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Agrin Regulates Neuronal Responses to Excitatory Neurotransmitters *in Vitro* and *in Vivo*

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Agrin mediates motor neuron-induced differentiation of the postsynaptic apparatus of the neuromuscular junction but its function in brain remains unknown. Here we report that expression of *c-fos*, induced by activation of nicotinic or glutamatergic receptors, was significantly lower in cortical neurons cultured from agrin-deficient mutant mouse embryos compared to wildtype. Agrin-deficient neurons also exhibited increased resistance to excitotoxic injury. Treatment with recombinant agrin restored glutamate-induced *c-fos* expression and excitotoxicity of the agrin-deficient neurons to near wild-type levels, confirming the agrin dependence of the phenotype. The observation that *c-fos* induction by activation of voltage-gated Ca^{2+} channels is also reduced in agrin-deficient neurons raises the possibility that agrin may play a wider role by regulating responses to Ca^{2+} -mediated signals. Consistent with the decline in response of cultured mutant neurons to glutamate, decreases in kainic acid-induced seizure and mortality were observed in adult agrin heterozygous mice. Together, these data demonstrate that agrin plays an important role in defining neuronal responses to excitatory neurotransmitters both *in vitro* and *in vivo*.

INTRODUCTION

Agrin, an extracellular matrix heparan sulfate proteoglycan, was originally isolated on the basis of its ability to induce clustering of acetylcholine receptors (AChR) on cultured muscle fibers (Nitkin *et al.*, 1987). Subsequent studies demonstrated that agrin mediates the motor neuron-induced aggregation of AChR in the postsynaptic apparatus of the developing neuromuscu-

lar junction (for review see Sanes, 1997; Hoch, 1999). Accumulation of other postsynaptic components at the neuromuscular junction (Wallace *et al.*, 1985; Wallace, 1989; Nitkin and Rothschild, 1990; Sharp and Caldwell, 1996; Rimer *et al.*, 1998) and expression of synapse-specific muscle genes (Jones *et al.*, 1996; Gramolini *et al.*, 1998) has also been shown to be agrin-dependent. Thus, agrin seems to play a general role in directing differentiation of postsynaptic components of the muscle endplate.

The postsynaptic membranes of neuron–neuron synapses, like the neuromuscular junction, are characterized by a high density of neurotransmitter receptors (Kneussel and Betz, 2000; Lee and Sheng, 2000). Many populations of neurons in the central nervous system also express agrin (O'Connor *et al.*, 1994), raising the possibility that agrin plays a role in the formation or maintenance of neuron–neuron synapses. Consistent with this hypothesis, agrin is localized at neuronal synapses (Mann and Kröger, 1996; Koulen *et al.*, 1999; Gingras and Ferns, 2001) and peak levels of agrin expression in developing brain coincide with periods of synapse formation (Li *et al.*, 1997). Agrin's function in brain, however, is controversial. Agrin-deficient mice show no gross abnormalities in brain development (Gautam *et al.*, 1996). Moreover, synapses formed between agrin-deficient neurons in cell culture appear morphologically and functionally normal (Li *et al.*, 1999; Serpinskaya *et al.*, 1999), leading to the conclusion that interneuronal synapse formation does not share the same agrin dependence evident at the neuromuscular junction. Recent studies, however, have demonstrated that suppression of agrin expression in cultured hippocampal neurons with antisense oligonucleotides inhibits the appearance of both pre- and postsynaptic specializations and alters synaptic function (Ferreira,

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1999; Böse *et al.*, 2000). This suggests that agrin does influence some aspect of the formation or stability of neuron–neuron synapses. Further evidence of a role for agrin in regulating neuron–neuron interactions has come from studies demonstrating the existence of an agrin signal transduction pathway in CNS neurons. Agrin triggers phosphorylation of the transcription factor CREB (cAMP response element binding protein) in cultured hippocampal neurons (Ji *et al.*, 1998) and induces the immediate-early gene *c-fos* in cortical and other CNS neurons (Hilgenberg *et al.*, 1999). These findings predict that mutations that disrupt agrin signaling could perturb neuronal function and/or organization.

To determine if we could find evidence to support this hypothesis, we used induction of *c-fos* to compare the responses of wild-type and agrin-deficient cultured cortical neurons to a variety of extracellular stimuli. The results of these studies reveal that signaling by ligand-gated ion channels and voltage-gated Ca^{2+} channels is attenuated in agrin-deficient neurons. Consistent with these findings, seizure activity and induction of *c-fos* in brains of agrin heterozygous mice following systemic administration of kainic acid were also reduced. Together these findings suggest that agrin regulates rapid communication between cortical neurons, both in cell culture and *in vivo*.

RESULTS

Nicotine-induced expression of Fos is reduced in agrin-deficient cortical neurons. In addition to regulating the spatial distribution of AChR in muscle, agrin influences AChR function by regulating expression of the ϵ subunit (Jones *et al.*, 1996). Nicotinic AChR are also expressed by neurons (nAChR) in the CNS, where they act as both mediators and modulators of fast synaptic transmission (reviewed in Jones *et al.*, 1999). Previous studies have demonstrated that activation of nAChR with nicotine induces *c-fos* (Greenberg *et al.*, 1986; I. Santos, personal communication). To determine if nAChR signaling is influenced by agrin, we compared levels of nicotine-induced Fos immunoreactivity in cultured cortical neurons prepared from mouse embryos homozygous for a mutation in the agrin gene that blocks neuromuscular synaptogenesis (Gautam *et al.*, 1996) or their wild-type siblings. Neuronal Fos immunoreactivity was markedly increased in both wild-type and agrin-deficient neurons following treatment with 30 μM nicotine (Fig. 1A). This increase was a specific response to activation of nAChR since it was inhibited by 100 μM *d*-tubocurarine (dTbC; data not shown) but

unaffected by a cocktail of drugs containing 1 μM tetrodotoxin (TTX), 50 μM dizocilpine (MK801), 10 μM 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzoquinoline-7-sulfonamide (NBQX), and 10 μM bicuculline methchloride (BMC), to block Na^{+} -dependent action potentials and activation of glutamate and GABA receptors. However, Fos immunoreactivity was noticeably less intense in agrin-deficient compared to wild-type neurons, suggesting that agrin is involved in mediating neuronal responses to nicotine.

To examine the genotype-specific difference in response to nicotine in more detail, cultured neurons were treated for 5 min with 30 μM nicotine, conditions shown in preliminary studies to cause a half-maximal induction of *c-fos* in wild-type neurons. As for the immunohistochemical studies above, experiments were performed in the presence of TTX, NBQX, MK801, and BMC. The level of Fos expression at 2 h following nicotine treatment was determined by *in situ* ELISA (Hilgenberg *et al.*, 1999). Consistent with the immunohistochemical analysis, nicotine-dependent induction of *c-fos* in agrin-deficient cultures was significantly decreased, by about 40%, compared to wild-type cultures (Fig. 1B). An intermediate, although not significantly different, level of Fos expression was evident in neurons prepared from heterozygous embryos, a trend consistent with the possibility that nicotine response might be correlated with agrin gene dosage. Fos expression in wild-type and agrin-deficient cultures treated with nicotine in the presence of 100 μM dTbC was indistinguishable from that in control cultures treated with vehicle alone (data not shown), confirming that increased Fos immunoreactivity is specifically and directly related to activation of nicotinic nAChR on the surface of the neurons.

One simple explanation for the apparent decrease in response to nicotine of agrin-deficient cortical neurons is that nAChR expression is agrin-dependent. Although individual nAChR are generated by assembly of distinct α and β subunits (Cordero-Erausquin *et al.*, 2000), greater than 70% of the nicotine-dependent increase in Fos in cultured cortical neurons can be blocked by α -bungarotoxin (BTX; I. Santos, personal communication), indicating that $\alpha 7$ -subunit-containing receptors are the predominant nAChR on the neurons. No significant difference in the number of ^{125}I -BTX binding sites in agrin-deficient and wild type cultures was evident (Fig. 1C; $+/+$ vs $-/-$ $P = 0.87$; $+/+$ vs $+/-$ $P = 0.33$, paired *t* test). In addition, cell counts, performed blind with respect to genotype, revealed no difference between the number of neurons in agrin-deficient and wild-type cultures (data not shown). Thus, differences

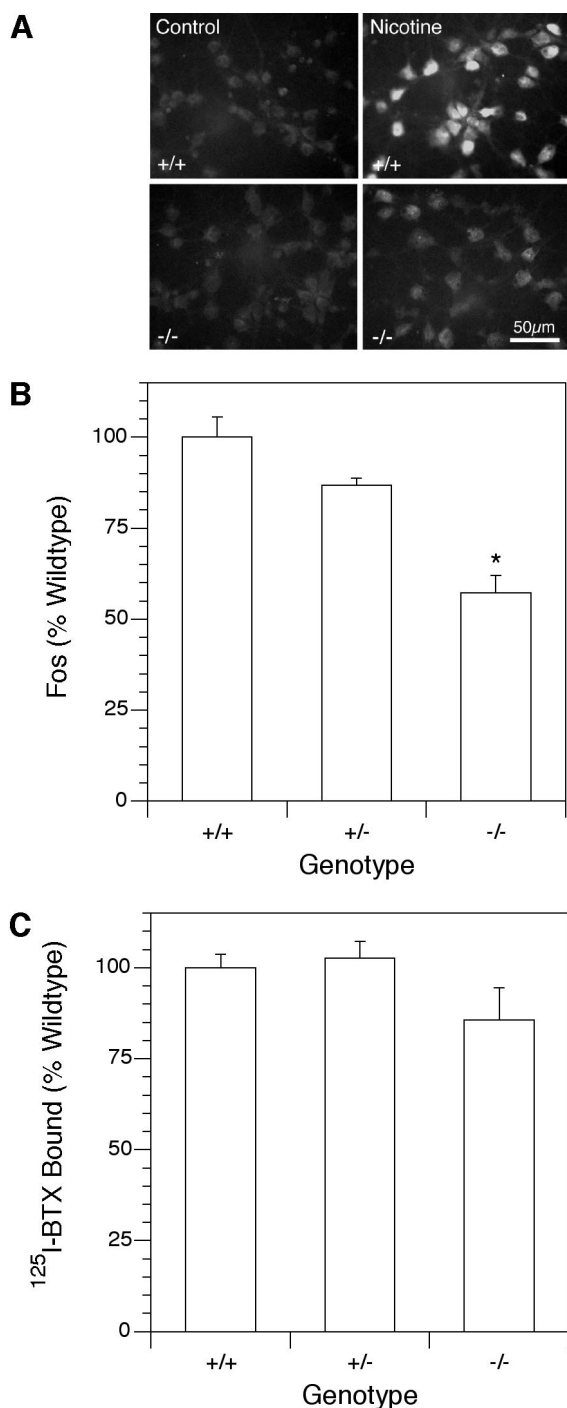


FIG. 1. Nicotine-induced expression of *c-fos* is reduced in agrin-deficient neurons. 10- to 14-day-old cultures of wild-type (+/+) and homozygous agrin-deficient (-/-) cortical neurons were treated with 30 μ M nicotine for 5 min and Fos expression was assayed 2 h later. (A) Although exposure to nicotine caused an increase in Fos immunoreactivity in both wild-type and agrin-deficient neurons, the level of *c-fos* induction was noticeably lower in agrin-deficient compared to wild-type cultures. (B) Similar results were obtained when an *in situ* ELISA was used to quantitate nicotine induction of

in nicotine-induced expression of Fos exhibited by agrin-deficient and wild-type cortical neurons seem unlikely to be accounted for by genotype-specific differences in nAChR expression, neuronal plating efficiency, or survival.

Glutamate-induced expression of Fos is reduced in agrin-deficient cortical neurons. To explore the possibility that agrin plays a wider role in modulating neuronal neurotransmitter receptor function, we examined glutamate receptor-mediated induction of *c-fos*. Cortical cultures were treated for 5 min with 100 μ M glutamate in the presence of TTX, dTbC, and BMC and then returned to the incubator for 2 h prior to fixation and staining for Fos immunoreactivity. Irrespective of genotype, Fos expression was clearly increased in neurons treated with glutamate compared to control cultures treated with glutamate in the presence of the NMDA and non-NMDA antagonists 2-amino-5-phosphonopivalate (APV) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Fig. 2A). However, similar to the results of experiments using nicotine, the relative intensity of the staining suggested that Fos expression levels were reduced in agrin-deficient neurons compared to wildtype. In contrast, Fos immunoreactivity in GFAP-positive astrocytes following glutamate treatment was indistinguishable from that in control cultures (data not shown).

The role of agrin in modulating the response of cortical neurons to glutamate was further investigated by quantitative *in situ* ELISA for Fos (Hilgenberg *et al.*, 1999). The contribution of Na^+ -dependent action potential-driven changes in Fos expression was eliminated by performing experiments in the presence of TTX either alone or in the same cocktail of drugs used for the immunohistochemical studies (no difference). Consistent with the results of our immunohistochemical analysis, glutamate induced a concentration-dependent increase in Fos expression that was attenuated in homozygous agrin-deficient, compared to wild-type,

c-fos in the cultures. Nicotine-induced Fos levels were significantly lower in agrin-deficient compared to wild-type cultures ($*P \leq 0.05$, two tailed paired *t* test) with a similar trend evident in heterozygous cultures. (C) In contrast, no significant difference was evident in the number of $\alpha 7$ -containing nAChR expressed on the surface of the neurons as determined by binding of 10 nM ^{125}I -BTX. Data are expressed as percentages of wildtype to facilitate comparison between experiments. Charts summarize data obtained from three independent platings and at least three embryos/genotype. Nonspecific induction of *c-fos* or ^{125}I -BTX binding observed in the presence of excess dTbC has been subtracted. Bars show means \pm SEM.

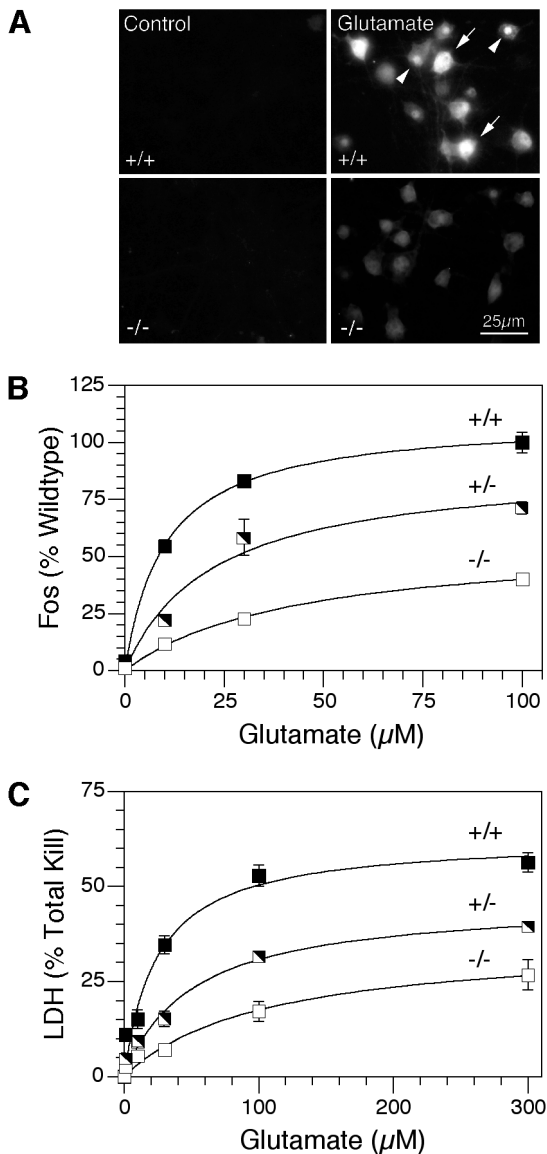


FIG. 2. Glutamate-induced expression of *c-fos* is reduced in agrin-deficient neurons. (A) Two-week-old cortical cultures, prepared from wild-type or agrin-deficient littermate embryos, were treated for 5 min with 100 μ M glutamate either alone (Glutamate) or in the presence of 50 μ M APV and 10 μ M CNQX (Control). Cultures were fixed 2 h later and processed for Fos immunoreactivity. All experiments were performed in the presence of 1 μ M TTX, 100 μ M dTbC, and 10 μ M BMC. Fos expression was increased in neurons of both genotypes following glutamate treatment but labeling of neuronal somata (arrows) and nuclei (arrowheads) was noticeably more intense in wild-type compared to agrin-deficient cultures. (B) The concentration dependence of glutamate-mediated induction of *c-fos* was examined by quantitative *in situ* ELISA. To facilitate comparison between experiments, data are expressed as percentages of Fos levels in wild-type neurons treated with 100 μ M glutamate. Consistent with the results of the immunohistochemistry, wild-type neurons are more responsive to glutamate than agrin-deficient neurons. Cultures prepared from cortices of heterozygous embryos exhibit an intermediate response.

neurons (Fig. 2B). Fos expression curves were well fit by a single-site nonlinear regression model ($R^2 > 0.95$) predicting an EC_{50} of 12.1 ± 2.6 and 41.0 ± 2.6 μ M for wild-type and agrin-deficient neurons, respectively. Intermediate EC_{50} values were obtained for cultures prepared from heterozygous embryos (25.6 ± 6.3 μ M), consistent with an effect of gene dosage. Similar results were also obtained using a second excitatory amino acid, kainate (data not shown). Apparent genotypic differences in the maximal levels of *c-fos* induction are probably due to the range of glutamate concentrations tested. In two experiments (three embryos of each genotype), in which the glutamate concentration was increased to 200 μ M, Fos expression in agrin-deficient neurons increased to $87 \pm 7.0\%$ of wildtype. Given these results, the decreased response to glutamate of the agrin-deficient neurons is unlikely to be due to a decrease in transcription and/or translation of the *c-fos* gene (see also Fig. 5).

Agrin-deficient cortical neurons are resistant to excitotoxic damage. Overactivation of ionotropic glutamate receptors is excitotoxic to cortical neurons (Choi et al., 1987). The reduction in glutamate-induced Fos expression in agrin-deficient cortical neurons predicts a concomitant resistance to glutamate-mediated excitotoxicity. Release of the cytosolic enzyme lactate dehydrogenase (LDH) has been used in the past as a reliable index of excitotoxic damage in cultured CNS neurons (Koh and Choi, 1987). Accordingly, we monitored LDH levels in the medium surrounding wild-type, homozygous, and heterozygous agrin-deficient cortical cells 4 h following treatment with different concentrations of glutamate. To facilitate comparison between experiments, data were expressed as a fraction of total neuronal LDH (total kill) determined by overnight exposure of sister cultures to 300 μ M glutamate. As expected, LDH levels in the culture media increased with increasing glutamate concentration for all geno-

(C) A similar differential response was observed for glutamate-mediated excitotoxicity. Measurement of LDH levels in the medium 4 h after a 5-min treatment with different concentrations of glutamate showed agrin-deficient < heterozygous < wild-type neurons. Data are expressed as percentages of total neuronal kill, defined as the LDH level observed in sister cultures following overnight exposure to 300 μ M glutamate. Basal levels of Fos expression and LDH observed in sister cultures treated with glutamate in the presence of APV and CNQX have been subtracted. Data have been fit by nonlinear regression using a single-site model. Each data point shows the mean \pm SEM of five (B) or four (C) experiments representing a minimum of four embryos in each genotype.

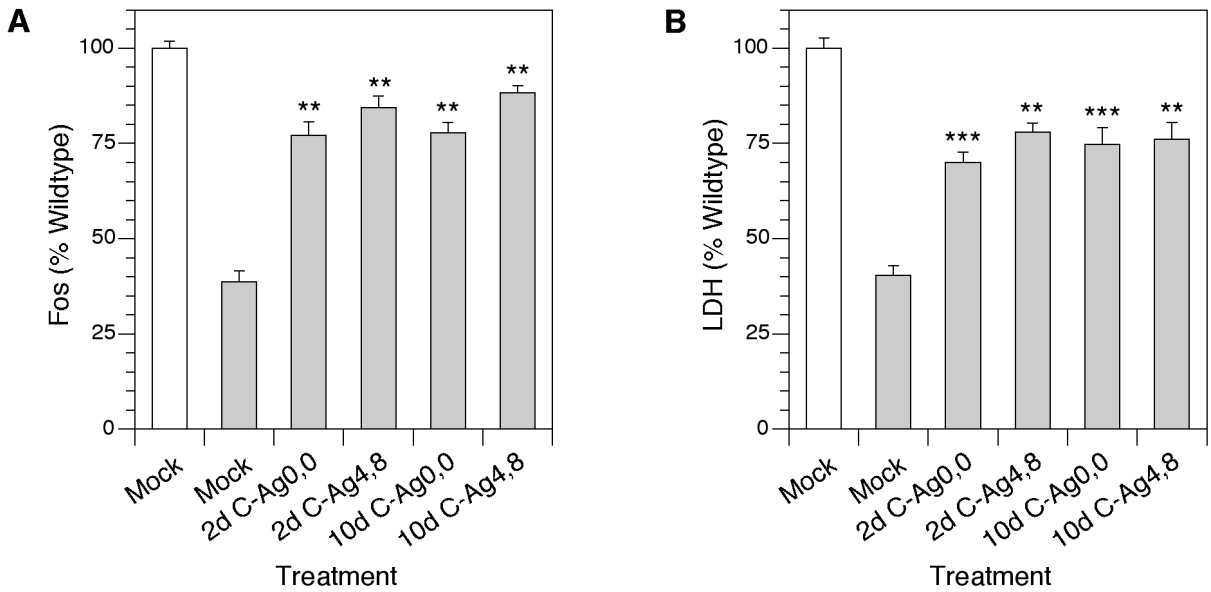


FIG. 3. Decreased response to glutamate of agrin-deficient neurons is agrin specific. Beginning at 2 or 10 days after plating, cortical cultures were maintained in medium supplemented with conditioned medium from untransfected COS cells (Mock) or COS cells expressing the soluble C-terminal half of agrin_{y0z0} or agrin_{y4z8} for either 2 or 10 days. At 12 days of culture, neuronal response to glutamate was determined by measuring (A) *c-fos* induction and (B) LDH release following 5 min treatment with 100 μ M glutamate. Data are expressed as percentages of the response observed in wild-type neurons (open bars) treated with conditioned medium from untransfected COS cells. Treatment with agrin for as little as 2 days produced a significant increase in the glutamate-dependent responses of agrin-deficient neurons (gray bars) measured in terms of either Fos expression or LDH release (** $P \leq 0.01$; *** $P \leq 0.001$; paired *t* test, mock versus agrin-treated agrin-deficient neurons). No difference was apparent in the efficacy of the two agrin isoforms tested. Bars indicate means \pm SEM obtained from four (A) or three (B) experiments representing a minimum of three embryos in each genotype.

types (Fig. 2C). However, homozygous agrin-deficient neurons were about fourfold less sensitive to excitotoxic damage than their wild-type siblings ($EC_{50} -/-$, $104.9 \pm 11.2 \mu$ M; $EC_{50} +/+$, $24.5 \pm 8.6 \mu$ M), whereas heterozygous neurons exhibited an intermediate level of sensitivity ($EC_{50} +/-$, $50.4 \pm 3.3 \mu$ M). Interestingly, the agrin deficiency does not afford absolute protection against the excitotoxic effects of glutamate since genotypic differences in LDH levels diminished with increasing glutamate exposure time to the point at which, within any single plating, total kill LDH values obtained for each genotype were indistinguishable from one another (data not shown). Together with the results of the cell counts, these data suggest that neither neuronal survival nor growth is affected by mutation of the agrin gene but support the hypothesis that neuronal response to glutamate is agrin-dependent.

Application of exogenous agrin rescues the agrin-deficient phenotype. To determine whether the attenuation of glutamate responses observed in agrin-deficient neurons was a direct result of decreased agrin expression, rather than a nonspecific effect caused by mutation of the agrin gene, we tested the ability of

exogenous agrin to rescue the deficiency. Beginning at either 2 or 10 days after plating, homozygous agrin-deficient cortical cultures were fed medium containing recombinant soluble isoforms of rat agrin (C-Ag; Ferns *et al.*, 1993) at a concentration shown to maximally induce *c-fos* (Hilgenberg *et al.*, 1999). Levels of glutamate-induced Fos expression and LDH were determined at 12 days of culture. Following agrin treatment, glutamate induction of *c-fos* was restored to near wild-type levels (Fig. 3A). Interestingly, treatment with agrin for 2 days (from 10 to 12 days in culture) was as effective as treatment for 10 days (from 2 to 12 days in culture). Thus, not only was agrin able to prevent development of the deficient phenotype, it was also able to reverse it once established. Moreover, the “neural” agrin_{y4,z8} and “nonneural” agrin_{y0z0} isoforms were found to be equally potent in rescuing the agrin deficiency. Similar results were also obtained in a parallel series of experiments examining the effects of agrin treatment on glutamate-mediated excitotoxicity of agrin-deficient cortical neurons (Fig. 3B). Together these studies demonstrate that agrin has a direct influence on

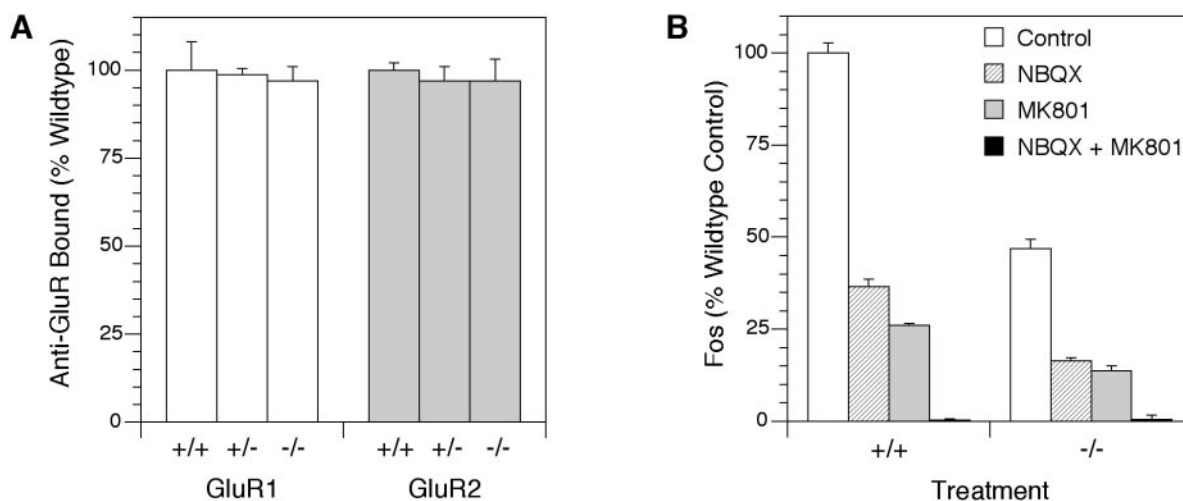


FIG. 4. Glutamate receptor expression is normal in agrin-deficient neurons. (A) 10- to 14-day cortical cultures were incubated in primary antibody to either GluR1 or GluR2 and then washed and fixed. Subsequently, the amount of primary antibody bound was determined using an alkaline phosphatase-conjugated second antibody and *in situ* ELISA as described for estimation of Fos expression. Bar chart summarizes data obtained from two experiments which included three wild-type, two heterozygous, and four homozygous agrin-deficient embryos. Data in each experiment were normalized to level of GluR1 or GluR2 expression observed in wild-type littermates. Nonspecific or background alkaline phosphatase activity, observed in control cultures not exposed to the primary antibody, has been subtracted. No genotype-specific difference in levels of GluR1 (open bars) or GluR2 (gray bars) expression is apparent. (B) To examine the relative contribution of NMDA and AMPA/KA type receptors to glutamate induction of *c-fos*, 10- to 14-day cultures were treated with 100 μ M glutamate for 5 min in the absence or presence of either NBQX or MK801 or both NBQX and MK801 and levels of Fos expression were determined. The fraction of total glutamate-induced Fos mediated by activation of NMDA or non-NMDA receptors is similar in wild-type and agrin-deficient neurons. Little or no glutamate induction of *c-fos* was observed in cultures treated with both NBQX and MK801, suggesting that activation of metabotropic glutamate receptors does not contribute to Fos expression under these conditions. Data for each experiment were normalized to Fos expression in wild-type neurons in sister cultures treated with glutamate alone. Bars show means \pm SEM for three separate platings from a total of four wild-type and seven agrin-deficient embryos.

mechanisms which shape the responses of cortical neurons to glutamate.

Glutamate receptor expression and function appear normal in agrin-deficient neurons. Immunohistochemical staining and electrophysiological analysis of miniature postsynaptic currents (Li *et al.*, 1999; Serpin-skaya *et al.*, 1999) argue that the density of synaptic glutamate receptors in agrin-deficient neurons is normal and unlikely to account for the difference in response to glutamate. However, CNS neurons express a significant population of extrasynaptic glutamate receptors (Lee and Sheng, 2000) and treatment of cultured hippocampal neurons with antisense oligonucleotides directed against agrin has been reported to decrease expression of several neurotransmitter receptor subunits (Ferreira, 1999). To investigate the possibility that changes in glutamate receptor expression might contribute to the altered glutamate-dependent responses of agrin-deficient neurons, cell surface expression of GluR1 and GluR2 AMPA receptor subunits was quantitated by *in situ* ELISA (Fig. 4A). Levels of GluR1 and GluR2 subunit expression in wild-type and agrin-defi-

cient cultures were indistinguishable from one another and, therefore, unlikely to contribute to the observed differences in response to glutamate.

We next considered the possibility that a reduction in response to glutamate might correlate with altered expression or function of different classes of ionotropic or metabotropic glutamate receptors in the neurons. To examine this hypothesis we used pharmacological agents that selectively block different receptor subtypes to determine the relative contribution of NMDA, non-NMDA, and metabotropic receptors in driving glutamate-induced expression of *c-fos* (Fig. 4B). Incubation with both the NMDA receptor antagonist MK801 and the non-NMDA antagonist NBQX completely blocked glutamate-induced Fos expression in agrin-deficient and wild-type cultures, ruling out variation in metabotropic glutamate receptors as an underlying cause. In contrast, incubation in either MK801 or NBQX alone produced a partial blockade of glutamate induction of *c-fos* in both wild-type and agrin-deficient neurons. Interestingly, the relative levels of inhibition of *c-fos* induction produced by each antagonist were similar in

both genotypes. For example, activation of NMDA receptors accounts for about 70% of the response in both wild-type and agrin-deficient neurons. The results of these experiments, therefore, suggest that NMDA and non-NMDA receptor expression and function are not affected by mutation of the agrin gene.

Evidence for altered calcium signaling in agrin-deficient neurons. The immediate second messenger responsible for glutamate induction of *c-fos* and excitotoxicity is a transient increase in intracellular Ca^{2+} . Increased intracellular Ca^{2+} is also believed to be responsible for signaling events that accompany activation of neuronal nicotinic receptors (reviewed in Ghosh and Greenberg, 1995). However, in addition to Ca^{2+} influx through the receptor channels themselves, activation of voltage-gated Ca^{2+} channels also represents an important pathway contributing to neurotransmitter-dependent changes in intracellular Ca^{2+} (Rajadhyaksha *et al.*, 1999). Therefore, to test for possible genotype-specific differences in voltage-gated Ca^{2+} channel expression and/or function we examined the response of agrin-deficient neurons to Ca^{2+} channel activation. Cultured cortical neurons were briefly depolarized by incubation in 60 mM KCl for 5 min in the presence of different external concentrations of Ca^{2+} and returned to the incubator in normal growth medium for 2 h and the level of Fos expression assayed. To block action potential- and neurotransmitter-dependent induction of *c-fos*, KCl treatment was performed in the presence of TTX, dTbC, BMC, MK801, and NBQX. Under these conditions only voltage-gated Ca^{2+} channels were activated by the KCl treatment since co-incubation with 1 mM CdCl_2 blocked Fos expression (data not shown). Plots of the Ca^{2+} concentration dependence of Fos expression for wild-type and agrin-deficient neurons were well fit by a single exponential nonlinear regression model ($R^2 +/+ = 0.96$; $R^2 -/- = 0.92$). However, the EC_{50} values predicted by these data ($+/+ = 86 \mu\text{M}$; $-/- = 554 \mu\text{M}$) suggest that the agrin-deficient neurons require higher concentrations of Ca^{2+} to achieve the same level of Fos expression as wild-type neurons (Fig. 5A). Consistent with gene dosage, levels of Fos expression in heterozygous neurons were intermediate to those of homozygous cultures (data not shown).

The decreased extent of *c-fos* induction by Ca^{2+} flux through voltage-gated channels in the mutant neurons is consistent with the idea that agrin is required for the organization or maintenance of one or more components of the biochemical cascade responsible for translating a change in intracellular Ca^{2+} into Fos expression. One possibility is that agrin is required for *c-fos* tran-

scription and translation, a relatively late event in the signal pathway. Our observation that high concentrations of extracellular Ca^{2+} offset differences in Ca^{2+} channel-mediated responses of agrin-deficient and wild-type neurons (Fig. 5A) seems inconsistent with a direct effect of the mutation on Fos expression. However, to address this issue more carefully, we examined Fos expression in neurons in which intracellular Ca^{2+} was clamped at known levels by bathing the cultures in medium containing different Ca^{2+} concentrations in the presence of the Ca^{2+} ionophore ionomycin. Under these conditions, the Ca^{2+} dependence of *c-fos* induction in agrin-deficient cultures was identical to that in wild-type cultures (Fig. 5B). Since neither Ca^{2+} -dependent transcription nor translation of *c-fos* is affected by mutation of the agrin gene, these data suggest that perturbation of the signaling pathway upstream of this step is responsible for the agrin-deficient phenotype.

We next examined the possibility that altered expression or function of Ca^{2+} channels might account for the reduced response of agrin-deficient neurons. Whole-cell recording was employed to examine currents flowing through voltage-gated Ca^{2+} channels in randomly selected neurons. Ca^{2+} currents recorded under normal external Ca^{2+} concentration of 1.8 mM were relatively small. Therefore, to increase the signal-to-noise ratio in these experiments, currents flowing through Ca^{2+} channels were recorded in the presence of 4 mM Ba^{2+} . Under these conditions, Ba^{2+} currents were observed in the majority of both wild-type and mutant neurons in response to depolarizing voltage steps. These currents had both rapidly and slowly decaying components and no obvious differences were evident in the kinetics of the currents between genotypes (Fig. 5C). To determine if the agrin gene mutation results in changes in current density, differences in cell size were accommodated by normalizing the peak current amplitude in each neuron to its whole-cell capacitance. No genotype-dependent difference was apparent in either neuronal size ($+/+$, $95.2 \pm 6.1 \text{ pF}$; $+/-$, $84.4 \pm 16.9 \text{ pF}$; $-/-$, $89.6 \pm 15.3 \text{ pF}$) or current density (Fig. 5D). Taken together, it seems unlikely that mutation of the agrin gene affects either expression or function of voltage-gated Ca^{2+} channels.

Altered seizure activity in mice heterozygous for agrin gene mutation. In light of reports that neuromuscular junctions of heterozygous agrin-deficient mice appear normal (Gautam *et al.*, 1996), we were struck by the observation that cultured cortical neurons from heterozygous embryos exhibit reduced responses to glutamate, implying the existence of a corresponding phenotype *in vivo*. Overstimulation of glutamate recep-

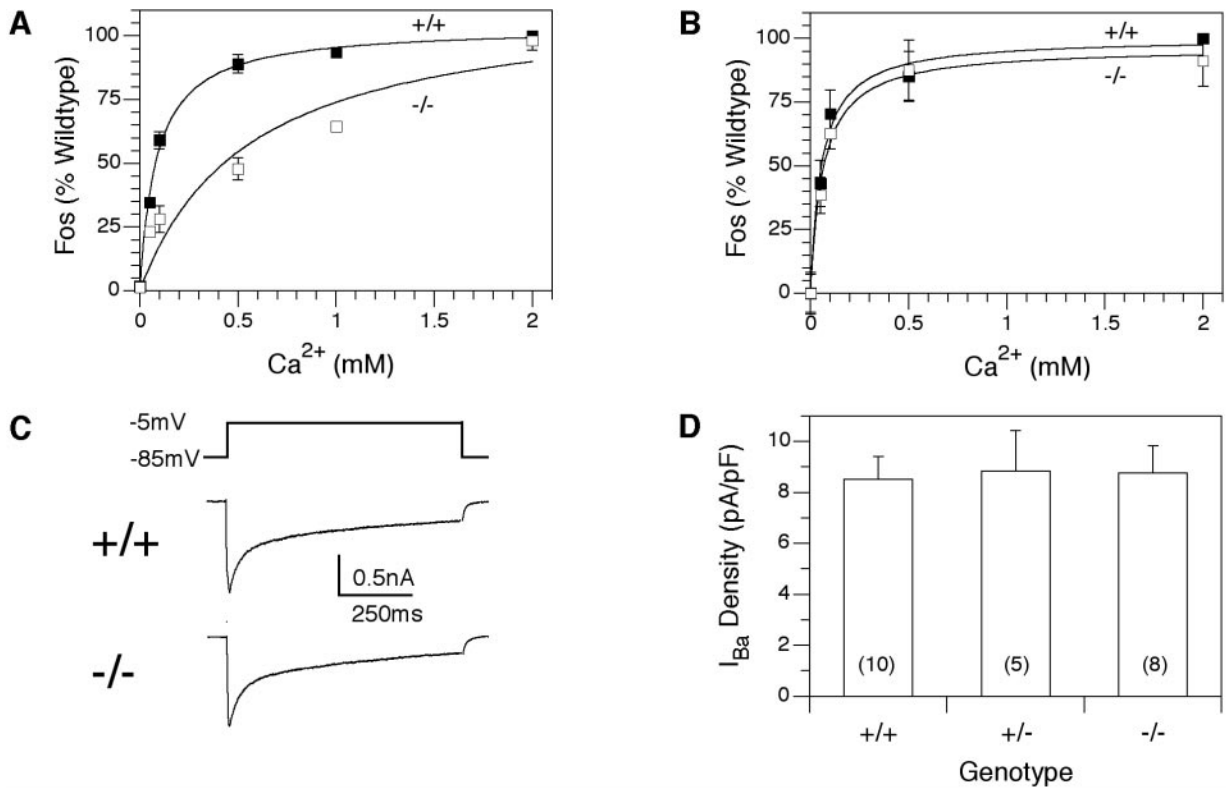


FIG. 5. Mutation of the agrin gene is associated with altered Ca^{2+} -dependent signaling. (A) 10- to 14-day cortical cultures were depolarized for 5 min by treatment with 60 mM KCl in a balanced salt solution at the indicated Ca^{2+} concentrations. Cultures were subsequently returned to normal medium for 2 h and the level of Fos expression was determined. Fos expression in control cultures preincubated with 1 mM CdCl₂ was indistinguishable from that in untreated cultures and has been subtracted. Agrin-deficient neurons are less responsive to induction of *c-fos* triggered by Ca^{2+} influx through voltage-gated channels than wildtype. Data, fit by a single-site nonlinear regression, predict EC₅₀ values of 554 and 86 μ M Ca^{2+} for agrin-deficient and wild-type neurons, respectively. (B) Genotypic differences in Ca^{2+} -dependent signaling are abolished by ionomycin. Neurons were preincubated in a cocktail of inhibitors to block action potential-dependent induction of *c-fos* followed by incubation in 5 μ M ionomycin at the indicated Ca^{2+} concentration. Ca^{2+} -dependent Fos expression measured 1 h following treatment with ionomycin appears identical in wild-type and agrin-deficient cultures. Fos is expressed as a percentage of wild-type Fos at 2 mM Ca^{2+} . Nonspecific induction of Fos by ionomycin at zero Ca^{2+} was similar to that in untreated cultures and has been subtracted. Bars show means \pm SEM of three independent platings. (C) Typical whole-cell currents observed in 10- to 14-day cultured cortical neurons in response to a depolarizing voltage step. Similar peak current amplitudes and decay times were observed in wild-type and agrin-deficient neurons. (D) Consistent with this observation, no evidence was found for a genotypic difference in Ba²⁺ current density when peak current amplitudes were normalized to cell capacitance. Number of neurons analyzed are shown in parentheses. Bars show means \pm SEM obtained from two separate platings.

tors *in vivo* by administration of chemoconvulsant drugs such as kainate induces seizure and increases expression of Fos in neurons in various brain regions, including cortex and hippocampus (reviewed in Herrera and Robertson, 1996). If agrin regulates glutamate sensitivity *in vivo* then agrin heterozygous mice should exhibit increased resistance to kainate. To test this hypothesis 9- to 12-week-old mice were injected subcutaneously with either 35 mg/kg kainic acid or vehicle alone and their behavior was scored at 5-min intervals for 2 h following the injection. At 3 h postinjection the surviving mice were anesthetized and their brains fixed by perfusion, sectioned, and stained for Fos expression.

Collection of behavioral data and counting of Fos-positive cells were performed blind with respect to genotype and treatment of the mice.

Dramatic differences were evident between agrin wild-type and heterozygous mice in behavioral response to kainate (Table 1). About 60% of the wild-type animals died within 2 h following administration of kainate. In contrast, only 10% mortality was observed in heterozygotes. Increased resistance to kainate toxicity was also reflected in a difference in severity of seizures. When scored on seizure scale of 0–5 (no seizure–continuous, tonic–clonic seizures; Schauwecker and Steward, 1997), heterozygous mice exhibited significantly

TABLE 1
Survival and Seizure Scores for Wild-Type and Agrin Heterozygous Mice Following Systemic Treatment with Kainic Acid

Genotype	N	Survival (%)	Seizure index
+ / +	15	40.0	3.63 ± 0.31
+ / -	9	88.9*	2.35 ± 0.44†

Note. The number of mice (*N*) and percentage survival in each genotype at 2 h following subcutaneous injection of 35 mg/kg kainic acid is shown (**P* = 0.02, χ^2). The seizure index represents the mean ± SEM of the average score for each animal in the group observed at 5-min intervals during the 2-h period postinjection (†*P* = 0.02, one-tailed Mann–Whitney *U* test).

less seizure activity during the 2-h monitoring period than wildtype. Further parallels to the findings in cell culture became apparent when we examined the pattern of Fos expression in the brains of surviving animals. Whereas basal levels of Fos expression observed in saline-treated mice were extremely low, Fos-positive neurons were present in several brain regions of both wild-type and heterozygous mice injected with kainate, including cerebral and pyriform cortex, amygdala, and

hippocampus. However, in line with the behavioral scores, Fos expression in brains of heterozygous mice were generally lower than wildtype, a difference that was particularly striking in the dentate gyrus (Fig. 6A). Quantitative analysis using cell counts supported this impression, showing that the density of Fos-positive neurons in the granule cell layer of wild-type mice was almost four times higher than in heterozygous animals following kainic acid treatment (Fig. 6B).

DISCUSSION

We have shown that nicotine- and glutamate-dependent induction of *c-fos* is reduced in agrin-deficient compared to wild-type cultured cortical neurons. Agrin-deficient neurons are also resistant to glutamate excitotoxicity and exhibit reduced levels of Fos expression following activation of voltage-gated Ca²⁺ channels compared to wild-type neurons. Mirroring these results in cell culture, mice heterozygous for the agrin mutation exhibited lower mortality and seizure activity in response to systemic administration of kainate, a

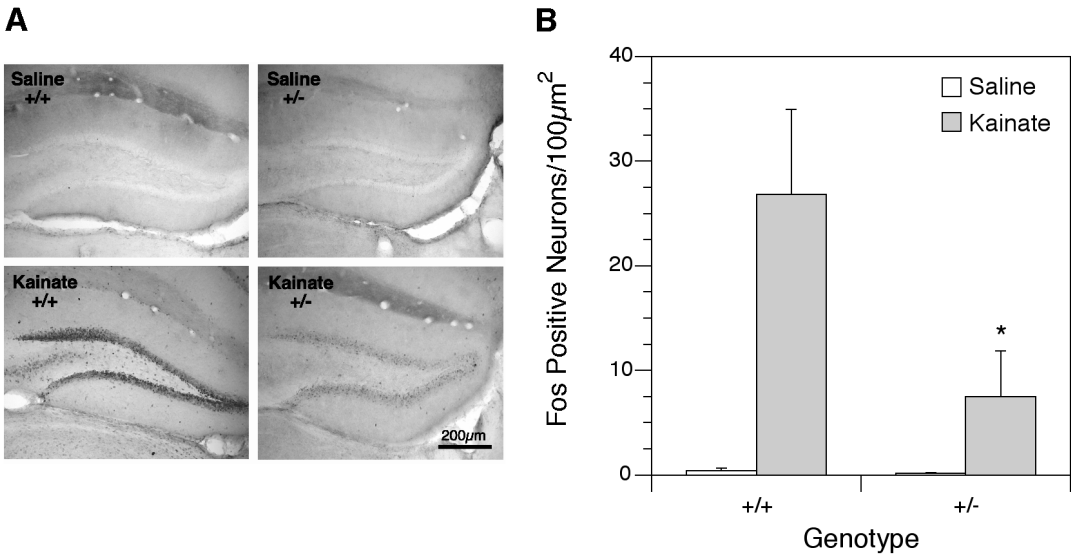


FIG. 6. Neuronal response to kainic acid is reduced in brains of heterozygous agrin-deficient mice. (A) Agrin wild-type and heterozygous mice were injected subcutaneously with 35 mg/kg kainic acid (Kainate) or vehicle alone (Saline). Three hours following injection, mice were anesthetized and fixed by perfusion and the brains were removed, sectioned, and stained for Fos immunoreactivity. Compared to control, in which few if any Fos-immunoreactive neurons were seen, increased Fos expression was observed throughout sections of the dentate gyrus of both wild-type and heterozygous agrin-deficient mice following kainate treatment. However, the number of Fos-immunoreactive neurons was noticeably higher in the wild-type compared to the heterozygous brains. (B) Consistent with this impression, cell counts, performed blind with respect to genotype, showed that the density of Fos-immunoreactive neurons in the dentate gyri of wild-type mice was significantly (**P* ≤ 0.05, one-tailed paired *t* test) higher than in heterozygotes. Bars show means ± SEM of sections taken from brains of a minimum of three wild-type and five heterozygous mice in each group.

potent glutamate receptor agonist. Consistent with these observations, induction of *c-fos* in brains of agrin-deficient mice following kainate treatment was also lower than wildtype. Taken together these data provide strong evidence that agrin plays an important role regulating the responses of cortical neurons to excitatory neurotransmitters both *in vitro* and *in vivo*.

Agrin-deficient neurons exhibited reduced responses to a number of different extracellular stimuli. Several lines of evidence suggest this is directly related to decreased agrin expression rather than a nonspecific effect of the agrin gene mutation. Early stages of synapse formation between agrin-deficient neurons in cell culture are apparently normal (Li et al., 1999; Serpinskaya et al., 1999) and no difference was found in either the number (cell counts) or the size (neuronal LDH and capacitance) of neurons in agrin-deficient and wild-type cultures. Most importantly, application of exogenous agrin resulted in a significant rescue of the decreased responses to glutamate characteristic of the mutant neurons. Our inability to effect a complete rescue in these experiments may have been due to the fact that while the initial concentration of agrin was sufficient to maximally induce *c-fos* acutely (Hilgenberg et al., 1999), loss of agrin from the medium by degradation or adsorption over time may have reduced its effectiveness. Partial rescue of the phenotype might also be an indication that structural domains present in agrin's N-terminal half, which includes regions involved in binding to extracellular matrix and cell surface proteins (Denzer et al., 1995; Tsen et al., 1995), may contribute to agrin function in neurons.

Agrin's AChR clustering activity in skeletal muscle is regulated by alternative splicing at the y and z sites: agrin isoforms with inserts at both sites have high AChR clustering activity, whereas agrin lacking inserts at the y and z sites exhibits little or no activity in this assay (Ferns et al., 1992, 1993). In contrast, agrin's ability to induce *c-fos* in cortical and other CNS neurons is not dependent on alternative splicing and the receptor mediating agrin function in neurons is distinct from that in muscle (Hilgenberg et al., 1999). The observation that agrin_{y4z8} and agrin_{y0z0} are equally effective in rescuing the agrin deficiency suggests that activation of this neuronal agrin receptor does indeed play a critical role in agrin signaling. Interestingly, mutation of the agrin gene used to create the agrin-deficient line used in these studies blocks expression of agrin isoforms containing inserts at the z site while permitting low but detectable levels of agrin_{z0} expression (Gautam et al., 1996; Li et al., 1999). Whereas endogenous agrin_{z0} may be sufficient for some aspects of synapse formation, such as cluster-

ing of neurotransmitter receptors (Li et al., 1999; Serpinskaya et al., 1999), other aspects of synaptogenesis, such as maturation and stabilization, may require higher levels of agrin or even depend on other agrin isoforms. Recent studies showing that synGAP, a component of a multimeric complex that includes the NMDA receptor, is gradually lost from synaptic contacts formed between agrin-deficient hippocampal neurons (Böse et al., 2000) support this hypothesis.

The diversity of extracellular stimuli affected by the agrin deficiency raises questions as to the biochemical site of agrin action. Just as accumulation of neurotransmitter receptors at synaptic sites is not agrin-dependent (Li et al., 1999; Serpinskaya et al., 1999), no evidence was found to support a role for agrin in regulating general expression of either glutamate receptors or nAChR in neuron surface membranes. This observation points to a defect in signaling as opposed to alterations in the fast ligand-gated events mediated by these neurotransmitter receptors. Increased intracellular Ca²⁺ is the primary second messenger driving both neurotransmitter induction of *c-fos* and glutamate excitotoxicity (Ghosh and Greenberg, 1995). That agrin is required for propagation of Ca²⁺ signals within neurons is supported by the finding that voltage-gated Ca²⁺ channel-mediated induction of *c-fos* was also attenuated in agrin-deficient neurons, even though the Ca²⁺ current density was similar to wildtype. The observation that Ca²⁺-dependent induction of *c-fos* was normal in agrin-deficient neurons treated with ionomycin rules out a direct effect on *c-fos* transcription and translation, suggesting instead that the agrin-deficient phenotype reflects an altered response to transient changes in intracellular Ca²⁺.

The complexity of the chain of events between channel activation and expression of Fos (or excitotoxic cell death) makes it difficult to pinpoint a specific agrin-dependent locus. Such an effect could be mediated by altered expression or function of any one of the myriad Ca²⁺ effector proteins within the MAPK/ERK pathway, as well as changes in the pumps and channels that control mobilization of Ca²⁺ from intracellular stores and its removal from the cell cytoplasm. Further biochemical analysis of Ca²⁺ signaling in agrin-deficient neurons may prove useful in this regard as will identification of differentially expressed genes in agrin-deficient and agrin-treated cortical neurons. Regardless of its exact locus of action, the demonstration here that agrin modulates long-term signaling through ligand-gated and voltage-gated channels suggests that agrin may modulate a wide range of processes in brain. That agrin is concentrated at neuron-neuron synapses

(Mann and Kröger, 1996; Gingras and Ferns, 2001) suggests that the most profound effects may be synaptic.

As predicted by the results of our studies in cell culture, the number of Fos-immunoreactive neurons was lower in brains of agrin heterozygous compared to wild-type mice following systemic administration of kainic acid. Consistent with this observation kainate-induced seizure activity and mortality were also lower in the heterozygotes. A significant fraction of agrin expressed in brain is associated with capillaries and other blood vessels, where it may play a role in regulating the permeability of the blood–brain barrier (Barber and Lieth, 1997). We cannot discount the possibility that differences we report here are due to decreased permeability of the blood–brain barrier to kainate in agrin heterozygotes; nevertheless it seems unlikely. Such an explanation would not account for differences in response to glutamate and nicotine observed in cell culture where no such barrier to diffusion exists. Moreover, metalloproteinase-mediated degradation of extracellular matrix components, likely to include agrin, is associated with increased rather than decreased permeability of the blood–brain barrier in CNS disease (Lukes *et al.*, 1999; VanSaun and Werle, 2000). More plausible is that mutation of the agrin gene attenuates glutamate receptor activation and/or the neural activity triggered by it. Consistent with this apparent decline in kainate sensitivity *in vivo* is the finding that evoked currents recorded from synapses on cultured hippocampal neurons formed at low levels of agrin expression are smaller and exhibit higher failure rates than normal (Böse *et al.*, 2000). In light of these observations, it will be important to learn whether morphological or functional changes can be detected at CNS synapses of agrin heterozygous mice.

Recent studies suggest that agrin may play a role in the etiology of Alzheimer's disease (AD). Agrin is expressed at high levels in AD brains, binds to β -amyloid, and is a major component of the neurofibrillar tangles and senile plaques characteristic of AD (Donahue *et al.*, 1999; Verbeek *et al.*, 1999; Cotman *et al.*, 2000). Several lines of evidence suggest that the protein products of immediate-early genes act as mediators of neurodegeneration. For example, expression of *c-fos* has been shown to be associated with developmentally regulated apoptosis in the brain (Smeyne *et al.*, 1993; Kasof *et al.*, 1995) and treatment with cycloheximide prevents both kainate excitotoxicity and *c-fos* expression (Schreiber *et al.*, 1993). We have previously demonstrated that agrin induces *c-fos* in cortical and other CNS neurons (Hilgenberg *et al.*, 1999). Decreased agrin expression is correlated with a reduction in the response of cortical

neurons to excitatory amino acids, measured in terms of Fos expression or neurodegeneration. This raises the possibility that high concentrations of agrin within senile plaques may contribute to the death of surrounding neurons both through direct activation of the *c-fos* induction cascade and by increasing neuronal sensitivity to glutamate and other neurotransmitters. Although it is not known whether agrin complexed with β -amyloid is still active, *c-fos* is upregulated in AD brain (Zhang *et al.*, 1992; Marcus *et al.*, 1998).

EXPERIMENTAL METHODS

Tissue culture. Cortical cultures were prepared from whole cortices of individual embryonic day 18 (E18)–E19 mouse fetuses that resulted from pairing of mice heterozygous for a mutation in the agrin gene (Gautam *et al.*, 1996). Cultures were prepared as described (Li *et al.*, 1997) and their genotypes determined by PCR analysis of genomic DNA obtained from tissue samples of each embryo (Li *et al.*, 1999). Neurons were plated into 24-well plastic tissue culture plates (Corning) or onto glass coverslips coated with poly-d-lysine (Collaborative Biomedical Products). Cultures were maintained in Neurobasal Medium + B27 supplements (NBM; Life Technologies) at 37°C in a humidified 5% CO₂ atmosphere overnight. After 24 h, the culture medium was replaced with cNBM (NBM that had been conditioned for 24 h by nonneuronal feeder cell cultures) and the cultures were fed with cNBM every 2–3 days thereafter (Hilgenberg *et al.*, 1999).

Immunocytochemistry. Cultures were prepared for immunocytochemistry as described (Hilgenberg *et al.*, 1999). To identify cells expressing Fos, cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) on ice for 1 h, followed by labeling with rabbit anti-Fos antibody (Ab-2; Oncogene Research Products) either alone or in combination with an anti-GFAP (G-A-5; Sigma) mouse monoclonal antibody diluted 1:400 in PBS-BTA (PBS containing 0.1% Triton X-100, 4% BSA, 0.02% sodium azide). Cultures were washed in PBS, followed by co-incubation with Texas red-labeled goat anti-rabbit IgG and/or FITC-labeled horse anti-mouse IgG (Vector Laboratories) diluted 1:200 in PBS-BTA. Finally, cultures were washed, mounted in Fluoromount (Southern Biotechnology), and viewed under epifluorescence illumination on a Nikon Optiphot-2 microscope. Images were captured using a SpotCam digital camera and software (Diagnostic Instruments, software version 1.2.1).

Quantitative analysis of Fos expression. Triplicate wells of 10- to 14-day-old cultures were exposed to the test stimulus (nicotine, glutamate, or KCl) for 5 min at room temperature in NBM, washed in fresh NBM, and returned to the incubator for 2 h at 37°C to allow *c-fos* transcription and translation to take place. Levels of Fos expression were determined by *in situ* ELISA as described (Hilgenberg et al., 1999). Briefly, cultures were fixed and incubated overnight in Ab-2 and diluted 1:1000 in PBS-BTA, followed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit antibody (Southern Biotechnology) diluted 1:1000 in PBS-BTA. Fos expression was determined by conversion of *p*-nitrophenyl phosphate (pNPP) substrate to a soluble yellow reaction product and the concentration in the supernatant determined by adsorption at 405 nm (Hilgenberg et al., 1999). Statistical analyses were performed on data from a minimum of three independent experiments.

Analysis of glutamate-induced excitotoxicity. Cortical cultures were incubated with glutamate in the presence of 1 μ M glycine (Johnson and Ascher, 1987; Finkbeiner and Stevens, 1988) in NBM for 5 min, rinsed in NBM, and returned to the incubator for 4 h at 37°C. Overall levels of neuronal cell death were assessed by counting phase-bright cells with neuronal morphology in randomly selected fields at 200 \times magnification. Quantitative analysis of neuronal injury was performed by measuring the level of LDH released into the culture medium (Koh and Choi, 1987). LDH values were normalized to total neuronal LDH determined from sister cultures exposed to 300 μ M glutamate overnight, a treatment that results in 100% neuronal cell death.

Analysis of nAChR expression. Cortical cultures were incubated in 10 nM 125 I-BTX in NBM containing 2% FBS for 1 h at 37°C. Unbound 125 I-BTX was removed by washing the cultures 4 \times 10 min in PBS. Cells were scraped into 0.5 ml of 0.5 N NaOH and the amount of 125 I-BTX bound was measured by gamma counting. Nonspecific binding of 125 I-BTX was determined by labeling in the presence of 0.5 mM dTbC. Each determination was performed on triplicate wells.

Analysis of AMPA receptor expression. AMPA receptor expression levels were determined by measuring binding of antibodies directed at extracellular epitopes on GluR1 and GluR2 AMPA receptor subunits in intact cultured cortical neurons by *in situ* ELISA (Hilgenberg et al., 1999). Cultures were incubated in anti-GluR1 or GluR2 goat antibody (Santa Cruz Biotechnology) diluted 1:1000 in a balanced salt solution (BSS) for 20 min at room temperature. Cultures were subsequently washed in BSS, fixed, and incubated with an alkaline

phosphatase-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories) diluted 1:1000 in PBS-BTA. The amount of alkaline phosphatase activity in each culture, corresponding to the amount of anti-GluR antibody bound, was determined by incubation with pNPP substrate and measuring the OD_{405 nm} of the resulting soluble yellow reaction product. Determinations were performed in triplicate for each experiment.

Electrophysiology. Electrophysiological recordings were obtained using the whole-cell recording technique (Hamill et al., 1981). Cells were bathed in an external recording solution containing 140 mM NaCl, 4 mM BaCl₂, 1 mM CaCl₂, 4 mM MgCl₂, 3 mM KCl, 5 mM Hepes, pH 7.2, and supplemented with 1 μ M TTX, 5 μ M CNQX, 50 μ M APV, and 10 μ M picrotoxin. Currents were elicited by stepping cells from a holding potential of -85 to -5 mV and recorded via glass pipettes filled with a solution containing 120 mM CsOH, 120 mM d-glucuronic acid, 0.1 mM CaCl₂, 2 mM MgCl₂, 20 mM NaCl, 1.1 mM EGTA, 4 mM ATP, and 10 mM Hepes, pH 7.2, and tip resistance of 2–4 M Ω . Currents were recorded with an Axopatch 200A amplifier (Axon Instruments), filtered at 2 kHz, and digitized at 10 kHz using a Digidata 1200 interface and pClamp 6 software (Axon Instruments). Cell capacitance was measured from the transient current elicited by a 10-mV step above the holding potential.

Kainic acid administration and behavioral monitoring. The effect of systemic administration of kainic acid was studied in 9- to 12-week-old heterozygous agrin mutant mice and their wild-type littermates. Mice were injected subcutaneously with 35 mg/kg kainic acid (Sigma) dissolved in saline or with saline alone. Animals were monitored every 5 min for 2 h postinjection for the onset and intensity of seizures. Seizures were rated according to a previously defined scale (Racine, 1972; Schauwecker and Steward, 1997): stage 1, immobility; stage 2, forelimb and/or tail extension; stage 3, repetitive movements, head shaking; stage 4, rearing and falling; stage 5, continuous tonic-clonic seizures. Collection of behavioral data was performed "blind" with respect to genotype and treatment of the animals.

Analysis of kainate induction of c-fos in brain. Animals surviving 3 h following injection of kainic acid or saline were anesthetized with pentobarbital (300 mg/kg ip) and perfused transcardially with 30 ml saline solution followed by 120 ml of 4% paraformaldehyde in PBS. Brains were removed and postfixed in 4% paraformaldehyde in PBS for 2 h and then kept in 30% sucrose-PBS at 4°C overnight. Coronal sections (50 μ m)

were cut on a sliding microtome and every sixth section was stained for Fos immunoreactivity by overnight incubation at 4°C with Ab-2 diluted 1:1000. Labeling was visualized using the horseradish peroxidase VectorStain ABC Elite protocol with nickel intensification according to the manufacturer's instructions (Vector Laboratories). Counts of Fos-positive cells in sections of the dentate gyrus were performed blind with respect to genotype at 250× magnification on digital images acquired using a SpotCam digital camera mounted on a Nikon Optiphot-2 microscope.

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