0022-3565/02/3021-163–173$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 302, No. 1
33076/988064
Printed in U.S.A.

JPET 302:163–173, 2002

A Novel *N*-Methyl-D-aspartate Receptor Open Channel Blocker with in Vivo Neuroprotectant Activity

ROSA PLANELLS-CASES, CARMINA MONTOLIU, MARC HUMET, ASIA M. FERNÁNDEZ, CAROLINA GARCÍA-MARTÍNEZ, ELVIRA VALERA, JAIME M. MERINO, ENRIQUE PÉREZ-PAYÁ, ANGEL MESSEGUER, VICENTE FELIPO, and ANTONIO FERRER-MONTIEL

Centro de Biología Molecular y Celular, Universidad Miguel Hernández, Alicante, Spain (R.P.-C., A.M.F., C.G.M., A.F.-M.); Laboratory of Neurobiology, Instituto de Investigaciones Citológicas, Fundación Valenciana de Investgaciones Biomédicas, Valencia, Spain (C.M., V.F.); Institut d'Investigacions Químiques i Ambientals de Barcelona, Consejo Superior de Investigaciones Cientificas, Barcelona, Spain (M.H., A.M.); Departamento de Bioquímica y Biología Molecular, Universidad de Extremadura, Badajoz, Spain (E.V., J.M.M.); and Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, Valencia, Spain (E.P.-P.)

Received January 16, 2002; accepted February 19, 2002 This article is available online at http://jpet.aspetjournals.org

ABSTRACT

Excitotoxicity has been implicated in the etiology of ischemic stroke, chronic neurodegenerative disorders, and very recently, in glioma growth. Thus, the development of novel neuroprotectant molecules that reduce excitotoxic brain damage is vigorously pursued. We have used an ionic current block-based cellular assay to screen a synthetic combinatorial library of trimers of *N*-alkylglycines on the *N*-methyl-D-aspartate (NMDA) receptor, a well known molecular target involved in excitotoxicity. We report the identification of a family of *N*-alkylglycines that selectively blocked the NMDA receptor. Notably, compound 3,3-diphenylpropyl-*N*-glycinamide (referred to as N20C) inhibited NMDA receptor channel activity with micromolar affinity, fast on-off blockade kinetics, and strong voltage dependence. Molecule N20C did not act as a competitive glutamate or glycine antagonist. In contrast, saturation of the blocker binding site with N20C prevented dizolcipine (MK-801) blockade of the NMDA receptor, implying that both drugs bind to the same receptor site. The *N*-alkylglycine efficiently prevented in vitro excitotoxic neurodegeneration of cerebellar and hippocampal neurons in culture. Attenuation of neuronal glutamate/NMDA-induced Ca^{2+} overload and subsequent modulation of the glutamate-nitric oxide-cGMP pathway seems to underlie N20C neuroprotection. Noteworthy, this molecule exhibited significant in vivo neuroprotectant activity against an acute, severe, excitotoxic insult. Taken together, these findings indicate that *N*-alkylglycine N20C is a novel, low molecular weight, moderate-affinity NMDA receptor open channel blocker with in vitro and in vivo neuroprotective activity, which, in due turn, may become a tolerated drug for the treatment of neurodegenerative diseases and cancer.

A recognized hallmark of excitotoxic neuronal death seems to be glutamate-mediated Ca^{2+} overload that, depending on the free intracellular Ca^{2+} concentration and the severity of the injury, leads to necrosis or apoptosis (Choi and Rothman, 1990; Lipton and Rosenberg, 1994; Garthwaite, 1995; Nicotera et al., 1997; Lee et al., 1999). Glutamate neurotoxicity has been implicated in the underlying neuronal damage found in cerebral ischemia, as well as in the pathogenesis of different neurodegenerative diseases, including amyotrophic lateral sclerosis and Huntington's, Alzheimer's, and Parkinson's diseases (Lipton and Rosenberg, 1994; Garthwaite, 1995; Chase et al., 2000; Heinzt and Zoghbi, 2000). Very recently, excessive glutamate in the brain parenchyma has been associated with brain tumor progression (Takano et al., 2001). Among the glutamate receptor family, the NMDA receptor plays a critical role in excitotoxicity due to its remarkable Ca^{2+} permeability (Garthwaite, 1995; Lee et al., 1999). Indeed, a widely held view is that prolonged activation of NMDA receptors mediates a massive influx of Ca^{2+}, leading to an imbalanced cellular homeostasis that results in cell death (Schinder et al., 1996; Kroemer et al., 1998; Lee et al., 1999). As a consequence, NMDA receptors have been considered prime therapeutic targets for the development of useful neuroprotective strategies (Bräuner-Osborne et al., 2000). Accordingly, a significant effort has been made to develop

This work was supported by grants from La Fundación La Caixa (to A.F.-M.), Fundació La Marató de TV3 (to A.M. and V.F.), the Spanish Interministerial Commission of Science (Centro de Investigación Científica y Tecnológica) and Technology and the European Commission (to A.F.-M. and E.P.-P.), and the Centro de Investigación Científica y Tecnológica (to A.F.-M. and A.M.).

ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; N20C, 3,3-diphenylpropyl-*N*-glycinamide; NMDAR, *N*-methyl-D-aspartate receptor; L-Glu, L-glutamate; BSS, basic saline solution; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; VR1, vanilloid receptor subunit 1; N20-19-7C, [(3,3-diphenylpropyl)glycyl]-[[3-(*N*,*N*-diethylamino)propyl]glycyl]-[2-(methylcarbonylamino)ethyl] glycinamide; N20-20-7C, [(3,3-diphenylpropyl)glycyl]-[(3,3-diphenylpropyl)glycyl]-[2-(methylcarbonylamino)ethyl] glycinamide; N20-19-12C, [(3,3-diphenylpropyl)glycyl]-[[3-(*N*,*N*-diethylamino)propyl]glycyl]-[3-(imidazolyl)ethyl]glycinamide.

high-affinity and selective NMDA and glycine competitive antagonists (Fischer et al., 1997; Chenard and Menniti, 1999; Bräuner-Osborne et al., 2000), as well as novel vaccine-based strategies (During et al., 2000). Although most of these molecules efficiently reduce glutamate neurotoxicity in vitro, their in vivo utility has been heavily questioned due to serious cognitive side effects at clinically effective doses (Garthwaite, 1995; Lee et al., 1999). The high receptor affinity of known NMDA receptor antagonists, along with their lack of discrimination between pathologically and physiologically acting receptors, seems to be a major shortcoming because these compounds also suppress glutamate neurotransmission. Because NMDA receptors are implicated in learning and memory, inhibition of glutamate neurotransmission may underlie the cognitive deficits provoked by high-affinity, competitive antagonists of these receptors (Lipton and Rosenberg, 1994; Lee et al., 1999). Therefore, there is a necessity to develop therapeutic strategies that target overactivated receptors but do not arrest synaptic transmission. Uncompetitive NMDA antagonists such as channel blockers are promising leads for neuroprotectant drug discovery (Lipton and Rosenberg, 1994; Ferrer-Montiel et al., 1998a; Parsons et al., 1999a,b; Le and Lipton, 2001; Tai et al., 2001). A clear advantage of this kind of compounds is that they bind preferentially to pathologically active receptors. Drugs such as dizolcipine (MK-801) and phencyclidine are nanomolar affinity open channel blockers that efficiently protect neurons but display significant side effects. Submicromolar affinity blockers such as memantine exhibit a better therapeutic profile, although it has been reported that chronic administration of this antagonist enhances neuronal death (Ikonomidou et al., 2000). Thus, the development of novel NMDA receptor open channel blockers of low molecular weight, moderate-to-low receptor affinity, and fast on/off blockade kinetics is actively pursued. These new compounds may be devoid of the adverse in vivo effects of well established, high-affinity NMDA antagonists (Parsons et al., 1999b; Le and Lipton, 2001).

We have used a channel blockade-based cellular assay to identify NMDA receptor blockers from a combinatorial library of oligo *N*-substituted glycines, also known as peptoids (García-Martínez et al., 2002). We report the identification of a family of *N*-alkylglycines that selectively block the NMDA receptor channel activity with micromolar activity in a voltage-dependent manner. Stepwise size reduction of the original trimers of *N*-alkylglycines led to the identification of compound 3,3-diphenylpropyl-*N*-glycinamide (N20C), a low molecular weight molecule that acts as an open channel blocker. The *N*-alkylglycine N20C exhibited important neuroprotection in vitro and in vivo. Because this neuroprotectant molecule blocks the NMDA receptor with moderate affinity and fast on/offset kinetics, it may minimize the psychotropic effects displayed by high-affinity antagonists of this ionotropic receptor.

Materials and Methods

Synthesis of *N*-Trialkylglycine-Based Combinatorial Mixtures and Individual Compounds. The library and individual oligo *N*-alkylglycines were prepared by simultaneous multiple solid phase synthesis as described previously (García-Martínez et al., 2002). Briefly, the mixture positions (Fig. 1, X) were incorporated by coupling a mixture of 22 selected amines with the relative ratios adjusted to yield equimolar incorporation. Briefly, starting from Rink amide resin (0.7 mEq/g; Rapp Polymere, Tübingen, Germany) the eight-step synthetic pathway involved the initial release of the Fmoc protecting group. Thereafter, the successive steps of acylation with chloroacetyl chloride followed by the corresponding amination of the chloromethyl intermediate, using the selected individual amine (Fig. 1, O) or the mixture of amines (Fig. 1, X) was conducted. The final step involved the release of the library components from the resin by treatment with a trifluoroacetic acid/CH_2Cl_2/H_2O cocktail. Analytical reverse phase-high-performance liquid chromatography, laser desorption time of flight mass spectrometry, and NMR were used to determined the purity and identity of the individual oligo *N*-alkylglycine compounds.

Synthesis and Characterization of N20C. A solution of 3,3-diphenylpropylamine (0.25 g, 1.2 mmol) in anhydrous dioxane was treated with chlorbetamide (0.11 g, 1.2 mmol) and K_2CO_3 (0.33 g, 2.4 mmol) for 24 h at 90°C. The crude reaction mixture was cooled down, filtered, and washed with ethyl acetate. The joined organic fractions were evaporated to dryness to give a residue, which was purified by column chromatography on silica gel eluting with 200:10:5 ethyl acetate/MeOH/NH_4OH to give a pale yellow oil, which solidified on standing. Recrystallization from hexane/CH_2Cl_2 afforded pure N20C in 60% yield. Compound N20C had the following properties: melting point, 103°C; IR (KBr), ν: 3000 to 3400 (NH), 1683 (CO) cm^{-1}; ^1H NMR ($CDCl_3$), δ: 7.1 to 7.3 (10H), 6.9 (NH), 66 (NH), 3.97 (t, 1H, J = 7.8 Hz), 3.11 (s, 2H), 2.51 (t, 2H, J = 6.8 Hz), 2.20 (t, 2H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$) δ: 175.1 (CO), 144.2 (Ar), 128.3 (Ar), 127.4 (Ar), 126.0 (Ar), 52.04 (C-2), 48.61 (CH), 48.14 (CH_2-NH), 35.51 ($\underline{C}H_2$-CH); GC-MS (e.i.), *m/z*: 269 (M + 1), 224 (M − 45), 165, 91.

Recombinant Rat NMDAR Channel Expression in *Xenopus* Oocytes and Channel Blockade. All the procedures have been described in detail previously (Ferrer-Montiel et al., 1998b; Ferrer-Montiel and Montal, 1999). Heteromeric NMDARs composed of rat NR1 and NR2A subunits were used. Whole-cell currents from NMDAR-injected oocytes were recorded in Ba^{2+}-Ringer solution (115 mM NaCl, 2.8 mM KCl, 1.8 mM $BaCl_2$, and 10 mM HEPES, pH 7.4) with a two-microelectrode voltage-clamp amplifier at 20°C. NMDAR channels were activated by application of 100 μM L-glutamate/10 μM glycine (L-Glu) in the absence or presence of increasing concentrations of peptoid mixtures or individual compounds at a holding potential (V_h) of −80 mV. Dose-response curves for individual peptoids were fitted to a Hill equation:

$$\frac{I}{I_{max}} = \frac{1}{1 + \left(\frac{[\text{blocker}]}{IC_{50}}\right)^{n_H}}$$

where IC_{50} denotes the channel blocker concentration that inhibits half of the response obtained in its absence (I_{max}), and n_H denotes the Hill coefficient, which is an estimate of the number of drug binding sites. I-V characteristics were recorded using a ramp protocol (Ferrer-Montiel et al., 1998b; Ferrer-Montiel and Montal, 1999). Oocytes were depolarized from −80 to 20 mV in 5 s (20 mV/s). Leak currents were measured in the absence of agonist in the external bath medium and subtracted from the ionic current recorded in the presence of the ligand. Voltage dependence of channel blockade was studied as described previously (Ferrer-Montiel et al., 1998b). Experimental data were fitted to either the Hill or Woodhull equations with a nonlinear least-squares regression algorithm using MicroCal ORIGIN version 5.0 (MicroCal Software, Amherst, MA).

Hippocampal Cultures and Excitotoxic Death of Hippocampal Neurons in Culture. Mixed hippocampal neuronal/glial cultures were prepared as described previously (Schinder et al., 1996; Ferrer-Montiel et al., 1998a). Briefly, hippocampi from E17 to E19 rat embryos were incubated at 37°C in basic saline solution (BSS) containing 137 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 0.33 mM $Na_2HPO_4 \cdot 7H_2O$, 5 mM TES pH 7.4, and 10 mM glucose with 0.25% trypsin for 15 min. Trypsin was diluted by rinsing the tissue

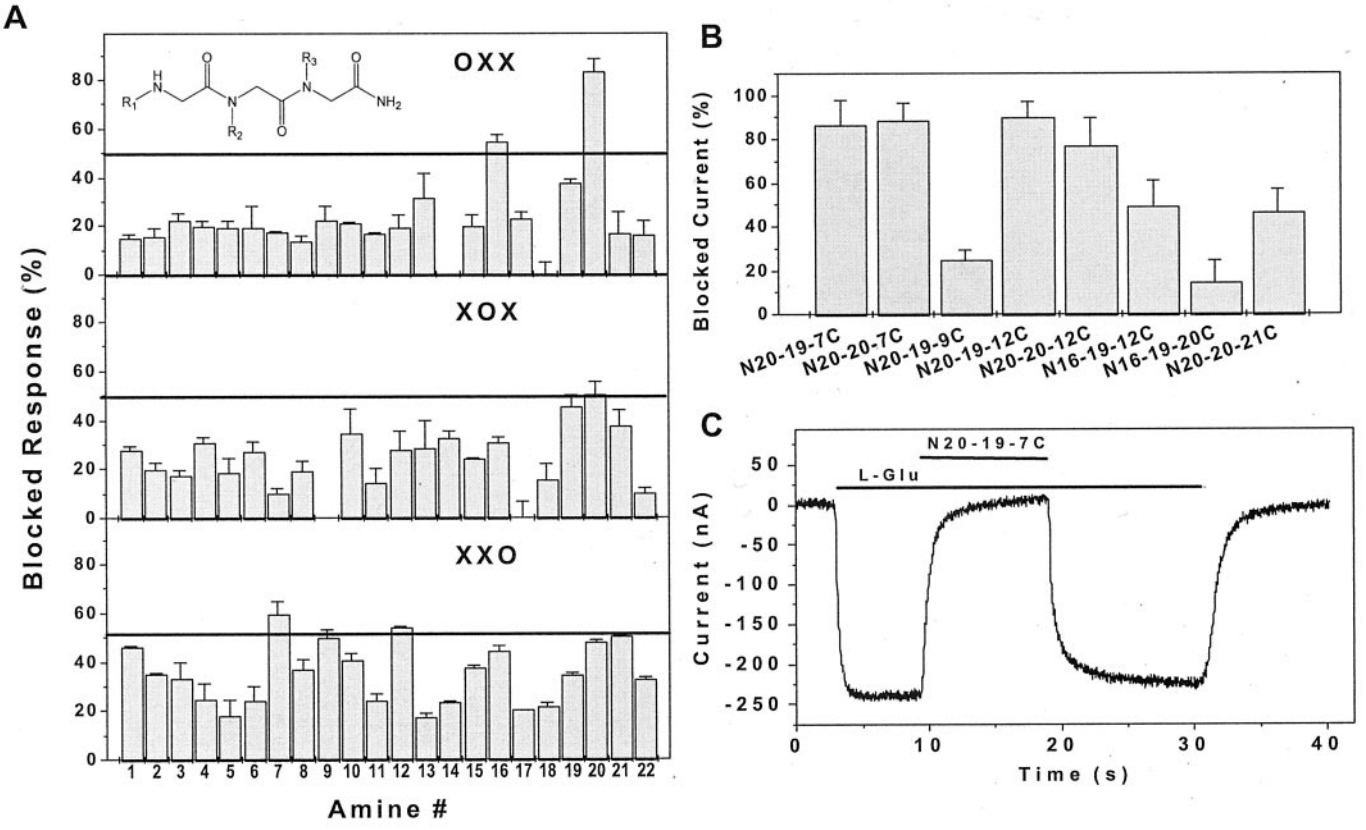


Fig. 1. Screening of an oligo *N*-substituted glycine combinatorial library to identify NMDA receptor blockers. A, NMDAR blockade profile of the 66 library mixtures. Each graph represents the blocking activity for each of the three positions that compose the library. The bars denote the activity of each mixture as a function of the number of the defined amine used to generate the chemical diversity. Library mixtures were assayed at 100 μg/ml. Horizontal bar denotes the cutoff blocking activity (set to 50%). Inset, generic formula of the *N*-trialkylglycine combinatorial library, where R_1, R_2, and R_3 denote the sites where chemical diversity was introduced. B, representative blockade activity exerted by 100 μM of selected *N*-trialkylglycines. Responses were normalized with respect to that in the absence of peptoids. C, kinetics of channel blocking activity of 100 μM *N*-trialkylglycine N20-19-7C. Downward deflection denotes inward current. NMDA receptor responses were elicited with 100 μM L-glutamate/10 μM glycine (referred to as L-Glu), at a holding potential of −80 mV. Results are given as mean ± S.E.M. for at least three oocytes.

3 × 5 min with BSS. Tissue was dissociated by several passes through a siliconized Pasteur pipette. Cells were subjected to centrifugation (5 min at 200*g*) and pellets resuspended in BSS. Cells were plated (1×10^5 viable cells/cm^2) in minimal essential medium (Earle's salts) supplemented with 10% heat-inactivated horse serum (Hyclone Laboratories, Logan, UT), 10% fetal bovine serum (Hyclone Laboratories), 1 mM glutamine, and 22 mM glucose. Cells were maintained as described previously (Ferrer-Montiel et al., 1998a).

Neurons cultured 15 to 17 days in vitro were used. Culture medium was removed and neurons rinsed with BSS, 1 mM $CaCl_2$, and 10 μM glycine (control conditions). Cultures were challenged with 200 μM NMDA in the absence and presence of drugs or peptoids for 20 min at 22°C at the indicated concentrations. The excitotoxic insult was terminated by removal of BSS medium, followed by addition of culture medium supplemented with 20 μM MK-801 or peptoids to prevent activation of NMDA receptor attributable to residual NMDA or glutamate released from synapses. Cultures were returned to the incubator and cell death was blindly assessed 18 to 24 h postinsult using the trypan blue exclusion assay. The fraction of dead cells in cultures treated with vehicle was subtracted. Neuroprotective activity is expressed as the percentage of net neuronal survival with respect to that elicited by the NMDA insult alone. A minimum of 500 cells was counted on each culture dish. Three cultures were used for each experiment.

Cerebellar Cell Cultures and Glutamatergic Cell Death. Primary cultures of cerebellar neurons were prepared using cerebella from 7- to 8-day-old Wistar rats. Neurons were grown at 37°C in 5% CO_2 atmosphere (Miñana et al., 1998). To prevent proliferation of non-neuronal cells, 10 μM cytosine arabinoside was added 24 h after plating. Glucose, 5.6 mM final, was added to the culture medium twice a week.

Glutamate toxicity in cerebellar neurons was assayed after 11 to 15 days of culture. Briefly, culture medium was removed and kept at 37°C (conditioned medium). Cells were washed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, and 5 mM HEPES pH 7.4) and incubated with 10 μM glycine for 20 min at 37°C. Upon glycine removal, neuronal cultures were incubated with 1 mM L-glutamate in the absence or presence of peptoids or drugs for 4 h at 37°C. Cells were washed with Locke's solution and the conditioned medium was added back. Cell viability was measured 24 h later, by staining with fluorescein diacetate and propidium iodide (Molecular Probes, Eugene, OR) as described previously (Marcaida et al., 1995). The percentage of surviving neurons was calculated by assessing the ratio of fluorescein diacetate/propidium iodide (green/red) staining directly under the microscope. At least 1200 cells were counted for each point.

Determination of cGMP in Cultured Cerebellar Neurons. Cerebellar neurons were used 11 to 15 days after seeding. To measure NMDA-induced cGMP formation, neuronal cultures were washed three times with prewarmed Locke's solution, and challenged with 1 mM NMDA for 5 min at 37°C, and cGMP levels were determined using the BIOTRAK cGMP enzyme immunoassay kit (Amersham Biosciences AB, Uppsala, Sweden) as described previously (Hermenegildo et al., 1998; Montoliu et al., 1999). For each experiment, samples were measured in duplicate, deviations within

samples in the same experiment was always less than 10% of the mean value.

Determination of Free Intracellular Calcium. Changes in intracellular free Ca^{2+} were monitored in single cerebellar neurons using an ACAS 570 confocal laser cytometer (Meridians Instruments, Okemos, MI) (Marcaida et al., 1995). Primary cultures of cerebellar neurons were prepared as described above using 35-mm-diameter tissue culture dishes. Cells were loaded with 20 pM Fluo-3/AM for 1 h at 37°C. Dye-loaded neurons were incubated with peptoids for 10 min, and Ca^{2+} influx was triggered with 250 pM NMDA. Each experiment was repeated at least four times using three different neuronal cultures.

Prevention of Ammonia-Induced Excitotoxicity in Mice. Acute ammonia intoxication leads to excessive activation of NMDA receptors in brain (Marcaida et al., 1995; Hermenegildo et al., 1998), which is responsible for ammonia-induced excitotoxic lethality (Hermenegildo et al., 1996). Male Swiss mice (25–35 g) were injected i.p. with 14 mmol/kg (3 μl/g) ammonium acetate. To assess the protective effect of peptoids, these were injected i.p. 10 min before ammonium injection. The population of animals surviving the acute excitotoxic insult was assessed 24 h postinsult.

Results

Screening of an *N*-Trialkylglycine-Based Combinatorial Library to Identify Novel NMDAR Channel Blockers. We previously identified that arginine-rich hexapeptides block NMDA receptors with high efficacy and, in addition, exhibit remarkable neuroprotectant activity in vitro (Ferrer-Montiel et al., 1998a). When in vivo assayed in animal models of neurodegeneration, these peptides exhibited high toxicity at therapeutically relevant doses (data not shown). To circumvent this shortcoming a focused, mixture-based combinatorial library made of trimers of *N*-alkylglycines in a positional scanning format was designed and synthesized to identify nontoxic blockers of the NMDA receptor channel activity (Fig. 1A, top, inset). The library consisted of three separate positions each having a single position defined with one of the 22 primary amines used (a total of 66 separate mixtures), and the remaining two positions had an equimolar mixture of these amines (García-Martínez et al., 2002). Each mixture contained 484 molecules, and the library chemical diversity comprised 10,648 individual trimers. The set of 22 amines included aliphatic and aromatic groups to improve the bioavailability and permeation through the blood-brain barrier of the active *N*-trialkylglycines. We also considered using primary amines bearing an additional tertiary amino group because of the positive charge preference exhibited by the NMDA receptor (Ferrer-Montiel et al., 1998a).

Peptoid mixtures were assayed for blockade activity of recombinant rat brain NMDAR channels composed of the rat NR1 and NR2A subunits heterologously expressed in amphibian oocytes. Screening of the complete library identified several mixtures that blocked ≥50% of the L-glutamate-evoked ionic current (Fig. 1A). The preferred chemical functionalities at the R_1 position were 2-[2-(*N*-methyl)pyrrolidinyl]ethyl and 3,3-diphenylpropylamine; at the R_2 position were 3-(*N,N*-diethylamino)propyl and 3,3-diphenylpropylamine; and at the R_3 position were 2-(methylcarbonylamino)ethyl, 2-(2-pyridyl)ethyl, 3-(imidazolyl)ethyl, 3,3-diphenylpropyl, and 3-(*N,N*-dimethylamino)propyl.

When used in concert, the data derived from the screening suggest the chemical identity of the bioactive *N*-trialkylglycines in the library (Ferrer-Montiel et al., 1998a; García-Martínez et al., 2002). Thus, a family of individual *N*-trialkylglycines, resulting from all possible combinations of the active functional groups identified in the deconvolution process, was synthesized. The extent of NMDA blockade activity of representative members of the *N*-trialkylglycine family is illustrated in Fig. 1B. Three compounds [(3,3-diphenylpropyl)glycyl]-[[3-(*N,N*-diethylamino)propyl]glycyl]-[2-(methylcarbonylamino) ethyl]glycinamide, [(3,3-diphenylpropyl) glycyl]-[(3,3-diphenylpropyl)glycyl]-[2-(methylcarbonylamino) ethyl] glycinamide, and [(3,3-diphenylpropyl)glycyl]-[[3-(*N,N*-diethylamino) propyl]glycyl]-[3-(imidazolyl)ethyl]glycinamide (referred to as N20-19-7C, N20-20-7C, and N20-19-12C, respectively)] inhibited NMDAR channel activity by ≥85% at 100 μM (Fig. 1B). As displayed in Fig. 1C, NMDAR blockade by these compounds seems to be rapid and readily washable, exhibiting fast k_{on} and k_{off} kinetic constants of channel blockade. Note that active *N*-trialkylglycines have a 3,3-diphenylpropylamine group (amine no. 20) at the N-end position, implying that this chemical functionality is essential for function.

To further characterize the blockade activity of these molecules, we selected the *N*-trialkylglycines N20--19-7C and N20-20-7C. The dose response of both compounds shows that N20-19-7C inhibited the NMDA receptor channel activity with an IC_{50} of 24 ± 3 μM, whereas N20-20-7C had an IC_{50} of 22 ± 2 μM (Fig. 2). The hill coefficients for N20-19-7C and N20-20-7C were 0.9 ± 0.1 (n = 6) and 1.1 ± 0.3 (n = 4), respectively. These results indicate a low blockade efficacy and the presence of a single binding site for both *N*-trialkylglycines. Analysis of current-to-voltage relationships of receptor blockade by these molecules revealed a weak voltage dependence (data not shown), suggesting that these mole-

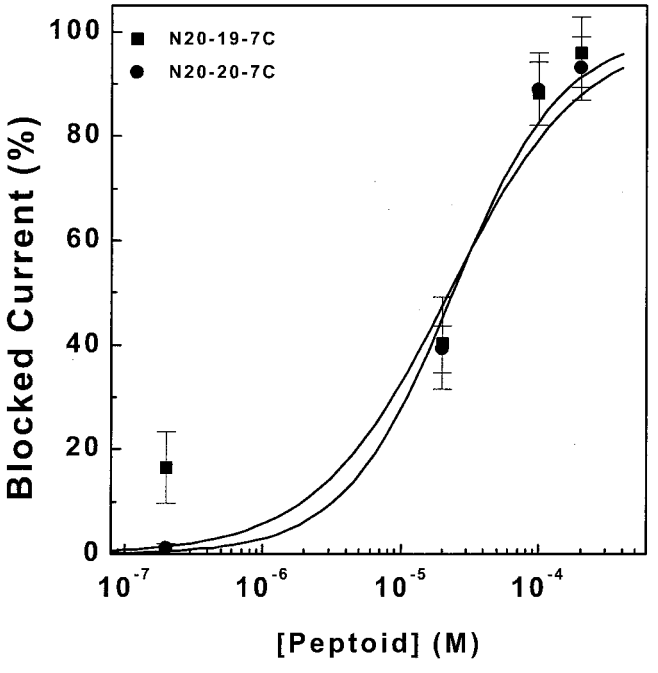


Fig. 2. Efficacy of identified *N*-trialkylglycines blocking NMDA receptor channel activity. Dose-response curves for compounds' N20-19-7C and N20-20-7C blockade activity of glutamate-activated NMDA receptor channels expressed in amphibian oocytes. Responses were normalized with respect to that in the absence of *N*-trialkylglycines. Solid lines depict the theoretical fits to a Michaelis-Menten binding isotherm. Each point represents the mean ± S.E.M., with $n \geq 4$.

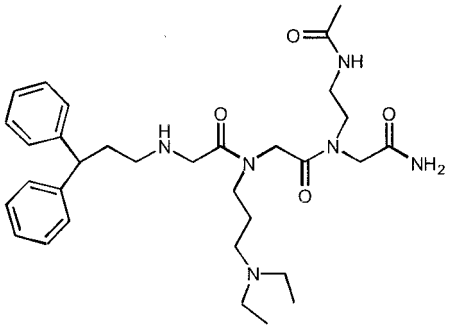
cules do not reach deep into the pore electrostatic field to reach their binding site.

An *N*-Alkylglycine Derived from Identified *N*-Trialkylglycines Inhibits NMDA Receptor Responses with Higher Efficacy and Selectivity. Because the 3,3-diphenylpropylamine group seems to play a central role in function, we reasoned that a reduction in the size of these newly identified channel blockers may provide molecules with improved blockade efficacy. Thus, we synthesized and investigated the inhibitory activity of the *N*-dialkylglycines N20-19C and N20-20C, and the *N*-alkylglycine N20C (Fig. 3). Both *N*-dialkylglycines N20-19C and N20-20C were chemically unstable, giving rise to the corresponding 1,4-diketopiperazines, which did not significantly block the NMDA receptor (20 ± 5%, $n = 3$) at concentrations as high as 50 μM (data not shown). In contrast, the *N*-alkylglycine N20C was chemically stable, and inhibited the NMDA receptor with moderate micromolar efficacy. As illustrated in Fig. 4A, agonist-elicited ionic currents from oocytes heterologously expressing the NMDA receptor were rapidly reduced by ≥80% when challenged with 50 μM of N20C. Channel unblocked was fast, as evidenced by the full recovery of agonist-operated responses after blocker removal (Fig. 4A). The dose-response relationship of N20C inhibitory activity is shown in Fig. 4B. The IC_{50} of receptor inhibition was 5.0 ± 0.2 μM ($n = 5$), which is ~4-fold lower than that exhibited by *N*-trialkylglycines. The hill coefficient was 0.8 ± 0.1, suggesting the presence of a single binding site.

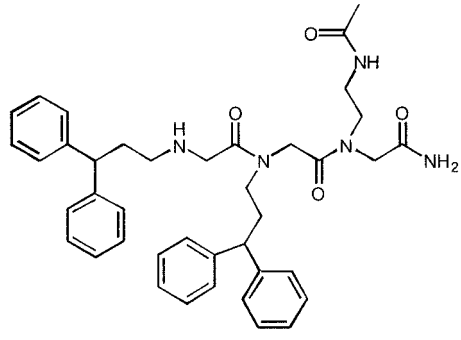
We next examined the receptor selectivity of these molecules. As illustrated in Fig. 4C, 100 μM N20-19-7C inhibited 87 ± 5% ($n = 10$) of the NMDAR channel activity, 9 ± 2% ($n = 3$) of the homomeric rat brain GluR1, and 25 ± 4% ($n = 4$) of the capsaicin-activated receptor VR1 from rat dorsal root ganglion. Notably, 50 μM *N*-alkylglycine N20C reduced the NMDA receptor activity by 92 ± 4% ($n = 10$), the GluR1-mediated ionic currents by 5 ± 2% ($n = 4$), and VR1 responses by 7 ± 2% ($n = 4$). In addition, compound N20C did not block recombinant, voltage-dependent Ca^{2+} channels nor Na^+ channels nor K^+ channels (data not shown). Taken together, these findings suggest that newly identified *N*-alkylglycines, especially N20C, selectively block the NMDA receptor.

***N*-Alkylglycine N20C Is a Noncompetitive Antagonist That Binds to Receptor Permeation Pathway.** To characterize the inhibitory activity of this compound, we studied the mechanism of channel blockade. Because N20C is a glycine derivative, we first questioned whether the *N*-alkylglycine acted as a competitive glycine antagonist. As depicted in Fig. 5A, the glycine-dependent activation of the NMDA receptor exhibited an EC_{50} of 0.8 ± 0.1 μM ($n = 5$) that was not significantly changed by the presence of 20 μM N20C in the medium (EC_{50} = 1.1 ± 0.3 μM, $n = 5$). These data imply that the *N*-alkylglycine N20C does not recognize the glycine binding site. Similarly, this compound did not act as a competitive L-glutamate antagonist (data not shown). Accordingly, N20C seems to be a noncompetitive NMDA antagonist.

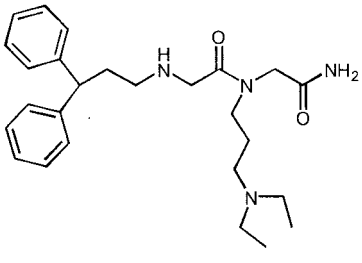
Compound N20C has a secondary amine group with a pK_a of 7.8, indicating that at neutral pH there is a population of positively charged molecules that could be sensing the pore electrostatic field. To evaluate whether the *N*-alkylglycine is a channel blocker, we studied the voltage dependence of channel blockade. Current-to-voltage relationships depict that molecule N20C inhibited glutamate/glycine-operated responses exclusively at negative membrane potentials in the range of −80 to −40 mV (Fig. 5B). The reversal potential of the ionic currents was not altered by the compound (Fig. 5B, $V_r = -7 \pm 3$ mV). These results indicate that NMDA receptor



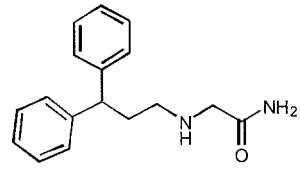
N20-19-7C



N20-20-7C



N20-19C



N20C

Fig. 3. Representative chemical structures of *N*-trialkylglycines selected and characterized in this study. Chemical structures were drawn with ChemDraw software package.

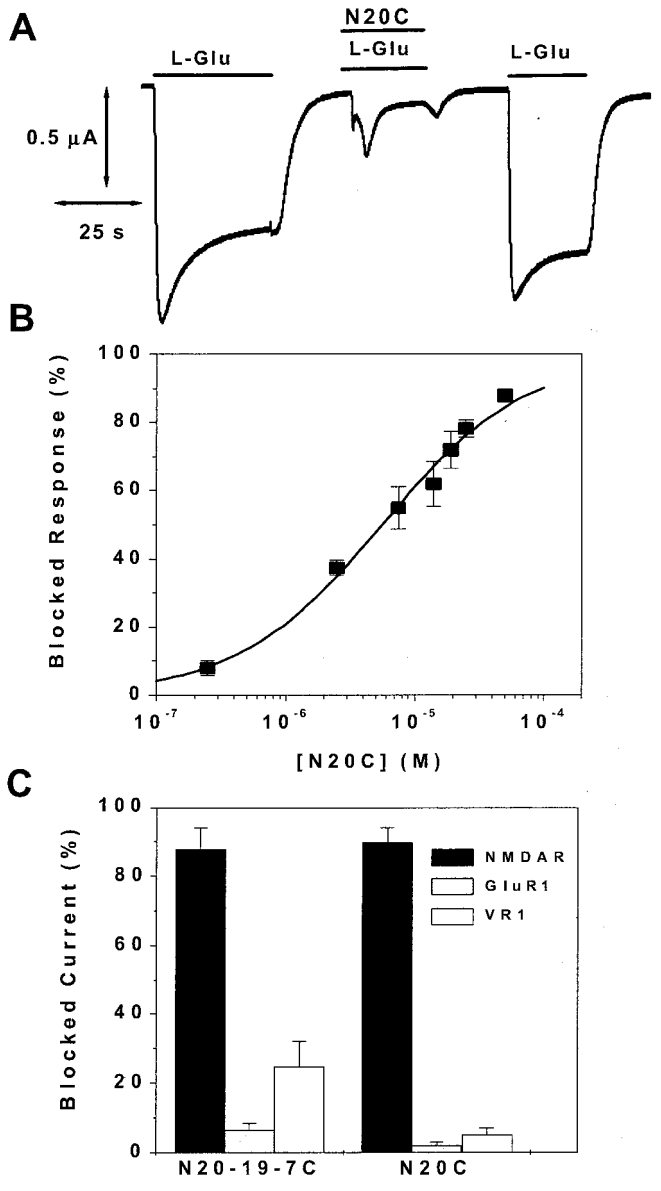


Fig. 4. *N*-Alkylglycine N20C is an efficient and selective blocker of the NMDA receptor. A, representative blockade of the NMDA receptor elicited by 25 μM *N*-alkylglycine N20C. Solid lines depict pulse length. Downward defection denotes inward currents. B, dose-response curve for compound N20C blockade activity of glutamate-activated recombinant NMDA receptor channels. Responses were normalized with respect to that in the absence of *N*-alkylglycine. Solid lines depict the theoretical fits to a Michaelis-Menten binding isotherm. Each point represents the mean ± S.E.M. with $n \geq 4$. C, blockade activity of 100 μM *N*-alkylglycine N20-19-7C and 50 μM N20C on the heteromeric NMDA receptor, the non-NMDA receptor GluR1, and the vanilloid receptor VR1. NMDA receptors were activated with 100 μM L-glutamate/10 μM glycine, GluR1 receptors with 200 μM kainate, and VR1 receptors with 10 μM capsaicin. Ionic currents were elicited at −80 mV and normalized with respect to that obtained in the absence of blockers.

blockade by N20C is voltage-dependent, and suggest that the *N*-alkylglycine binding site senses the pore electrostatic field. To further substantiate this observation, we obtained the fraction of unblocked response ($I_{blocker}/I_{control}$) as a function of the membrane potential (Fig. 5B, inset). The fraction of unblocked response-voltage relationship is related with the location of the blocker binding site within the membrane electrostatic field (Woodhull, 1973; Hille, 1992; Zarei and Dani, 1995; Premkumar and Auerbach, 1996). Experimental data exhibited a dependence on the applied membrane voltage in the range of −80 to −45 mV, consistent with a rather internal location of the drug binding site. Indeed, considering the occurrence of a single binding site within the pore electrostatic field and a negligible multiple ion occupancy of this site (Woodhull, 1973; Hille, 1992; Zarei and Dani, 1995), the inferred electrical distance of the N20C binding site from the mouth of the channel, δ, was ~0.55 (Fig. 5B, inset). In agreement with this observation, saturation of the superficial cation binding site ($\delta \leq 0.10$; Premkumar and Auerbach, 1996; Ferrer-Montiel et al., 1998a) with 10 mM extracellular Ba^{2+} did not alter the percentage of NMDA receptor inhibition by 10 μM N20C [62 ± 4% at 2 mM $[Ba^{2+}]_o$ ($n = 4$) versus 58 ± 5% at 10 mM $[Ba^{2+}]_o$ ($n = 4$)]. Together, these results imply that the drug binding site is located within the aqueous pore, and hint that N20C acts as an NMDAR open channel blocker with moderate affinity.

To further investigate the mechanism of channel blockade by compound N20C, we evaluated whether it prevented MK-801 blockade of the NMDA receptor. The rational of the experiment was based on the remarkably slow dissociation of MK-801 bound to the receptor at negative potentials (Huettner and Bean, 1988; Ferrer-Montiel et al., 1998a). As illustrated in Fig. 6, blocking activity of 500 μM N20C was readily washable (75 ± 5% response recovery, $n = 5$), whereas that of MK-801 was virtually irreversible (10 ± 3% response recovery, $n = 3$). Saturation of the N20C binding site with 500 μM *N*-alkylglycine notably prevented MK-801 interaction with the receptor as evidenced by the nearly complete recovery of the glutamate-evoked ionic current upon blocker removal (75 ± 4% response recovery) (Fig. 6B). This finding suggests that both channel blockers compete for the same binding site, thus implying that compound N20C binds deep into the channel permeation pathway.

Identified *N*-Alkylglycines Have Significant in Vitro Neuroprotectant Activity. NMDA receptor open channel blockers with low-to-moderate blockade efficacy are considered promising therapeutic leads to reduce the devastating effects of excitotoxic neuronal death (Parsons et al., 1999a). We subsequently assessed whether compound N20C displayed neuroprotectant activity. For these experiments, we evaluated the extent of protection exerted by N20C on primary cultures of cerebellar neurons exposed to 1 mM L-glutamate for 4 h. This insult gave rise to considerable neural death (≥80%) that was significantly attenuated by the presence of N20C in the extracellular medium (Fig. 7A). The dose-response relationship shows that N20C-mediated neuroprotectant activity increased as a function of the drug concentration, until a maximal 85 ± 6% ($n = 4$) neuronal survival at 30 μM (Fig. 7B). Higher concentrations of the *N*-alkylglycine were toxic to the neuronal cultures. Similar neuroprotection efficacy of 20 μM N20C was obtained when assayed on hippocampal neurons exposed to 200 pM NMDA for 20 min (Fig. 7A). The *N*-trialkylglycines N20-19-7C and N20-20-7C were also neuroprotectants against these excitotoxic insults, although they exhibited lower neuroprotective efficacy, as evidenced by the higher peptoid concentrations required (Fig. 7A). The level of neuroprotectant activity exhibited by these new compounds was comparable with that exerted by 10 μM MK-801.

Neuroprotection by *N*-alkylglycine N20C correlated well

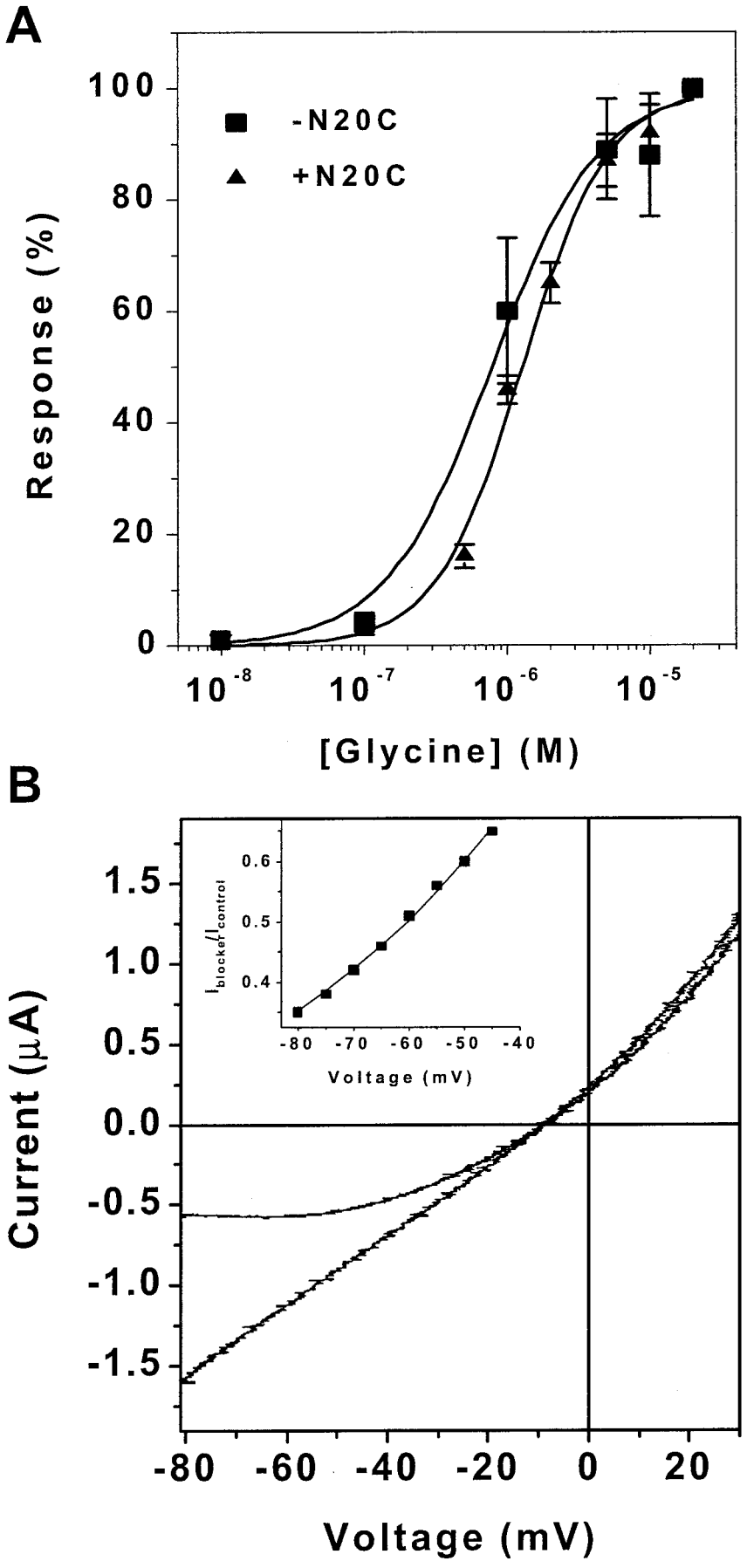


Fig. 5. *N*-Alkylglycine N20C is a noncompetitive antagonist that senses the pore electrostatic field. A, dose-response relationship for glycine-dependent activation of recombinant NMDA receptor in the absence (−N20C) and presence (+N20C) of 20 μM N20C. L-Glutamate concentration was 100 μM, and holding potential was −80 mV. B, voltage dependence of NMDA receptor block by *N*-alkylglycine N20C. Current-voltage relationship obtained with agonist in the absence (bottom trace) and presence (top trace) of 5 μM N20C. Reversal potential of ionic currents was −7 ± 3 mV. Inset, fraction of unblocked response (I/I_{max}) as a function of the membrane potential. Solid line depicts the best fit to a Woodhull-type model considering a single site and negligible multiple ion occupancy of this site. The estimated electrical distance was $\delta \sim 0.55$.

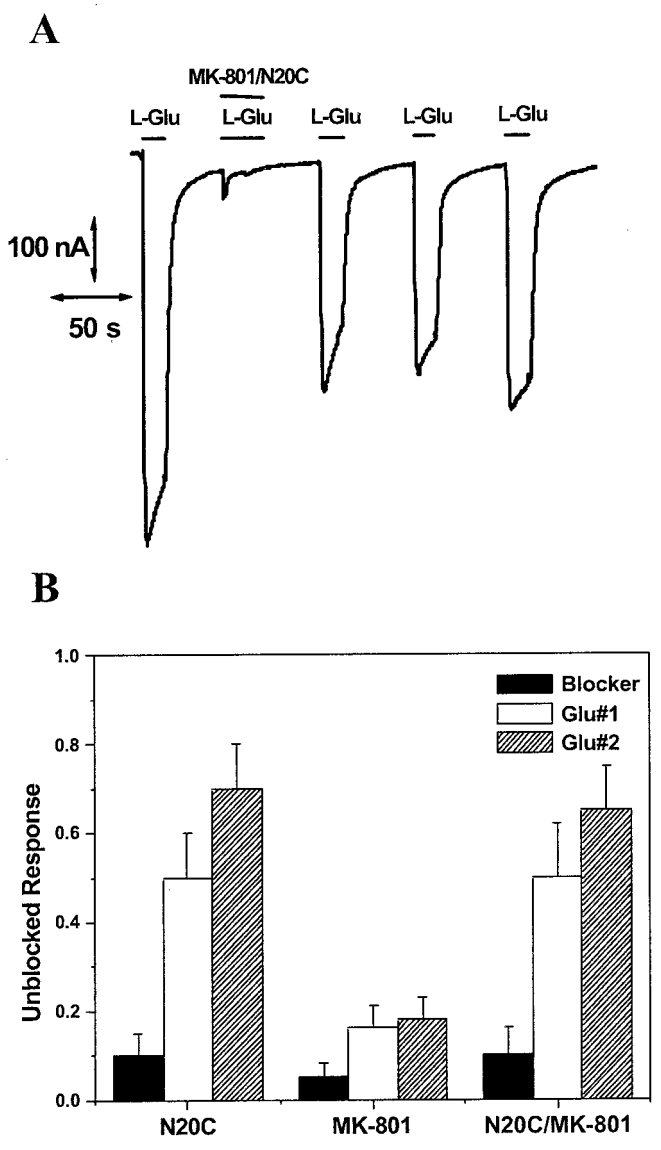


Fig. 6. Compound N20C prevents MK-801 binding to recombinant NMDA receptors. Sequentially perfused oocytes with agonist pulses in the absence and the presence of 500 μM *N*-alkylglycine N20C plus 1 μM MK-801 (A), followed by pulses of agonist alone. Pulse duration is indicated by solid lines. B, extent of channel blockade (solid columns) and recovery (stripped column) for compound N20C, MK-801, and N20C in combination with MK-801. Concentrations were as indicated in the sequential pulse protocol illustrated in A. L-Glutamate-evoked currents, at −80 mV, were normalized with respect to that obtained without blocker (first pulse in the protocol). Glu#1 and Glu#2 are the two sequential agonist pulses after blocker(s) application. Rundown current was ≤15%. Values are indicated as mean ± S.E.M., with $n \geq 3$.

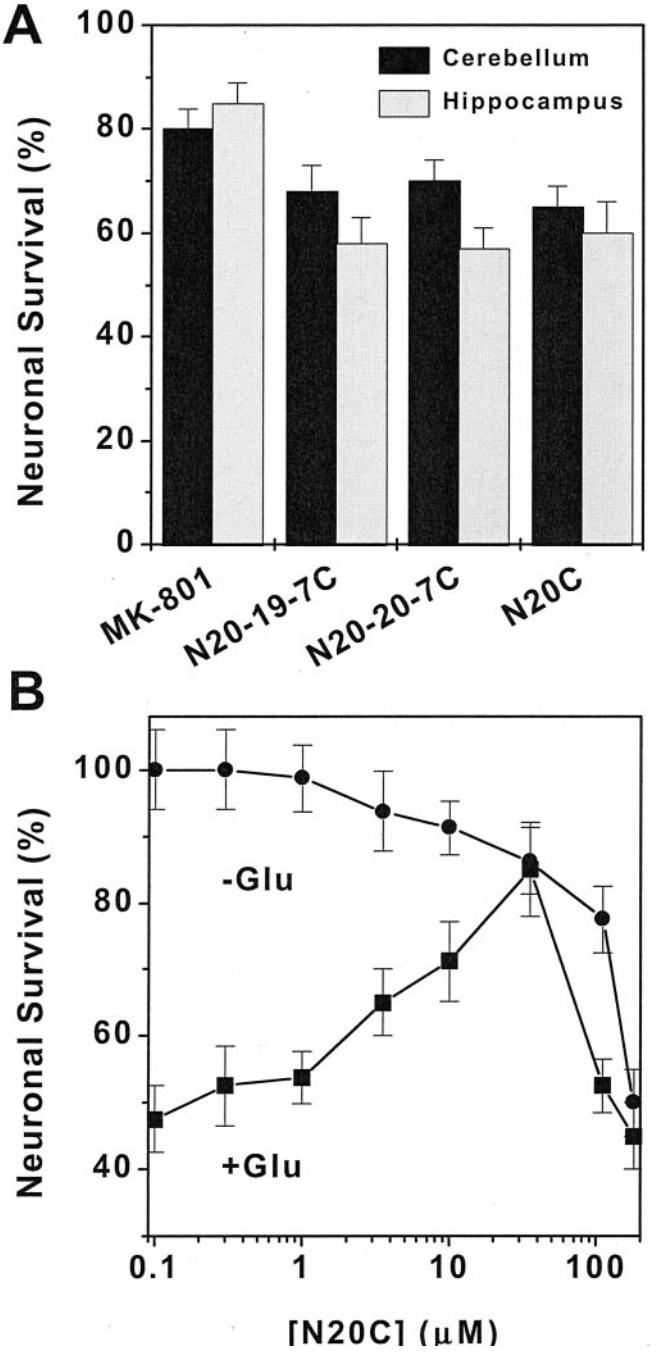


Fig. 7. Identified *N*-alkylglycines protect primary neuronal cultures from an excitotoxic insult. Normalized neuronal survival induced by L-glutamate (cerebellar neurons) or NMDA (hippocampal neurons) in the presence of blockers. Cerebellar primary neuronal cultures were challenged with 1 mM L-glutamate for 4 h. Hippocampal primary neurons were exposed to 200 μM NMDA for 20 min. Neuronal survival was evaluated 24 h after insult and normalized with respect to that obtained in the absence of channel blockers. MK-801 was assayed at 10 μM, N20C at 20 μM, and N20-19-7C and N20-20-7C at 100 μM. B, dose-response relationship of in vitro neuroprotectant activity of *N*-alkylglycine N20C. Neuronal survival was evaluated at increasing concentrations of compound N20C. Two conditions were used: −L-Glu, where no agonist or coagonist was used; and +L-Glu, where cultures were exposed to 1 mM L-glutamate for 4 h. Values are given as mean ± S.E.M., with $n \geq 1500$. Each condition was tested on a minimum of three different cultures.

with inhibition of NMDA-induced cGMP formation that results from Ca^{2+} influx through the receptor (Fig. 8A). In agreement with this finding, compound N20C significantly reduced the L-glutamate-induced intracellular Ca^{2+} increase in cerebellar neurons at concentrations that show significant neuroprotection (Fig. 8B). Thus, the *N*-alkylglycine N20C arrests L-glutamate-induced neuronal death by blocking the NMDA receptor channel activity, preventing neuronal Ca^{2+} overload, and subsequent nitric oxide and cGMP formation.

Identified *N*-Alkylglycine Exhibit in Vivo Neuroprotectant Activity. To determine the potential in vivo antineurodegenerative activity of the identified *N*-alkylglycine N20C, we next investigated whether this compound prevented the excitotoxic death characteristic of an animal model of hepatic encephalopathy, a neurological disorder

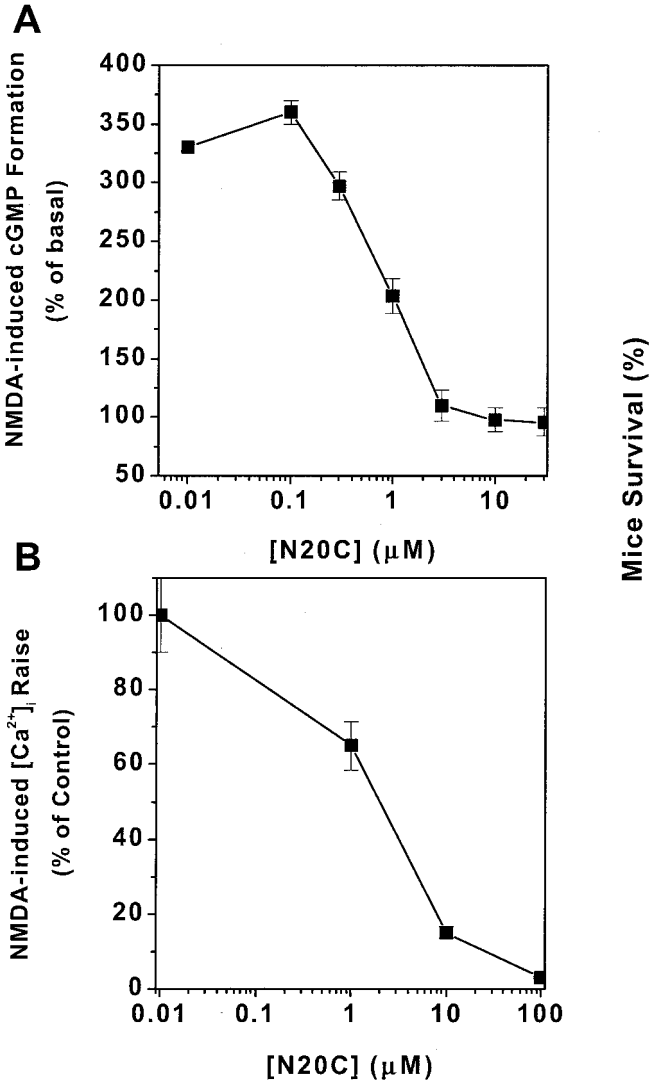


Fig. 8. Down-regulation of the glutamate-nitric oxide-cGMP pathway underlies N20C neuroprotection. A, inhibition of NMDA-induced cGMP formation by increasing concentrations of compound N20C cGMP formation was measured in cerebellar neurons as described under *Materials and Methods*. Data were normalized with respect to that of basal conditions. Data are given as mean ± S.E.M. with $n \geq 4$. B, blockade of NMDA-induced Ca^{2+} entry into cerebellar neurons as a function of the *N*-alkylglycine concentration. Neurons were loaded with Fluo-3, incubated with N20C for 10 min, and Ca^{2+} influx was triggered with 250 μM NMDA. Values are mean ± S.E.M., with $n \geq 4$.

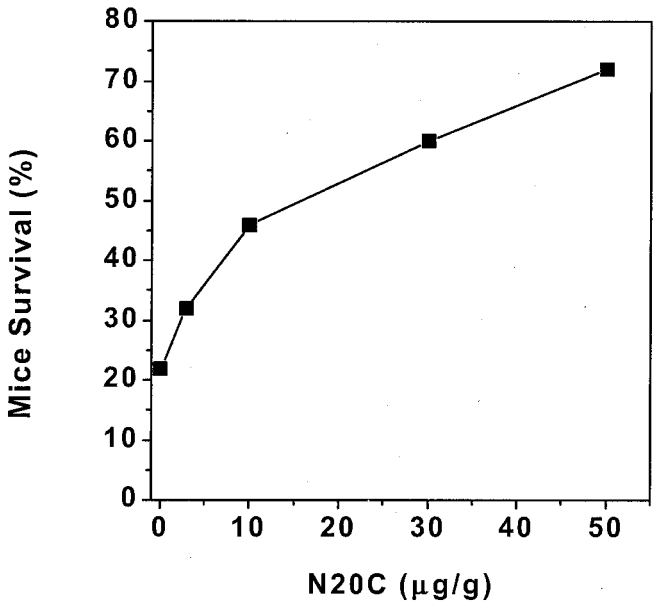


Fig. 9. *N*-Alkylglycine N20C exhibits remarkable in vivo neuroprotectant activity. Dose-response relationship of the protection exerted by compound N20C against the severity of ammonia excitotoxicity. *N*-Alkylglycine N20C was injected intraperitoneally 10 min before ammonium injection. Ammonium acetate (14 mmol/kg) was also injected intraperitoneally. Stock solutions were adjusted to reach the desired dose by injecting 3 μl/g of body weight. The number of surviving mice was counted 24 h after the acute excitotoxic insult. The number of mice used in each condition was eight.

caused by high levels of ammonia (Hermenegildo et al., 1998). Intraperitoneal administration of ammonium acetate provokes a hyperammonemic condition that triggers an acute, lethal, excitotoxic insult in mice brain. Hyperammonemia excitotoxicity is completely and specifically prevented by antagonists of the NMDA receptor, implying that activation of this ionotropic receptor significantly contributes to this form of severe neurodegeneration (Hermenegildo et al., 1996). Thus, this model of acute and intense excitotoxicity represents a reliable and reproducible assay to assess the in vivo neuroprotectant activity of newly developed molecules. As depicted in Fig. 9, intraperitoneal injection of N20C, before the acute excitotoxic insult, prevented ammonia excitotoxicity in a dose-dependent manner, as evidenced by the increasing population of unaffected mice. The minimum dose that produced a protective effect was 5 μg/g, and the maximal protection against the excitotoxic insult was obtained at 50 μg/g. Overall, the in vivo neuroprotective efficacy of N20C was higher than that depicted by parenteral *N*-trialkylglycines N20-19-7C and N20-20-7C that required doses as high as 200 μg/g to protect animals against hyperammonemia excitotoxicity (data not shown). Taken together, these findings suggest that newly identified *N*-alkylglycine N20C exhibits significant in vivo neuroprotectant activity.

Discussion

Glutamate antagonists have been shown to exhibit important neuroprotectant activity in vitro against excitotoxic insults that kill neurons, as well as in experimental models of stroke, head trauma, and tumor growth (Lee et al., 1999; Ikonomidou et al., 2000; Rzeski et al., 2001; Takano et al., 2001). The NMDA subtype of glutamate receptors is considered a central player in glutamate neurotoxicity because of its large Ca^{2+} permeability (Lee et al., 1999). Indeed, prolonged activation of this receptor overloads neurons with Ca^{2+} prompting necrotic cell death, although a contribution of apoptosis has not been ruled completely out (Garthwaite, 1995; Lee et al., 1999; Ikonomidou et al., 2000). Accordingly, NMDA receptor-specific blockers may be useful compounds to reduce excitotoxic neurodegeneration. Because of their preferential interaction with active receptors, uncompetitive NMDA antagonists are considered promising neuroprotectants to ameliorate the devastating effects of neurodegeneration. Particular emphasis is being directed toward uncompetitive NMDA antagonists acting as open channel blockers with moderate-to-low efficacy, rapid blockade kinetics, and reduced blocker trapping when the channel closes upon the

ligand is removed (Huettner and Bean, 1988; Blanpied et al., 1997; Parsons et al., 1999a). Compared with high-affinity blockers such as MK-801, these drugs cause few channels to accumulate in the trapped state. As a consequence, the population of channels blocked during synaptic activity would release the drug rather than trap it, thus resetting synapses for incoming activity. In contrast, overstimulated receptors by high and/or persistent levels of L-glutamate will be effectively tuned down by these types of drugs.

We have screened a restricted, oligo *N*-substituted, glycine-based combinatorial library to find novel antagonists of the NMDA receptor. Oligomers of *N*-substituted glycines provide a class of small, non-natural molecules that are proteolytically stable and have potent biological activities (Ostergaard and Holm, 1997; Heizmann et al., 1999). A major advantage of using short oligomers is that low molecular mass molecules (≤600 Da) usually display acceptable tissue penetration properties and better pharmacological conformities (Ostergaard and Holm, 1997; Newton, 1999; Pardridge, 1999; Lipinski et al., 2001). To identify channel blockers, the inhibitory activity of peptoid mixtures was evaluated at saturating concentrations of L-glutamate and glycine and a negative holding potential (Ferrer-Montiel et al., 1998b). The salient contribution of this work is the identification of a family of trimers of *N*-alkylglycines bearing a 3,3-diphenylpropylamino moiety that blocked the NMDA receptor channel activity. Structure-activity relationship studies on the trimers selected from the library deconvolution led to a stepwise size reduction strategy that resulted in the identification of the low molecular mass *N*-alkylglycine N20C (268.2). This compound selectively inhibited NMDA channel activity with micromolar affinity, fast on/offset kinetics, modest trapping, and strong voltage dependence ($\delta \sim 0.55$). Compound N20C did not compete with L-glutamate nor glycine. In contrast, saturating concentrations of this molecule completely prevented MK-801 blockade of the NMDA receptor. This observation, along with the finding that the N20C binding site is located deep into the pore electrostatic field, indicates that the *N*-alkylglycine interacts with the channel permeation pathway. Consequently, the *N*-alkylglycine N20C can be considered a novel open channel blocker of the NMDA receptor.

With the exception of memantine, which has shown promising results for the treatment of Alzheimer's dementia and Parkinson, the majority of channel blockers of the NMDA receptor has failed in clinical trials (Chen et al., 1998; Lee et al., 1999; Parsons et al., 1999b; Le and Lipton, 2001). Thus, there is an urgency for developing novel neuroprotectant molecules that target the NMDA receptor. Recently, an *N*-benzylated triamine was described as a highly selective and potent NMDA receptor blocker with in vitro neuroprotectant activity, although an in vivo activity was not demonstrated (Tai et al., 2001). The *N*-alkylglycine N20C also exhibited significant in vitro neuroprotection, as evidenced by the prevention of glutamate-induced neuronal death of primary neuronal cultures from hippocampus and cerebellum. The in vitro neuroprotective potency rivaled with that shown by MK-801 and memantine (Table 1). Prevention of neuronal death correlated with attenuation of sustained Ca^{2+} influx through NMDA receptors, as well as subsequent activation of downstream signaling cascades such as glutamate-nitric oxide-cGMP pathway. More significantly, the neuroprotectant activity of compound N20C was also seen in vivo in an experimental animal model of acute, severe excitotoxicity such as hepatic encephalopathy. Administration of 50 μg/g N20C protected ≥70% mice from acute hyperammonemia excitotoxicity, a condition triggered by massive and sustained activation of the NMDA receptor (Hermenegildo et al., 1996, 1998). This finding suggests that the *N*-alkylglycine N20C readily crosses the blood-brain-barrier to reach the brain, consistent with its moderate hydrophobicity (log P = 2.04) (Lipinski et al., 2001). Notably, the extent of in vivo neuroprotection of N20C is significantly higher than that characteristic of memantine (Table 1), which suggests a better therapeutic profile for the *N*-alkylglycine. It is also remarkable to note that compound N20C did not exhibit toxicity at concentrations as high as 100 μg/g, and that animals treated with the *N*-alkylglycine did not display conspicuous behavioral or motor deficits. However, further work is required to determine the therapeutic index of this new neuroprotectant. Taken together, the in vitro and in vivo beneficial properties of compound N20C emphasize its therapeutic potential for clinical use in the treatment of neurodegenerative diseases that have an excitotoxic component as well as in glioma growth. In conclusion, this novel open channel blocker of the NMDA receptor should be considered a lead compound for drug development.

TABLE 1
Comparison of functional properties of N20C with MK-801 and memantine

	IC_{50}	δ	In Vitro Neuroprotection	In Vivo Prevention Acute Excitotoxicity
	μM		%	
N20C	5.0 ± 0.3	≥0.55	65	65
MK-801	0.025 ± 0.008[a]	≥0.65	90	75[b]
Memantine	0.3 ± 0.1[c]	≥0.65[a]	70[a]	40[b]

[a] Values taken from Garcia-Martinez et al. (2002). N20C and memantine concentrations were 20 and 10 μM, respectively.
[b] Values taken from Hermenegildo et al. (1999). N20C was i.p. injected at 30 μg/g. MK-801 and memantine were i.p. administered at 2 and 30 μg/g, respectively.
[c] Values taken from Ferrer-Montiel et al. (1998b).

Acknowledgments

We are indebted to S. Nakanishi for providing rat NR1 and NR2A subunits cDNA, D. Julius for rat VR1 channel cDNA, and P. Seeburg for rat GluR1 cDNA. We thank R. Torres for technical assistance with cRNA preparation, oocyte manipulation, and injection, and C. Carreño, Wim van der Nest, M. Delgado, and J. Messeguer for the preparation of *N*-alkylglycine samples.

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Address correspondence to: Dr. Antonio Ferrer-Montiel, Centro de Biología Molecular y Celular, Universidad Miguel Hernández, Ed. Torregaitán, Avenida. Ferrocarril s/n, 03202 Elche (Alicante), Spain. E-mail: aferrer@umh.es