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CP-101,606, a potent neuroprotectant selective for forebrain neurons

Frank Menniti *, Bertrand Chenard, Mary Collins, Mary Ducat, Ismail Shalaby, Frost White

Pfizer Inc., Central Research Division, Eastern Point Road, Groton, CT 06340, USA

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Abstract

The neuroprotective activity of (1*S*,2*S*)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol (CP-101,606), an *N*-methyl-D-aspartate (NMDA) receptor antagonist structurally similar to ((±)-(*R**,*S**)-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidineethanol (ifenprodil), was investigated in neurons in primary culture. CP-101,606 potently and efficaciously protected hippocampal neurons from glutamate toxicity but was > 900-fold less effective for cerebellar granule neurons. The neuroprotective activity in the hippocampal neurons is mediated through a high affinity binding site distinct from the agonist and thienylcyclohexylpiperidine (TCP) binding sites of the NMDA receptor. Autoradiography indicates the CP-101,606 binding site is localized in forebrain, most notably in hippocampus and the outer layers of cortex. The functional selectivity for hippocampal neurons, forebrain localization of binding sites, and structural relation to ifenprodil suggest that CP-101,606 is an NMDA antagonist highly selective for NR2B subunit containing receptors. © 1997 Elsevier Science B.V.

Keywords: NMDA receptor antagonist; Neurotoxicity; Neurodegenerative disease

1. Introduction

Glutamate receptors play a pivotal role in the pathway leading to neuron death in a variety of pathological conditions. In acute central nervous system (CNS) ischemia or insult such as occurs in stroke or head trauma, extracellular glutamate levels in the CNS are elevated leading to aberrant and toxic glutamate receptor activation (McCulloch, 1994; Bullock et al., 1992). It has also been hypothesized that aberrant glutamate receptor activity plays a causal role in the neuron loss associated with the chronic neurodegenerative conditions such as Alzheimer's and Parkinson's disease (Greenamyre, 1986; Maragos et al., 1987). Thus, glutamate receptor inhibition is an actively investigated therapeutic strategy to prevent both acute and chronic neurodegeneration in man.

Several lines of evidence suggest that the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor is the principal mediator of glutamate toxicity. Neurons in primary culture are exquisitely sensitive to the toxic effects of NMDA receptor activation and NMDA receptor antagonists protect cultured neurons from both NMDA and glutamate toxicity (Choi et al., 1988; Rosenberg and Aizenman, 1989). NMDA receptors are also implicated as mediators of neurotoxicity in vivo since NMDA receptor antagonists can reduce neuron loss in animal models of focal ischemia (McCulloch, 1994) and head trauma (Bullock et al., 1992). The neuroprotective effect of NMDA receptor inhibition is realised with several different classes of compounds which target different sites on the NMDA receptor-channel complex. These include competitive antagonists at the glutamate binding site such as (*R*,*E*)-4-(3-phosphonoprop-2-enyl) piperazine-2-carboxylic acid (d-CPPene) (Lowe et al., 1994) and *cis*-4-phosphonomethyl-2-piperidine carboxylic acid (CGS-19,755) (Bennett et al., 1990; Murphy et al., 1988) and competitive antagonists at the glycine co-agonist (Johnson and Ascher, 1987; Kemp and Leeson, 1993) binding site such as 5,7-dichloro-4*S*-(3-phenylureido)-1,2,3,4-tetrahydro-quinoline-2*R*-carboxylic acid (L-689,560) and 5-nitro-6,7-dichloro-1,4-dihydro-2,3-quinoxalinedione (ACEA-1021) (Leeson and Iversen, 1994). Compounds have also been identified which block

* Corresponding author. Tel.: (1-860) 441-5939; Fax: (1-860) 441-6973; e-mail: mennif@pfizer.com

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the NMDA receptor-gated ion channel, including phencyclidine (PCP), (+)-5-methyl-10,11-dihydro-5-*H*-dibenzo[*a*,*d*]cycloheptan-5,10-imine (MK-801) (Kemp et al., 1987) and *C*-(1-naphthyl-*N*′-(3-ethyl phenyl)-*N*′-methyl guanidine hydrochloride (CNS-1102) (Reddy et al., 1994). However, in contrast to the similar range of neuroprotective activity, NMDA receptor antagonists of different mechanistic classes have distinct effects on behaviour and neuronal metabolism, at least in rodents (Schmidt, 1994; Hargreaves et al., 1994); this may affect the therapeutic utility of these different compounds (Muir and Lees, 1995).

(±)-(R^*,S^*)-α-(4-Hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidineethanol (ifenprodil) and (±)-α-(4-chlorophenyl)-4-[(4-fluorophenyl)methyl]-1-piperidineethanol (eliprodil) represent a fourth class of NMDA receptor antagonist (Carter et al., 1988) which may interact at the polyamine modulatory site on the NMDA receptor (Carter et al., 1990; Schoemaker et al., 1990). These compounds are of particular interest since they also display selectivity for a subset of NMDA receptors. As with other ligand gated ion channels, the functional NMDA receptor is composed of multiple protein subunits (Mori and Mishina, 1995). Five subunits have been cloned to date, NR1 (of which there are eight splice variants) and NR2A–D. Expression studies indicate a composition of at least one NR1 subunit and one or more of the NR2 subunits (Monyer et al., 1992; Kutsuwada et al., 1992; Chazot et al., 1994). In situ hybridization and immunohistochemistry studies indicate that subunits are widely and differentially distributed throughout the brain (Monyer et al., 1994; Kutsuwada et al., 1992; Ishii et al., 1993; Wenzel et al., 1995). Ifenprodil has been found to be functionally selective for NMDA receptors containing the NR2B subunit (Williams et al., 1993; Williams, 1995). Thus, the fact that this class of NMDA receptor antagonist is neuroprotective in animal models of focal ischemia (Gotti et al., 1988) and head trauma (Toulmond et al., 1993) suggests that NR2B containing NMDA receptors are prominently involved in the toxic cascade elicited by these types of insults. However, ifenprodil and eliprodil also interact with σ receptors (Karbon et al., 1990; Contreras et al., 1990) and inhibit high voltage activated Ca^{2+} channels (Biton et al., 1994, 1995; Bath et al., 1996), activities which may also contribute to the neuroprotective efficacy of these compounds in vivo.

(1*S*,2*S*)-1-(4-Hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol) (CP-101,606) is a structural analogue of ifenprodil which is a potent NMDA receptor antagonist but lacks α_1-adrenoceptor activity (Chenard et al., 1995). We report here on the neuroprotective activity of CP-101,606 for neurons in primary culture and the characterization of the binding site through which this activity is derived. We show that CP-101,606 binds to a high affinity site localised to forebrain structures and is selectively neuroprotective for forebrain neurons. This selectivity is consistent with a specific interaction with NMDA receptors containing an NR2B subunit. This work has previously been presented as abstracts (Menniti et al., 1995; White et al., 1995).

2. Materials and methods

2.1. Materials

CP-101,606 was synthesized as described by Chenard et al. (1995). Racemic [^{3}H]CP-101,606 was prepared by Chemsyn Science Laboratories (Lenexa, KS, USA) by palladium catalysed tritium reduction of a halogenated precursor (Chenard and McCarthy, data not shown). [^{3}H]Glycine, [^{3}H]TCP and [^{3}H]CGS-19,755 were purchased from DuPont-NEN (Wilmington, DE, USA). Cell culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA) unless otherwise noted. All other reagents were purchased from commercial sources.

2.2. Neuron primary cultures

The effects of CP-101,606 on glutamate-induced neurotoxicity were characterized in rat hippocampal and cerebellar granule neurons in primary culture. Primary cultures of rat hippocampal neurons were prepared as described previously (Shalaby et al., 1992). Briefly, hippocampi were dissected from embryonic day 17 rat brains, minced into 1 mm pieces and incubated for 30 min in Ca^{2+}-Mg^{2+}-free Tyrode's solution containing 0.1% trypsin. The digested tissue was triturated with a fine bore Pasteur pipette and the cell suspension seeded onto 96-well Falcon Primaria tissue culture plates (Becton Dickinson, Franklin Lakes, NJ, USA) at 80 000 cells per well. Medium was composed of minimal essential medium with Earle's salts, 10% fetal calf serum (Hyclone Laboratories, Logan, UT, USA), 5% equine serum (Hyclone Laboratories), L-glutamine (2 mM), penicillin-streptomycin (100 units per ml) and glucose (21 mM). Cell division was blocked at 6 days in vitro by incubating for 2 days in medium containing 0.035 mg/ml uridine and 0.015 mg/ml 5-fluoro-2′-deoxyuridine. Medium, minus the fetal calf serum, was thereafter changed twice per week. Cultures were used at 14–21 days in vitro.

Primary cultures of rat cerebellar granule neurons were prepared as described previously (Parks et al., 1991). Cerebella were removed from 8-day-old CD rats, minced into 1 mm pieces and incubated for 15 min at 37°C in Ca^{2+}-Mg^{2+}-free Tyrode's solution containing 0.1% trypsin. The tissue was then triturated using a fine bore Pasteur pipette. The cell suspension was plated onto poly-D-lysine coated 96-well tissue culture plates at 10^5 cells per well. Medium consisted of minimal essential medium with Earle's salts, 10% fetal calf serum (Hyclone Laboratories), 2 mM L-glutamine, penicillin-streptomycin (100 units per ml) and 25 mM KCl. After 24 h, the medium was replaced with fresh medium containing 10 μM cytosine arabinoside

to inhibit cell division. Cultures were used at 6–8 days in vitro.

2.3. Glutamate-induced neurotoxicity

The ability of CP-101,606 and other compounds to inhibit glutamate-induced neurotoxicity in hippocampal cultures was assessed using two different paradigms as previously described (Shalaby et al., 1992). For the protection paradigm, cultures were exposed simultaneously to 1 mM glutamate and various compounds for 15 min at 37°C. The cultures were then washed twice in serum-free medium and incubated a further 20–24 h in the absence of compounds and added glutamate. Viability was then assessed by measuring the lactate dehydrogenase activity (LDH) released into the medium (Koh and Choi, 1987). Alternatively, for the rescue paradigm, the cultures were incubated in a chloride-free solution (in mM: 69 Na_2SO_4, 2.67 K_2SO_4, 0.33 NaH_2PO_4, 0.44 KH_2PO_4, 1.0 $NaHCO_3$, 1.0 $MgSO_4$, 10.0 Hepes, 22.0 glucose and 71.0 sucrose at pH 7.4) containing 1 mM glutamate for 15 or 20 min at 37°C. The glutamate-containing solution was then removed and test compounds were added in serum-free medium. The cultures were incubated 20–24 h and the viability assessed by measuring LDH release.

The effects of compounds on glutamate-induced neurotoxicity in cerebellar granule neurons in culture was also investigated. The medium was replaced with a glucose-free salt solution (in mM: 25 Hepes buffer pH 7.4, 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$ and 1.8 $CaCl_2$). After a 5 min preincubation, cultures were incubated for 30 min with an identical solution containing test compound. Glutamate was then added to a final concentration of 1 mM and cultures were incubated for a further 60 min at 37°C. Viability was then assessed by exclusion of the vital dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983). Cultures were washed and then incubated for 2–4 h at 37°C in serum-free medium containing 0.1% MTT. The formazin product was then dissolved 0.08 N HCl in isopropanol and absorbance read at 550 nm and 650 nm. Cultures with a higher absorbance contained more viable cells.

2.4. CP-101,606 binding site characterization

The binding of racemic [^{3}H]CP-101,606 was characterized in rat forebrain and cerebellar membranes. Forebrains or cerebella of adult male CD rats were homogenized in 0.32 M sucrose at 4°C. The crude nuclear pellet was removed by centrifugation at $1000 \times g$ for 10 min, and the supernatant centrifuged at $17\,000 \times g$ for 25 min. The resulting pellet was resuspended in 5 mM Tris acetate pH 7.4 at 4°C for 10 min to lyse cellular particles and again centrifuged at $17\,000 \times g$. The resulting pellet was washed twice in Tris acetate, resuspended at 10 mg protein/ml and stored at −20°C until use.

For binding assays, membranes were thawed, homogenized and diluted to 0.5 mg protein/ml with 50 mM Tris HCl, pH 7.4. Compounds under study were added at various concentrations followed by racemic [^{3}H]CP-101,606 (specific activity 42.8 Ci/mmol, 5 nM final concentration). Following incubation for 20 min at 30°C in a shaking water bath, samples were filtered onto Whatman GFB glass fiber filters using a MB-48R Cell Harvester (Brandel Research and Development Laboratories, Gaithersburg, MD, USA). Filters were washed for 10 s with ice-cold Tris HCl buffer and the radioactivity trapped on the filter quantified by liquid scintillation spectroscopy. Nonspecific binding was determined in parallel incubations containing 100 μM racemic CP-101,606. Specific binding was defined as total binding minus nonspecific binding.

2.5. CP-101,606 receptor autoradiography

The distribution in rat brain of racemic [^{3}H]CP-101,606 binding sites was compared to that of [^{3}H]glycine, [^{3}H]TCP and [^{3}H]CGS-19,755 using an autoradiographic technique. Brains from adult male CD rats were frozen on dry ice, sectioned at 20 μm on a cryostat and mounted on microscope slides coated with gelatin-chrom alum. Slides were prepared such that serial adjacent sections from an individual animal were exposed to each of the four ligands used. After incubation with ligand, slides were exposed to Hyperfilm ^{3}H (Amersham Life Sciences, Arlington Heights, IL, USA) in X-ray cassettes at −80°C. The film was developed in Kodak Dektol developer, fixed in Kodak Fixer (Rochester, NY, USA), and washed under running water for 30 min. Autoradiograms were analysed using an MCID Image Analysis system (Imaging Research, St. Catharines, Ontario, Canada); optical densities were quantified or converted to a pseudocolor scale and the resulting images photographed. Binding reactions for each ligand were as follows:

2.5.1. Racemic [^{3}H]CP-101,606

Sections were incubated for 20 min in 50 mM Tris HCl pH 7.4 containing 10 nM racemic [^{3}H]CP-101,606 at 30°C. Sections were rapidly washed at 4°C in Tris HCl followed by distilled water to remove salts and rapidly dried under a stream of anhydrous air. Nonspecific binding was determined on adjacent sections by including 100 μM racemic CP-101,606 in the incubation.

2.5.2. [^{3}H]Glycine

Sections were preincubated for 45 min in 50 mM Tris citrate pH 7.4 at 23°C, followed by incubation for 20 min at 4°C in Tris citrate containing 100 nM [^{3}H]glycine. Sections were rapidly washed in 2 changes of Tris citrate followed by distilled water to remove salts and rapidly dried under a stream of anhydrous air. Nonspecific binding

was determined on adjacent sections by including 200 μM glycine in the incubation.

2.5.3. [^{3}H]TCP

Sections were preincubated for 2 h in 50 mM Tris HCl, 1 mM EDTA, 0.1% formalin pH 7.4 at 30°C followed by 5 min in Tris HCl, 0.1% formalin pH 7.4 at 23°C. Sections were then incubated for 20 min at 23°C in 2 nM [^{3}H]TCP in buffer containing 100 μM glutamate and 10 μM glycine. Unbound radioactivity was removed by a 5 min wash in Tris HCl pH 7.4 at 4°C, the sections were then rapidly washed in distilled water to remove salts and dried under a stream of anhydrous air. Nonspecific binding was determined on adjacent sections by including 100 μM PCP in the incubation.

2.5.4. [^{3}H]CGS-19,755

Sections were preincubated for 30 min in 50 mM Tris HCl pH 8.0 at 23°C, followed by incubation for 20 min in Tris HCl containing 10 nM [^{3}H]CGS-19,755 at 23°C. Sections were rapidly washed at 4°C in 3 changes of Tris HCl followed by distilled water to remove salts and rapidly dried under a stream of anhydrous air. Nonspecific binding was determined on adjacent sections by including 1 mM glutamate in the incubation.

3. Results

3.1. Neuroprotective activity in neuronal cultures

The ability of CP-101,606 to protect against glutamate-induced neurotoxicity was examined in primary cultures of rat neurons. CP-101,606 inhibited the neurotoxicity induced by 1 mM glutamate in hippocampal neurons in culture when the compound was added simultaneously with glutamate (protection paradigm, Fig. 1). CP-101,606 was also neuroprotective when added after the glutamate insult (rescue paradigm, Fig. 1). The potency in the protection paradigm was approximately three-fold greater than in the rescue paradigm (IC_{50} values of 11 ± 4 nM vs. 35 ± 7 nM, two-tailed Student's $t = 2.98$, df = 6, $P < 0.05$). The neuroprotective activity of CP-101,606 was compared to that of several NMDA receptor antagonists. CP-101,606 was approximately equipotent to MK-801 in hippocampal cultures and was considerably more potent than the other agents examined (Table 1).

In contrast to the potent neuroprotective activity of CP-101,606 in the hippocampal neuron cultures, the compound weakly protected cerebellar granule neurons from glutamate toxicity (Fig. 1 and Table 1). The potency ratio of CP-101,606 for neuroprotection in the hippocampal vs. for cerebellar granule neurons was > 900. In contrast, this ratio was 3, 6 and 12 for MK-801, eliprodil and ifenprodil, respectively.

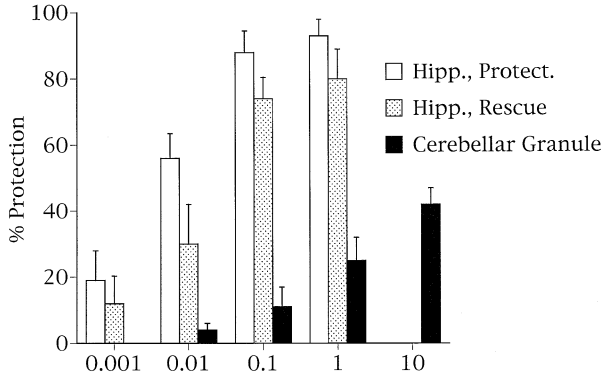

Fig. 1. CP-101,606 protects neurons in culture from glutamate-induced toxicity. Hippocampal or cerebellar granule neurons were exposed to 1 mM glutamate and the indicated concentrations CP-101,606 as described in Section 2. For hippocampal neurons, CP-101,606 was added simultaneously with (Hipp., Protect) or after (Hipp., Rescue) glutamate. For hippocampal neurons toxicity was measured by LDH release into the medium and for cerebellar neurons toxicity was measured by formation of the formazan product of MTT: 0% protection was defined as the LDH/MTT level in the presence of 1 mM glutamate alone and 100% protection as LDH/MTT level in the absence of added glutamate or compound. Each bar is the mean from 4 separate cell preparations (plus S.E.M.). For each experiment, determinations were made on at least 8 separate wells of a 96-well plate.

3.2. Binding site characterization

Racemic [^{3}H]CP-101,606 binds to a saturable site on rat forebrain membranes (Fig. 2). Binding affinity (K_d) is 10 ± 1 nM and the B_{max} is 1.7 ± 0.2 pmol/mg protein. Equilibrium binding is best fit by a model which assumes interaction at a single population of sites; however, interaction with multiple sites of similar affinity cannot be excluded. In contrast, racemic [^{3}H]CP-101,606 fails to bind with high affinity to rat cerebellar membranes ($K_d > 10$ μM).

The only compounds found to displace specifically bound racemic [^{3}H]CP-101,606 were close structural analogues and polyamines (Table 2). Ifenprodil and eliprodil displaced racemic [^{3}H]CP-101,606 binding with potencies

Table 1
EC_{50} concentrations for CP-101,606 and other compounds for inhibiting the glutamate-induced death of neurons in primary culture

Compound	Hippocampal	*n*	Cerebellar	*n*
CP-101,606	0.011 ± 0.004	4	> 10	3
MK-801	0.027 ± 0.004	6	0.083 ± 0.016	3
7-Cl-Kynurenate	6.4 ± 1.4	3	58 ± 35	3
AP-7	200 ± 260	3	67 ± 25	3
Eliprodil	1.0 ± 0.2	3	5.7 ± 1.3	3
Ifenprodil	0.26 ± 0.06	4	3.1 ± 0.7	3

Hippocampal and cerebellar granule neurons were exposed to 1 mM glutamate as described in Section 2. Compounds were added simultaneously with glutamate. Values are the mean ± S.E.M. of the concentration of compound (μM) that inhibited the release of LDH by 50%. The number of experiments (*n*) is indicated.

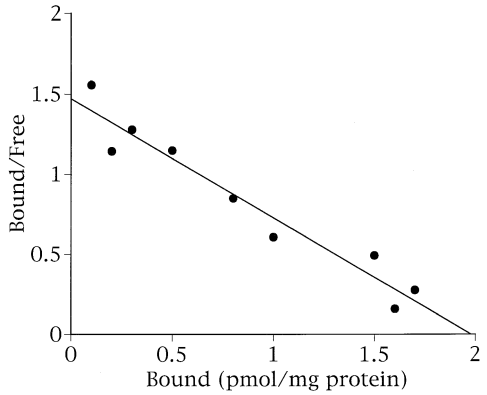

Fig. 2. Scatchard analysis of the binding of racemic [^{3}H]CP-101,606 to rat forebrain membranes For this experiment, $K_d = 10$ nM, $B_{max} = 1.6$ pmol/mg protein. Similar results were obtained in 3 independent experiments with triplicate measures at each concentration.

Table 2
Affinity of CP-101,606 and other compounds for displacement of the specific binding of racemic [^{3}H]CP-101,606 to rat forebrain membranes

Compound	K_i (nM)	Hill slope	n
CP-101,606	13 ± 4	−0.93 (−1.11, −0.73)	8
Ifenprodil	70 ± 25	−0.64 (−0.93, −0.45)	6
Eliprodil	450 ± 130	−0.90 (−1.07, −0.73)	5
Spermine	7000 ± 2000	−0.83 (−0.95, −0.73)	5
Spermidine	30000 ± 15000	−0.41 (−0.54, −0.32)	3
Putrescine	> 100000		3

The listed compounds were added at six concentrations surrounding the K_i. K_i was calculated using linear regression with the Cheng-Prusoff correction, each value is the mean ± S.E.M. The Hill slope was also calculated and is expressed as the mean with the range in parentheses. The number of experiments (n) is indicated.

much lower than CP-101,606 but similar to their potency for displacement of [^{3}H]ifenprodil binding as reported by others (Schoemaker et al., 1990). Spermine and spermidine

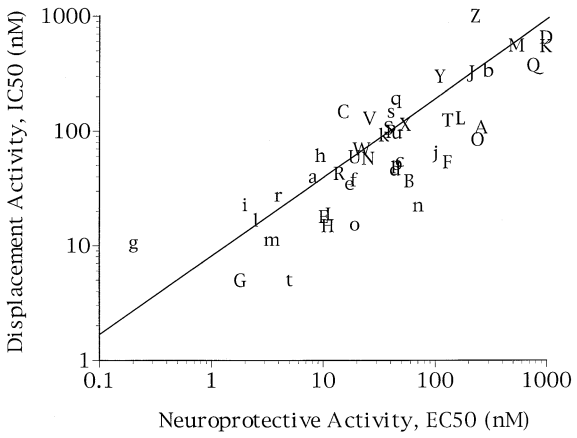

Fig. 3. Correlation between displacement activity at the racemic [^{3}H]CP-101,606 binding site and neuroprotective activity against glutamate-induced toxicity in hippocampal neurons in culture for CP-101,606 and analogs. $y = 0.69x + 0.66$, $r^2 = 0.7$. The compounds used were: **A**, 4-[2S^*-(4-benzyl-piperidin-1-yl)-1R^*-hydroxy-propyl]-phenol; **B**, 4-benzyl-1-[2R^*-hydroxy-2-(4-hydroxy-phenyl)-1R^*-methyl-ethyl]-piperidin-4-ol; **C**, 4-[1R^*-hydroxy-2R^*-(3-phenylsulfanyl-8-aza-bicyclo[3.2.1]oct-8-yl)-propyl]-phenol; **D**, 1-(4-chloro-phenyl)-2-[4-(4-fluoro-benzyl)-piperidin-1-yl]-ethanole; **E**, 1-[2R^*-hydroxy-2-(4-hydroxy-phenyl)-1R^*-methyl-ethyl]-4-phenyl-piperidin-4-ol; **F**, 3S^*-(4-hydroxy-4-phenyl-piperidin-1-yl)-chroman-4R^*,7-diol; **G**, 4-(4-methyl-phenyl)-1-[2R^*-hydroxy-2-(4-hydroxy-phenyl)-1R^*-methyl-ethyl]-piperidin-4-ol; **H**, 1-[2R-hydroxy-2-(4-hydroxy-phenyl)-1R-methyl-ethyl]-4-phenyl-piperidin-4-ol; **I**, CP-101,606; **J**, 5-[2R^*-(4-benzyl-4-hydroxy-piperidin-1-yl)-1R^*-hydroxy-propyl]-1,3-dihydro-indol-2-one; **K**, 1-[2R^*-hydroxy-2-(4-hydroxy-phenyl)-1R^*-methyl-ethyl]-4-(4-phenyl-butyl)-piperidin-4-ol; **L**, 3S^*-(4-benzyl-piperidin-1-yl)-chroman-4R^*,7-diol; **M**, 4-{1R^*-hydroxy-2R^*-[3-(hydroxy-phenyl-methyl)-piperidin-1-yl]-propyl}-phenol; **N**, 5-[1R^*-hydroxy-2R^*-(4-hydroxy-4-phenyl-piperidin-1-yl)-propyl]-1,3-dihydro-indol-2-one; **O**, 2R^*-(4-hydroxy-4-phenyl-piperidin-1-yl)-indan-1S^*,5-diol; **P**, 1-[2-hydroxy-2-(4-hydroxy-phenyl)-1-methyl-propyl]-4-phenyl-piperidin-4-ol; **Q**, 2R^*-(4-hydroxy-4-phenyl-piperidin-1-yl)-indan-1R^*,5-diol; **R**, 6-[1R^*-Hydroxy-2R^*-(4-hydroxy-4-phenyl-piperidin-1-yl)-propyl]-3,4-dihydro-1H-quinolin-2-one; **S**, 5-{2R^*-[3-(4-chloro-phenylsulfanyl)-8-aza-bicyclo[3.2.1]oct-8-yl]-1R^*-hydroxy-propyl}-1,3-dihydro-indol-2-one; **T**, 4-benzyl-1-[2-hydroxy-2-(4-hydroxy-phenyl)-1-methyl-propyl]-piperidin-4-ol; **U**, 4-{1R^*-hydroxy-2R^*-[4-(1H-indol-3-yl)-3,6-dihydro-2H-pyridin-1-yl]-propyl}-phenol; **V**, 4-{1-hydroxy-2-[4-(1H-indol-3-yl)-3,6-dihydro-2H-pyridin-1-yl]-ethyl}-phenol; **W**, 6-[1R^*-hydroxy-2R^*-(3-phenylsulfanyl-8-aza-bicyclo[3.2.1]oct-8-yl)-propyl]-3,4-dihydro-1H-quinolin-2-one; **X**, 5-[1R^*-hydroxy-2R^*-(4-hydroxy-4-phenyl-piperidin-1-yl)-propyl]-3-methyl-1,3-dihydro-indol-2-one; **Y**, 5-[2R^*-(4-benzyl-piperidin-1-yl)-1R^*-hydroxy-propyl]-1,3-dihydro-indol-2-one; **Z**, 5-[2R^*-(4-benzyl-piperidin-1-yl)-1S^*-hydroxy-propyl]-1,3-dihydro-indol-2-one; **a**, 6-[1R^*-hydroxy-2R^*-(4-hydroxy-4-p-tolyl-piperidin-1-yl)-propyl]-3,4-dihydro-1H-quinolin-2-one; **b**, 4-{2R^*-[4-(benzothiazol-2-yl-methyl-amino)-piperidin-1-yl]-1R^*-hydroxy-propyl}-phenol; **c**, 5-[1R^*-hydroxy-2R^*-(4-hydroxy-4-phenyl-piperidin-1-yl)-propyl]-1,3-dihydro-indol-2-one; **d**, 5-[1R^*-hydroxy-2R^*-(4-hydroxy-4-phenyl-piperidin-1-yl)-propyl]-1,3-dihydro-indol-2-one; **e**, 6-[2-(4-benzyl-oxy-piperidin-1-yl)-1-hydroxy-ethyl]-3,4-dihydro-1H-quinolin-2-one; **f**, 1-[2R^*-hydroxy-2-(4-hydroxy-phenyl)-1R^*-methyl-ethyl]-4-(1H-indazol-3-yl)-piperidin-4-ol; **g**, 6-[1-hydroxy-2-(4-phenoxymethyl-piperidin-1-yl)-ethyl]-3,4-dihydro-1H-quinolin-2-one; **h**, 5-[1-hydroxy-2-(4-phenoxymethyl-piperidin-1-yl)-ethyl]-1,3-dihydro-indol-2-one; **i**, 5-[1R^*-hydroxy-2R^*-(4-phenoxymethyl-piperidin-1-yl)-ethyl]-1,3-dihydro-benzoimidazol-2-one; **j**, 5-[2-(4-benzyloxy-piperidin-1-yl)-1-hydroxy-ethyl]-1,3-dihydro-benzoimidazol-2-one; **k**, 5-{1R^*-hydroxy-2R^*-[4-hydroxy-4-(1H-indazol-3-yl)-piperidin-1-yl]-propyl}-1,3-dihydro-indol-2-one; **l**, 6-[1-hydroxy-2-(4-hydroxy-4-phenoxymethyl-piperidin-1-yl)-ethyl]-3,4-dihydro-1H-quinolin-2-one; **m**, 5-[1-hydroxy-2-(4-hydroxy-4-phenoxymethyl-piperidin-1-yl)-ethyl]-1,3-dihydro-benzoimidazol-2-one; **n**, 5-[1-hydroxy-2-(4-hydroxy-4-phenoxymethyl-piperidin-1-yl)-ethyl]-1,3-dihydro-indol-2-one; **o**, 6-[1R^*-hydroxy-2R^*-(4-hydroxy-4-phenoxymethyl-piperidin-1-yl)-propyl]-3,4-dihydro-1H-quinolin-2-one; **p**, 5-[1R^*-hydroxy-2R^*-(4-hydroxy-4-phenoxymethyl-piperidin-1-yl)-propyl]-1,3-dihydro-indol-2-one; **q**, 5-[1R^*-hydroxy-2R^*-(3-phenylsulfanyl-8-aza-bicyclo[3.2.1]oct-8-yl)-propyl]-1,3-dihydro-indol-2-one; **r**, 6-[1R^*-hydroxy-2S^*-(4-hydroxy-4-phenoxymethyl-piperidin-1-yl)-propyl]-3,4-dihydro-1H-quinolin-2-one; **s**, 5-[1R^*-hydroxy-2S^*-(3-phenylsulfanyl-8-aza-bicyclo[3.2.1]oct-8-yl)-propyl]-1,3-dihydro-indol-2-one; **t**, 5-[1R^*-hydroxy-2R^*-(4-hydroxy-4-phenoxymethyl-piperidin-1-yl)-propyl]-1,3-dihydro-benzoimidazol-2-one; **u**, 7-fluoro-5-[1R^*-hydroxy-2R^*-(4-hydroxy-4-phenyl-piperidin-1-yl)-propyl]-1,3-dihydro-indol-2-one.

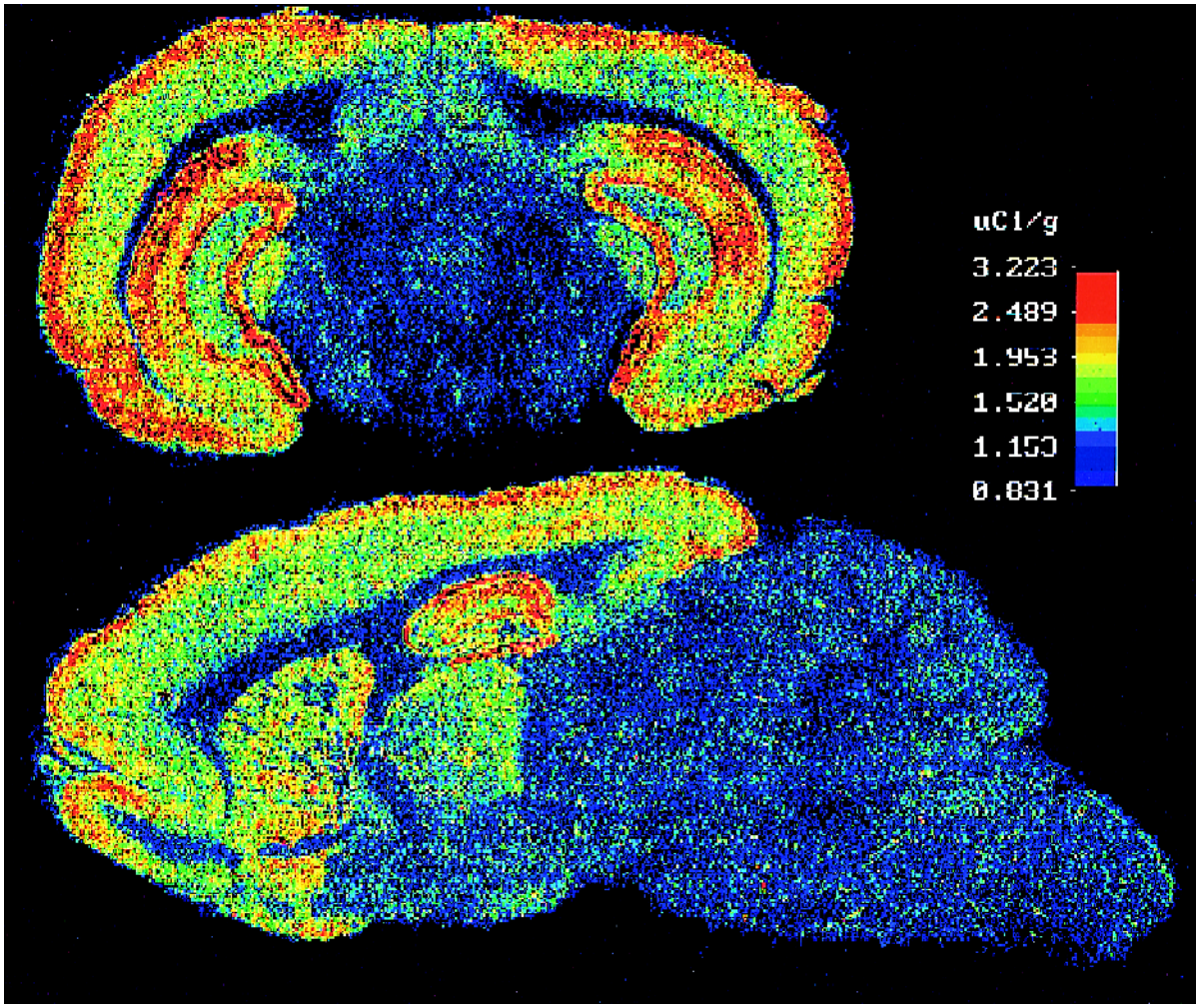

Fig. 4. The distribution of binding sites for racemic [^{3}H]CP-101,606 in coronal and parasagittal sections of rat brain. Sections, taken at 1.90 mm lateral to the midline (bottom) or 5.60 mm posterior to bregma (top), were processed as described in Section 2. Color scale indicates highest (top) to lowest (bottom) level of specific binding (see Table 3).

also displaced racemic [^{3}H]CP-101,606 binding with potencies similar to those reported for displacement of [^{3}H]ifenprodil binding (Schoemaker et al., 1990), whereas putrescine had no activity at concentrations up to 100 μM. No other of 35 tested compounds, including ligands for other glutamate, aminergic, cholinergic, opiate, or GABAergic receptors or Ca^{2+} channels, displaced racemic [^{3}H]CP-101,606 binding at pharmacologically relevant concentrations [1].

CP-101,606 had no effect on the binding of ligands to the three well characterized sites on the NMDA receptor: the glutamate recognition site labelled with [^{3}H]CGS-19,755, the glycine recognition site labelled with [^{3}H]glycine, or the PCP noncompetitive site labelled with [^{3}H]TCP (IC_{50} values > 10 μM). CP-101,606 also failed to displace binding of ligands for a range of other central nervous system sites [2]. The exception was binding to the σ site labelled with [^{3}H](3-(3-hydroxyphenyl)*N*-(1-propyl)-piperidine (3-PPP) where the IC_{50} for CP-101,606 was 60 nM. However, the σ ligands (at 10 μM) 3-PPP, *N*-allylnormetazocine (SKF-10,047), (1*S*,4*S*)-4-(3,4-dichloro-

[1] All compounds were screened at 10 μM for inhibition of racemic [^{3}H]CP-101,606 binding: glutamate, NMDA, quisqualate, kainate, ibotinate, MK-801, PCP, Arg-636, kynurenate, 7-chlorokynurenate, sertraline, serotonin, ketanserin, ritanserin, clozapine, DPAT, norepinephrine, propranolol, phentolamine, clonidine, pindolol, pargyline, reserpine, GABA, diazepam, hemicholinium, THA, QNB, atropine, pyrilamine, naloxone, cyclozocine, 4-aminopyridine, nitrendipine.

[2] Ligand binding studies for the following ligands were performed using published protocols and a CP-101,606 concentration of 1 μM: *N*-methylscopolamine, nicotine, prazosin, *p*-aminoclonidine, dihydroalprenolol, SCH 23390, *N*-propylnorapomorphine, 8-hydroxy-DPAT, mesulergine, serotonin, ketanserine, ICS 205,930, mepyramin, tiotidine, N^6-cyclohexyladenosine, N^6-ethylcarboxamideadenosine, muscimol, flunitrazepam, naloxone, substance P, neurokinin-A, eledoisin, TCP, AMPA, kainate, desmethoxyverapamil, nitrendipine.

phenyl)-1,2,3,4-tetrahydro-*N*-methyl-1-naphthylamine (sertraline) and 1,3-di-*o*- tolylguanidine (DTG) failed to significantly displace racemic [^{3}H]CP-101,606 binding.

3.3. Correlation between neuroprotection and affinity at the racemic [^{3}H]CP-101,606 binding site

The neuroprotective potency of CP-101,606 for hippocampal neurons (IC_{50} = 11 nM) is similar to its affinity for the forebrain binding site (K_d = 10 nM). Furthermore, there is a close correlation between neuroprotective activity for hippocampal neurons and binding site affinity for a range of CP-101,606 analogues (Fig. 3).

3.4. Binding site distribution

The distribution of the binding site for racemic [^{3}H]CP-101,606 within the CNS was investigated using autoradiographic techniques. The highest densities of the CP-101,606 site were seen in the hippocampal formation and the cortex (Fig. 4 and Table 3). Within the hippocampus binding is sharply defined and most dense in CA1 stratum radiatum and the molecular layer of the dentate gyrus. In the cortex binding is concentrated in the outer layers of the neocortex. Binding is low in the cingulate/retrosplenial cortex. Moderate to high levels of binding are seen in the striatum and low levels are found in the thalamus, brainstem and cerebellum.

The restricted localization of racemic [^{3}H]CP-101,606 binding contrasts with the widespread distribution of the PCP noncompetitive antagonist binding site measured using [^{3}H]TCP (Table 3). The PCP site is found throughout the cortex, is amorphously distributed in hippocampus, is moderately high in both diencephalon and cerebellum and has a low but defined distribution throughout the brainstem. Both [^{3}H]CGS-19,755 binding to the glutamate site and [^{3}H]glycine binding to the glycine site have a distribution intermediate to that for racemic [^{3}H]CP-101,606 and [^{3}H]TCP (Table 3).

4. Discussion

The present results demonstrate that CP-101,606 is a potent neuroprotective agent against glutamate-induced, NMDA receptor mediated toxicity. The unique feature of this compound is a high degree of selectivity for forebrain neurons, indicative of a selective interaction with NMDA receptors containing an NR2B subunit as discussed below.

In the present study, brief exposure to glutamate of hippocampal and cerebellar granule neurons in culture caused significant neuron loss. In both types of cultures,

Table 3
Density of binding sites in different regions of rat brain for racemic [^{3}H]CP-101,606, [^{3}H]glycine, [^{3}H]CGS-19,755 and [^{3}H]TCP

Structure	Specific binding, % CA1			
	CP-101,606	Glycine	CGS-19,755	TCP
Central grey	2.2	26.3	23.6	61.4
Substantia nigra	11.1	19.6	14.4	147.7
Caudate nucleus	39.8	43.3	6.1	196.9
Medial geniculate	40.7	44.9	43.0	110.8
Hypothalamus	13.1	8.1	0.0	80.2
Ventral thalamus	30.2	19.8	28.6	112.1
Mesencephalon	3.4	10.7	0.0	0.0
PRN	1.8	15.4	0.0	51.7
Cerebellum	7.1	30.7	4.8	244.1
Subiculum	26.7	54.8	40.9	97.2
Dentate gyrus	80.0	70.3	79.9	110.8
Hilus	48.1	47.2	45.7	211.0
CA1	100.0	100.0	100.0	100.0
CA2	94.1	87.3	76.4	121.0
CA3	87.4	66.7	76.4	134.1
Amygdala	68.9	57.8	45.8	56.8
Entorhinal cortex	62.5	59.1	52.9	130.7
Neocortex, outer	82.2	66.2	64.1	138.1
Neocortex, inner	40.7	50.9	51.8	155.1
Cingulate cortex	32.6	60.0	39.4	147.2

Binding site densities were determined by autoradiography as described in Section 2. The nmol binding sites per g wet weight tissue were calculated from optical densities for each ligand at each brain region (after subtraction of the optical densities associated with nonspecific binding, see Section 2). For each ligand in each brain region, the specific binding is presented as a percentage of that in the CA1 region. Specific binding in CA1 (nmol/g, mean ± S.E.M.) was: racemic [^{3}H]CP-101,606, 0.91 ± 0.11; [^{3}H]glycine, 3.66 ± 0.34; [^{3}H]CGS-19,755, 0.096 ± 0.009; [^{3}H]TCP, 0.019 ± 0.002. Each data point is a mean from 4–8 slices. Coronal slices were taken at 5.60 mm posterior to bregma and sagittal slices were taken at 1.90 mm lateral to the midline (Paxinos and Watson, 1986). PRN: parvocellular reticular nucleus.

this neurotoxicity was efficaciously inhibited by the channel blocking NMDA receptor antagonist MK-801, the glutamate binding site competitive antagonist 2-amino-7-phosphonoheptanoic acid (AP-7) and the glycine site competitive antagonist 7-Cl-kynurenate. The neuroprotective potencies of these compounds were similar to that reported previously for inhibition of NMDA-induced neurotoxicity in vitro (Shalaby et al., 1992; Graham et al., 1992). These compounds were also efficacious when added after the glutamate challenge (data not shown) consistent with the hypothesis that brief stimulation of neurons in culture with exogenous glutamate causes release of endogenous NMDA receptor agonists which contribute to neurotoxicity (Choi et al., 1988). These data indicate that the glutamate-induced toxicity investigated in the present study was mediated by NMDA receptor activation.

CP-101,606 efficaciously inhibited glutamate-induced neurotoxicity in hippocampal neurons in culture with potency similar to that of MK-801. CP-101,606 was slightly more potent when added simultaneously with glutamate compared to immediately after the glutamate challenge. Receptor binding studies showed that CP-101,606 does not interact directly with the well characterized glutamate, glycine and channel binding sites of the NMDA receptor. Instead, the neuroprotective effect of CP-101,606 is hypothesized to be mediated through a distinct binding site labelled with racemic [^{3}H]CP-101,606, based on two observations. First, affinity at the racemic [^{3}H]CP-101,606 binding site for CP-101,606 and a range of CP-101,606 analogs correlates closely with neuroprotective activity in the hippocampal cultures. Second, CP-101,606 was only very weakly neuroprotective against glutamate toxicity in cerebellar granule neurons in culture, consistent with the lack of a high affinity racemic [^{3}H]CP-101,606 binding site in cerebellum detected in receptor binding and autoradiographic studies. Thus, these results indicate that CP-101,606 is a highly potent NMDA receptor antagonist which inhibits receptor activity through an allosteric site. This site is expressed by neurons from hippocampus but not from cerebellum.

The binding site for CP-101,606 appears to be closely related, if not identical, to the ifenprodil binding site on the NMDA receptor. Ifenprodil and eliprodil displace racemic [^{3}H]CP-101,606 binding to forebrain membranes with activities similar to that for displacement of [^{3}H]ifenprodil binding (under conditions where the binding of [^{3}H]ifenprodil to σ sites is excluded (Schoemaker et al., 1990; Hashimoto et al., 1994), see further discussion below). Furthermore, the polyamines spermine and spermidine, but not putrescine, displace both racemic [^{3}H]CP-101,606 (present study) and [^{3}H]ifenprodil (Schoemaker et al., 1990; Hashimoto et al., 1994) binding with similar activity. This activity of the polyamines matches closely that for potentiation of [^{3}H]TCP binding to the NMDA receptor channel (Ransom and Stec, 1988). In contrast to the activity of ifenprodil, eliprodil and the polyamines, no other of the various classes of compounds examined displaced racemic [^{3}H]CP-101,606 from its binding site. Together with the structural similarity between ifenprodil and CP-101,606, these data strongly suggest that the CP-101,606, ifenprodil and eliprodil interact with a common site on the NMDA receptor.

The interaction of ifenprodil with the NMDA receptor appears to be specific to the NR2B subunit. Williams and co-workers (Williams et al., 1993; Williams, 1995) have shown that ifenprodil is a functionally selective inhibitor of recombinant NMDA receptors containing the NR2B subunit expressed in *Xenopus* oocytes. More recently, Gallagher et al. (1996) used recombinant chimeras of NMDA receptor subunits and site directed mutagenesis to map the ifenprodil binding site to the N-terminal region of the NR2B subunit. Thus, it is reasonable to hypothesize that the binding site for CP-101,606 also resides with NMDA receptors assembled with an NR2B subunit. In support of this hypothesis, preliminary evidence indicates that CP-101,606 is significantly more efficacious for inhibition of ion flux in *Xenopus* oocytes expressing NR2B containing NMDA receptors vs. those containing the NR2A subunit (D.D. Mott, S. Zhang, M.S. Washburn, M. Fendley, R. Dingledine, W. Volberg and S.B. Sands, unpublished observations). This hypothesis is also supported by the analysis of the racemic [^{3}H]CP-101,606 binding site distribution determined using autoradiographic techniques. The highest densities of racemic [^{3}H]CP-101,606 binding sites are observed in the hippocampal region in CA1 stratum radiatum and the molecular layer of the dentate gyrus. In the cortex binding is concentrated in the outer layers of the neocortex. Moderate to high levels of binding are also seen in the striatum whereas only low levels are found in the thalamus, brainstem and cerebellum. This distribution overlaps to a striking degree to the distribution of mRNA for the NR2B subunit (Monyer et al., 1992; Kutsuwada et al., 1992; Ishii et al., 1993) and NR2B immunoreactivity (Wenzel et al., 1995), consistent with a specific interaction of CP-101,606 with the NR2B containing NMDA receptor complex.

Although CP-101,606, ifenprodil and eliprodil apparently interact at a shared site on the NMDA receptor, quantitative differences in both potency and functional selectivity were observed for these compounds. The potency of ifenprodil and eliprodil for protection of hippocampal neurons in culture from glutamate toxicity reported here is similar to that reported by others for hippocampal (Shalaby et al., 1992) and cortical (Graham et al., 1992) neurons. CP-101,606 was found to be 20- and 90-fold more potent than these compounds as a neuroprotectant against glutamate toxicity in hippocampal cultures. These potency differences are consistent with similar potency differences at the racemic [^{3}H]CP-101,606 binding site. CP-101,606 also displays greater functional selectivity for forebrain neurons compared to ifenprodil and eliprodil. CP-101,606 is over 900-fold more potent for protection of

hippocampal neurons compared to cerebellar granule neurons whereas the selectivity ratio is only 12- and 6-fold for ifenprodil and eliprodil. As noted above, the functional selectivity of CP-101,606 for forebrain neurons correlates with the distribution of binding sites which are restricted to forebrain regions. In contrast, the distribution of ifenprodil binding sites (at low temperature and in the presence of ligands to block interaction with σ sites) is reported to be considerably more widespread and parallels the distribution of sites labelled with [^{3}H]TCP (Hashimoto et al., 1994; Dana et al., 1991), a compound which nonselectively labels all NMDA receptors.

CP-101,606, ifenprodil and eliprodil also differ with regard to interactions with other molecular entities within the CNS. In contrast to ifenprodil, CP-101,606 lacks significant interaction with α_1-adrenoceptors (Chenard et al., 1995). CP-101,606 and ifenprodil differ in interaction with σ sites in the CNS. Ifenprodil displaces with high potency binding of the σ site ligands [^{3}H]3-PPP (Contreras et al., 1990; Karbon et al., 1990) and [^{3}H]DTG (Karbon et al., 1990). A variety of σ site ligands also effectively displace [^{3}H]ifenprodil binding (Benavides et al., 1992; Hashimoto and London, 1993; Hashimoto et al., 1994). CP-101,606 was found to displace [^{3}H]3-PPP binding with a potency 5-fold lower than that for displacement of racemic [^{3}H]CP-101,606 binding. However, the σ site ligands 3-PPP, sertraline, SKF-10,047 and DTG failed to displace the binding of racemic [^{3}H]CP-101,606. Furthermore, the distribution of racemic [^{3}H]CP-101,606 binding sites (determined by autoradiography at 30°C in the absence of compounds to block binding to σ sites) was clearly distinct from that reported for σ site ligands and for [^{3}H]ifenprodil under experimental conditions in which σ sites were not masked (Hashimoto et al., 1994). These data indicate that CP-101,606, in contrast to ifenprodil, fails to interact significantly with σ sites in the CNS. Finally, ifenprodil and eliprodil have been reported to inhibit voltage operated Ca^{2+} channels (Biton et al., 1994, 1995; Bath et al., 1996); under some experimental conditions the potency of these compounds against Ca^{2+} channel activity was found to be similar to that for inhibition of NMDA receptor-mediated currents (Biton et al., 1994, 1995). Preliminary results suggest that CP-101,606 at concentrations up to 10 μM does not significantly inhibit ion flux mediated by N- or P-type Ca^{2+} channels (Siok and Ganong, personal communication). However, it appears that recording conditions can significantly affect the potency of ifenprodil for inhibition of Ca^{2+} channels (Biton et al., 1995); thus, experimental parameters must be carefully considered prior to drawing conclusions regarding the activity of CP-101,606 in this regard.

In summary, the results presented here indicate that CP-101,606 is a potent, selective, noncompetitive NMDA receptor antagonist. Furthermore, several lines of evidence suggest that CP-101,606 is highly selective for NMDA receptors assembled with the NR2B subunit. As such, CP-101,606 may represent an important new tool to study the function of the NR2B-containing NMDA receptor and to probe this receptor subtype as a therapeutic target.

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