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Sustained exposure to a glycine receptor partial agonist differentially alters NMDA receptor agonist and antagonist potencies in cultured spinal cord neurons

Yu Lin [a], Linda H. Fossom [b,1], Phil Skolnick [b,2], Joseph B. Long [a,*]

[a] *Division of Neurosciences, Walter Reed Army Institute of Research, Washington, DC, 20307-5100, USA*

[b] *Laboratory of Neuroscience, National Institute for Diabetes and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA*

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Abstract

Sustained (20 h) exposure to the glycine partial agonist 1-aminocyclopropanecarboxylic acid (ACPC) significantly reduced *N*-methyl-D-aspartate (NMDA)-induced neurotoxicity in cultured spinal cord neurons when the NMDA (25 and 100 μM) was added to the cultures 30 min after removal of the ACPC (1 mM). In contrast, ACPC preexposure failed to protect against kainate-induced neuronal injury. The magnitude of neuronal protection against NMDA (100 μM) was further enhanced if the neurons pretreated with ACPC were reexposed to this drug during the NMDA challenge. In addition, the potencies of both the competitive NMDA antagonist AP5 and the noncompetitive antagonist dizocilpine to block NMDA toxicity were significantly increased following ACPC preexposure, while the potency of the competitive glycine receptor antagonist 7-chlorokynurenate (7-CK) was unchanged. Analysis of Northern blots suggest that ACPC-induced changes in NMDA receptor function were not associated with alterations in the levels of the mRNAs encoding the NMDAR-1, -2A, -2B, or -2C subunits. These results indicate that sustained exposure to ACPC modifies NMDA receptors in a manner that diminishes NMDA receptor-mediated neurotoxicity while selectively enhancing the potencies of several NMDA receptor antagonists. These effects do not appear to be related to changes in expression of specific NMDA receptor subunits, and may instead involve a post-translational modification of one or more subunit proteins.

Keywords: ACPC (1-Aminocyclopropanecarboxylic acid); Neuroprotection; Excitotoxicity; NMDA (*N*-methyl-D-aspartate); Neuronal injury; Glycine; Excitatory amino acid

1. Introduction

1-Aminocyclopropanecarboxylic acid (ACPC) is a high affinity partial agonist at the strychnine-insensitive site of the NMDA receptor complex (Marvizon et al., 1989; Watson and Lanthorn, 1990). ACPC antagonizes a variety of NMDA receptor-mediated actions in vivo and in vitro, presumably under conditions in which synaptic concentrations of glycine are at or near saturation (Skolnick et al., 1992; Boje et al., 1993; Fossom et al., 1995a,b; Lin and Long, 1996; Zapata et al., 1996). In addition to protecting against ischemic brain and spinal cord injury in vivo when administered at the time of the insult, ACPC has also been shown to be protective when repeatedly administered prior to the injury (Von Lubitz et al., 1992; Long and Skolnick, 1994). In the latter situation, the therapeutic effects of ACPC were evident following a one day washout period despite undetectable levels of drug in brain and plasma. These findings prompted the hypothesis that chronic administration of ACPC reversibly desensitized the NMDA receptor by uncoupling glycine and glutamate binding sites (Von Lubitz et al., 1992).

In a parallel manner, sustained exposure to ACPC has been shown to produce similar protective effects in primary neuronal cultures. Specifically, in experiments with spinal cord, forebrain and cerebellar neurons, a 20-h preexposure to ACPC reduced the subsequent toxic effects of either NMDA or glutamate without disrupting the response of these neurons to acute application of glycine receptor antagonists (Lin and Long, 1996, 1997). In contrast, a

* Corresponding author. Tel.: +1-202-782-4259; Fax: +1-202-782-6910.

[1] Present addresses: Department of Anatomy, Uniformed Services University of the Health Sciences, Bethesda, MD 20814, USA.

[2] Present address: Lilly Research Laboratories, Neurosciences Discovery, Drop Code 0510, Indianapolis, IN 46285, USA.

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sustained preexposure of cultures enriched in rat cerebellar granule cells to ACPC paradoxically sensitized the cells to NMDA receptor agonists while significantly reducing the response to acutely applied ACPC and other glycine partial agonists (Boje et al., 1993; Fossom et al., 1995a,b). In these latter experiments, the increased sensitivity of granule cell neurons to glutamate was associated with a selective 2.5-fold increase in NMDA receptor 2C mRNA levels, suggesting that the changes in cellular responsiveness could be attributed to an ACPC-induced alteration in NMDA receptor composition (Fossom et al., 1995a).

The divergent effects of sustained exposure to ACPC in these neuronal cultures of different origins might reflect either preexisting neuroanatomical heterogeneity or ensuing differences in neuronal development and organization in culture. Since the effects of sustained exposure to ACPC on primary cultures of spinal cord neurons appear to resemble those of chronic ACPC on ischemic and excitotoxic neuronal injuries in vivo, in the present study we further examined the effects of this drug on the pharmacological responsiveness of cultured spinal cord neurons, specifically seeking to identify if the salutary effects of ACPC preexposure are selective to excitotoxicity mediated through the NMDA receptor complex, and if the acute actions of other ligands targeting the NMDA receptor complex are altered following sustained ACPC pretreatment. In addition, we attempted to establish whether any functional pharmacological alterations are associated with ACPC-induced changes in the expression of NMDA receptor subunit mRNA.

2. Materials and methods

The experimental methods used in this study have been reported previously (Lin and Long, 1996). In brief, primary neuronal cell cultures were prepared from prenatal day 15 Sprague–Dawley rat embryos. After a 48-h incubation, cytosine arabinoside (10 μM) was added to inhibit non-neuronal cell division. Cells were used in experiments after 7 days in culture. For drug preexposures, ACPC was dissolved in neuronal culture medium and was added to half of the wells of each 48 well plate 20–24 h prior to the neurotoxicity assays. The remaining wells served as vehicle-pretreated controls. Cell damage was quantitatively assessed 24 h after exposure to NMDA (30 min) or kainate (60 min) using a tetrazolium salt colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA). This dye is converted to an insoluble blue formazan product by living cells, which is then dissolved in acidified isopropanol and quantitated by absorbance intensity. Changes in cell viability are expressed relative to untreated control cells that were maintained on each plate. Each experimental condition was replicated in a minimum of 15 wells using cells obtained from at least three independent preparations.

For mRNA measurements, the spinal cord neurons were plated in 6-well plates (35-mm wells) at a density of 4×10^6 cells per well and were subsequently treated in a manner identical to that used to treat the cells used in viability assays. Twenty h after ACPC treatment, total cellular RNA was isolated by the acid guanidinium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi (1987). RNA concentrations were estimated by absorbance spectrometry. Specific RNAs for four NMDAR subunits (NR1, NR2A, NR2B, and NR2C) were measured by hybridization to specific cDNA probes as previously described (Fossom et al., 1995a). Briefly, amounts of specific RNAs were analyzed in 5 μg aliquots of RNA from control and ACPC-treated cells by slot–blot hybridization to specific cDNA probes that had been radiolabelled with [^{32}P]dCTP by random primer procedure. Probe that hybridized to slot blots was visualized by exposure to X-ray film and quantified by densitometric scanning of the autoradiograms.

Differences in the cell viability among treatment groups were determined using one-way analysis of variance and

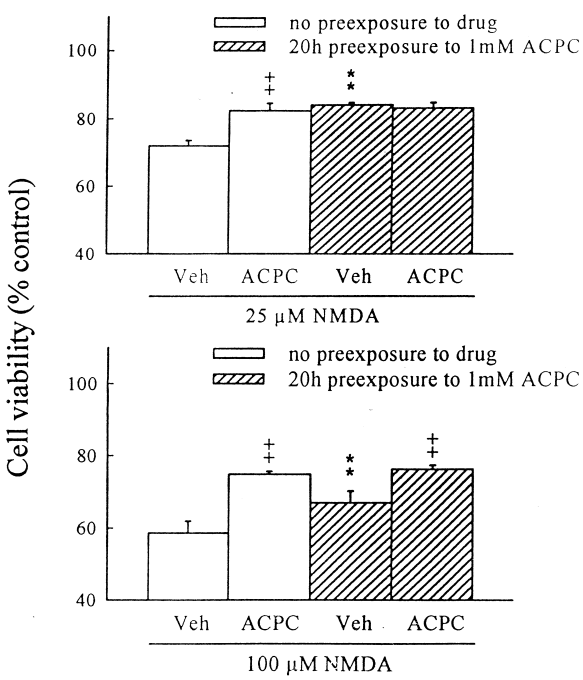

Fig. 1. ACPC reduction of NMDA toxicity. In this and the following figures, cell viability is expressed relative to the MTT measurements made in control cells maintained on each plate. The neuroprotective effects of 1 mM ACPC were evident following either ACPC cotreatment with NMDA (second column) or preexposure and removal of ACPC before NMDA addition (third column). ACPC cotreatment or pretreatment were equally effective with 25 μM NMDA (columns 2 and 3, top), whereas ACPC pretreatment was less effective with 100 μM NMDA (columns 2 and 3, bottom). Maximal protection against 100 μM NMDA was restored with reexposure of these neurons to ACPC along with the 100 μM NMDA (bottom panel, fourth column). ++, $P < 0.01$, NMDA toxicity is diminished following ACPC cotreatment relative to vehicle cotreatment. **, $P < 0.01$, NMDA toxicity is diminished following ACPC pretreatment relative to vehicle pretreatment.

the Newman–Keuls test (GB-STAT V6.0, Dynamic Microsystems, Silver Spring, MD, USA). Comparisons of mRNA levels were made using the Student's *t*-test.

3. Results

A 20-h preexposure as well as a coexposure of cells to 1 mM ACPC significantly attenuated the cell damage caused by 25 or 100 μM NMDA (Fig. 1). For example, in contrast to the 28% and 42% reductions in viability measured in control cells after 30 min exposure to 25 or 100 μM NMDA, respectively, coapplication of ACPC with these concentrations of NMDA resulted in corresponding 18% and 26% losses in viability. Similarly, cells pretreated with ACPC (which was removed 30 min before addition of NMDA) had 16% or 33% losses in viability produced by 25 or 100 μM NMDA, respectively. Although both preexposure and coexposure to ACPC yielded comparable degrees of protection against 25 μM NMDA, preexposure to ACPC was less effective than cotreatment against 100 μM NMDA (Fig. 1). In contrast to its effects on NMDA toxicity, ACPC pretreatment failed to alter the neurotoxic effects of kainic acid (Fig. 2).

When ACPC was present during both the pretreatment and cotreatment phases, it caused an additional significant reduction in the neurotoxicity produced by 100 μM NMDA relative to that produced in the cells receiving only the ACPC pretreatment (Fig. 1). Under this condition, in comparison to the 42% and 33% losses in viability caused by 100 μM NMDA in the control cells and cells pretreated with ACPC, respectively, the cells that received the combined pre- and co-treatments with ACPC had a 24% loss in viability after exposure to NMDA. Although significantly greater than that produced by ACPC pretreatment alone, the protection resulting from the combined treatments did not differ significantly from that produced by ACPC cotreatment alone. In addition, ACPC caused comparable, significant degrees of protection against 25 μM NMDA whether administered as a pretreatment, cotreatment, or as a combined treatment (Fig. 1).

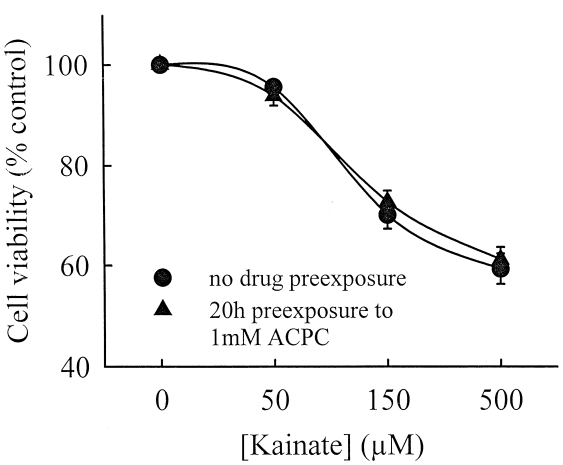

Fig. 2. Sustained preexposure to 1 mM ACPC had no effect on kainate toxicity. Kainate caused dose-dependent toxicity which was not altered in neurons preexposed to ACPC.

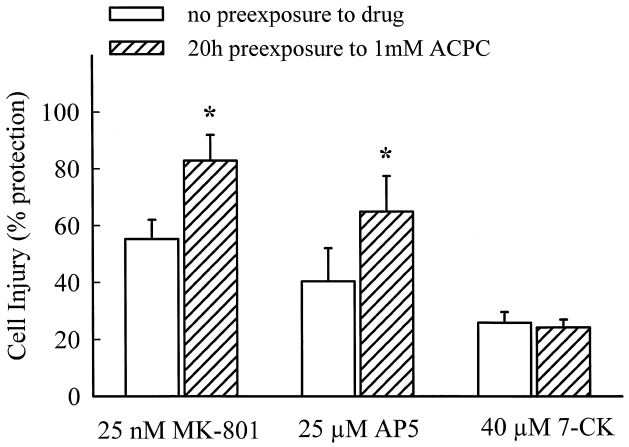

Fig. 3. The protective effects of low concentrations of dizocilpine (MK-801) and AP5 (but not 7-CK) against 100 uM NMDA toxicity were significantly increased after sustained preexposure to ACPC. For these comparisons, the % protection due specifically to the acute protective effects of these agents (i.e., protection beyond that resulting from the ACPC pretreatment alone) was determined and compared to that seen following application of these drugs to cells pretreated with vehicle. The percentage protection resulting from dizocilpine or AP5 treatment of cells preexposed to vehicle was calculated from MTT absorbance values as $100 \times [(\text{NMDA} + \text{Drug}) - (\text{NMDA} + \text{Vehicle})] \div [\text{Control} - (\text{NMDA} + \text{Vehicle})]$. In contrast, for measurements made in cells after pretreatment with ACPC, the protection specifically attributable to the acute effects of AP5 and dizocilpine was distinguished by calculating percentage protection as $100 \times [(\text{ACPC pretreated, NMDA} + \text{Drug}) - (\text{ACPC pretreated, NMDA} + \text{Vehicle})] \div [(\text{ACPC pretreated control} - (\text{ACPC pretreated, NMDA} + \text{Vehicle})]$. In this manner, the acute protective effects of dizocilpine and AP5 are calculated relative to a baseline of NMDA toxicity that is appropriate to eliminate from consideration the protective contributions arising from the sustained preexposure to ACPC. As can be seen from the data calculated in this manner, the percentage protection specifically resulting from cotreatment with either AP5 or dizocilpine was significantly greater in cells preexposed to ACPC than was measured in the cells preexposed to vehicle alone, indicating that potencies of these agents had increased. $**$, $P < 0.01$ when compared to the protective effects recorded in cells not preexposed to ACPC.

If, after ACPC pretreatment, cells were exposed to dizocilpine or AP5 during the 30 min incubation with 100 μM NMDA, the potencies of these NMDA antagonists were significantly increased relative to that recorded in sister cells that did not receive the ACPC pretreatment. As seen in Fig. 3, the protection specifically attributable to the acute effects of each of these drugs (i.e., the protection beyond that resulting from the ACPC pretreatment alone) was significantly greater in the cells pretreated with ACPC than was produced by these drugs in the absence of ACPC pretreatment. In contrast to its effects on acute actions of dizocilpine and AP5, pretreatment with ACPC failed to significantly change the acute protective effects of 7-CK (Fig. 3). In addition, ACPC did not alter the maximally-

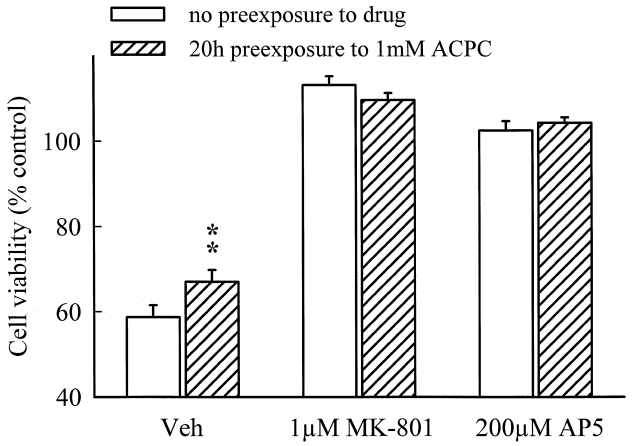

Fig. 4. The maximally protective effects of NMDA antagonists are not diminished after sustained preexposure of cells to ACPC. At these drug concentrations, the injury induced by 100 μM NMDA was completely blocked by cotreatment with dizocilpine (MK-801) or AP5 in neurons preexposed to either vehicle or 1 mM ACPC. **, $P < 0.01$ when compared to cell viability measured in cells preexposed to vehicle.

protective effects of higher concentrations of these agents (Fig. 4).

The NMDA receptor-related pharmacological alterations resulting from sustained pretreatment with ACPC were not associated with altered expression of NMDA receptor subunit mRNAs. The levels of RNA encoding the NMDAR-1, -2A, and -2B subunits were unchanged after 20 h exposure of cells to 1 mM ACPC (Fig. 5). The signal for the mRNA encoding the NMDAR-2C subunit was not reliably above background in these cultures.

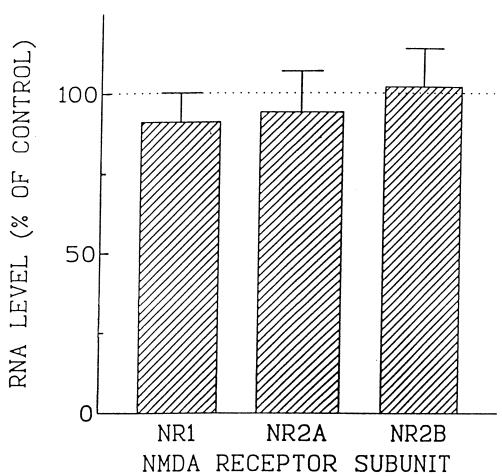

Fig. 5. Sustained exposure to ACPC does not alter levels of RNAs encoding NMDA receptor subunits NR1, NR2A, or NR2B. Total cellular RNA was isolated by acid guanidinium–phenol–chloroform method of Chomczynski and Sacchi (1987). Amounts of specific RNAs were analyzed in 5 μg aliquots of RNA from control and ACPC-treated cells by slot–blot hybridization to specific cDNA probes that had been radiolabelled with [^{32}P]dCTP by random primer procedure. Probe that hybridized to slot blots was visualized by exposure to X-ray film and quantified by densitometric scanning of the autoradiograms. The signal for a particular RNA from cells treated with ACPC was expressed as a percentage of the signal for the same RNA measured in control cells from the same preparation. Values are expressed as means ± SEM for nine spinal cord culture preparations.

4. Discussion

The results of this study are consistent with earlier findings with spinal, forebrain and mixed cerebellar neuronal cultures in which ACPC antagonized NMDA receptor-mediated excitotoxicity following both acute and sustained preexposure (Lin and Long, 1996, 1997). The diminished NMDA toxicity following sustained exposure to ACPC appears to result from a drug-induced alteration in the responsiveness of the cells because the ACPC was removed 30 min prior to addition of NMDA, and in earlier work the effects of sustained exposure to ACPC were shown to be time-dependent (Lin and Long, 1996). While the overall impact of acute and sustained exposures to ACPC in the present study was quite similar to that reported previously with cultured spinal cord neurons (Lin and Long, 1996), there were several minor differences. Most notably, in the earlier report cotreatment with ACPC protected against 25 but not 100 μM NMDA, whereas in the present study significant protection was evident against both concentrations of NMDA (Fig. 1). In addition, in the present study we observed that, relative to the cells receiving only ACPC pretreatment, protection against 100 μM NMDA was enhanced by the subsequent coexposure to ACPC (Fig. 1). Since kainate-induced cell damage was unaffected by preexposure to ACPC, the diminished NMDA toxicity is not readily attributable to an alteration in the general responsiveness of cells to excitotoxic insult, and is instead more likely the consequence of ACPC-induced alterations in responses to NMDA receptor activation.

These results clearly differ in several respects from the previous findings (Boje et al., 1993; Fossom et al., 1995a,b) with enriched cerebellar granule cell cultures in which sustained (24 h) exposure to ACPC was not neuroprotective, and instead diminished the acute protective effects of ACPC and other glycine partial agonists (e.g., D-cycloserine, HA 966). Moreover, following ACPC pretreatment of cerebellar granule cells the potencies of NMDA receptor agonists (e.g., glutamate and NMDA) to increase intracellular calcium and cGMP and to injure cells were enhanced. In contrast, in the present study with spinal cord neurons we noted that NMDA toxicity was diminished rather than enhanced following ACPC pretreatment, and that the acute protective effects of ACPC were also retained after ACPC preexposure (Fig. 1). Additionally, after ACPC pretreatment of spinal cord neurons two antagonists acting at other loci on the NMDA receptor complex (AP5 and dizocilpine) ameliorated NMDA toxicity with an increased potency, whereas in cultured cerebellar granule cells the effects of these drugs were unaltered after sustained exposure to ACPC (Boje et al., 1993).

Subunit composition has been shown to determine ligand affinities for a wide variety of ligand gate ion channels, including NMDA receptors. For example, the respective potencies of glycine and glutamate have been shown to be approximately 25 and 6.5 times greater in *Xenopus* oocytes expressing NMDAR1 and 2C subunits than are seen with receptors comprised of NMDAR1 and 2A subunits (Wafford et al., 1993). Thus, differences in the composition of NMDA receptors induced by sustained exposure to ACPC in these different neuronal cell cultures might, in part, account for the differing pharmacological outcomes observed. Specifically, the pharmacological alterations induced in cerebellar granule cells by ACPC were previously shown to be associated with an approximately 2.5-fold increase in the expression of the mRNA encoding the NMDAR-2C subunit without concomitant changes in levels of mRNA encoding the NMDAR-1, -2A, or -2B subunits (Fossom et al., 1995a). If the measured mRNA changes are associated with a corresponding change in NMDAR-2C protein, then this observation provides a potential explanation for the concommitant increased sensitivity of cerebellar granule cells to glutamate and/or glycine and diminished neuroprotective effects of ACPC after sustained exposure. Conversely, chronic administration of ACPC to mice caused regional increases in the mouse homolog of NMDAR-2A mRNA and decreases in the mouse homologs of NMDAR-2B and -2C mRNAs (Bovetto et al., 1997). If accompanied by parallel alterations in the incorporation of the corresponding encoded subunits into NMDA receptors, one would predict from these changes in mRNA levels that the resultant receptor populations would have lower affinities for glycine and glutamate and would as a result be less likely to mediate excitotoxic damage. This speculation is supported by the demonstration that chronic ACPC treatment reduced by approximately 2-fold the potency of glycine to inhibit [^{3}H]5,7-dichlorokynurenic acid binding to strychnine-insensitive glycine binding sites in cortical membranes (Nowak et al., 1993). However, in contrast to these earlier findings, we were unable to distinguish ACPC-induced changes in NMDA receptor subunit mRNA expression in cultured spinal cord neurons (Fig. 5). Thus, alterations in subunit composition cannot readily account for the pharmacological effects of sustained exposure to ACPC recorded in the present experiments. Receptor alterations resulting from post-translational modifications might provide potential alternative explanations for these drug-induced functional changes.

The protective effects resulting from sustained preexposure of spinal cord neurons to ACPC parallel those described following the chronic administration of this agent in vivo and indicate that, along with cultured forebrain and mixed cerebellar neurons (Lin and Long, 1997), cultured spinal cord neurons provide a useful means to model the neuroprotective desensitization described with ischemic or excitotoxic injury (Skolnick et al., 1989; Von Lubitz et al., 1992; Long and Skolnick, 1994). As a partial agonist, ACPC does not completely block NMDA receptor activity and should most effectively antagonize NMDA receptor function when and where synaptic concentrations of glycine are at or near saturation, such as are found with stroke and brain ischemia (Hillered et al., 1989; Graham et al., 1990; Globus et al., 1991; Takagi et al., 1993). Consequently, NMDA receptor activity may be therapeutically modulated with a minimal disruption of NMDA receptor function elsewhere in the brain. Moreover, after sustained exposure to ACPC, spinal cord neurons did not display a pronounced tolerance to ACPC or 7-CK and actually responded to AP5 and dizocilpine with an enhanced potency (Fig. 3). If these in vitro responses to ACPC mirror those occurring in vivo, they point to the possible therapeutic utility of this drug following chronic administration. If used as an adjunct to other therapies targeting the NMDA receptor complex, ACPC might reduce dose requirements and in turn minimize the side effect liabilities. As a result, chronic ACPC-induced modification of NMDA effector mechanisms might provide a therapeutic approach relevant to disorders linked to excessive activation of this family of ligand-gated ion channels.

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