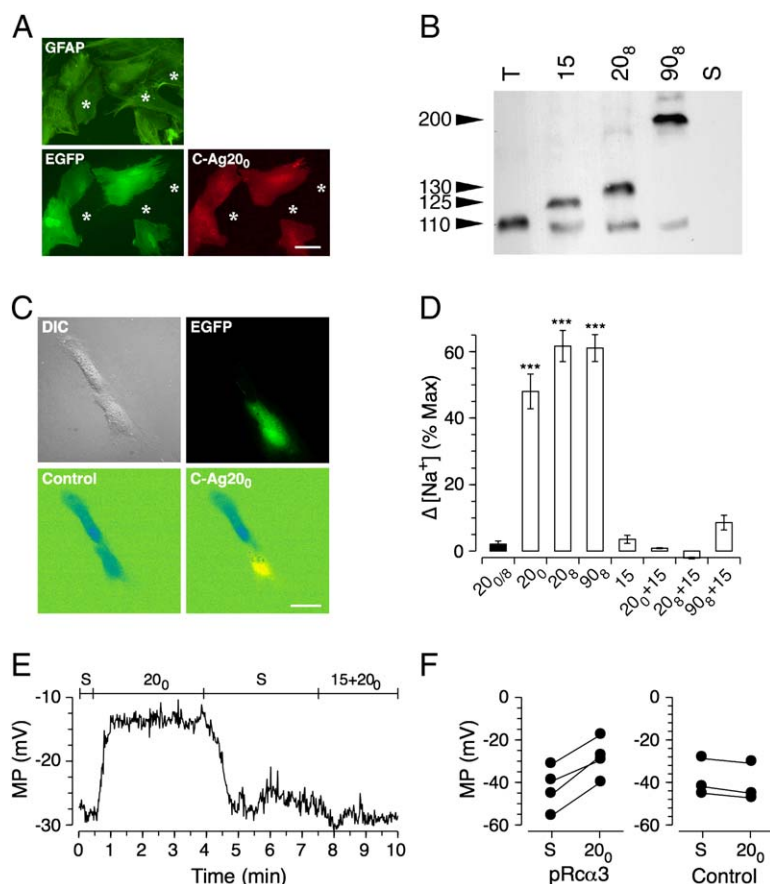
Parallel observations were obtained when whole-cell current-clamp measurements were made to examine the effects of agrin on the electrophysiological properties of nonneuronal cells expressing the  $\alpha 3$  construct (Figures 4E and 4F). The mean resting potential of nonneuronal cells was higher and more variable than in neurons. Nevertheless, nonneuronal cells expressing the rat  $\alpha 3\text{NKA}$  subunit were reversibly depolarized ( $14.1 \pm 1.7$  mV) by treatment with either of the C-Ag20 fragments. In contrast, no change in the membrane potential was evident upon agrin treatment of nontransfected nonneuronal cells or cells transfected with pEGFP-C1 alone (Figure 4F), indicating that the response to agrin was specific for the pRc $\alpha 3$  construct. Taken together, these findings provide strong evidence that the  $\alpha 3$  subunit of the NKA is the receptor responsible for agrin's effects in cortical neurons.

#### Agrin- $\alpha 3\text{NKA}$ Interactions Regulate Neuronal Activity In Situ

The electrogenic activity of the NKA and its role in maintaining gradients of counter-ions necessary for the function of other transport proteins suggest that changes in  $\alpha 3\text{NKA}$  activity will have profound effects on neuronal function and excitability. To test this hypothesis, we examined the effects of different agrin fragments on the firing properties of cultured cortical neurons bathed in normal external solution.

In line with our earlier observations, neurons were rapidly depolarized by treatment with C-Ag20<sub>0</sub>. However, in the absence of TTX and neurotransmitter receptor antagonists, the agrin-induced depolarization was accompanied by a significant and reversible increase in the frequency of spontaneous action potentials (Figures 5A and 5B). Similar results were also obtained when neurons were exposed to C-Ag20<sub>8</sub>. Coincubation with C-Ag15 blocked both the depolarization and increase in action potential frequency induced by either C-Ag20 isoform (data not shown).

The response to exogenously applied agrin prompted us to ask whether the basal level of activity normally present in cultured neurons might be dependent upon endogenous agrin- $\alpha 3\text{NKA}$  interactions. To address this question, we tested the effects of C-Ag15 on spontaneous action potentials in cultured cortical neurons (Figures 5C and 5D). In contrast to C-Ag20, C-Ag15 inhibited spontaneous activity in cortical neurons. The effect of C-Ag15



**Figure 4. Expression of the  $\alpha 3$  Subunit of the NKA in Nonneuronal Cells Confers Binding and Functional Response to Agrin**

(A) Transfected and nontransfected cells were visualized by postlabeling for GFAP. Only EGFP-positive cells cotransfected with pEGFP-C1 and pRc $\alpha 3$  bind agrin (C-Ag20<sub>0</sub>). Asterisks indicate nontransfected cells. Scale bar = 10  $\mu$ m.

(B) An immunoblot probed with an antibody to the  $\alpha 3$ NKA. The  $\alpha 3$  subunit is expressed in transfected (T) but not sham-transfected (S) control cells and can be crosslinked to agrin.

(C) The DIC image shows a pair of cells in which only the lower cell has been transfected with pRc $\alpha 3$ , indicated by expression of EGFP. The pseudocolor images show that, compared to saline control, treatment with C-Ag20<sub>0</sub> results in a marked increase in intracellular Na<sup>+</sup> concentration in the transfected but not the nontransfected cell. Scale bar = 10  $\mu$ m.

(D) Bar chart shows mean response to different agrin fragments of nonneuronal cells transfected with either pEGFP-C1 alone (filled bar) or in combination with pRc $\alpha 3$ . Responses of pEGFP-C1-transfected cells to C-Ag20<sub>0</sub> and C-Ag20<sub>8</sub> have been pooled. Bars show mean  $\pm$  SEM. \*\*\* $p$  < 0.001; ANOVA with Bonferroni post hoc comparison to pEGFP-C1 control.

(E) pRc $\alpha 3$  expression in nonneuronal cells confers an agrin-inducible reversible membrane depolarization that can be blocked by C-Ag15. (F) Nonneuronal cells expressing the  $\alpha 3$  subunit of the NKA but not control (nontransfected or pEGFP-C1-transfected) cells are consistently depolarized ( $p$  < 0.001, paired Student's  $t$  test) by treatment with agrin.

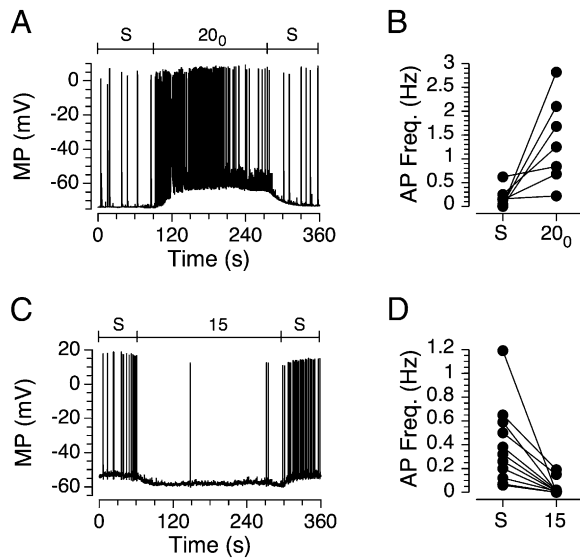
was reversible in that the frequency of spontaneous action potentials returned to basal levels upon washing with normal external solution (Figure 5C).

A simple explanation for the effect of C-Ag15 on neuronal activity is that some  $\alpha 3$ NKAs are normally inhibited by native agrin; competition by C-Ag15 removes this inhibition, decreasing the probability of firing. Consistent with this hypothesis, pull-down experiments on detergent extracts of cultured neurons crosslinked with BS<sup>3</sup> revealed a protein complex with an apparent molecular weight of  $\geq 300$  kDa recognized by both agrin and  $\alpha 3$ NKA antibodies (Figure 6A). Moreover, formation of the endogenous agrin- $\alpha 3$ NKA complex was blocked by crosslinking in the presence of C-Ag15, providing evidence that endogenous agrin can be displaced from its receptor by C-Ag15. Omission of the crosslinking step in control cultures resulted in the appearance of a 110 kDa band characteristic of the native  $\alpha 3$ NKA.

Similar results were obtained when the same experimental paradigm was used to crosslink endogenous agrin- $\alpha 3$ NKA complexes in cortical slices prepared from 12-day-old mice (Figure 6A), providing evidence that agrin might be regulating neuronal activity in vivo. To examine

this possibility, we tested the effects of C-Ag20 and C-Ag15 on the electrophysiological properties of layer V neurons in acute cortical slices prepared from 6- to 11-day-old mice. Only about 10% of neurons in the slice preparations exhibited spontaneous action potentials. Nevertheless, similar to its effects on cultured neurons, bath application of C-Ag20<sub>0</sub> resulted in rapid depolarization ( $\Delta 9.6 \pm 2.22$  mV,  $p$  < 0.001, paired Student's  $t$  test) and appearance of high-frequency action potentials (Figures 6B and 6C). In line with the results on cultured neurons, the response to C-Ag20<sub>0</sub> was also blocked by C-Ag15 (data not shown), arguing that the effect of agrin is specifically mediated through inhibition of the  $\alpha 3$ NKA.

The low frequency of spontaneously active neurons in the cortical slices limited our ability to examine the effects of C-Ag15 on ongoing activity, but C-Ag15 reversibly inhibited action potentials in two neurons that were found to be spontaneously active (data not shown). For a more robust test of the role of endogenous agrin in regulating neuronal activity, we examined the ability of C-Ag15 to inhibit action potentials induced by the glutamate-receptor agonist kainic acid. As expected, treatment with kainic acid (0.5  $\mu$ M) induced a rapid depolarization accompanied



**Figure 5. Frequency of Spontaneous Action Potentials in Cultured Neurons Is Agrin Dependent**

(A) A typical record showing the reversible membrane depolarization and increased frequency of spontaneous action potentials in a neuron treated with C-Ag20<sub>0</sub>.

(B) Increase in mean frequency of spontaneous action potentials of individual neurons in normal saline followed by C-Ag20<sub>0</sub> ( $p < 0.02$ , Wilcoxon signed-rank test).

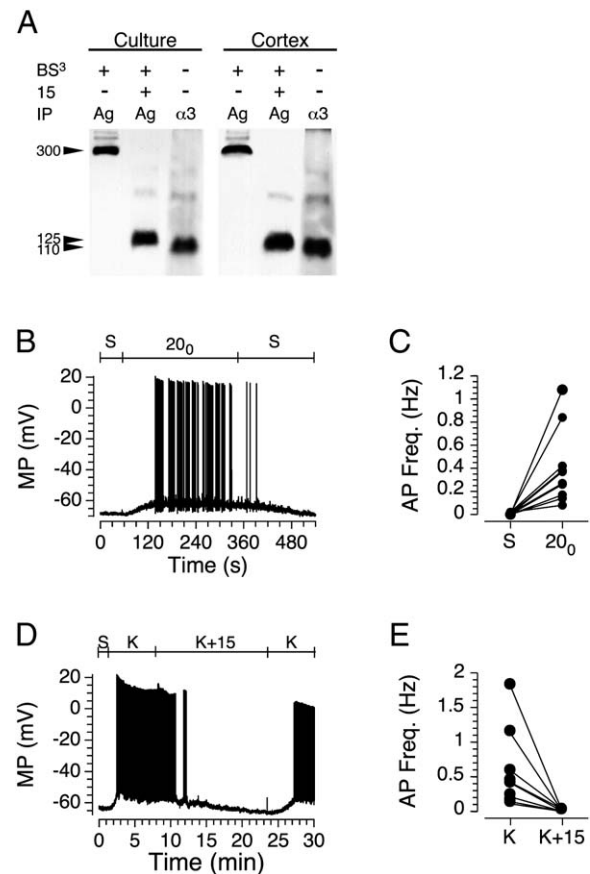
(C) Bath application of C-Ag15 results in a reversible decrease in the frequency of spontaneous action potentials. Slight hyperpolarization of the resting membrane potential is also apparent.

(D) The mean frequency of action potentials in individual neurons is consistently reduced by treatment with C-Ag15 ( $p < 0.001$ , Wilcoxon signed-rank test).

by the appearance of sustained high-frequency action potentials in most neurons (Figures 6D and 6E). However, within 10 min of the addition of C-Ag15, neuron membrane potentials became increasingly more negative to a point where they were comparable to normal resting membrane potentials measured prior to kainic acid/C-Ag15 treatment ( $-56.2 \pm 1.3$  mV in kainic acid versus  $-66.3 \pm 1.8$  mV in kainic acid + C-Ag15,  $p < 0.01$ , paired Student's *t* test;  $-70.4 \pm 1.6$  mV in saline). Action potential frequency also declined over a similar time course, from a mean of  $0.6 \pm 0.2$  Hz in kainic acid to  $0.01 \pm 0.005$  Hz in kainic acid + C-Ag15. The fact that C-Ag15's effects on membrane potential and action potential frequency were reversible is evidence of the specificity of C-Ag15 action.

## DISCUSSION

Agrin has been implicated in a wide range of functions in central and peripheral neurons, including organization of pre- and postsynaptic specializations, process growth, calcium homeostasis, and now neuronal activity. However, a general mechanism of agrin action has been elusive, in large part due to a lack of knowledge concerning



**Figure 6. Neuronal Activity In Vivo Is Regulated by Endogenous Agrin- $\alpha$ 3NKA Interactions**

(A) Detergent-solubilized membranes of BS<sup>3</sup>-crosslinked cultured cortical neurons or cortical slice were immunoprecipitated (IP) with antibodies against either agrin (Ag) or the  $\alpha$ 3NKA ( $\alpha$ 3) and then immunoblotted for  $\alpha$ 3NKA. BS<sup>3</sup> crosslinking of either cultured neurons or cortex results in the formation of an  $\sim 300$  kDa adduct, indicative of the presence of native agrin- $\alpha$ 3NKA complexes. Endogenous agrin- $\alpha$ 3NKA interactions can be disrupted by incubation with C-Ag15, resulting in the appearance of a 125 kDa C-Ag15- $\alpha$ 3NKA band. For comparison, the 110 kDa  $\alpha$ 3NKA visualized by immunoprecipitation and immunoblotting with the same  $\alpha$ 3NKA antibody in the absence of crosslinking is shown.

(B) A whole-cell current-clamp record from layer V neuron in a slice of motor cortex. Treatment with agrin results in reversible membrane depolarization and appearance of high-frequency action potentials.

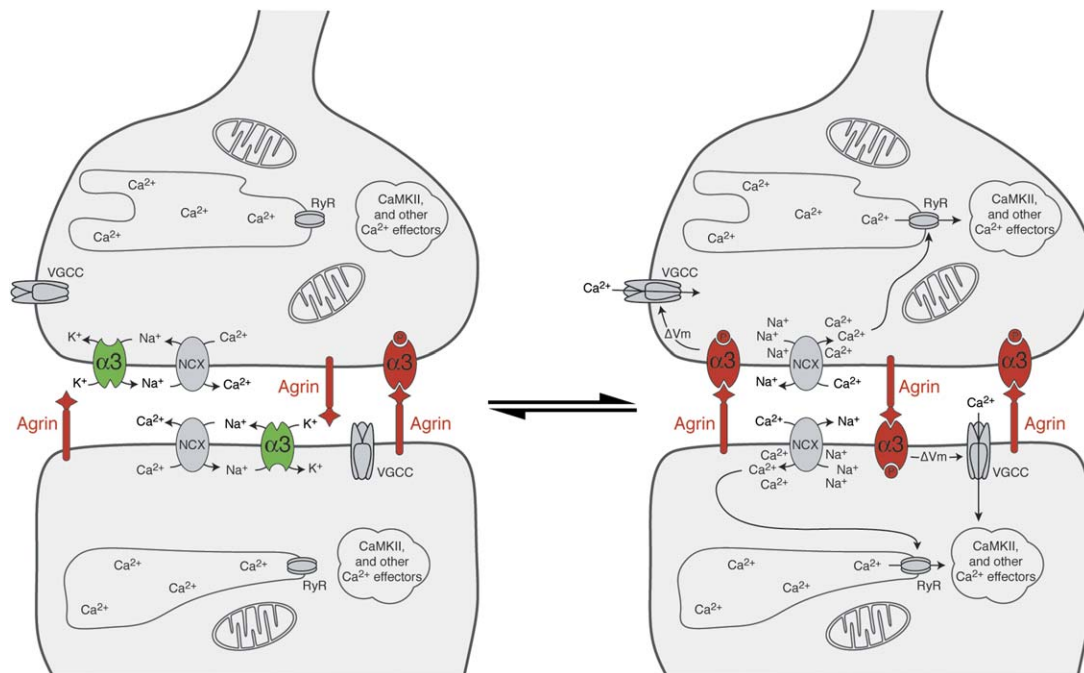
(C) The mean action potential frequency of individual neurons was significantly increased by treatment with C-Ag20<sub>0</sub> ( $p < 0.01$ , Wilcoxon signed-rank test).

(D) A typical current-clamp record showing action potentials induced by treatment with kainic acid (K). Addition of C-Ag15 results in reversible membrane repolarization and blockade of action potentials.

(E) C-Ag15 consistently inhibited firing of kainate-induced action potentials in individual neurons ( $p < 0.01$ , Wilcoxon signed-rank test).

the identity of the receptor (or receptors) on neurons that binds agrin. The results presented here show that agrin acts as an endogenous ouabain-like molecule targeted specifically to the  $\alpha$ 3NKA, a member of the NKA family selectively expressed in neurons. NKAs are responsible for





**Figure 7. Model of Agrin Function at the Synapse**

The resting  $\text{Na}^+/\text{K}^+$  electrochemical gradient depends on activity of local  $\alpha 3\text{NKAs}$ ; some  $\alpha 3\text{NKAs}$  are bound to and inhibited by agrin at rest. Further inhibition of  $\alpha 3\text{NKAs}$  by agrin results in collapse of the  $\text{Na}^+$  gradient within a diffusion-restricted physiological space, leading to slowing or even reversal of the NCX and a rapid rise in cytoplasmic  $\text{Ca}^{2+}$  concentration. Depolarization of synaptic membranes associated with the increase in intracellular  $\text{Na}^+$  concentration following  $\alpha 3\text{NKA}$  inhibition also triggers  $\text{Ca}^{2+}$  influx through voltage-gated channels. Increased cytoplasmic  $\text{Ca}^{2+}$ , augmented by  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release from intracellular stores via ryanodine receptors (Hilgenberg and Smith, 2004), activates CaMKII and other  $\text{Ca}^{2+}$  effectors known to regulate a variety of synaptic functions such as neurotransmitter release and neurotransmitter receptor turnover. Agrin and the  $\alpha 3\text{NKA}$  are shown on both pre- and postsynaptic membranes; however their precise subcellular location remains to be determined.

maintaining the  $\text{Na}^+/\text{K}^+$  ion gradient that underlies the membrane potential and provides the driving force for a variety of secondary cellular processes necessary for normal cell function. Whereas studies of  $\alpha 3\text{NKA}$  mutant mice will be needed to determine whether additional agrin receptors are present in neural tissues, the results of these experiments indicate that many of agrin's effects in neurons are driven by local and/or global changes in the  $\text{Na}^+/\text{K}^+$  ion gradient.

An early response to agrin is an increase in cytoplasmic  $\text{Ca}^{2+}$ , a composite of  $\text{Ca}^{2+}$  release from intracellular stores and influx through voltage-gated channels (Hilgenberg and Smith, 2004). The finding that agrin antagonizes the  $\alpha 3\text{NKA}$  provides a simple explanation for these observations (see model, Figure 7). It is well known that the plasma-membrane sodium/calcium exchanger (NCX) plays a key role in  $\text{Ca}^{2+}$  homeostasis. However, because of its dependence on the  $\text{Na}^+$  ion gradient, activity of the NCX is largely governed by the NKA: Under normal conditions, the pump operates in forward mode, transporting  $\text{Ca}^{2+}$  ions out of the cell, but the direction of transport reverses as the  $\text{Na}^+$  ion gradient declines (Annunziato et al., 2004). In neurons, the  $\alpha 3\text{NKA}$  and NCX colocalize within plasma-membrane domains, juxtaposed by elements of the endoplasmic reticulum, creating a diffusion-restricted

cytoplasmic space that enhances the functional linkage between them (Blaustein et al., 2002). Thus, one component of the agrin-induced increase in intracellular  $\text{Ca}^{2+}$  is likely to be due to changes in NCX activity driven by inhibition of the  $\alpha 3\text{NKA}$ , a hypothesis that could be tested by examining the agrin response of NCX mutant neurons. Whereas we previously speculated that an agrin-induced membrane conductance might be responsible for  $\text{Ca}^{2+}$  influx through voltage-gated channels, the model suggests that opening of voltage-gated  $\text{Ca}^{2+}$  channels in response to membrane depolarization associated with the agrin-induced decline in  $\alpha 3\text{NKA}$  activity is more likely.

The present study provides strong evidence for a role for agrin in regulating neuronal activity. Action potential frequencies were dramatically increased by exogenous agrin; more importantly, C-Ag15, an agrin fragment that acts as an agrin antagonist, disrupted native agrin- $\alpha 3\text{NKA}$  interactions, blocking spontaneous action potentials in both cultured neurons and acute-slice preparations. Agrin behaves as an endogenous ouabain-like molecule, and mechanisms of ouabain-induced hyperexcitability have been studied in hippocampal neurons, where changes in both intrinsic membrane properties and synaptic transmission are important (Vaillend et al., 2002). Neurons express multiple NKA subunits, and, given agrin's

exquisite specificity for the  $\alpha$ 3NKA, only a subset of the effects attributed to ouabain may be agrin dependent. Nevertheless, the observation that agrin and its receptor are concentrated at synapses suggests the synapse is an important site of agrin action. Neurotransmitter release and/or spike threshold are both dependent on membrane potential; functional coupling between the  $\alpha$ 3NKA and NCX, which plays a role in vesicle cycling and neurotransmitter release (Bouron and Reuter, 1996), may also be important. Consistent with the latter possibility, suppression of agrin expression in cultured hippocampal neurons is associated with a decrease in synaptic vesicle cycling (Böse et al., 2000). Not surprisingly, behavioral studies have shown that ouabain inhibits memory formation (Gibbs and Ng, 1978; Xia et al., 1997; Sato et al., 2004), while a decline in NKA activity is responsible for a form of long-term plasticity in hippocampal interneurons (Ross and Soltesz, 2001). Agrin expression is activity dependent (O'Connor et al., 1995), and it is tempting to speculate that agrin regulation of the  $\alpha$ 3NKA might play a role in synaptic plasticity. Given the functional link between the  $\alpha$ 3NKA and NCX, studies showing enhanced learning and memory in mice lacking NCX2 (Jeon et al., 2003) support this hypothesis. Clearly, more detailed studies of the spatiotemporal changes in agrin expression and the effects of conditional knockouts of agrin and the  $\alpha$ 3NKA on learning and memory are required.

Dysfunction of the  $\alpha$ 3NKA has been strongly linked with pathological changes in the brain. Intraventricular infusion of ouabain causes seizures (Davidson et al., 1978), and loss of  $\alpha$ 3NKA activity potentiates excitotoxic injury and neuronal-cell death (Brines et al., 1995; Xiao et al., 2002). In addition, mutation of the  $\alpha$ 3NKA has been shown to be responsible for rapid-onset dystonia parkinsonism, an autosomal-dominant movement disorder in human (de Carvalho Aguiar et al., 2004). Paralleling these observations, heterozygous agrin-deficient mice are less sensitive to kainic acid than their wild-type siblings, while agrin-deficient neurons exhibit decreased responses to excitatory stimuli and resistance to excitotoxic insult (Hilgenberg et al., 2002). Interestingly, these effects of mutating the *Agrn* gene are predicted by our model: Decreased agrin expression, functionally equivalent to treatment with C-Ag15, translates into an increase in total  $\alpha$ 3NKA activity that lowers neuronal activity while enhancing the ability to buffer potentially damaging increases in intracellular  $\text{Ca}^{2+}$  ions by sustaining activity of the NCX. Together, these studies suggest that dysregulation of agrin expression will have a significant impact on brain function. It is noteworthy that agrin is concentrated in both amyloid plaques and tangles characteristic of Alzheimer's disease (Verbeek et al., 1999) and Lewy bodies found in Parkinson's disease (Liu et al., 2005) and may, therefore, contribute to the etiology of these diseases. The ability of C-Ag15 to relieve inhibition of the  $\alpha$ 3NKA by endogenous agrin suggests that it will be a useful starting point for the development of therapeutic agents that might alleviate or reverse the progress of these and other diseases of the CNS.

Agrin was originally identified at the neuromuscular junction, where it mediates the motor neuron-induced accumulation of AChR in the postsynaptic muscle-fiber membrane. Curiously, agrin molecules present at the junction are functionally heterogeneous and distinct in cellular origin: Alternatively spliced  $z^+$  isoforms, equivalent to C-Ag90<sub>8</sub> and C-Ag20<sub>8</sub>, have high AChR clustering activity and originate from motor neurons;  $z_0$  agrin, like C-Ag20<sub>0</sub>, has no AChR clustering activity and is synthesized by muscle (Sanes and Lichtman, 2001). The function of  $z_0$  agrin is unclear, but its location and origin are consistent with a role as a retrograde signal agent. The fact that the  $\alpha$ 3NKA is expressed on motor neuron axon terminals (Zahler et al., 1996) suggests it may be the target for muscle agrin. Possible roles for this retrograde signal would include tuning neurotransmitter release to the muscle fiber's action potential threshold or matching growth of the axon terminal to the muscle fiber. Further studies will be needed to examine the effects of agrin on synaptic transmission at the neuromuscular junction. However, guidance of developing axons is known to depend on translation of local cues to changes in intracellular  $\text{Ca}^{2+}$  within the growth cone (Zheng, 2000), which is also a site of  $\alpha$ 3NKA concentration (Brines and Robbins, 1993). Thus, agrin regulation of  $\alpha$ 3NKA provides an attractive explanation for the observation that motor neurons overgrow their target muscle in agrin mutant mice (Gautam et al., 1996) and that  $z_0$  agrin inhibits growth and stimulates axon-terminal differentiation in cultured neurons (Campagna et al., 1997).

Finally, because of its positive inotropic effect, the major therapeutic use of ouabain and related compounds is in the treatment of congestive heart failure. Although cardiac muscle expresses multiple NKA isoforms, the low effective dose of ouabain suggests that its therapeutic effects are mediated by the high-affinity  $\alpha$ 2- and  $\alpha$ 3NKAs (Glitsch, 2001). Agrin, which acts as an endogenous ouabain, is also expressed in heart (Godfrey et al., 1988; Hoch et al., 1993). While a role for agrin in heart function remains to be determined, understanding the structural basis of agrin's specificity for a single member of a family of such closely related proteins and the mechanisms by which it regulates  $\alpha$ 3NKA function is likely to contribute to the understanding and development of improved therapies for cardiac disease.

## EXPERIMENTAL PROCEDURES

### Cell Culture

Primary cultures of mouse 1- to 2-day postnatal cortical neurons were prepared as described (Hilgenberg et al., 1999; Hilgenberg and Smith, 2004). Experiments were performed between 10 and 16 days in culture.

Nonneuronal cells were prepared by growing dissociated rat cortical cells in minimal essential medium supplemented with 10% fetal bovine serum. Contaminating neurons were removed by treating the cultures briefly with 0.5% trypsin, and the cells were resuspended by trituration followed by dilution and replating. No neurons were detectable following two replatings, as indicated by staining with a MAP-2 antibody (SMI-52, Sternberger Monoclonals).

All handling and treatment of animals complied with the guidelines of the Institutional Animal Care and Use Committee of the University of California, Irvine.

### Immunohistochemistry

Neuronal and nonneuronal cells were identified by double staining with a mouse antibody directed against MAP-2 and a rabbit antibody against GFAP (DAKO) as described (Hilgenberg et al., 1999). Agrin binding sites and  $\alpha$ 3NKA were visualized by double labeling with C-Ag208 (Hoover et al., 2003) and monoclonal antibody XVIF9-G10 (Novus Biologicals) against the  $\alpha$ 3NKA (see Supplemental Data for details). The concentration of agrin used here and in other parts of the study was based on bioactivity determined by  $\text{Ca}^{2+}$  imaging of agrin-induced increases in intracellular  $\text{Ca}^{2+}$  in cultured cortical neurons (Hilgenberg and Smith, 2004).

### Biochemistry

Membrane-impermeant chemical crosslinking agents BS<sup>3</sup> and DMA (Pierce) were used to stabilize the bond between agrin and its binding sites on cell surface membranes. Cultured neurons were washed briefly in phosphate-buffered saline (PBS) containing 10 mM EDTA followed by preincubation with one or more agrin fragment in PBS containing 1.8 mM  $\text{Ca}^{2+}$  for 30 min at room temperature and then cooled on ice prior to addition of a  $10\times$  solution of crosslinking agent to a final concentration of 0.1 mM. The crosslinking reaction was allowed to proceed for 30 min, after which any unreacted crosslinker was quenched and removed by washing with ice-cold PBS<sup>2+</sup> containing 50 mM ethanolamine. For immunoblot analysis, cells were scraped into ice-cold TI buffer (20 mM Tris [pH 7.4], 10 mM EDTA, and protease inhibitors [Sigma, P8340]) and then Dounce homogenized, and membrane fractions were recovered by centrifugation. For immunoprecipitation studies, cells were scraped and homogenized in TI buffer containing 150 mM NaCl and 0.5% Triton X-100. Cell extracts were cleared by centrifugation, and aliquots of the detergent-soluble fraction were incubated with the appropriate antibody at 4°C overnight. Antigen-antibody complexes were precipitated with either protein A or protein G and resuspended in SDS-PAGE sample buffer for immunoblot analysis.

### Na<sup>+</sup> Imaging

Intracellular Na<sup>+</sup> was monitored by ratiometric imaging of the membrane-permeant sodium binding fluorescent dye SBFI-AM (Molecular Probes) by essentially the same methods described for Fura-2 imaging of agrin-induced changes in neuronal  $\text{Ca}^{2+}$  (Hilgenberg and Smith, 2004). For quantitative analyses, responses of individual cells were normalized to their maximal response to treatment with 5  $\mu\text{M}$  gramicidin, a potent ionophore.

### Electrophysiology

Standard whole-cell current-clamp techniques were used to measure membrane potentials and spontaneous action potentials in cortical neurons in culture and acutely prepared slices (see Supplemental Data for details). Only neurons with stable pretreatment resting potentials of  $-55$  mV or less that also exhibited a reversible response to agrin or ouabain were accepted for analysis. Action potential frequency measurements were performed on 3–5 min of data during each phase (pretreatment, treatment, posttreatment) of the experiment. All recordings were carried out at room temperature.

### Expression Studies

The plasmid pRc $\alpha$ 3-AAC (a generous gift of J. Lingrel and T. Pressley) encodes an ouabain-insensitive rat  $\alpha$ 3 subunit of the NKA when expressed in eukaryotic cells. To generate a wild-type  $\alpha$ 3 expression construct, an 805 bp SacII/Bsu36I restriction fragment was excised from pRc $\alpha$ 3-AAC and replaced with the corresponding fragment isolated by RT-PCR from rat brain RNA to form pRc $\alpha$ 3 used for expression of the native  $\alpha$ 3NKA subunit. Nonneuronal cells prepared from

rat or mouse cortex were cotransfected with pRc $\alpha$ 3 and the enhanced green fluorescent protein vector pEGFP-C1 (Clontech) to facilitate identification of transfected cells using the Effectene reagent (QIAGEN) according to the manufacturer's instructions. Agrin responses of non-neuronal cells were determined by Na<sup>+</sup> imaging and whole-cell current-clamp as for cultured neurons.

### Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and can be found with this article online at <http://www.cell.com/cgi/content/full/125/2/359/DC1/>.

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