# Regulation of Ionotropic Glutamate Receptor Subunits in Different Rat Brain Areas by a Preferential Sigma$_1$ Receptor Ligand and Potential Atypical Antipsychotic

*Xavier Guitart, Ph.D., Raúl Méndez, B.Sc., Sergio Ovalle, Ph.D., Francesc Andreu, Alicia Carceller, Antoni J. Farré, Ph.D., and Daniel Zamanillo, Ph.D.*

The effect of chronic administration of the putative atypical antipsychotic E-5842, a preferential sigma$_1$ receptor ligand, on ionotropic glutamate receptor subunit levels of mRNA and protein, was studied. The repeated administration of E-5842 differentially regulated levels of the NMDA-2A and of GluR2 subunits in a regionally specific way. Levels of immunoreactivity for the NMDA-2A subunit were up-regulated in the medial prefrontal cortex, the frontoparietal cortex, the cingulate cortex, and in the dorsal striatum, while they were down-regulated in the nucleus accumbens. Levels of the GluR2 subunit of the AMPA receptor were up-regulated in the medial prefrontal cortex and the nucleus accumbens and down-regulation was observed in the dorso-lateral striatum. Regulation of the levels of mRNA for the different subunits was also observed in some cases. The results show that E-5842, through a mechanism still unknown, is able to modify levels of several glutamate receptor subunits and these changes could be related to its antipsychotic activity in pre-clinical tests. **[Neuropsychopharmacology 23:539–546, 2000]** © 2000 American College of Neuropsychopharmacology. Published by Elsevier Science Inc.

Schizophrenia is a central nervous system disorder with an aetiology still unknown. Altered dopaminergic activity in different brain areas may be responsible for some of the symptoms of the illness (Meltzer and Stahl 1976; Seeman 1987). On the other hand, these same symptoms can be prompted in normal subjects by administration of amphetamine (Angrist et al. 1974). Drugs effective in treating schizophrenia are also able to block dopamine receptors (Anden et al. 1970; Creese et al. 1976; Seeman et al. 1976), thus suggesting an increase in dopaminergic tone. Recently, the dopaminergic hypothesis has been reformulated and a dysfunctional integration between cortical and subcortical dopaminergic activity, with reduced dopaminergic function in the cortex and increased dopaminergic function in the striatum has been postulated (Weinberger et al. 1988; Davis et al. 1991). A quite novel hypothesis, based in part on the schizophrenic symptoms produced by NMDA antagonists (Javitt and Zukin 1991; Ellison 1995), postulates that a dysfunction in the main excitatory amino acid system of the brain could subserve the pathophysiology of schizophrenia (Deutsch et al. 1989; Carlsson and Carlsson 1990; Moghaddam 1994; Olney and Farber 1995; Tamminga 1995).

From the Department of Neuropharmacology, Research Center, Laboratoris Esteve, S.A., Barcelona, Spain.

Address correspondence to: Xavier Guitart, Department of Neuropharmacology, Research Center, Laboratoris Esteve, S.A., Verge de Montserrat, 221, 08041-Barcelona, Spain. Tel.: + 34 93 446.60.63; Fax: + 34 93 446.62.20; E-mail: xguitart@esteve.es

Received January 24, 2000; revised April 28, 2000; accepted May 8, 2000.

It is well known that glutamate is the principal excitatory neurotransmitter in the brain. NMDA and AMPA receptors are two important families of ionotropic glutamate receptors. Both receptors are composed of a combination of several subunits (NMDAR-1, NMDAR-2A-D for the NMDA receptor, and GluR1-GluR4 for the AMPA receptor) encoded by different genes (Hollmann and Heinemann 1994). Both receptor subtypes are voltage-gated ion channels, subserving rapid neurotransmission. A role for these receptors has been proposed in models of neuronal plasticity, learning and excitotoxicity (Nakanishi et al. 1998; Tang et al. 1999; Zamanillo et al. 1999).

Recently, a number of laboratories have demonstrated regulation of several glutamate receptor subunits by typical and atypical antipsychotics, using different experimental approaches (Fitzgerald et al. 1995; McCoy et al. 1996; Healy and Meador-Woodruff 1997; Tascedda et al. 1999). Post mortem investigation of glutamate receptor subunits in human brain, in treated or untreated schizophrenics, have not yielded consistent results (Akbarian et al. 1996; Healy et al. 1998; Grimwood et al. 1999).

E-5842 (4-(4-fluorophenyl)-1,2,3,6-tetrahydro-1-[4-(1,2,4-triazol-1-il)butyl]pyridine citrate) is a new putative atypical antipsychotic with a preferential binding affinity for the sigma$_1$ receptor ($K_i$ = 4 nM) and moderate to low affinity for other central nervous system receptors, including the dopamine D$_1$, D$_2$, D$_3$, and D$_4$ receptors and the serotonin 5-HT$_2$ and 5-HT$_{1A}$ receptors (Guitart et al. 1998). E-5842 is active in different animal models predictive of antipsychotic activity (Guitart et al. 1998), and in biochemical (Guitart and Farré 1998) and electrophysiological approaches (Sánchez-Arroyos and Guitart 1999). Based on the effects observed with other antipsychotics on the regulation of different glutamate receptor subunits, in the present study we investigated the possible modulation of protein levels of several AMPA and NMDA receptors and the mRNA encoding for these subunits in response to a repeated administration of E-5842. The regulation of these subunits in different brain areas may account for some of the effects of psychotropic drugs.

## METHODS

### Experimental Protocol

Male Sprague-Dawley rats (Iffa Credo, L'Arbresle, France) weighing 250–300 g were used in the experiments. Animals were maintained on a 12-h light/12-h dark cycle with food and water available *ad libitum.* For the chronic treatment studies, saline or E-5842 (20 mg/kg, 1 ml/kg) were administered intraperitoneally to rats once daily for 21 days. Two different experiments were performed, including between 10 and 16 rats in

each experimental group. All the experiments in rats adhered to the European Community Guide for the Care and Use of Laboratory Animals.

### Western Blot Studies

Rats were killed 24 h after the last injection, brains quickly removed and immediately cooled in ice-cold physiological buffer. Coronal sections (1 mm thick) were obtained in various brain regions, and punches of medial prefrontal cortex (mPFC), frontoparietal cortex (FPC), cingulate cortex (CgC), nucleus accumbens (NAc) and dorsolateral striatum (DLS) were excised [sections were taken based on the atlas of Paxinos and Watson (1986)]. The brain areas that were excised from different sections are shown as dark circles in Figure 1. The hippocampus was obtained by gross dissection. Bilateral punches were pooled from individual rats. Brain samples were immediately homogenized by sonication in a small volume of 2% SDS, and protein content determined by the method of Lowry et al. (1951). Aliquots of
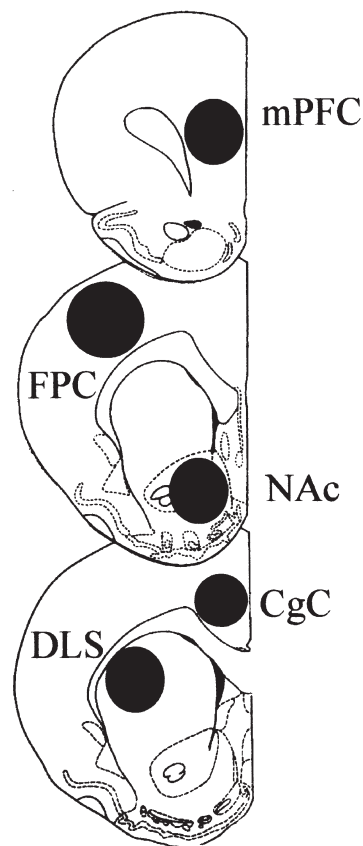


**Figure 1.** Drawing of rat brain slices used for the immunoblotting studies to detect NR-1, NR-2A, GluR1, and GluR2/3 glutamate receptor subunits. Dark circles show the brain regions that were punched out. Medial prefrontal cortex (mPFC), frontoparietal cortex (FPC), nucleus accumbens (NAc), cingulate cortex (CgC), and dorsolateral striatum (DLS).

brain extract (containing 5, 10 or 30 $\mu$g of protein) were subjected to SDS/polyacrylamide gel electrophoresis.

After electrophoretic transfer to nitro-cellulose, different glutamate receptor subunits were immunodetected. GluR1 and GluR2/3 subunits of the AMPA receptor were immunolabelled using rabbit polyclonal antibodies (1:1000, Chemicon, Temecula, CA). NMDAR-1 (NR-1) and NMDAR-2A (NR-2A) subunits were immunolabelled using rabbit polyclonal antibodies (1:1000, Calbiochem, San Diego, CA) followed by goat anti-rabbit immunoglobulins (IgG) (1:2000) conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA). Immunocomplexes were visualized by using the Supersignal Chemiluminescent substrate (Pierce, Rockford, IL). The relative levels of proteins were analyzed using a Bio-Rad Fluor-S MultiImager.

For each experiment, gels contained saline-treated samples and E-5842-treated samples running under the same conditions. Band density values were analyzed by Student's unpaired $t$-tests. Given that the intensity of the bands varies across experiments, results were expressed as mean percentage of control (saline treated rats) values ($\pm$S.E.M.).

### RNA Isolation

Rats were killed 24 h after the last administration, the brains quickly removed, frozen on dry ice and stored at $-80°$C. Total RNA was isolated from frontal cortex, hippocampus, striatum and cerebellum. Tissues were homogenized directly in a guanidine thiocyanate solution (Trizol, Gibco RBL) and total RNA was isolated by phenol-chloroform, precipitated with isopropanol and quantified by spectrophotometry.

### Preparation of Probes

Five micrograms of total RNA were reverse transcribed in a total volume of 50 $\mu$l including 50 mM Tris buffer pH 8.3, 50 mM KCl, 4 mM MgCl$_2$, 0.4 mM deoxynucleoside triphosphates, 10 mM DL-dithiothreitol, 0.5 $\mu$g oligo (dT)$_{18}$ primer and 500 units M-MLV reverse transcriptase. The reaction was carried out at 37°C for 1 h. GluR1, GluR2, and $\beta$-actin probes were obtained from PCR product amplification of the reverse transcribed material using 10 pmol of each specific primer. 5'-GGA ATA TGC CGT ACA TCT TTG-3' and 5'-AAG TCA TCT CAA AGC TGT CGC-3' were used as sense and antisense oligo to amplify a specific mRNA GluR1 223 bp PCR fragment from cDNA. 5'-TAC CCT GGA GCA CAC ACA GCG-3' and 5'-GTG TCA TTT CCT GAT GGG AGC-3' were used as sense and antisense oligo to amplify a specific mRNA GluR2 370 bp PCR fragment from cDNA. 5'-CCA GAT CAT GTT TGA GAC CT-3' and 5'-TAG AGG TCT TTA CGG ATG TCA-3' were

used respectively as sense and antisense oligo to obtain specific mRNA $\beta$-actin 522 bp fragment.

NR-2A probe was obtained from a 0.6 Kb Pst I cDNA fragment specific to the rat NR-2A mRNA; NR2B probe was from a 1.5 Kb Pst I cDNA fragment specific to the rat NR-2B mRNA, and GluR3 probe was obtained from a 0.4 Kb BamHI-Pst I cDNA segment specific to the rat GluR3 mRNA. All the fragments were selected in order to obtain the cDNA region with less homology to the rest of the members of the receptor family. The cDNAs for NR2A, NR2B and GluR3 were kindly provided by Prof. P.H. Seeburg (Max-Planck Institute for Medical Research, Heildelberg, Germany).

### Northern Analysis

Ten $\mu$g of each RNA were size fractionated by 1% agarose/formaldehyde gel electrophoresis. Before transfer to nylon membranes the gel was stained with 50 $\mu$g/ml ethidium bromide for 30 min and destained in water for 1 h. The quality of the RNA preparation and the position of ribosomal bands were established at that step. Gels were blotted overnight in 10 $\times$ SSC. Filters were crosslinked by UV and prehybridized for 30 min in 10 ml of Quichyb solution (Stratagene, La Jolla, CA). Hybridization was performed for 1 h at 68°C in the same solution with 1 mg denatured salmon testes DNA and 50 ng denatured DNA probe (radiolabelled using a Random Primed DNA labelling kit, Roche Molecular Biochemical, Rotkreuz, Switzerland). Filters were washed to final stringency of 0.1 $\times$ SSC, 0.1% SDS at 60°C. Autoradiography was for several days at $-80$°C with Hyperfilm $\beta$-max. Quantification of mRNA was carried out by densitometric analysis of the autoradiograms.

### RESULTS

Immunoblotting studies show that, as we hypothesized, repeated treatment for 21 days with E-5842, a preferential sigma receptor ligand, induced a differential regulation of NMDA and AMPA glutamate receptor subunits in several rat brain areas. Figure 2 shows the regulation of NR-2A subunit. E-5842 treatment significantly increased NR-2A immunoreactivity levels compared to saline treated-rats in three cortex areas, the mPFC, the FPC, and the CgC (Figure 2). No change was observed in the hippocampus. In contrast, E-5842 treatment had differential effects on the subdivision of the striatum: levels of NR2A immunoreactivity were decreased in the nucleus accumbens while a tendency to increase was observed in the dorsolateral striatum (Figure 2), although such increase did not reach statistical significance. No changes were observed in the immu-
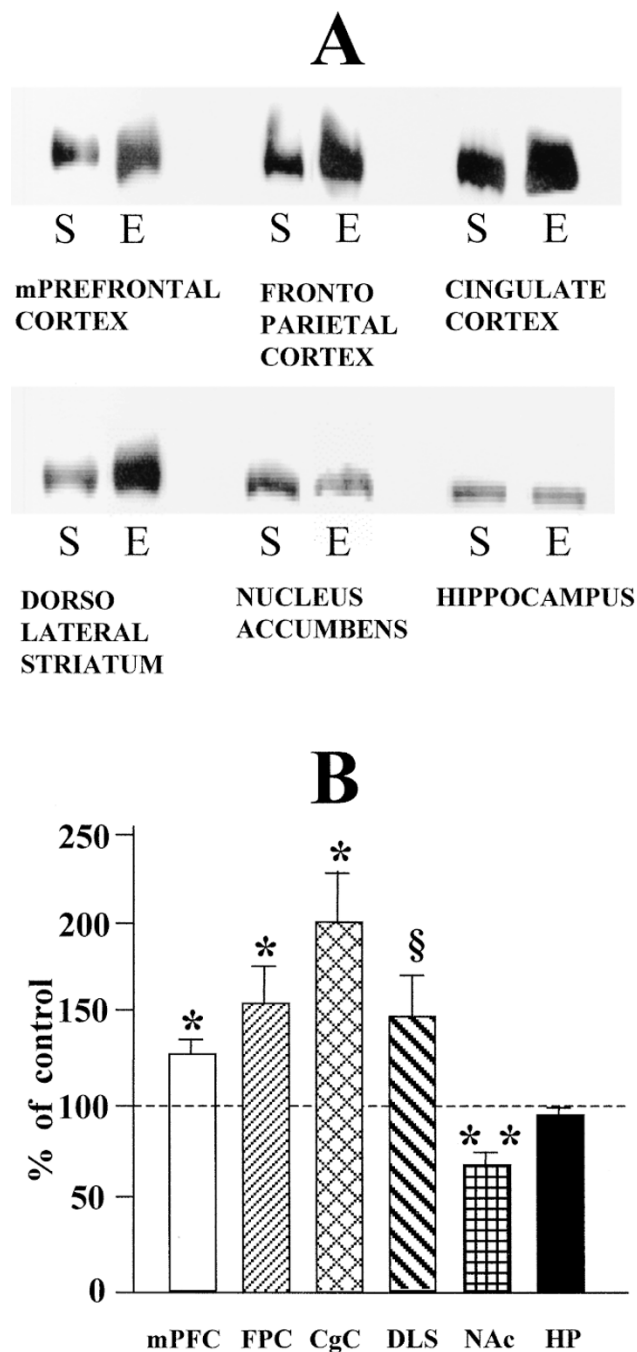
## A



S E    S E    S E

mPREFRONTAL    FRONTO        CINGULATE
CORTEX         PARIETAL       CORTEX
               CORTEX

S E    S E    S E

DORSO          NUCLEUS       HIPPOCAMPUS
LATERAL        ACCUMBENS
STRIATUM

## B



**Figure 2.** Regulation of NR-2A glutamate receptor subunit in different regions of rat brain. **(A)** Immunoreactivity levels of the subunit in saline-treated (S) and E-5842 treated (E) rats (20 mg/kg, i.p., 21 days). **(B)** Representation of NR-2A regulation by E-5842. mPFC (medial prefrontal cortex), FPC (frontoparietal cortex), CgC (cingulate cortex), DLS (dorsolateral striatum), NAc (nucleus accumbens), HP (hippocampus). Each bar represents the mean ± S.E.M. of data from 12–14 animals from two different experiments. Statistically different from saline-treated rats *$p < .05$, **$p < .01$, §$p \simeq .08$.

**Table 1.** NMDAR-1 and GluR1 Glutamate Receptor Subunit Immunoreactivity as Measured by Western Blotting After Chronic Treatment with E-5842

| Region | NMDAR-1 | GluR1 |
|---|---|---|
| Medial prefrontal cortex | 105 ± 5 | 103 ± 6 |
| Frontalparietal cortex | 97 ± 5 | 115 ± 10 |
| Cingulate cortex | 105 ± 4 | 104 ± 8 |
| Nucleus accumbens | 94 ± 4 | 98 ± 3 |
| Striatum | 100 ± 5 | 95 ± 7 |
| Hippocampus | 95 ± 3 | 96 ± 3 |

Data are expressed as percentage of control (saline-treated) ± S.E.M. and are derived from two different experiments with a total $n = 12–14$ animals per group of treatment.

noreactivity levels of NR-1 subunits in any of the different brain areas that were studied (Table 1).

The effects of chronic administration of E-5842 on GluR1 and GluR2/3 subunits the AMPA receptor were also studied. Figure 3 shows the regionally specific regulation of the levels of GluR2/3 subunits. We found that the levels of GluR2/3 are clearly increased in the mPFC, slightly decreased in the FPC, and no significant change was apparent in the CgC. On the other hand, it is interesting to note that levels of Glur2/3 are regulated in an opposite way in the DLS and the NAc, as observed for the NR-2A subunit. In the case of GluR2/3, levels of immunoreactivity are up-regulated in the NAc while a significant decrease of the immunoreactivity is seen in the DLS. It is interesting to note that levels of these two different excitatory aminoacid receptor subtypes are both regulated in opposite ways in the dorsolateral striatum and the nucleus accumbens. Levels of GluR1 subunit were not changed after repeated treatment with E-5842 (Table 1).

Levels of mRNA expression for the NR-1, NR-2A, NR-2B, GluR1, GluR2, and GluR3 subunits were also studied by Northern blotting. NR-1, NR-2A, NR-2B and GluR3 probes hybridized in all cases with a single band of different size as it has been previously shown (Kutsuwada et al. 1992; Meguro et al. 1992; Durand and Zukin 1993), whereas GluR1 and GluR2 detection showed hybridization with several bands (Durand and Zukin 1993). GluR2 probe hybridized with two major species, approximately 5.9 and 3.9 Kb in size. GluR1 showed hybridization with a major band of approximately 5.2 Kb in size, and two smaller species of approximately 3.9 and 3.2 Kb. For analysis purposes the data on the 3.9- and 3.2-Kb species were pooled (Table 2). Comparable levels of loaded total RNA were confirmed by re-hybridizing blots with a probe directed to β-actin. As shown in Table 2, levels of mRNA for NR-1 and GluR1 were unchanged after chronic treatment with E-5842. In the case of NR-2A, a significant increase in the mRNA level for this protein in the striatum was indicated by Northern blotting analysis, in parallel with
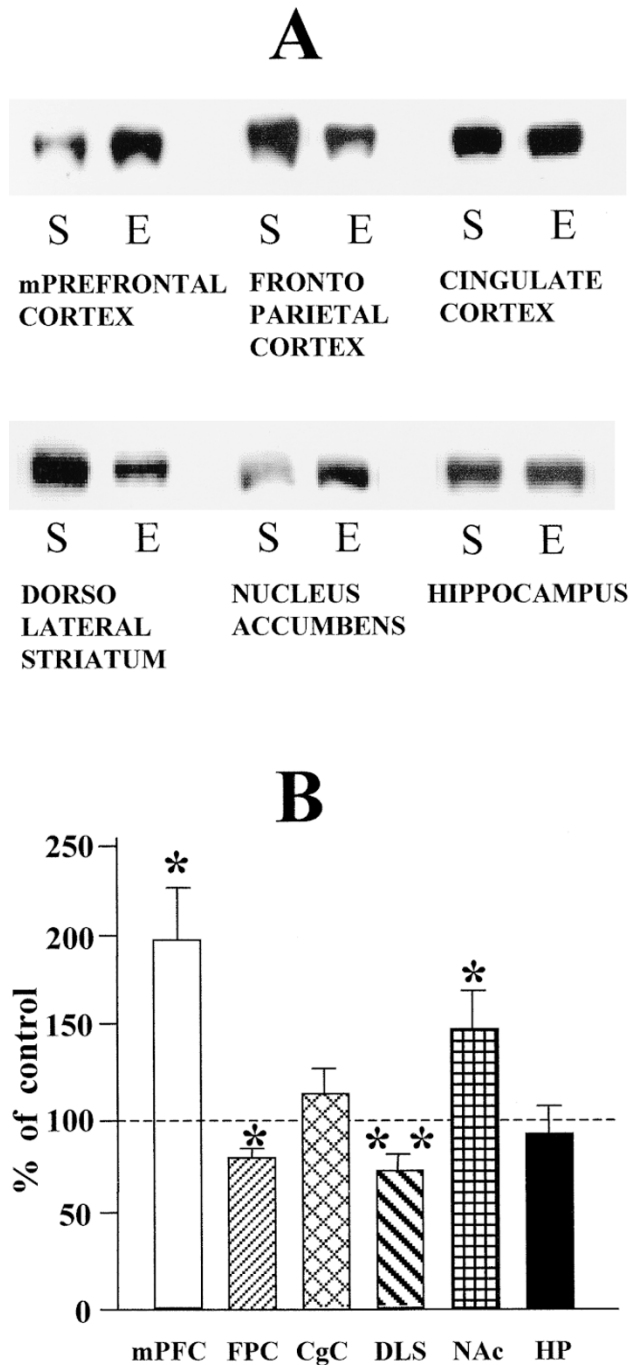
**Figure 3.** Regulation of GluR2/3 glutamate receptor subunit in different regions of rat brain. **(A)** Immunoreactivity levels of the subunit in saline-treated (S) and E-5842 treated (E) rats (20 mg/kg, i.p., 21 days). **(B)** Representation of NR-2A regulation by E-5842. mPFC (medial prefrontal cortex), FPC (frontoparietal cortex), CgC (cingulate cortex), DLS (dorsolateral striatum), NAc (nucleus accumbens), HP (hippocampus). Each bar represents the mean ± S.E.M. of data from 10–14 animals from two different experiments. Statistically different from saline-treated rats *$p < .05$, **$p < .01$.

the increase seen in the level of protein. In the frontal cortex, a tendency to increased levels of mRNA was observed, although it did not reach statistical significance. mRNA levels for NR-2B were unchanged in the studied brain regions. Table 2 also shows the regulation of the expression of three AMPA receptor subunits. GluR1 mRNA was unchanged after E-5842 treatment, while a small but significant increase was detected for the GluR2 5.9-Kb mRNA subunit (a tendency to increase in the 3.9-Kb mRNA was also observed) in the prefrontal cortex. The unchanged expression levels of GlurR3 in the prefrontal cortex might suggest that the increase in immunoreactivity observed with Western blotting using the GluR2/3 antibody could correspond mainly to the GluR2 subunit.

## DISCUSSION

The data reported in this article show that repeated administration of the putative atypical antispychotic E-5842 is able to modulate the levels of immunoreactivity of several NMDA and AMPA receptor subunits and, in some cases, the levels of mRNAs for these subunits. Our results also show that there is a differential regulation of these subunits in several brain regions.

Regulation of glutamate receptor gene expression has been shown by different laboratories using either typical or atypical antipsychotics such as haloperidol, clozapine and quetiapine (Fitzgerald et al. 1995; McCoy et al. 1996; Healy and Meador-Woodruff 1997; Tascedda et al. 1999). Given that the binding profile of these antipsychotics differs substantially from each other, it is difficult to assign the capacity to regulate the expression of glutamate receptors to a single interaction with a given (or some) neurotransmitter receptor. We suggest that regulation of glutamate receptor subunits is somehow linked to antipsychotic activity. E-5842, although having a neurotransmitter receptor binding profile different from the binding profile of other antipsychotics, behaves as an atypical compound in several behavioral tests and biochemical approaches (Guitart et al. 1998; Guitart and Farré 1998). The present data show that E-5842 is also able to regulate different glutamate receptor subunits.

One of the major findings of this work is that levels of NR-1 mRNA and NR-1 immunoreactivity are not affected after a chronic treatment with E-5842 in any of the brain regions studied. Although it is difficult to assign a clear correlation between a given receptor and the regulation of glutamate receptors, these results would be in accordance with a postulated regulatory effect on NR-1 as being mediated by dopamine D$_1$/D$_2$ receptor mechanisms (Fitzgerald et al. 1995). These authors have described that haloperidol, raclopride and SCH23390 differentially affect levels of the NR-1 sub-

**Table 2.**   Effect of Chronic E-5842 Administration on NMDA and AMPA Receptor Subunit mRNA Levels in Rat Brain Regions as Measured by Northern Blotting

| Region | NR-1 | NR-2A | NR-2B | GluR1 | | GluR2 | | GluR3 |
| | | | | 3.9+3.2Kb RNA | 5.2Kb RNA | 3.9Kb RNA | 5.9Kb RNA | 5.2Kb RNA |
|---|---|---|---|---|---|---|---|---|
| Prefrontal cortex | 99 ± 15 | 116 ± 7 | 97 ± 12 | 90 ± 7 | 102 ± 6 | 118 ± 9# | 114 ± 5* | 101 ± 13 |
| Striatum | 94 ± 15 | 141 ± 14* | 111 ± 15 | 98 ± 17 | 100 ± 13 | 101 ± 8 | 103 ± 7 | 102 ± 17 |
| Hippocampus | 91 ± 12 | 118 ± 10 | 100 ± 17 | 88 ± 11 | 97 ± 6 | 101 ± 8 | 102 ± 16 | 109 ± 10 |
| Cerebellum | 107 ± 20 | 101 ± 13 | – | 96 ± 12 | 105 ± 12 | 109 ± 7 | 100 ± 14 | 101 ± 15 |

Data are expressed as percentage of control (saline-treated) ± S.E.M. and derived from two different experiments with a total $n = 12$–16 animals per treatment group.
* Significantly different from controls, $p < .05$; #$p = .08$ (Student's $t$-test).

unit in the striatum after chronic administration. Given that E-5842 has very weak affinity for the dopamine $D_1/D_2$ receptors (and other dopamine receptors), a lack of regulation of the NR-1 subunit would be expected. In any case, and independently of this consideration, a likely interaction of E-5842 with other receptors should not be discarded, especially after a repeated treatment.

NR-2A subunit was clearly up-regulated in several cortical regions after chronic treatment with E-5842. Levels of immunoreactivity in the striatum showed a tendency to increase, although this did not reach statistical significance, while a decrease in NR-2A levels were observed in the nucleus accumbens. Given that levels of NR-1 and NR-2B mRNA remain unchanged, it can be hypothetized that more NR-1/NR-2A complexes are formed in the brain regions where an up-regulation of NR-2A is observed. In this sense, and apart from conflicting binding data after chronic treatment with several antipsychotics (Tarazi et al. 1996; Gandolfi and Dall'Olio 1996), an increased binding affinity of [3H]CGP39653 in several cortical regions after antipsychotic treatment has been shown (Ossowka et al. 1999). This radioligand exhibits the highest affinity for a combination of NR-1 and NR-2A, and the NR-1 and NR-2B subunits (Laurie and Seeburg 1994). Although the increase in levels of immunoreactivity for NR-2A in the striatum did not reach statistical significance, it is interesting to note the opposite direction of subunit regulation in the striatum as compared to the nucleus accumbens, a potential site for antipsychotic drugs.

Regarding GluR1 expression, no statistically significant change was observed either at the mRNA or at the immunoreactivity level in any of the six brain regions studied. These results resemble the reported effect of the new atypical antipsychotic quetiapine at mRNA level (Tascedda et al. 1999) and of haloperidol and clozapine (Healy and Meador-Woodruff 1997). Regulation of GluR2/3 subunit after chronic treatment with E-5842 also follows a characteristic pattern: levels of the subunit immunoreactivity are clearly increased in the medial prefrontal cortex, while a small decrease is observed in the frontoparietal cortex and no significant

change is seen in the posterior cingulate cortex. The unchanged expression levels of GlurR3 in the prefrontal cortex suggest that the increase in immunoreactivity observed with Western blotting using the GluR2/3 antibody could correspond to the GluR2 subunit. Interestingly, subunit levels are regulated in an opposite way in the striatum and the nucleus accumbens.

GluR2/3 is significantly down-regulated in the striatum and up-regulated in the nucleus accumbens, thus showing, an opposite pattern of expression as compared to the expression of NR-2A. This is a puzzling result, but contradictory published results are found, with up- and down-regulation of mRNA for GluR2 and GluR3 in different cortical and subcortical brain regions, using both typical and atypical antipsychotics (Fitzgerald et al. 1995; Healy and Meador-Woodruff 1997; Tascedda et al. 1999). In any case, E-5842, repeatedly administered, is able to differentially regulate levels of immunoreactivity of GluR2/3 in brain areas that have been involved in the pathophysiology of schizophrenia (while levels of mRNA for GluR2 in the frontal cortex of treated rats were also increased). The significance of such results remains unknown, although it has been described that AMPA receptor characteristics are dependent on the GluR2 subunit. Channels containing the GluR2 subunit are impermeable to $Ca^{2+}$, while channels formed by any combination of GluR1, GluR3, or GluR4, are substantially $Ca^{2+}$-permeable. It has been suggested (Fitzgerald et al. 1995) that the increase in GluR2 induced by clozapine leads to an increased number of calcium-impermeable channels in selected groups of neurons. This effect could subserve some of the characteristics of atypical compounds, although further work is needed to ascertain such relationship.

Based on the results of the present work, it seems that the preferential sigma$_1$ receptor ligand E-5842 is able to regulate the expression of NMDA and AMPA receptors in different brain regions. This effect is shared by other antipsychotics, although the glutamate receptor subunits that are regulated in some cases are not the same. Taking together our results and results from other groups it seems clear that antipsychotic medica-

tion (regardless of the mechanism of action of the compound) leads to a clear interaction with the glutamatergic system. Based on human post mortem studies it seems that such regulation exists, but reported results are puzzling (Simpson et al. 1992; Akbarian et al. 1996; Porter et al. 1997; Healy et al. 1998; Grimwood et al. 1999). Although chronic treatment with commercially available antipsychotics produces regulation of several glutamate receptor subunits, more work is needed in order to clarify the functional significance of such changes either in cortical or subcortical brain structures that have been related to the development of schizophrenia.

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