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The Anticonvulsants Lamotrigine, Riluzole, and Valproate Differentially Regulate AMPA Receptor Membrane Localization: Relationship to Clinical Effects in Mood Disorders

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A growing body of data suggests that the glutamatergic system may be involved in the pathophysiology and treatment of severe mood disorders. Chronic treatment with the antimanic agents, lithium and valproate, resulted in reduced synaptic expression of the AMPA(-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor subunit GluR1 in the hippocampus, while treatment with an antidepressant (imipramine) enhanced the synaptic expression of GluR1. The anticonvulsants, lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine) and riluzole (2-amino-6-trifluoromethoxybenzothiazole), have been demonstrated to have efficacy in the depressive phase of bipolar disorder. We therefore sought to determine the role of these anticonvulsants, compared to that of the predominantly antimanic anticonvulsant valproate, on AMPA receptor localization. We found that the agents with a predominantly antidepressant profile, namely lamotrigine and riluzole, significantly enhanced the surface expression of GluR1 and GluR2 in a time- and dose-dependent manner in cultured hippocampal neurons. By contrast, the predominantly antimanic agent, valproate, significantly reduced surface expression of GluR1 and GluR2. Concomitant with the GluR1 and GluR2 changes, the peak value of depolarized membrane potential evoked by AMPA was significantly higher in lamotrigine- and riluzole-treated neurons, supporting the surface receptor changes. Phosphorylation of GluR1 at the PKA (cAMP-dependent protein kinase) site (S845) was enhanced in both lamotrigine- and riluzole-treated hippocampal neurons, but reduced in valproate-treated neurons. In addition, lamotrigine and riluzole, as well as the traditional antidepressant imipramine, also increased GluR1 phosphorylation at GluR1 (S845) in the hippocampus after chronic *in vivo* treatment. Our findings suggest that regulation of GluR1/2 surface levels and function may be responsible for the different clinical profile of anticonvulsants (antimanic or antidepressant), and may suggest avenues for the development of novel therapeutics for these illnesses.
Neuropsychopharmacology (2007) **32,** 793–802. doi:10.1038/sj.npp.1301178; published online 16 August 2006

Keywords: AMPA receptors; phosphorylation; lamotrigine; riluzole; valproate; imipramine

INTRODUCTION

Bipolar disorder is a common, severe, and often life-threatening disorder. The recognition of the significant morbidity and mortality of patients with severe mood disorders, as well as the growing appreciation that a significant percentage of patients respond poorly to existing treatments has made the task of discovering new therapeutic agents increasingly more important. In recent years, a

growing number of anticonvulsants have been utilized in the treatment of the disorder (Ernst and Goldberg, 2003; Evins, 2003). Indeed, based on the ability of anticonvulsants to reduce neural excitability, there has been a growing assumption that most anticonvulsants would have some utility in the treatment of the manic phase of the disorder; such a contention is supported by the widespread use of valproate for the treatment of the manic phase of the disorder. This assumption was called into question by the demonstration—in a double-blind, placebo-controlled study—that lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine) was effective in acute bipolar *depression* (Calabrese *et al*, 1999); indeed, lamotrigine is now increasingly accepted as a very effective agent for the depressed phase of bipolar disorder, but its use is limited by its side-effect profile. Lamotrigine is a comparatively novel antiepileptic agent used primarily in the treatment of

generalized and partial seizures (Bazil, 2002; Kwan and Brodie, 2001); its mechanism of action is considered to be a reduction in glutamate release following inhibition of Na$^+$ channels and consequent neurotransmitter glutamate exocytosis (Ahmad et al, 2004; Lees and Leach, 1993). Riluzole (2-amino-6-trifluoromethoxybenzothiazole), an inhibitor of sodium channel and thus glutamate release, is approved by the US Food and Drug Administration for the treatment of amyotrophic lateral sclerosis (Bensimon et al, 1994). Similar to lamotrigine, it also has anticonvulsant and neuroprotective properties. Recent studies show that riluzole was effective in patients with major (unipolar) and bipolar depression (Zarate et al, 2004; Zarate et al, 2005). Since acute regulation of Na$^+$ channels, calcium channels, or glutamate release (Rogawski and Loscher, 2004) does not adequately explain the differing clinical profile of these agents in the treatment of mood disorders, we sought to investigate additional potential mechanisms.

The potential role of glutamatergic system in the pathophysiology of and treatment of mood disorders has been investigated in earnest only recently (Bonanno et al, 2005; Coyle and Duman, 2003; Du et al, 2004; Krystal et al, 2002; Sun et al, 2005). It is noteworthy that a growing body of data suggests that AMPA (-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptor trafficking (including receptor insertion, internalization, and delivery to synaptic sites) plays a critical role in regulating activity-dependent regulation of synaptic strength, as well as various forms of neural and behavioral plasticity (Bonanno et al, 2005; Carlezon and Nestler, 2002; Kendell et al, 2005; Malenka, 2003; Rumpel et al, 2005; Sun et al, 2005; Wolf et al, 2004). Three major classes of ionotropic glutamate receptors are expressed throughout the mammalian central nervous system (CNS), including AMPA, kainate, and N-methyl-D-aspartate (NMDA) receptors. AMPA receptors mediate the majority of excitatory synaptic transmission in the CNS. The AMPA receptor channel is composed of the subunits GluR1, GluR2, GluR3, and GluR4. Emerging data suggest that a modification of the levels of synaptic expression of these subunits is a critically important mechanism for regulating postsynaptic responsiveness at many different synapses. AMPA receptor subunit GluR1 and GluR2 trafficking is regulated by cAMP-dependent protein kinase (PKA), calcium-calmodulin-dependent protein kinase II (CAMKII), PKC, and ERK through phosphorylation of specific sites (Esteban et al, 2003; Lee et al, 1998; Malinow and Malenka, 2002). Notably, many of these cascades are targets for mood-stabilizing agents and antidepressants (Donati and Rasenick, 2003; Manji and Lenox, 2000; Shao et al, 2005). We therefore sought to investigate concurrently the effects of lamotrigine, riluzole, and valproate on surface AMPA receptors, AMPA phosphorylation, and AMPA function.

We have found that the two anticonvulsants lamotrigine and riluzole (with a predominantly antidepressant profile, but possessing structural dissimilarity), when administered chronically at therapeutically relevant concentrations, enhanced AMPA receptor GluR1 and GluR2 distribution on the neuronal surface. The increase in surface GluR1 and GluR2 levels was accompanied by enhanced depolarization of membrane potential. In dramatic contrast, the anticonvulsant valproate (with a predominantly antimanic

profile) exerted opposite effects on GluR1 and GluR2 localization. In addition, phosphorylation of GluR1 at S845 site was significantly increased after treatment with lamotrigine and riluzole in vitro and in vivo, while valproate demonstrated an opposite effect at the same site. Finally, chronic in vivo treatment of rodents with the traditional antidepressant imipramine, or riluzole, or lamotrigine, all resulted in increased GluR1 phosphorylation at the S845 site. Together, these data raise the intriguing possibility that chronic effects on AMPA receptor localization may play a role in conferring a predominantly antidepressant or antimanic profile to anticonvulsant agents.

MATERIALS AND METHODS

Neuronal Culture Preparations

Cultures of hippocampal neurons were prepared utilizing previously published procedures with minor modifications (Du et al, 2000). Briefly, whole hippocampi were dissected from embryonic day 18 (E18) rats, dissociated in Ca^{2+}- and Mg^{2+}-free Hank's balance salt solution containing 0.125% trypsin for 15 min, triturated in DMEM (Invitrogen)/10% fetal bovine serum, and plated at 0.4 million cells/well in six-well plates. Cells were grown at 37°C, 5% CO$_2$, and 95% humidity, first in 10% fetal bovine serum/DMEM, and switched 1 day later to serum-free medium, neurobasal plus B27 (B27NB, Invitrogen). The cultures were grown in serum-free medium for 8–10 days before the start of experiments, and the medium was changed every 3 days. Fresh medium was applied 24 h before each experiment. These cultures yielded virtually pure neurons (Supplementary Figure 2). Doses of drugs chosen were based on their therapeutic plasma concentrations clinically (Groeneveld et al, 2003; Hirsch et al, 2004; Zarate et al, 2005). Thus, concentrations of lamotrigine (20 μM), riluzole (2.0 μM), and valproate (1.0 mM) were chosen for our studies.

Surface Biotinylation and Western Blot Analysis of GluR1 and GluR2

Surface GluR1 and GluR2 receptors were detected by biotinylation assay followed by Western blot analysis using either an anti-GluR1 or anti-GluR2 antibody (Chemicon), as described. After a 3-day treatment with lamotrigine, riluzole, or valproate, ice-cold phosphate buffer saline (PBS with calcium and magnesium, pH 7.4, Invitrogen) was added to the cultures to prevent receptor internalization. The cells were washed three times with ice-cold PBS and incubated in Sulfo-NHS-LC-biotin (0.25 mg/ml in cold PBS, Pierce) for 30 min. Surface biotinylation was stopped by removing above solution and incubating the cells in 10 mM ice-cold glycine in PBS for 20 min. Cells were then washed three times with cold PBS and harvested with RIPA buffer, containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM Na orthovanadate, 50 mM NaF, 1% NP-40, 1% deoxycholate, 0.1% SDS, Protease Inhibitor Cocktail (Sigma), Phosphotase Inhibitor Cocktail I (Sigma), and Phosphotase Inhibitor Cocktail II (Sigma). Protein concentrations were determined by BCA protein assay kit (Pierce). Biotinylated proteins were precipitated with 100 μl of ImmunoPure Immobilized Streptavidin (Pierce), separated

on 10% SDS-PAGE gel, and transferred to nitrocellulose membrane. The membranes were probed with a polyclonal anti-GluR1 or anti-GluR2 antibodies (1:200, Chemicon), followed by peroxidase-conjugated goat anti-rabbit IgG (1:3000, Vector). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham). The ECL signal intensities were quantified with the Kodak Image program.

In order to measure the GluR1 phosphorylation of GluR1 at the PKA site and the total amount of GluRs, cultured hippocampal neurons were harvested with RIPA buffer containing protease inhibitors and phosphatase inhibitors after treatment with drugs with the concentrations indicated. The protein concentrations were determined by BCA protein assay kit (Pierce). Equal amounts of cell homogenates were separated by electrophoresis. We used 10 μg for the cell lysates which is within the linear rage (Supplementary Figure 1). Western blot analysis was performed with anti-phospho-GluR1 (S845) antibody (Covance) or anti-actin antibody (Sigma). The membrane was then stripped and Western blot analysis of GluR1 was performed. Quantitation for each experimental condition was based on 3–6 independent experiments (samples); each was repeated at least 2–3 times. The results were pooled and averaged, and presented as mean ± SE. The data were analyzed by Student's *t*-test.

Measurement of Membrane Potential Changes by DiBAC$_4$(3)

Hippocampal neurons were cultured for 10 days and treated with either lamotrigine (20 μM) or riluzole (2 μM) for additional 3 days. The neurons were then preincubated in MIC buffer (NaCl 130 mM, KCl 5.3 mM, MgSO$_4$ 0.8 mM, Na$_2$HPO$_4$ 1 mM, glucose 2 mM, HEPES 20 mM, Na-pyruvate 1 mM, NaHCO$_3$ 2.5 mM, ascorbic acid 1.0 mM, CaCl$_2$ 1.5 mM, BSA 1.5 mg/ml) containing the corresponding drugs such as lamotrigine or riluzole for 30 min. The buffer was then changed to 1350 μl of 500 nM DiBAC$_4$(3) (Anaspec) containing buffer. After a 10-min incubation, images were acquired by Zeiss LSM 510 confocal microscope. One hundred and fifty microliters of 10 μM AMPA solution were applied to the neurons to reach the final concentration of 1 μM in the solution. Fields for image were random. Images were captured every 10 s for 7–14 min by Zeiss LSM 510 confocal microscopy. Fluorescence was excited at 488 nm and emission was measured at 505 nm. The intensity of each neuron was quantified by Zeiss LSM 510 program. The experiments were repeated independently for three times and peak value data were combined for all three experiments. Data were analyzed by Dunnett's multiple comparison test.

## Animal Treatment and Sample Analysis

C57BL/6 mice were obtained from a breeding colony from Taconic Farm. Animals were group housed (*n* = 4/cage) in polypropylene cages, and maintained on a 12-h light/dark cycle at 25°C and had free access to water and chow. Lamotrigine (25 mg/kg, once daily), riluzole, (10 mg/kg, once daily), or imipramine (10 mg/kg, twice daily) were dissolved in 3% of total volume of DMSO and then 0.5% of

Tween 80 in saline and injected (intraperitoneally) into the animals for 10 days (*N* = 6 animals per group for lamotrigine and riluzole; and *N* = 11 animals for imipramine (impramine treatment was undertaken for another biochemical assay as well, resulting in the larger sample size)) (Boireau *et al*, 2000; Szymczyk and Zebrowska-Lupina, 2000). The hippocampi from the mouse brains were removed immediately after decapitation, immersed in liquid nitrogen, placed in labeled Eppendorf tubes on dry ice, and then stored and kept frozen at −70°C until processing. The samples were sonicated in homogenization buffer A (50 mM Tris-Cl, pH 7.5, containing 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 50 μM 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride, 50 mM KF, 50 nM okadaic acid, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 0.1% NP-40, and 5 μg/ml each of leupeptin, aprotinin, chymostatin, and pepstatin A), spun in the Eppendorf 5810R centrifuge for 20 min at 4°C at 20 000 r.c.f., and the clear homogenate used for (1) protein determination, (2) immunoblot analysis of phosphorylated, and total GluR1 levels in the hippocampus. Protein concentrations were determined using the BCA assay kit; the linearity of the protein concentration for immunoblotting was ascertained by resolution of selected concentrations of protein. Equal amounts of proteins were subjected to 10% SDS-PAGE gels and separated by electrophoresis. Proteins were then electrophoretically transferred to nitrocellulose membranes. Nonspecific binding on the nitrocellulose was blocked with *T*ris-*b*uffered *s*aline plus *T*ween 20 (TBST), 10% nonfat dry milk, and then incubated with anti-phospho-GluR1S845 antibody. The secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-mouse IgG or HRP-anti-rabbit IgG. The ECL plus kit was used as a detection system. Of note, in these experiments, nitrocellulose membranes were first probed with anti-phospho-GluR1S845 antibody and then stripped with stripping buffer and re-probed with anti-GluR1 antibody. The bottom part of the same membrane was used for Western blot analysis of actin (Sigma) to assure that equal amount of protein was loaded to the wells. The significance of each groups were tested by Student's *t*-test.

RESULTS

Lamotrigine and Riluzole Treatments Enhance Surface Expression of GluR1 in Cultured Hippocampal Neurons, while Valproate Treatment Reduces it

Hippocampal neuronal cultures were prepared from E18 embryo to yield a pure neuron population and cultured for 8–10 days to gain synaptic neuronal connections. We chose therapeutically relevant concentrations of 20 μM for lamotrigine and 2.0 for riluzole, based on patient's serum concentrations. Different doses of lamotrigine (4, 20, 100 μM), riluzole (0.4, 20, 100 μM), or valproate (1.0 mM) were applied to cultured hippocampal neurons, and the surface GluR1 levels were then determined by biotinylation assay. Both lamotrigine and riluzole significantly enhanced GluR1 surface expression after 3 days of treatment, with maximum increase of 168 and 156%, respectively (Figure 1a–e). We also determined the time course for riluzole and lamotrigine in therapeutically relevant concentrations. Cells
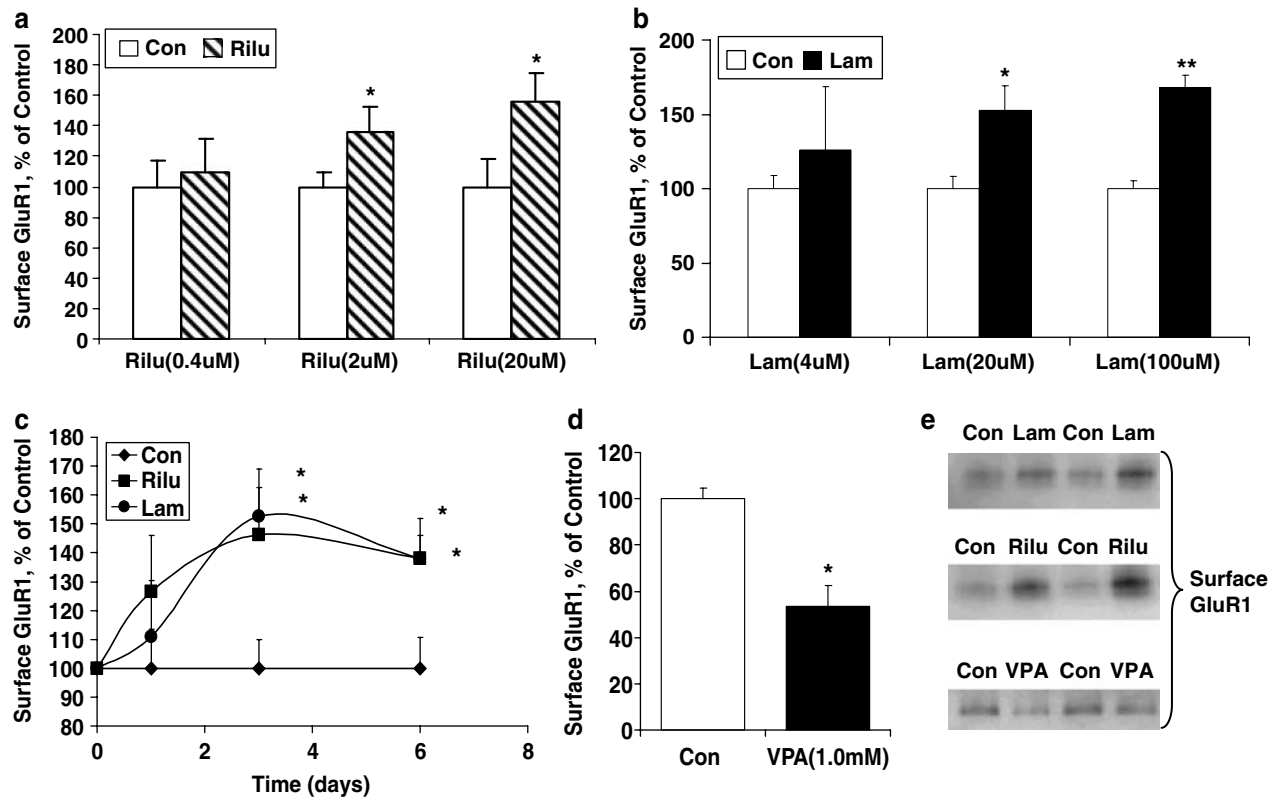
Figure 1 Lamotrigine and riluzole treatments enhance surface expression of GluR1 in cultured hippocampal neurons, while valproate treatment reduces it. Hippocampal primary culture neurons were prepared from E18 Sprague–Dawley embryos. After 8–10 days of culturing in B27-neurobasal media, neurons were treated with lamotrigine (Lam), or riluzole (Ril), or valproate (VPA) in the concentrations and for the times indicated. Surface proteins of the neurons were labeled with biotin and the cells were then harvested with RIPA buffer. Biotinylated surface proteins were precipitated by immobilized-avidin and analyzed by Western blot analysis with anti-GluR1 antibody. Data were analyzed by Kodak imaging system and were pooled from 2–3 independent experiments and presented as mean $\pm$ SE ($n = 9$–11, Student's t-test, $*p < 0.05$, $**p < 0.01$). (a) Dose dependency for riluzole treatment on surface GluR1 in cultured hippocampal neurons after 3 days of treatment. (b) Dose dependency for lamotrigine treatment on surface GluR1 in cultured hippocampal neurons after 3 days of treatment. (c) Time course after treatment of lamotrigine (20 μM) and riluzole (2.0 μM) on surface GluR1 in cultured hippocampal neurons. (d) Effect of valproate (1.0 mM) on surface GluR1 after 3-day treatment in cultured hippocampal neurons. (e) Samples of Western blot analysis of surface GluR1 after therapeutically relevant concentrations of lamotrigine (20 μM), riluzole (2.0 μM), and valproate (1.0 mM) treatments.

treated with lamotrigine (20 μM) and riluzole (2 μM) significantly enhanced GluR1 on the surface after 3 days, effects that were sustained through 6 days (Figure 1c). In dramatic contrast, valproate reduced surface GluR1 levels to 53.6% after 3 days of treatment (Figure 1d and e).
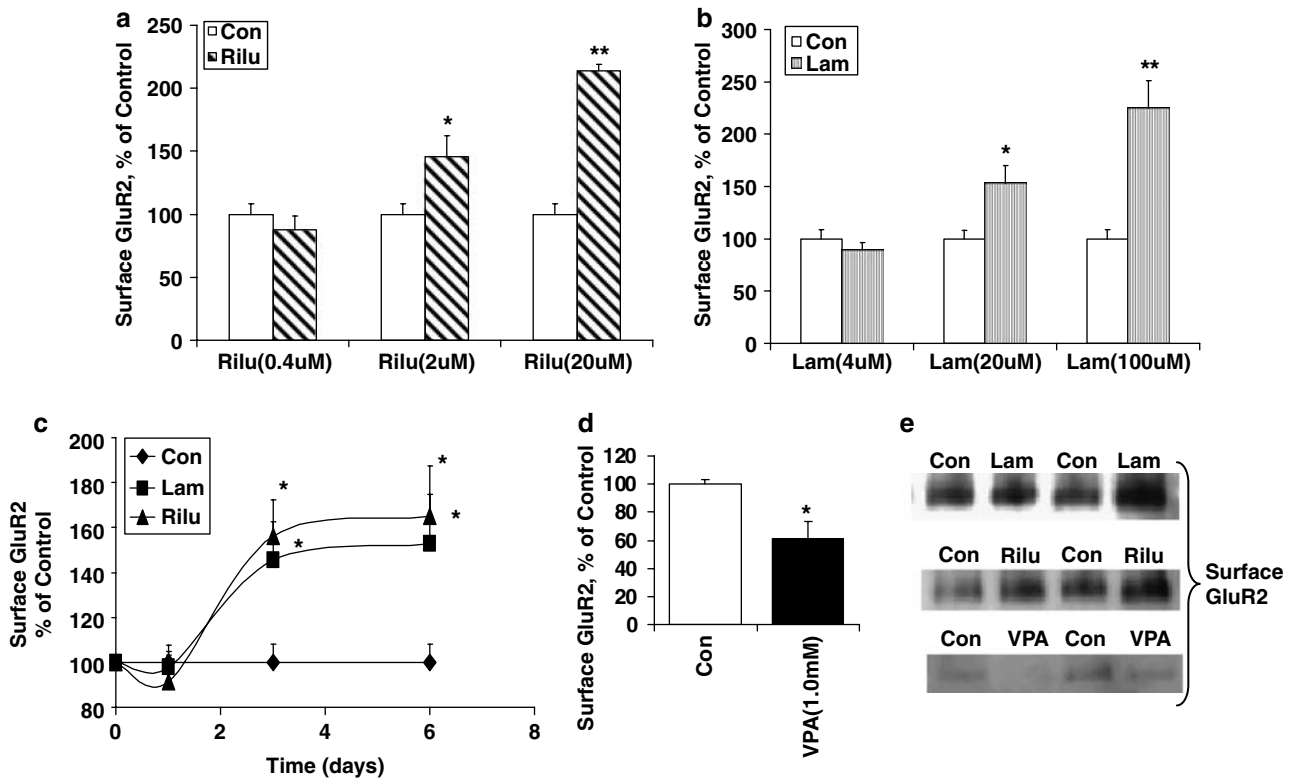
Lamotrigine and Riluzole Treatments Enhance Surface Expression of GluR2 in Cultured Hippocampal Neurons, while Valproate Treatment Reduces it

GluR1 and GluR2 form heterotetramers; thus, we next sought to determine whether GluR2 surface expression is also altered by these treatments. Hippocampal neurons were treated with lamotrigine (4, 20, 100 μM), or riluzole (0.4, 2.0, 20 μM), or valproate (1.0 mM) for 3 days. Surface GluR2 levels were determined by biotinylation assay. Both lamotrigine and riluzole significantly enhanced GluR2 surface expression in a dose- and time-dependent manner, with the maximum increase of 225 and 213%, respectively (Figure 2a, b, and e). Notably, lamotrigine and riluzole enhanced GluR2 surface expression in the therapeutically relevant concentrations to 153 and 146%, respectively, after 3 days of treatment, and these effects sustained through 6

days (Figure 2c). Once again, in dramatic contrast, valproate (1 mM) reduced surface GluR2 levels to 60.9% after 3 days of treatment (Figure 2d and e).

Membrane Depolarization is Significantly Increased with AMPA Stimulation after Riluzole and Lamotrigine Treatments

Next, we investigated the functional consequence of the increased number of AMPA receptors on the neuronal surface after treatment with either lamotrigine or riluzole. Hippocampal neurons were cultured for 10 days and treated with riluzole (2.0 μM) and lamotrigine (20 μM) for 3 additional days. The cells were incubated with DiBAC$_4$(3) dye and their membrane potentials were determined by fluorescent color changes after AMPA stimulation. TTX (10 μM) was applied to the hippocampal neurons to stop the spontaneous activity. AMPA-induced depolarization was significantly higher in the lamotrigine- and riluzole-treated groups (Figure 3a and b). The peak values of depolarization in riluzole- and lamotrigine-treated group were 150.6 and 130.6% of control, respectively (Figure 3c).

Figure 2 Lamotrigine and riluzole treatments enhance surface expression of GluR2 in cultured hippocampal neurons, while valproate treatment reduces it. Hippocampal neurons were treated with lamotrigine, or riluzole, or valproate for the times and doses indicated. Biotinylated surface proteins were precipitated by immobilized-avidin and analyzed by Western blot analysis with anti-GluR2 antibody. Data were analyzed by Kodak imaging system and were pooled from 2–3 independent experiments and presented as mean $\pm$ SE ($n = 6$–10, Student's t-test, $*p < 0.05$, $**P < 0.01$). (a) Dose dependency for riluzole treatment on surface GluR2 in cultured hippocampal neurons after 3 days of treatment. (b) Dose dependency for lamotrigine treatment on surface GluR2 in cultured hippocampal neurons after 3 days of treatment. (c) Time course after treatment of lamotrigine ($20\,\mu M$) and riluzole ($2.0\,\mu M$) on surface GluR2 in cultured hippocampal neurons. (d) Effect of valproate ($1.0\,mM$) on surface GluR2 after 3-day treatment in cultured hippocampal neurons. (e) Samples of Western blot analysis of surface GluR2 after therapeutically relevant concentrations of lamotrigine ($20\,\mu M$), riluzole ($2.0\,\mu M$), and valproate ($1.0\,mM$) treatments.

Phosphorylation of GluR1 S845 at PKA Site is Significantly Enhanced after Lamotrigine or Riluzole Treatment, but Reduced by Valproate Treatment

Previous studies show that the phosphorylation of GluR1 at the PKA site (S845) is an essential mechanism to regulate GluR1/2 receptor insertion onto the neuronal surface (Esteban *et al*, 2003; Smith *et al*, 2006). We therefore sought to determine whether the phosphorylation of GluR1 at the PKA site was altered, or not, after the treatment. We found that both lamotrigine ($20\,\mu M$) and riluzole ($2.0\,\mu M$) treatments significantly enhanced the GluR1 phosphorylation at the PKA site to 156 or 130%, respectively, whereas valproate significantly reduced GluR1 phosphorylation at PKA site to 58.5% (Figure 4). Actin was utilized as a loading control (Figure 4c).

Total GluR1 and GluR2 Expression Levels are Increased in Riluzole-Treated but not Lamotrigine-Treated Hippocampal Neurons

In order to determine if the increased surface levels of GluR1 and GluR2 were accompanied by increases in total GluR1 and GluR2 protein levels, the total levels of these proteins were determined after chronic lamotrigine or riluzole. Total GluR1 levels were enhanced after riluzole treatment (145%), but remained unchanged after lamotrigine and valproate treatments (Figure 5a). Similarly, total GluR2 levels were also increased significantly in riluzole-treated cortical neurons (145%), but remained unchanged in lamotrigine- and riluzole-treated cortical neurons (Figure 5b). In conclusion, while both riluzole and lamotrigine increased GluR1 phosphorylation and surface levels, only riluzole enhanced total GluR1 and GluR2 expression.

Phosphorylation of GluR1 (S845) is also Significantly Increased *In Vivo* after Lamotrigine and Riluzole Treatments, as well as Traditional Antidepressant Imipramine Treatments

Finally, we sought to determine if riluzole or lamotrigine regulated GluR1 levels after chronic *in vivo* treatment of rodents. Furthermore, we compared the effects of these anticonvulsants to the effects of the traditional antidepressant, imipramine. Phosphorylation of hippocampal GluR1 (S845) was significantly increased after *in vivo* treatment with lamotrigine (128%), riluzole (147%), or imipramine (140%) treatment (Figure 6a). Total GluR1 levels remained unchanged *in vivo* (Figure 6b).
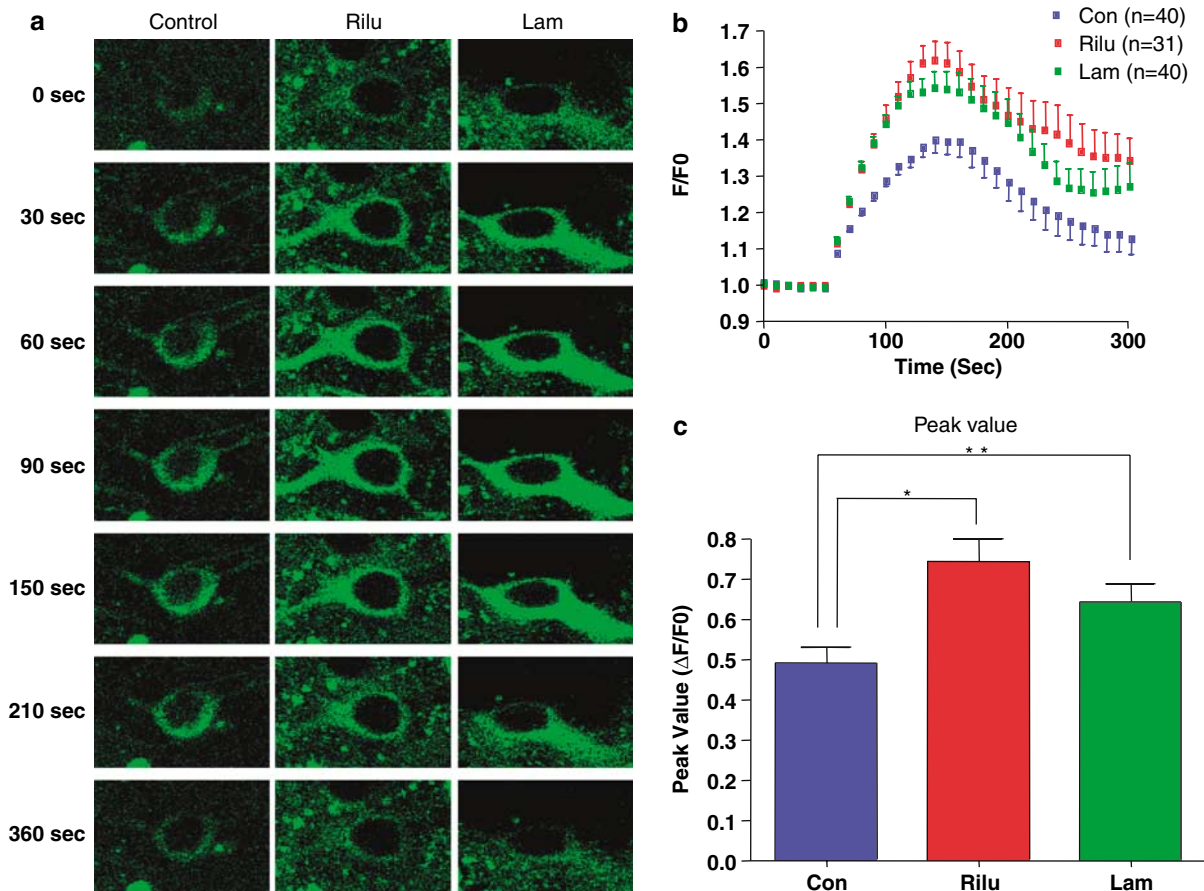
Figure 3 Membrane depolarization is significantly increased with AMPA stimulation after riluzole and lamotrigine treatments. Hippocampal neurons were cultured for 10 days and treated with lamotrigine and riluzole for additional 3 days. The AMPA ($1.0\,\mu M$)-induced depolarization of membrane potential was determined by a fluorescent dye $DiBAC_4$ (3) in the presence of TTX ($10\,\mu M$) to control spontaneous activity. Data were analyzed by Dunnett's multiple comparison test (number of experiments (N) = 3; number of cells (n) = 31–40, *$p < 0.05$, **$p < 0.01$) (a) The time course of membrane depolarization after AMPA stimulation in lamotrigine- or riluzole-treated hippocampal neurons. (b) The quantification of membrane depolarization after AMPA stimulation in lamotrigine- or riluzole-treated neurons. (c) Peak values of membrane depolarization after AMPA stimulation in lamotrigine- and riluzole-treated hippocampal neurons.

DISCUSSION

In this study, we sought to determine the effects of three anticonvulsants on surface AMPA receptors and the relationship to the clinical profile of these agents in the treatment of mood disorders. We found that the agents with a predominantly antidepressant profile, namely lamotrigine and riluzole, significantly enhanced surface expression of GluR1 and GluR2 in a time- and dose-dependent manner. By contrast, the anti-manic agent valproate significantly reduced surface expression of GluR1 and GluR2. Furthermore, the increase in surface GluR1 and GluR2 receptors was accompanied by increased AMPA-mediated membrane depolarization in the lamotrigine- or riluzole-treated hippocampal neurons. Phosphorylation of GluR1 at PKA site was enhanced in both lamotrigine- and riluzole-treated hippocampal neurons, whereas it was reduced in valproate-treated hippocampal neurons. In addition, phosphorylation of hippocampal GluR1 at its PKA site (often viewed as an indicator for GluR1 membrane insertion in neurons) was significantly increased after chronic *in vivo* treatment with lamotrigine, riluzole, and the traditional antidepressant, imipramine. These results raise the intriguing possibility that delayed changes in GluR1/2 phosphorylation and surface levels may play a role in conferring antidepressant/antimanic profiles to these agents.

Anticonvulsants as Drugs for Mood Disorders: Diverse Effects and Clinical Relevance
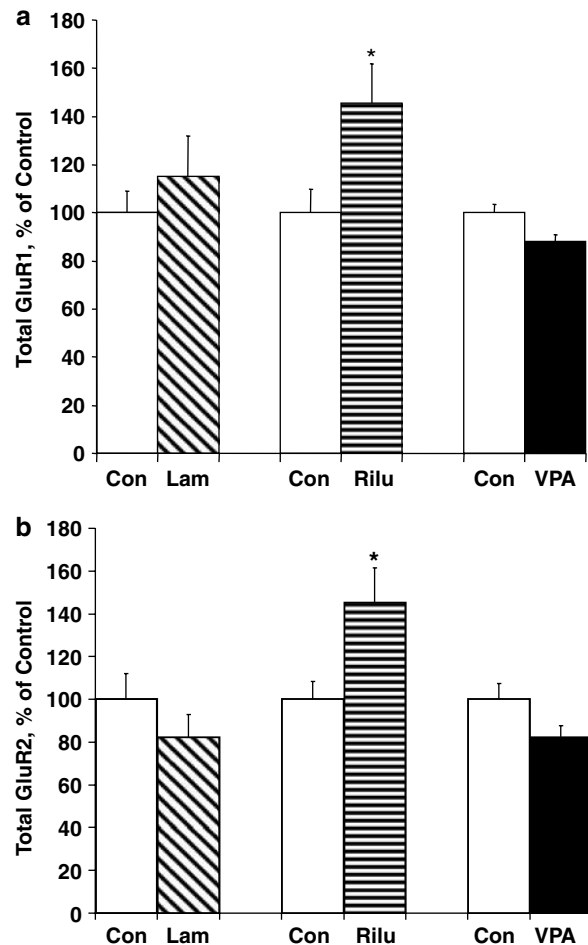
The anticonvulsant drugs studied all inhibit excessive neuronal activity; this acute effect appears to be produced by several mechanisms including blockage of voltage-gated sodium channels, or inhibition of excitatory glutamatergic neurotransmission. It is quite likely that many of the acute beneficial effects of these agents in the treatments of epilepsy can be explained by these direct-acting mechanisms. By contrast, the fact that these agents require chronic administration for therapeutic effects, and the fact that they have fairly distinct clinical profiles in the treatment of mood disorders suggests that other mechanisms must also be operative.

In view of the critical role of AMPA receptor localization in regulating various forms of plasticity, our laboratory has
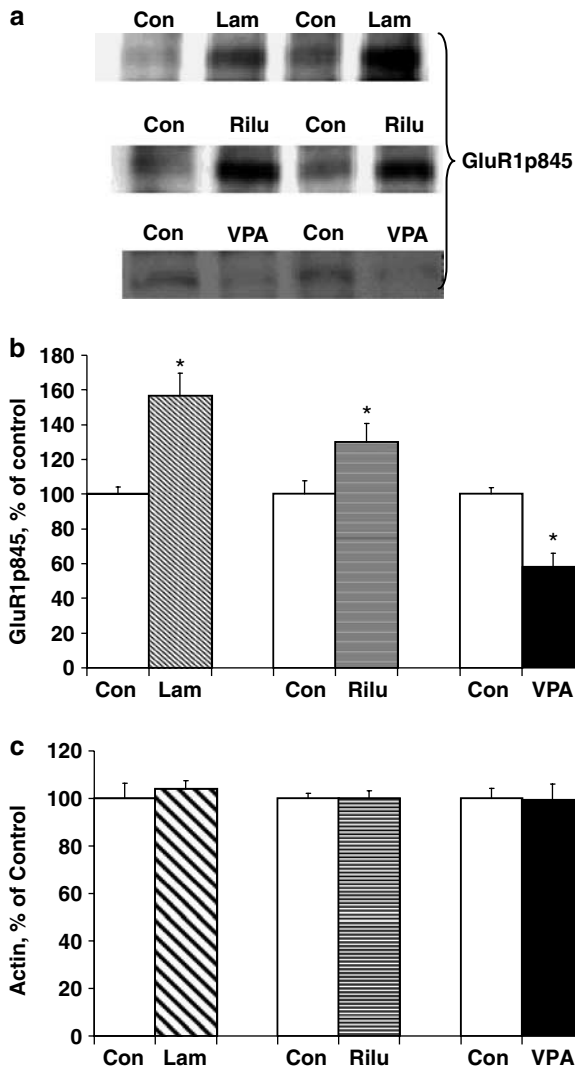
Figure 4 Phosphorylation of GluR1 at PKA site S845 is enhanced after lamotrigine or riluzole treatment, but reduced after valproate treatment. Hippocampal neurons were treated with lamotrigine (20 μM), or riluzole (2.0 μM), or valproate (1.0 mM) for 3 days. Proteins were harvested and equal amounts of proteins were loaded on the gel for electrophoresis. Western blot analysis was performed with anti-GluR1p845 antibody and anti-actin antibody. (a) Phosphorylation of GluR1 (S845) after lamotrigine, riluzole, and valproate treatments. (b) Actin was not changed in samples among the three treatment groups. Data were pooled from 2–3 independent experiments and presented as mean±SE ($n=7–11$, Student's t-test, $*p<0.05$).


Figure 5 Total GluR1 and GluR2 levels in cultured hippocampal neurons after riluzole and lamotrigine treatments. Hippocampal neurons were treated with lamotrigine (20 μM), riluzole (2.0 μM), or valproate (1.0 mM) for 3 days. Proteins were harvested and equal amount of proteins were loaded onto the gel for electrophoresis. Western blot analysis was performed with and anti-GluR1 (a) and anti-GluR2 (b) antibody. Data were pooled from 2–3 independent experiments and presented as mean±SE ($n=8–14$, Student's t-test, $*p<0.05$).

been investigating the potential role of AMPA receptor trafficking in mediating the therapeutic effects of antimanic agents. We found that the structurally highly dissimilar antimanic agents, namely lithium and valproate, have a common effect on downregulating AMPA GluR1 synaptic expression in the hippocampus after prolonged treatment with therapeutically relevant concentrations as assessed both *in vitro* and *in vivo* (Du *et al*, 2004). Notably, the antidepressant imipramine has an opposite effect as it upregulates AMPA receptors in the hippocampus (Du *et al*, 2003; Gray *et al*, 2003). These latter results are also consistent with the studies from the Greengard laboratory, demonstrating that chronic fluoxetine significantly enhanced GluR1 phosphorylation (S845) of GluR1 in the hippocampus (Svenningsson *et al*, 2002).

AMPA Receptor Phosphorylation at PKA Site Regulates Membrane Insertion of AMPA Receptors and Enhances the Depolarization of the Neuronal Membranes

A number of studies indicate that the synaptic delivery of different subunit combinations is governed by molecular rules encoded in the intracellular C-terminals of the receptor subunits. Phosphorylation of serines 831 and 845 (S831 and S845) in the GluR1 subunit by CaMKII and PKA, respectively, regulates the ion channel properties and synaptic trafficking of GluR1-containing AMPA receptors during hippocampal long-term potentiation (Roche *et al*, 1996; Barria *et al*, 1997; Mammen *et al*, 1997; Derkach *et al*, 1999; Banke *et al*, 2000; Lee *et al*, 2000; Esteban *et al*, 2003). Dephosphorylation of the GluR1 at the PKA site by protein
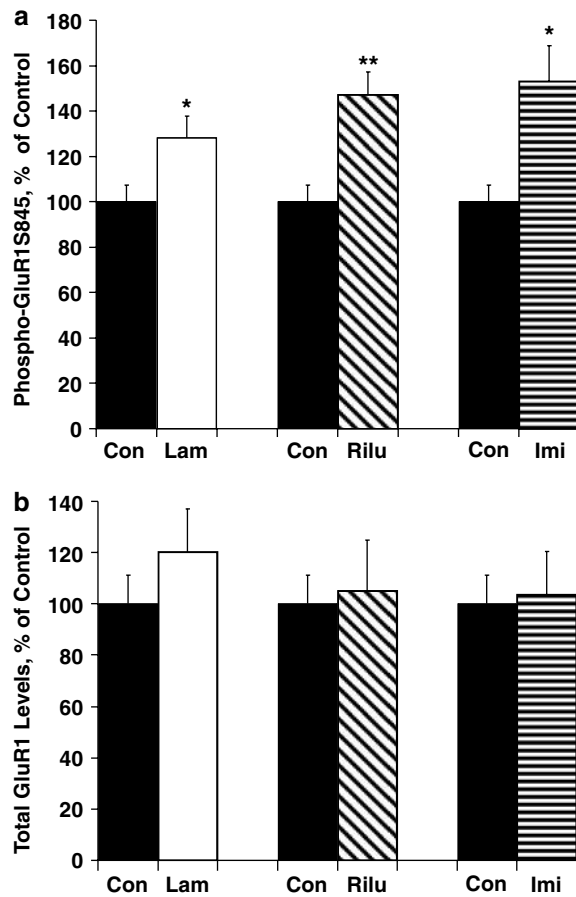
Figure 6 Chronic treatment with lamotrigine, riluzole, and imipramine *in vivo* significantly enhance GluR1 phosphorylation at S845. C57/BL/6 mice were treated with lamotrigine (Lam (6), 25 mg/kg, once daily), riluzole (Rilu (6), 10 mg/kg, once daily), and imipramine (Imi (11), 10 mg/kg, twice daily) for 10 days. Hippocampi were isolated from the brain tissue and protein samples were prepared from these brain tissues. Equal amount of proteins were loaded on 10% SDS-PAGE. Phosphorylation of GluR1 S845 was determined by Western blot analysis with an anti-GluR1S845 antibody (a). Total GluR1 were determined and showed no changes among three groups (b). Chronic lamotrigine-, riluzole-, or imipramine-treated animals all showed significantly increased levels of pGluR1 at the S845 site (*$p < 0.05$, **$p < 0.01$).

phosphatases (eg, calcineurin and PP1) targets GluR1 for recycling endosomes during long-term depression, while re-phosphorylation by PKA may target the receptor for reinsertion into the membrane (Banke *et al*, 2000). In activity-dependent regulation of subunit-specific AMPA receptor trafficking, GluR1 is the critical subunit that 'drives' AMPA receptors to the surface and to synapses in response to NMDA receptor stimulation and activation of CaMKII, resulting in synaptic potentiation (Hayashi *et al*, 2000). Specifically, activity-dependent movement into synapses were governed by two steps: (1) insertion of GluR1/GluR2 onto the neuronal surface is governed by phosphorylation of GluR1 on its PKA site (GluR1, S845); and (2) GluR1/GluR2 movement into synapses is regulated by the synaptic activity via activation of CaMKII and MAP kinase (Hayashi *et al*, 2000; Lee *et al*, 2000). In this study, we found that phosphorylation of GluR1 at PKA site is

significantly increased *in vitro* and *in vivo* in lamotrigine- and riluzole-treated groups, effects that may mechanistically underlie the enhancement of GluR1 and GluR2 membrane localization.

AMPA Receptors and Affective-Like Behavior

Interestingly, emerging animal behavioral studies also suggest that facilitating AMPA receptor throughput may be associated with antidepressant-like effects, whereas attenuating it results in antimanic-like effects (as assessed by stimulant-induced hyperactivity and measures of hedonic activity—cardinal symptoms of the manic syndrome) (Choi *et al*, 2005; Kaddis *et al*, 1995; Li *et al*, 1997; Ossowska *et al*, 2004; Vanover, 1998). Several classes of compounds can allosterically modulate AMPA receptors. These compounds (so-called AMPA receptor-positive modulators or AMPA receptor potentiators, ARPs) do not activate AMPA receptors themselves, but slow the rate of receptor desensitization and/or deactivation in the presence of an agonist (eg, glutamate and AMPA). Studies have shown that the biarylpropylsulfonamide ARPs (LY392098 and LY451616) have antidepressant effects in animal models of depression (including the application of inescapable stressors, forced-swim test, and tail-suspension-induced immobility tests), in learned-helplessness models of depression, and in animals exposed to chronic mild stress procedure (Alt *et al*, 2005; Li *et al*, 2001), Moreover, Ampalex was reported to have more rapid effect (during the first week of treatment) than fluoxetine (after 2 weeks) (Knapp *et al*, 2002). It is note worthy that lamotrigine has been shown to have an antidepressant effect in forced-swim test animal models (Szymczyk and Zebrowska-Lupina, 2000).

In conclusion, we have shown that chronic administration of two structurally dissimilar anticonvulsants with a predominantly antidepressant profile increases surface AMPA receptor levels and GluR1 phosphorylation at its PKA site *in vitro* and *in vivo*, whereas an agent with greatest efficacy in the treatment of mania reduces it. *In toto*, combined with the behavioral data (*vide supra*), biochemical data suggest that AMPA receptor localization may play an important role in the antidepressant actions. It is thus noteworthy that clinical studies of CNS-penetrant AMPA-kines are currently underway (Black, 2005). In view of the critical roles of AMPA receptors in regulating neural and behavioral plasticity, agents capable of directly modulating AMPA receptor throughput may represent exciting novel agents for the treatment of severe mood disorders.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)