Chronic Administration of a Glycine Partial Agonist Alters the Expression of N-Methyl-D-aspartate Receptor Subunit mRNAs

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Accepted for publication August 26, 1997

ABSTRACT

Both acute and chronic treatments with the glycine partial agonist 1-aminocyclopropanecarboxylic acid (ACPC) are neuroprotective in animal models of focal, global and spinal ischemia. After a chronic regimen of ACPC, brain and plasma levels were undetectable at the time of ischemic insult, which suggests that the neuroprotective effects of acute and chronic ACPC are mediated by different mechanisms. To investigate the possibility that chronic administration of ACPC alters N-methyl-D-aspartate (NMDA) receptor composition, the levels of mRNAs encoding z and epsilon subunits were quantified by in situ hybridization histochemistry with 35S-labeled riboprobes. Chronic ACPC administered to mice (200 mg/kg for 14 days) increased the level of epsilon-1 mRNA in the hippocampus (particularly CA1 and CA2 regions) and cerebral cortex (frontal, parietal and occipital regions), without altering levels in cerebellum. In contrast, this regimen decreased epsilon-3 subunit mRNA levels in the hippocampus (especially CA1 and dentate gyri) and frontal and occipital cortices. Decreases in epsilon-2 subunit mRNA levels in cerebral cortex (especially frontal and parietal cortices) were also observed without accompanying alterations in the cerebellum, hippocampus or dentate gyri. The levels of z subunit mRNA (determined with a probe that detects all splice variants) were not altered in any brain areas examined. Based on studies in recombinant receptors, these region-specific changes in mRNAs produced by a chronic regimen of ACPC could result in NMDA receptors with reduced affinities for glycine and glutamate. It is hypothesized that such alterations in NMDA receptor subunit composition may explain the neuroprotective effects produced by chronic ACPC.

Received for publication March 25, 1997.

ABBREVIATIONS: ACPC, 1-aminocyclopropanecarboxylic acid; NMDA, N-methyl-D-aspartate; EDTA, ethylenediaminetetraacetic acid; nt, nucleotide; DTT, dithiothreitol; ANOVA, analysis of variance.
Chronic treatment with ACPC was also neuroprotective in several of these in vivo models (Long and Skolnick, 1994; Lopez and Lanthorn, submitted; Von Lubitz et al., 1992). However in chronic studies, the final dose of ACPC was administered 24 h before ischemia, and plasma and brain concentrations of ACPC were undetectable at the time of ischemic insult (Von Lubitz et al., 1992). This finding suggests that the neuroprotective effects of acute and chronic administration of ACPC are mediated by different mechanisms (Von Lubitz et al., 1992).

Although the mechanism responsible for the neuroprotective effects of chronic ACPC is unknown, two recent observations suggest that sustained exposure to this glycine partial agonist can produce an alteration in the subunit composition of NMDA receptors. Thus, alterations in radioligand binding to cortical NMDA receptors have been demonstrated after chronic (14-day) administration of ACPC to mice (Nowak et al., 1993). Moreover, in primary cultures of rat cerebellar granule neurons, the levels of mRNA encoding the epsilon-3 subunit homolog were increased after a 24-h exposure to ACPC (Fossom et al., 1995a). The hypothesis that ACPC-induced alterations in subunit composition are responsible for these neuroprotective effects is consistent with the demonstrations that the physiological and pharmacological properties of NMDA receptors are largely defined by their subunit composition (Mori and Mishina, 1995). To further explore this hypothesis, we examined the effects of chronic ACPC administration on the levels of mRNAs that encode NMDA receptor ζ and epsilon subunits by in situ hybridization. We now report that this chronic regimen of ACPC produces robust region-specific changes in mRNA levels encoding the epsilon family of NMDA receptor proteins.

### Methods

#### Animals

Male NIH-Swiss mice (20–25 g) were housed in wire bottom cages (five per cage) with free access to a standard diet and tap water. The vivarium was maintained at 22–25°C on a 12-h light/dark cycle (lights on at 6:00 A.M.). All in vivo procedures were performed in accordance with NIH Animal Care and Use Committee guidelines.

During the week preceding drug administration, mice were handled briefly each day to reduce the stress associated with injection. Mice received daily intraperitoneal injections of either ACPC (200 mg/kg) or an equal volume of saline (0.2 ml) for 14 days. Injections were administered between 9:00 and 10:00 A.M.

#### Tissue collection and fixation

Twenty-four hours after the last injection, mice were deeply anesthetized with pentobarbital and perfused transcardially with saline followed by a standard fixative containing 4% paraformaldehyde in 0.1 M sodium borate. The brains were removed, postfixed in 4% paraformaldehyde in 0.1 M sodium borate for 1 h at 4°C, and subsequently placed in 4% paraformaldehyde-0.1 M sodium borate solution containing 10% sucrose overnight at 4°C. The brains were then frozen in isopentane and stored at −70°C until sectioned. Frozen brains were cut into 15-μm sagittal sections. The slices were collected in a cold cryoprotectant solution (50 mM sodium phosphate buffer, pH 7.3, 30% ethylene glycol, 20% glycerol) and stored at −20°C.

#### In situ hybridization histochemistry

Hybridization histochemical localization of each transcript was carried out on every sixth brain section with 35S-labeled cRNA probes. General protocols for riboprobe synthesis, probe hybridization and autoradiographic localization of mRNA signal were adapted from Simmons and co-workers (1989). Tissue sections mounted on poly-L-lysine-coated slides were fixed in 4% paraformaldehyde for 20 min. Sections were then: 1) digested with Proteinase-K (10 μg/ml in 100 mM Tris-HCl, pH 8, 50 mM EDTA for 30 min at 37°C); 2) rinsed in diethylpyrocarbonate water; 3) dipped in triethanolamine (0.1 M TEA, pH 8, for 10 min); 4) acetylated by dipping in 0.25% acetic anhydride in 0.1 M TEA, pH 8; and 5) dehydrated through graded concentrations (50, 70, 95 and 100%, 3 min each) of ethanol. The hybridization mixture (70 μl, ~107 cpm/ml) was spotted on each slide, sealed under a coverslip and incubated for 15 to 20 h at 60°C. The coverslips were removed and the slides rinsed (4× SSC: 0.6 M NaCl, 60 mM trisodium citrate buffer, pH 7), digested (RNase-A, 20 μg/ml, 37°C), washed (2× SSC for 10 min, 1× SSC for 10 min, 0.5× SSC for 10 min at 23°C and 0.1× SSC for 30 min at 65°C) and dehydrated by sequential dipping in 50 to 100% ethanol. The slides were exposed to BioMax MR film (Kodak) at 4°C for 24 to 72 h (depending on the probe), then defatted in xylene and dipped in LM1 nuclear emulsion. After 4- to 15-day exposures (ε probe, 4 days; epsilon-1 and epsilon-2 probes, 6 days; epsilon-3 probe, 15 days), emulsion-dipped slides were developed in D19 (Kodak) for 3.5 min at 14°C. Thereafter, tissues were rinsed in distilled running water for 1 h, dehydrated through graded concentrations of alcohol and embedded with DPX (Alrich Chemical Co., Milwaukee, WI).

#### cRNA probe synthesis and preparation

An antisense cRNA probe to detect RNAs encoding ε subunits was generated from the 1530-nt PsI fragment (+1330/+2860) of a cDNA encoding the rat homolog of the ε-1 subunit (denoted NMDAR1 in the rat; Boje et al., 1993), subcloned into pGEM-3zf (Promega Corporation, Madison, WI) and conserved in all splice variants of the ε subunit. An epsilon-1 cRNA probe was generated from the 1240-nt PsI fragment (+3110/+4350) of epsilon-1 cDNA, subcloned into pGEM-3zf (Promega Corporation, Madison, WI). An epsilon-2 cRNA probe was generated from the 760-nt Apol fragment (+3510/+4270) of epsilon-2 cDNA, subcloned into pGEM-11zf (Promega Corporation, Madison, WI). An epsilon-3 cRNA probe was generated from the 1020-nt NotI/HindIII fragment (+2630/+3650) of epsilon-3 cDNA, subcloned into pGEM-11zf (Promega Corporation, Madison, WI). These fragments of cDNA were chosen from regions that lack homology with other subunits. Furthermore, each of these fragments identified a single, appropriately sized RNA species, when used as a probe for Northern blot analysis of mouse brain RNA (data not presented). For the in situ studies reported here, sense-strand cRNA probes were also used to verify the specificity of each antisense-strand probe.

Radioactive riboprobe fragments were prepared by incubating 250 ng of plasmid, which had been linearized with an appropriate restriction enzyme, in 6 mM MgCl2, 35 mM Tris (pH 7.5), 2 mM spermidine, 10 mM DTT, 0.2 mM each of ATP, GTP and CTP and 200 μCi [35S]UTP, 40 U RNasin and 20 U SP6 polymerase (37°C, 60 min). Thereafter, SET-DTT (1% sodium dodecyl sulfate in 10 mM Tris, pH 7.4, 1 mM EDTA and 10 mM DTT) was added and the unincorporated nucleotides were removed with a spin-column (Boehringer Mannheim, Indianapolis, IN). Probe (10^5 cpm) was added to 1 ml of hybridization solution (50% formamide, 300 mM NaCl, 10 mM Tris, pH 8, 1 mM EDTA, pH 8, 1× Denhart’s solution, 10% dextran sulfate, 500 μg/ml rRNA, 10 mM DTT). This solution was heated for 5 min at 65°C before application to slide-mounted brain sections.

#### Quantitative analysis

Brain sections from slides dipped in nuclear emulsion were used to obtain hybridization signals. Sections were analyzed with Image I Software (Universal Imaging Corporation, West Chester, PA) with a Nikon optical system coupled to a PC. The optical density of the hybridization signals was measured under dark field illumination at 40× magnification. Brain areas of interest were digitized and subjected to densitometric analysis. The optical densities of each specific region were then corrected for the average background signal, which was determined by sampling cells located outside of the areas of interest. The analysis was performed on the following brain regions: frontal, parietal and occipital cortices (layers II–VI), hippocampal fields (CA1, CA2, CA3 and CA4), dentate gyrus and cerebellum.

#### Statistical analysis

Data were expressed as optical density (O.D.) values. Data for each subunit in each subfield of cortex were analyzed by two-way ANOVA, with treatment and layer as factors. Data for each subunit in hippocampus were analyzed by two-way...
ANOVA, with treatment and region (CA1, CA2, CA3, CA4 and dentate gyrus) as factors. Data for each subunit in cerebellum were analyzed by one-way ANOVA. Differences between saline and ACPC treatment for a subunit in a particular layer were determined by the least significant difference (LSD) test.

Results

Chronic ACPC differentially alters levels of mRNAs encoding epsilon subunits in cerebral cortex. Chronic treatment with ACPC produced significant changes in the levels of mRNAs encoding the NMDA receptor subunits designated epsilon-1 to epsilon-3 (Kutsuwada et al., 1992) in mice (corresponding to NMDAR2A-C in rats; Monyer et al., 1992; Moriyoshi et al., 1991). These effects depended on both the cortical subfield and layer. ACPC treatment increased levels of mRNA encoding epsilon-1 in frontal (13–20%), parietal (18–39%) and occipital (26–38%) cortex (fig. 1A). Figure 2 shows autoradiograms of epsilon-1 in frontal cortex from representative control (A) and ACPC-treated (B) animals. In contrast, ACPC treatment decreased levels of mRNAs encoding epsilon-2 and epsilon-3 in cortex. mRNA levels for epsilon-2 were decreased in frontal (25–35%) and parietal (10–26%) cortex (fig. 1B). Figure 2 shows autoradiograms of epsilon-2 in the frontal cortex from representative control (C) and ACPC-treated (D) animals. mRNA levels for epsilon-3 were decreased in frontal (18–27%) and occipital (10–42%) cortices and tended to be decreased in parietal cortex (15–21%, P < .075; fig. 1C). Figure 2 shows autoradiograms of epsilon-3 in frontal cortex from representative control (E) and ACPC-treated (F) animals.

Chronic ACPC differentially alters levels of mRNAs encoding epsilon subunits in hippocampus. Significant elevations in the levels of epsilon-1 mRNA were present in hippocampus (10–19%), particularly CA1 (19%) and CA2 (17%), after chronic ACPC treatment (fig. 3A). Figure 4 shows autoradiograms of epsilon-1 in hippocampus from representative control (A) and ACPC-treated (B) animals. epsilon-2 mRNA levels were not altered in hippocampus (fig. 3B). Figure 4 shows autoradiograms of epsilon-2 in hippocampus from representative control (C) and ACPC-treated (D) animals. Although epsilon-3 mRNA was present in relatively low abundance compared with levels of the other epsilon...

Fig. 1. Chronic ACPC treatment differentially alters expression of NMDA receptor subunit mRNAs in regions of cerebral cortex. Mice were injected daily for 14 days with saline (open bars) or 200 mg/kg ACPC (solid bars). Twenty-four hours after the final injection animals were sacrificed and RNA levels were quantified for epsilon-1 (panel A), epsilon-2 (panel B) and epsilon-3 (panel C) by in situ hybridization as described under "Methods." Data for each subunit in each region of cortex were analyzed by two-way ANOVA, with treatment and layer as factors. A significant main effect of ACPC treatment is indicated by a horizontal line above the data bars, with significance level noted. A significant effect of ACPC treatment for a subunit in particular layer was determined by two-tailed least significant difference (LSD) test (*) P < .05, **P < .01). Values represent mean ± S.E.M. (n = 5–10 mice).
species, ACPC treatment produced an overall reduction in $\epsilon$-3 mRNA levels, particularly in CA1 (30%) and dentate gyrus (26%) (fig. 3C). This is illustrated in autoradiograms from representative control and ACPC-treated animals in figure 4E, F.

Chronic ACPC does not alter levels of mRNAs encoding either $\epsilon$-1, $\epsilon$-2 or $\epsilon$-3 subunits in cerebellum or $\zeta$ subunits in any brain area studied. ACPC did not alter levels of $\epsilon$-1, $\epsilon$-2 or $\epsilon$-3 mRNAs in cerebellum (fig. 5).

Analysis of mRNA encoding $\zeta$ subunit(s), with a probe that detects all splice variants equally, revealed no significant differences between control and ACPC-treated animals in any brain regions analyzed, including cerebral cortex (frontal, parietal or occipital subfields), hippocampus (CA1, CA2 CA3–4 or dentate gyrus), cerebellum, striatum or thalamus (data not presented).

Discussion

In this study we demonstrate that chronic administration of ACPC, a glycine partial agonist, alters mRNA levels encoding NMDA $\epsilon$-1, $\epsilon$-2 and $\epsilon$-3 (but not $\zeta$) subunits in a region-specific manner. This investigation was prompted by the demonstration that chronic administration of ACPC was neuroprotective in animal models of global (Von Lubitz et al., 1992), focal (Lopez and Lanthorn, submitted) and spinal (Long and Skolnick, 1994) ischemia. ACPC was also reported to be neuroprotective when administered at the time of ischemic insult (Long and Skolnick, 1994; Zapata et al., 1996), but it was hypothesized that the neuroprotective effects produced by acute and chronic treatment were not mediated by an identical mechanism (Fossom et al., 1995b). Thus, chronic treatment regimens included a 24-h washout before the induction of ischemia, and at the time of insult, tissue levels of ACPC were below the limits of detection in gerbils that received seven daily injections of drug (Von Lubitz et al., 1992). Although drug levels were not measured in the focal and spinal ischemia studies (Long and Skolnick, 1994; Lopez and Lanthorn, submitted), both the short plasma half-life of ACPC in mice and rats (1.5 and 2.5 h, respectively) (Maccecchini, 1995) and the lack of identified metabolites (Cherkofsky, 1995) argue that no significant accumulation would occur in these species after chronic administration.

Excessive activation of NMDA receptors is one of a complex series of extra- and intracellular events in the “excitotoxic cascade” initiated by an ischemic insult (Choi, 1992; Greene and Greenamyre, 1996; Maccechini, 1995). Thus, it would not be necessary to invoke a direct link between the neuroprotection associated with chronic ACPC treatment and changes in the composition of NMDA receptors. Nonetheless, we examined mRNA levels encoding NMDA receptor subunits because two recent reports indicate that sustained exposure to ACPC can alter the properties of this family of ligand-gated ion channels. Thus, Nowak and co-workers (1993) reported an ~2-fold reduction in the potency of glycine to inhibit $[^{3}H]$5,7-dichlorokynurenic acid binding (an antagonist at strychnine-insensitive glycine receptors; Baron et al., 1991)
to NMDA receptors in cortical membranes after a chronic regimen of ACPC. Furthermore, we observed a 2.5-fold increase in epsilon-3 mRNA levels after a 24-h exposure of cerebellar granule cell neurons to ACPC in cell culture (Fossom et al., 1995a). The present demonstration of changes in mRNA levels encoding the epsilon family of NMDA receptor subunits is consistent with the hypothesis that sustained exposure to ACPC can alter NMDA receptor function in vivo. Future studies will be needed to determine whether the changes in mRNA levels that follow chronic ACPC administration are reflected in changes in NMDA receptor subunit protein levels. If these region-specific, bidirectional effects on mRNA levels reflect corresponding changes in the expression of NMDA receptor proteins, then these data offer some insight into the mechanism responsible for the neuroprotective effects produced by chronic treatment with ACPC.

The physiological and pharmacological properties of both wild-type and recombinant NMDA receptors are largely determined by subunit composition (Laurie and Seeburg, 1994; Lynch et al., 1994; Mori and Mishina, 1995; Wafford et al., 1993). Wild-type NMDA receptors are likely constituted as heterooligomers, assembled from combinations of z and one or more epsilon subunits (Sheng et al., 1994). ACPC-induced changes in the level of each specific epsilon mRNA were unidirectional among the brain regions examined. For example, ACPC treatment either increased or did not affect epsilon-1 mRNA levels, but in no instance were epsilon-1 levels decreased. Likewise, ACPC decreased (or did not significantly alter) epsilon-2 and epsilon-3 mRNA levels. In recombinant NMDA receptors expressed in Xenopus oocytes, the affinities of glutamate and glycine are both lower (10.5- and 2.4-fold, respectively) in receptors composed of z-1 and epsilon-1 subunits than those constituted by z-1 and epsilon-3 subunits (Kutsuwada et al., 1992). Similarly, the affinities of glycine and glutamate are 2.1- and 7-fold lower in receptors constituted with z-1 and epsilon-1 subunits than in those containing z-1 and epsilon-2 subunits. With the corresponding rat cRNAs, Wafford and co-workers (1993) reported that the affinities of glycine and glutamate are 24- and 6.6-fold lower in receptors composed of z-1 and epsilon-1 subunit homologs than in receptors constituted by z-1 and epsilon-3 homologs. Moreover, the affinities of glycine and glutamate at NMDA receptors containing both epsilon-1 and epsilon-3 with z-1 homologs were higher than at receptors containing only z-1 and epsilon-1 subunit homologs. If chronic administration of ACPC results in a higher proportion of NMDA receptors containing epsilon-1 and/or a lower proportion of receptors constituted with epsilon-2 and epsilon-3 subunits (as indicated by the changes in mRNA levels presented here), then the affinities of glycine and glutamate will be lower in this new receptor pool. Because ischemia results in sustained elevations of both glycine and glutamate levels (Globus et al., 1991), it would be predicted that receptor populations with a lower affinity for these agents would be less susceptible to excitotoxic damage. Although speculative, there is evidence to support this hypothesis. For example, the potency of glycine to inhibit [3H]5,7-dichlorokynurenic acid binding to strychnine-insensitive glycine sites in cortical membranes is reduced 2-fold after chronic ACPC treatment (Nowak et al., 1993). Conversely, sustained exposure of primary cultures of cerebellar neurons to ACPC, which results in significantly larger increases in both NMDA-stimulated changes in intracellular calcium levels and glutamate-induced cell death than in control cultures, is accompanied by 2.5-fold increases in levels of epsilon-3 mRNA (Fossom et al., 1995a). Although
these data demonstrate a clear contrast in the effects of sustained exposure to ACPC in vivo and in cell culture, changes in the levels of epsilon-3 mRNA apparently parallel the sensitivity to ischemic insult and NMDA (glutamate) exposure, respectively, in these two model systems. Whether this effect is caused by the partial agonist properties of ACPC or would also be observed with other ligands that occupy strychnine-insensitive glycine sites (i.e., without regard to the intrinsic efficacy) merits further investigation.

Because it is not possible to predict the occurrence of the most common causes of brain ischemia (i.e., stroke, heart attack and traumatic brain injury), a therapeutic strategy with use of chronic administration of a glycine ligand seems rather limited. However, such a strategy may prove suitable as prophylaxis for procedures such as coronary artery bypass grafting, in which the patient is hypoxic for extended periods. Moreover, excessive activation of NMDA receptors has been linked to the sensitivity to ischemic insult and NMDA (glutamate) receptor glutamate ionophore antagonists in ischemic injury. Hence, the development of novel pharmacological agents that may be effective in ameliorating ischemic cellular damage has been of great interest.

In this context, the NMDA receptor antagonist imipramine has been shown to be protective against ischemic cell death. However, it is not clear whether the protective effect of imipramine is mediated through the NMDA receptor or through other mechanisms. Therefore, the development of novel NMDA receptor antagonists with selective efficacy against ischemic injury would be of great significance.

Acknowledgments

The authors thank Dr. M. Mishina (Tokyo University, Tokyo, Japan) for the generous gift of plasmids containing cDNAs for epsilon-1, epsilon-2 and epsilon-3 subunits of the NMDA receptor from mouse. We also thank Drs. Y. Sei and G. Wong (previously from NIDDK/National Institutes of Health, Bethesda, MD) for contributing plasmid containing the entire coding region of the rat homolog of the 31- subunit of the NMDA receptor.

References


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