Identification and characterisation of novel NMDA receptor antagonists selective for NR2A- over NR2B-containing receptors

Ezio Bettini, Anna Sava, Cristiana Griffante, Corrado Carignani, Alberto Buson, Anna Maria Capelli, Michele Negri, Filippo Andreetta, Sergio A Senar-Sancho, Lorena Guiral and Francesca Cardullo

GlaxoSmithKline Medicines Research Centre, Screening and Compound Profiling (AB, EB, CC), Computational and Structural Science (AMC), Medicinal Chemistry (FC), Molecular and Cellular Biology (AS, CG, FA, MN), 37135 Verona, Italy
GlaxoSmithKline R&D Pharmaceuticals, Screening and Compound Profiling (LG, SAS-S), Tres Cantos, Spain
Running title: Identification of novel NR1/NR2A antagonists

Corresponding author: Ezio Bettini
GlaxoSmithKline Medicines Research Centre, Screening and Compound Profiling
Current address: Aptuit, via Fleming 4, 37135 Verona, Italy
Tel +390458218622, e-mail: ezio.bettini@aptuit.com

Number of text pages: 41
Number of tables: 5
Number of figures: 6
Number of references: 40
Words in Abstract: 219
Words in Introduction: 374
Words in Discussion: 1115

Abbreviations: ABD, agonist binding domain; AM, acetoxymethyl ester; CGP 39653, D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid; cLogP, calculated partition coefficient; CNS, central nervous system; CP-101,606, traxoprodil, or 1-[(1S,2S)-2-hydroxy-2-(4-hydroxyphenyl)-1-methylethyl]-4-phenyl-4-piperidinol; CRC, concentration response curve; compound 1, 3-chloro-4-fluoro-N-(4-[[2-(phenylcarbonyl)hydrazino]carbonyl]benzyl)benzenesulfonamide; compound 2, 3-chloro-N-[4-([2-[(2,5-dimethylfuran-3-yl)carbonyl]hydrazinyl]carbonyl)benzyl]-4-fluorobenzenesulfonamide; compound 3, 3-chloro-4-fluoro-N-(4-[[2-(pyridin-3-ylcarbonyl)hydrazinyl]carbonyl]benzyl)benzenesulfonamide; compound 4, 3-chloro-N-(4-[[2-(cyclohexylcarbonyl)hydrazino]carbonyl]benzyl)-4-fluorobenzenesulfonamide;
JPET #172544

compound 5, N-(4-[[2-(2-fluorobenzoyl)hydrazinyl]carbonyl]benzyl)-N,4-
dimethylbenzenesulfonamide; compound 6, 4-(((4-fluorophenyl)sulfonyl)amino)methyl)-N-
(pyridin-3-ylmethyl)benzamide; compound 7, 4-(((3-chloro-4-fluorophenyl)sulfonyl)amino)methyl)-N-(tetrahydrofuran-2-ylmethyl)benzamide; compound 8, 4-(((3-chloro-4-fluorophenyl)sulfonyl)amino)methyl)-N-(pyridin-4-ylmethyl)benzamide; compound 9, N-(4-chlorobenzyl)-4-(((4-fluorophenyl)sulfonyl)amino)methyl)benzamide; compound 10, 4-(((3-chloro-4-fluorophenyl)sulfonyl)amino)methyl)-N-(pyridin-2-ylmethyl)benzamide; compound 11, N-benzyl-4-(((4-fluorophenyl)sulfonyl)amino)methyl)-N-methylbenzamide; compound 12, 4-[benzenesulfonyl(methyl)amino]-N-(pyridin-3-ylmethyl)benzamide; compound 13, 2-[[5-(benzylamino)-1,3,4-thiadiazol-2-yl]sulfonyl]-N-(cyclohexylmethyl)acetamide; DMEM/F-12, Dulbecco’s modified Eagle medium: nutrient mixture F-12; DMSO, dimethyl sulfoxide; FLIPR, fluorometric imaging plate reader; GV196771A, sodium 4,6-dichloro-3-[(E)-(2-oxo-1-phenyl-3-pyrrolidinylidene)methyl]-1H-indole-2-carboxylate; GFP, green fluorescent protein; HBSSH, Hank’s balanced salt solution supplemented with HEPES; HEK, human embryonic kidney; HPLC, high performance liquid chromatography; HTS, high throughput screening; ifenprodil, 4-{(1S,2R)-1-hydroxy-2-[4-(phenylmethyl)-1-piperidinyl]propyl}phenol; LE, ligand efficiency; LLE, ligand lipophilicity efficiency; MDL 105,519, 3-[(E)-2-carboxy-2-phenylethenyl]-4,6-dichloro-1H-indole-2-carboxylic acid; memantine, 3,5-dimethyltricyclo[3.3.1.1^{3,7}]decan-1-amine; (+)-MK-801, dizocilpine, or (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; NMDA, N-methyl-D-aspartate; NMDAR, NMDA receptor; NTD, N-terminal domain; NVP-AAM077, ([R]-{[(1S)-1-(4-bromophenyl)ethyl]amino}(2,3-dioxo-1,2,3,4-tetrahydro-5-
quinoxalinyl)methyl]phosphoric acid; ketamine, ([RS]-2-(2-chlorophenyl)-2-(methylamino) cyclohexan-1-one; PCP, phencyclidine, or 1-(1-phenylcyclohexyl)piperidine; PEAQX, [([(1S)-1-(4-bromophenyl)ethyl]amino}(2,3-dioxo-1,2,3,4-tetrahydro-5-
quinoxalinylmethyl]phosphonic acid; perzinfotel, or EAA-090, {2-[8,9-dioxo-2,6-
diazabicyclo[5.2.0]non-1(7)-en-2-yl]ethyl}phosphonic acid; Pluronic F-127, ethane-1,2-diol:
propane-1,2-diol; Ro 25-6981, 4-{(1R,2S)-1-hydroxy-2-methyl-3-[4-(phenylmethyl)-1-
piperidiny]propyl}phenol; probenecid, 4-(dipropylsulfamoyl)benzoic acid; TCP, 1-(1-(2-
Thienyl)cyclohexyl)piperidine; TMD, transmembrane domain; U-2 OS, human
osteosarcoma.

**Recommended section:** Neuropharmacology
ABSTRACT

NR1/NR2A is a subtype of N-methyl-D-aspartate receptors (NMDARs), which are glutamate and glycine gated Ca\textsuperscript{2+} permeable channels highly expressed in the central nervous system. A high throughput screening (HTS) campaign using human osteosarcoma (U-2 OS) cells transiently transduced with NR1/NR2A NMDAR subunits, tested in a specifically designed fluorometric imaging plate reader (FLIPR)/Ca\textsuperscript{2+} assay, identified sulphonamide derivative series, exemplified by 3-chloro-4-fluoro-N-[4-[[2-(phenylcarbonyl)hydrazino]carbonyl]phenyl]methyl]benzenesulfonamide (compound 1), and thiodiazole derivative N-(cyclohexylmethyl)-2-{{5-[(phenylmethyl)amino]-1,3,4-thiadiazol-2-yl}thio}acetamide (compound 13) as novel NR1/NR2A receptor antagonists. Compounds 1 and 13 displayed submicromolar and micromolar potency at NR1/NR2A receptor, respectively, while they did not show activity at NR2B containing receptor up to 50 µM concentration. Addition of 1 mM glycine, but not 1 mM L-glutamate, was able to surmount compound 1 and 13 inhibitory effects in FLIPR NR1/NR2A assay. However, compounds 1 and 13 displaced a glutamate site antagonist [\textsuperscript{3}H]-D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid ([\textsuperscript{3}H]CGP 39653) to a greater extent than the glycine site antagonist [\textsuperscript{3}H]-3-[(E)-2-carboxy-2-phenylethenyl]-4,6-dichloro-1H-indole-2-carboxylic acid ([\textsuperscript{3}H]MDL 105,519), in rat brain cortex binding assay. Results of FLIPR cell based, electrophysiological and biochemical binding assays suggest that compounds 1 and 13 are the prototypes of novel classes of NMDAR ligands, which to the best of our knowledge are the first selective antagonists at NR1/NR2A over NR1/NR2B receptor, and might constitute useful tools able to elucidate the relative role of NR2A subunit in physiological and pathological conditions.
INTRODUCTION

N-methyl-D-aspartate receptors (NMDARs) are ligand-gated cation-selective channels that are highly expressed in the central nervous system (Köhr et al., 2006). NMDAR activity is crucial to brain functions such as circuit development (McKinney, 2010), learning and memory (Li and Tsien, 2009), and dysfunction of NMDAR activity has been implicated in a variety of neuropathological conditions including stroke, epilepsy, schizophrenia, depression, Huntington’s disease, Alzheimer’s disease, Parkinson’s disease, and multiple sclerosis (Large, 2007; Lipton, 2006; Milnerwood et al., 2010). NMDARs are unusual ligand-gated ion channels because their activation requires the relief of Mg$^{2+}$ block by membrane depolarization (Mayer et al. 1984), and the concomitant binding of two agonists: glycine (or D-serine) and L-glutamate (Johnson and Ascher, 1987; Mothet et al., 2000). The opening of NMDARs leads to an influx of cations including Ca$^{2+}$, which initiates the signal transduction cascade.

The majority of NMDARs are tetrameric complexes (Mayer, 2006), consisting of two glycine-binding NR1 subunits and two glutamate-binding NR2 subunits (Collingridge et al., 2009). NR1 is coded by a single gene, with at least 8 different splice variants; four different NR2 genes originate NR2A, NR2B, NR2C, NR2D subunits (Paoletti and Neyton, 2007). NMDARs containing different NR2 subunits have different pharmacological and kinetic properties (Vicini et al., 1998; Yuan et al., 2009).

NR2 subunits show distinct anatomical localization, providing an opportunity to target specific neuronal circuit using subunit-selective modulators. Ifenprodil has been the prototype of selective NR1/NR2B receptor antagonists (Williams, 1993), which are able to bind to the NR2B N-terminal domain (NTD), and have been used to evaluate the role of this subunit in brain function and diseases. Antagonists binding at NR2B NTD might have therapeutic potential in pain, mood disorders, and neurodegenerative diseases (Mony et al.,
2009). An involvement of NR1/NR2A receptor has been suggested in pathological conditions such as anxiety and depression (Boyce-Rustay and Holmes, 2006). Despite the importance of NMDA receptors in many neurological disorders, antagonists that are more than 10-fold selective for NR2A, NR2C, or NR2D have not yet been identified, hampering a deeper understanding of their physiological role in brain functions. To address the need for new pharmacological tools, a programme was established which included a high throughput screening (HTS) campaign on about 2 millions compounds aimed at the identification of NR2A selective compounds.
METHODS

Compound sources

The compounds used in this study were as follows: compounds 1-5, 7, 8, 10 and 13, were purchased from Enamine (Kiev, Ukraine); compounds 6, 9 and 11 were purchased from ChemDiv (San Diego, CA); compound 12 was purchased from Asinex (Moscow, Russia). NVP-AAM077 was obtained as the corresponding diammonium salt by HPLC separation of diasteroisomeric mixture PEAQX tetrasodium hydrate (Sigma-Aldrich, Milano, Italy). Memantine hydrochloride, (+)-MK-801 hydrogen maleate, PCP hydrochloride, and Ro 25-6981 maleate were purchased from Sigma-Aldrich. All other chemicals were of analytical grade and obtained from commercial sources.

Computational work

HTS concentration response curve (CRC) hits were prioritized according to their ligand efficiency (LE) and ligand lipophilicity efficiency (LLE) indexes as well as their selectivity compared to NR1/NR2B receptor. LE formula was derived from Hopkins et al. (2004), to normalize the potency of a compound with respect to its size:

\[ \text{LE} = \frac{\text{pIC}_{50}}{\text{number of non-hydrogen atoms}}. \]

Calculated partition coefficient (cLogP), obtained with the use of Daylight4.81 software (Daylight Chemical Information Systems, Laguna Niguel, CA) was used to calculate LLE, in order to maximise the minimally acceptable lipophilicity per unit of in vitro potency, according to Leeson and Springthorpe (2007):

\[ \text{LLE} = \text{pIC}_{50} - \text{cLogP}. \]
Hit expansion was performed by fishing out derivatives from in house repositories with the use of 2D similarity searches carried out with an in house developed tool, followed by visual compound inspection.

**cDNAs coding for NMDAR subunits**

cDNAs coding for human NMDAR subunits were transiently transduced in human osteosarcoma (U-2 OS) cells (ATCC HTB-96, Teddington, UK) through BacMam vectors (Condreay et al., 1999) for fluorometric imaging plate reader (FLIPR, Molecular Devices) assays, or transfected in human embryonic kidney (HEK) 293T cells (ATCC CRL-11268) through plasmid vectors for patch clamp experiments: pcDNA3.1topo plasmids were used for NR1, NR2A and NR2B subunits, while pCMV6-XL5 plasmids (Origene, Rockville, MD) were used for NR2D subunit.

pEGFP-N1 plasmid (Clontech, Mountain View, CA), encoding a red-shifted variant of wild-type GFP optimized for brighter fluorescence and higher expression in mammalian cells, was used to select transfected cells for patch clamp experiments.

**FLIPR/Ca^{2+} assay in U-2 OS cells expressing NMDAR**

FLIPR/Ca^{2+} experiments on recombinant receptors were conducted using U-2 OS cells, since they are efficiently transduced through BacMam vector (Condreay et al., 1999) and they adhere strongly to 384-well plates. Cryopreserved U-2 OS aliquots were thawed and suspended in DMEM/F-12 (Invitrogen, Milano, Italy), supplemented with 10% (v/v) dialyzed foetal calf serum (Invitrogen), and 0.3 mM MgCl₂. Suspended cells were added with 500 μM ketamine (Merial Italia, Milano, Italy), 3% (v/v) NR1 BacMam, 6% (v/v) NR2A (or NR2B) BacMam, and seeded in black-walled clear-base 384-well plates (Greiner Bio-One,
Frickenhausen, Germany) at a density of 18,000 cells/well in culture medium and maintained overnight (5% CO₂ at 37°C).

Cells grown overnight were directly added with the cytoplasmic calcium indicator, Fluo-4 (Invitrogen) in the acetoxylmethyl (AM) ester form (2 µM) in assay buffer at 37°C for 1 hour. Assay buffer contained 145 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 g/L D-(+)-glucose, 20 mM HEPES (pH adjusted to 7.3 with NaOH), and 2.5 mM probenecid (Sigma-Aldrich). Probenecid, an inhibitor of organic anion transport, was added to prevent intracellular Fluo-4 efflux.

After Fluo-4 AM loading, cell plates were washed twice or three times, for NR1/NR2A or NR1/NR2B receptor, respectively, with the assay buffer using the EMBLA 384 instrument (Molecular Devices, Sunnyvale, CA) to remove ketamine; 30 µl/well of assay buffer was left in each well at the end of the washing.

Cell plates were then placed into a FLIPR to monitor cell fluorescence (excitation wave length at 488 nm, emission wave length at 540 nm) 30 seconds before and till 5 minutes after 10 µl/well drug addition (Sullivan et al., 1999). Antagonist addition was able to induce a measurable decrease in fluorescence (see Supplemental data), similarly to what reported for other NMDARs (Hansen et al., 2010).

Compounds dissolved in DMSO were 50-fold diluted with assay buffer plus 0.01% Pluronic F-127 (Sigma-Aldrich) before the FLIPR/Ca²⁺ experiment. For concentration response curve experiments, serial dilution (1:3, 11 points) of compounds in DMSO was performed using a Biomek FX (Beckman-Coulter, Brea, CA). 100 mM L-glutamate and 400 mM glycine stock solutions were prepared in water and stored at -20°C, then diluted at the desired concentration in assay buffer on the experimental day. In mode of action studies, a second addition of 1mM glycine or L-glutamate was done by FLIPR instrument, five minutes after compound addition, and fluorescence measured for additional five minutes.
**FLIPR/Ca\(^{2+}\) assay in rat brain cortical cells**

Cortical neuronal cultures were obtained from embryonic day 18/19 Sprague-Dawley rats (Charles River, Como, Italy). Animal manipulations were performed according to Italian law (art.7, Legislative Decree No.116, 27 January 1992), which acknowledged the European Directive 86/609/EEC, and GlaxoSmithKline policy on the care and use of laboratory animals and related codes of practice. Rat brains were dissected out and cortices were quickly isolated at 4°C in Hank’s balanced salt solution supplemented with HEPES (HBSSH buffer), containing 5 mM KCl, 0.4 mM KH\(_2\)PO\(_4\), 138 mM NaCl, 0.3 mM Na\(_2\)HPO\(_4\), 5.5 mM D-glucose, 26 mM phenol red, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES at pH 7.3). Cortical cells were treated with HBSSH buffer added with 0.1% trypsin at 37°C for 10 min. In the last 5 min of incubation, 166 µg/ml of DNAse I (Sigma-Aldrich) was added. After a single wash with HBSSH buffer containing 10% foetal bovine serum (Invitrogen) and two additional washes with HBSSH buffer, cells were mechanically dissociated by triturating with Pasteur pipettes and placed in poly-L-lysine coated 384-well plates (Greiner Bio-one, Germany) at the density of 8,000 cells/well in serum-free Neurobasal medium (Invitrogen) added with B27 supplement (Invitrogen), 500 µM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were grown at 37°C, 5% CO\(_2\). Addition of fresh medium was made at 3 and 10 days, and the cells were used for intracellular Ca\(^{2+}\) measurements at 15 days after plating. Intracellular Ca\(^{2+}\) measurements were made using a FLIPR\(^{\text{TETRA}}\) (Molecular Devices). On the assay day, cultures were washed two times with physiological salt solution (PSS) buffer (145 mM NaCl, 1 g/L glucose, 5 mM KCl, 2 mM CaCl\(_2\), 20 mM HEPES) supplemented with 2.5 mM probenecid. Then, loading solution (PSS supplemented with 2.5 mM probenecid, 0.04% Pluronic F-127 and 2 µM Fluo-4 AM) was added. After 40 min at 37 °C, cells were washed 3 times as above, and transferred
to the FLIPRTETRA. Ca\textsuperscript{2+} responses were measured with excitation at 470-495 nM and emission at 515-575 nM. Camera gain and intensity were adjusted for each plate to yield a minimum baseline fluorescence of 300 relative light units. To assess the effect of NMDAR antagonists on the NMDA-triggered Ca\textsuperscript{2+} response, cells were exposed first to different concentrations of test drugs (master plate at 4 x concentration) for 10 min, then to a sub-maximal concentration of NMDA (EC\textsubscript{80}, 10-20 µM) (master plate at 5 x concentration) for 3 min. 5 fluorescence readings were taken to measure baseline, prior to compound addition.

**FLIPR/Ca\textsuperscript{2+} data analysis**

FLIPR/Ca\textsuperscript{2+} functional responses were measured in U-2 OS experiments as fluorescence intensity at 5 minutes after drug addition, normalized to the maximal response evoked by 30 µM (+)-MK-801 NMDAR blocker on the same plate. Iterative curve fitting and parameter estimations were carried out using a four parameter logistic model in Activity Base (IDBS, Guilford, UK) environment.

In rat cortical neurons, Ca\textsuperscript{2+} responses were quantified as area under the curve analysis of the time-response data over baseline and normalized to the response evoked by EC\textsubscript{80} of NMDA. To determine antagonists potencies, concentration-response curves were generated for each compound by fitting to a four-parameter logistic equation (GraphPad Software, La Jolla, CA). The percent block of the Ca\textsuperscript{2+} response and the calculated pIC\textsubscript{50} values are expressed as mean ± S.E.M. of ≥ 3 independent experiments, each performed at least in triplicate.

**Whole cells patch clamp electrophysiology in HEK 293T cells expressing NMDAR**

Whole cells patch clamp recording was performed on HEK 293T, cells transiently transfected with human NMDAR subunits and EGFP plasmid (see above). Electrophysiology experiments were carried out using an EPC-9 amplifier (HEKA Electronik, Germany). Each
cell was held at -60 mV throughout the recording, performed at a sampling rate of 1 kHz (no filter was applied). Intracellular solution contained 140 mM KCl, 2.5 mM MgCl₂, 11 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, 5 mM Na-phosphocreatine and 0.2 mM Na-GTP, pH adjusted to 7.25 with KOH. Extracellular solution contained 150 mM NaCl, 5 mM KCl, 0.2 mM CaCl₂, 3 μM glycine, 10 mM D-glucose, 10mM HEPES, pH adjusted to 7.4 using NaOH. Whole-cell voltage clamp currents were evoked by rapidly exchanging the extracellular solution to extracellular solution containing NMDA for a period of 5 s every 60 s by means of a fast step perfusion system (RSC 160, Biologic Science Instruments, France). Data were analysed using Pulse software (HEKA electronik) for current measurements and GraphPAD Prism 5 (GraphPAD software) for statistics. In CRC experiments, data were fitted to the Hill equation with fitted parameters values given ± S.E. and where \( n \) refers to the range of repetitions for each data point. In all other cases, statistical data are expressed as means ± S.E.M., where \( n \) represents the number of cells studied.

**Single concentration binding in rat brain cortical membranes**

Selected compounds were submitted for single concentration (10 µM) displacement binding assay in rat cortical membranes to MDS Pharma Services (Taipei, Taiwan). Tritiated ligands \[^{3}H\]CGP 39653, \[^{3}H\]MDL 105,519, \[^{3}H\]TCP and \[^{3}H\]ifenprodil binding were selected for their ability to bind glutamate site, glycine site, pore and NR2B NTD of NMDAR, respectively. \[^{3}H\]CGP 39653, \[^{3}H\]MDL 105,519, \[^{3}H\]TCP and \[^{3}H\]ifenprodil were used at 2, 0.33, 4, 2 nM, respectively, and nonspecific binding was defined by 1 mM L-glutamate, 10µM MDL 105,519, 1µM (+)-MK-801, 10µM ifenprodil, respectively. Incubation buffers for \[^{3}H\]CGP 39653, \[^{3}H\]MDL 105,519, \[^{3}H\]TCP and \[^{3}H\]ifenprodil single concentration binding studies were 50 mM Tris-HCl, pH 7.4, 50 mM HEPES pH 7.7, 10 mM Tris-HCl pH 7.4, 50 mM Tris-HCl pH 7.4, respectively.
Cerebral cortices from male Sprague-Dawley rats (200-250 g, Charles River) were dissected immediately after animal decapitations. Cortices were weighed and homogenised in 15 volumes of ice-cold 5 mM Tris-HCl (pH 7.4 at 25°C). The homogenate was then centrifuged at 40,000 g for 20 min at 4°C, and the pellet was washed additional 5 times by re-suspension and centrifugation as above. The pellet was then re-suspended in 15 volumes of ice-cold 5 mM Tris-HCl containing 10 mM disodium EDTA and incubated for 20 minutes at 37°C. After 3 further re-suspension and centrifugation steps in ice-cold 5 mM Tris-HCl, the final pellet was re-suspended in 3 volumes of ice-cold 5 mM Tris-HCl buffer and aliquots were frozen at -80°C until use. Protein concentration was determined by the Bio-Rad Protein assay (Milan, Italy) using bovine serum albumine as standard. Binding of \( ^{3}H \)CGP 39653 (Sills et al., 1991) was performed in 96 deep-well plate (Whatman, Piscataway, NJ) in a final volume of 0.4 ml of 50 mM Tris-HCl (pH 7.4 at 25°C) and 1% DMSO. In displacement experiments, 10 nM \( ^{3}H \)CGP 39653 (specific activity of 40.5 Ci/mmol, PerkinElmer, Boston, MA) was incubated for 30 minutes at 25°C with 24 µg of protein in the absence or presence of competing compounds previously diluted in DMSO. Nonspecific binding was defined by 1 mM L-glutamate. In saturation experiments, increasing concentrations of \( ^{3}H \)CGP 39653 (1.5–130 nM) were incubated as above. Reactions were stopped by filtration over GF/C filterplates (Perkin Elmer) followed by three washes with cold 50 mM Tris-HCl (pH 7.4 at 25°C) using a Packard cell harvester (Perkin Elmer). Retained radioactivity was counted by a Top Counter (Perkin Elmer) after the addition of 50 µl/well of Microscint 20 (Perkin Elmer).
Radioligand binding data were analyzed with non-linear regression using GraphPad Prism software. Determination of \( K_D \) and \( B_{\text{max}} \) of \([\text{H}]\text{CGP 39653}\) was assessed by elaborating saturation experiments using one site binding (hyperbola) equation, after checking with F test that this equation fitted better compared to two-site binding equation. Competition curves for the known competitive antagonist NVP-AAM077 were statistically analyzed for a best one-or two-site competition fit. Competition curves for the other compounds for which the site of binding within the receptor was unknown were analysed by using a four parameters dose-response curve. Results are expressed as mean pIC\(_{50}\) ± S.E.M.
RESULTS

**FLIPR/Ca\(^{2+}\) assay in U-2 OS cells expressing NMDAR**

Reference NMDAR antagonists binding at different sites were profiled in NR1/NR2A and NR1/NR2B FLIPR assays, obtaining pIC\(_{50}\) values in line with literature data (table 1). The preferential NR2A antagonist NVP-AAM077, binding at the glutamate site, showed pIC\(_{50}\) values of 7.8 and 6.9 in NR1/NR2A and NR1/NR2B FLIPR assays, respectively (table 1). Glycine antagonist GV196771A (Giacobbe et al., 1998; Quartaroli et al., 1999) inhibited NR2A- with a slightly higher pIC\(_{50}\) than NR2B-containing receptors (table 1), in agreement with reported 10-fold lower affinity of the agonist glycine for NR2A- over NR2B-containing receptors (Priestley et al., 1995). NR2B NTD ligands Ro 25-6981 (Fisher et al., 1997) and CP-101,606 (traxoprodil) showed detectable potency only in NR1/NR2B FLIPR assay (table 1). Pore blocker (+)-MK-801 (dizocilpine) had similar potency in both assays, while PCP (phencyclidine) and memantine pore blockers showed slight preference for NR1/NR2B receptor combination (table 1).

A cell-based HTS campaign on about 2 million compounds, using FLIPR/Ca\(^{2+}\) methodology in transiently transduced U-2 OS cells, identified 279 compounds active with pIC\(_{50}\) ≥ 5.0 on NR1/NR2A receptor in CRC experiments.

The HTS CRC hits prioritization performed with LE and LLE indexes and selectivity profile resulted in the selection of compounds 1-3, 6, 13 (figure 1 and table 2). These compounds were selective towards NR1/NR2A receptor with respect to NR1/NR2B subtype (table 2). Albeit showing molecular weight (377-480) and polar surface area (67-104, calculated according to Ertl et al., 2000) that are high for a CNS drug, they were further characterized. Subsequently, similarity searches were carried out in order to find more potent analogues possibly maintaining good selectivity. For this purpose 127 analogues were selected by using similarity searches, then reduced to eight compounds (4, 5, 7-12) after discarding derivatives.
carrying undesirable reactive chemical functionalities like oxyme or moieties unsuitable for a CNS drug like carboxylate, as a result of visual compound inspection. Selected derivatives were tested in the FLIPR NR1/NR2A and NR1/NR2B assays for pIC$_{50}$ determination. Results of this exploration have been reported together with original HTS hits in table 2.

Selected hits completely inhibited (92-100%) human recombinant NR1/NR2A receptor in FLIPR/Ca$^{2+}$ assay. Four hits (1-4) showed pIC$_{50}$ between 6.4 and 6.8; compounds 6 and 13 exhibited pIC$_{50}$ of 5.4 in NR1/NR2A assay. All hits resulted with pIC$_{50}$ < 4.3 in FLIPR NR1/NR2B assay (table 2).

In order to clarify the inhibitory mechanism of action of five selected hits (1-3, 6, 13) in FLIPR NR1/NR2A assay, 1 mM glycine or 1mM L-glutamate were added to U-2 OS expressing NR1/NR2A receptor five minutes after compound addition. Only 1 mM glycine, but not 1 mM L-glutamate, was able to surmount the effect of each of the five compounds (figure 2 and table 3). In the same test, NVP-AAM077 inhibitory effect was surmounted by 1 mM L-glutamate, but not by 1 mM glycine, while (+)-MK-801 inhibitory effect was not surmounted by any of the two agonists (table 3).

**FLIPR/Ca$^{2+}$ assay in rat cortical cells**

To confirm functional activity at native NMDAR, measurements of intracellular Ca$^{2+}$ were performed in rat cortical cultures. NMDA-evoked Ca$^{2+}$ response was completely blocked by pore blocker memantine with a pIC$_{50}$ value of 5.4 ± 0.1 (n=5) suggesting that the NMDA-stimulated Ca$^{2+}$ influx is truly mediated by activation of NMDA receptors, while it was partially blocked (77 ± 3 %) with pIC$_{50}$ value of 7.3 ± 0.1 (n = 6) by NR1/NR2B receptor selective antagonist Ro 25-6981, indicating that the majority of functional NMDAR contained NR2B subunit (figure 3A), similarly to what already described (Fisher et al., 1997, Zhong et al., 1994). Based on the known partial antagonism of Ro 25-6981 (Fisher et al.,
1997), like of any described selective NR2B antagonist at NTD site (Paoletti and Neyton, 2007), the remaining NMDA induced Ca\(^{2+}\) response might be attributable to a mixed population of NMDAR, including both NR2A- and NR2B-containing receptors (Zhong et al., 1994). The effect of compounds 1, 6, and 13 was studied in the presence of 1 \(\mu\)M Ro 25-6981 to minimize the contribution of NR2B-containing receptors (at 1 \(\mu\)M the antagonist is expected to give about 95% of its maximal effect). Compounds 1, 6 or 13 partially inhibited the NMDA-induced intracellular Ca\(^{2+}\) response in a concentration-dependent manner (figure 3B), with pIC\(_{50}\) values of 6.4 ± 0.4 (n = 3), 5.4 ± 0.2 (n = 3) and 5.8 ± 0.3 (n = 3) and to a maximal level of 39 ± 1, 46 ± 6, and 66 ± 8%, respectively.

**Patch clamp electrophysiology in HEK 293T cells expressing NMDAR**

Whole cell patch clamp experiments were carried out with selected compounds in recombinant HEK 293T cells transiently expressing human NMDA receptor for one to three days after transfection.

As shown in figure 4, compound 1 inhibited the current induced by 30 \(\mu\)M NMDA in presence of 3 \(\mu\)M glycine in a concentration dependent manner, in HEK 293T transiently transfected with NR1/NR2A subunits. Fitting of data gave a pIC\(_{50}\) value of 7.0 ± 0.1 (n = 3-9) corresponding to an IC\(_{50}\) of 109 nM and a Hill slope of 1.4 ± 0.3 (n = 3-9). The compound at 3 \(\mu\)M blocked 95.1 ± 0.3 % (n = 3) of the NMDA current in NR1/NR2A transfected cells. Compound 1 was also tested in HEK 293T transiently transfected with NR1/NR2B or NR1/NR2D subunits: it was inactive below a concentration of 30 \(\mu\)M on the current induced by 30 \(\mu\)M NMDA in presence of 3 \(\mu\)M glycine in either NR1/NR2B or NR1/NR2D transfected cells.

**Binding studies in rat cortical membranes**
To gain an insight into their site of action, three compounds (1, 6 and 13) were selected for single concentration (10µM) displacement binding test in rat cortical membranes of [³H]CGP 39653, [³H]MDL 105,519, [³H]TCP and [³H]ifenprodil, selective ligands for glutamate site (Sills at al., 1991), glycine site, pore and NR2B NTD of NMDAR, respectively. Highest inhibition values were obtained with compounds 1 (36%) and 6 (57%) in [³H]CGP 39653 assay. Displacement values lower than 25% were obtained in all other binding assays (table 4). Therefore, more detailed [³H]CGP 39653 radioligand binding assays were performed. In saturation experiments, [³H]CGP 39653 displayed single-site, saturable binding to rat cerebral cortex membranes. The negative logarithm of the apparent dissociation constant (pKₐ) was 7.5 ± 0.03 and the calculated maximum binding capacity (B_max) was 5745 ± 373 fmol mg⁻¹ protein (n = 3). In competition experiments, the known competitive NR1/NR2A preferring antagonist NVP-AAM077 showed a biphasic displacement curve with pIC₅₀ values of 8.5 ± 0.2 and 7.3 ± 0.2. The percentage of higher affinity binding sites, most likely corresponding to NR1/NR2A receptors, was 54 ± 9 %. Compounds 1 and 6 partially inhibited [³H]CGP 39653 specific binding showing a maximal radioligand displacement of 44 ± 3 and 31 ± 3% and pIC₅₀ values of 6.5 ± 0.1 and 5.6 ± 0.1, respectively. Similarly, compound 13 inhibited only 24 ± 4% of [³H]CGP 39653 specific binding with a pIC₅₀ value of 4.8 ± 0.4 (table 5 and figure 5).

Selectivity profile of selected hits

Compounds 1, 6 and 13 showed selectivity in a panel of over 30 targets targets, including ion channels and 7-transmembrane membrane receptors.

The high degree of specificity of compounds 1, 6 and 13 was also demonstrated in several additional assays. In a variety of binding and functional tests they did not interact with ion channels (Transient Receptor Potential Vanilloid 4, human Ether-à-go-go Related Gene
Channel, hNaV1.5 sodium channel), receptors of nonpeptide (adenosine, adrenergic,
cannabinoid, dopamine, histamine, acetylcholine, serotonin) or peptide ligands (neurokinin,
opioid, vasopressin) nor with enzymes (cyclooxygenase, phosphodiesterase) or transporter
(norepinephrine, serotonin) when tested at 10μM concentration.
DISCUSSION

Novel antagonists for human recombinant NR1/NR2A receptor were discovered through a FLIPR/Ca\(^{2+}\) HTS approach (table 2), the potency for selected compounds was confirmed in electrophysiology patch clamp experiments and rat brain cortical cells FLIPR/Ca\(^{2+}\) assay (figure 3B); in addition, displacement of radioligands at different NMDAR sites was verified in rat brain cortical membranes (figure 5, table 4).

All selected hits were able to fully block (92-100%, table 2) human recombinant NR1/NR2A receptor, the more potent compound 1 displaying submicromolar potency and higher than 300-fold selectivity compared to NR1/NR2B receptor in both FLIPR/Ca\(^{2+}\) and electrophysiology patch clamp experiments (tables 2 and 4). Patch clamp experiment also showed that compound 1 is inactive at NR1/NR2D receptors at 30 µM concentration, while the effect on receptors containing NR2C subunit has not yet been tested.

Replacing the phenyl group of sulphonamide derivative 1 with different R\(_5\) groups (compounds 2-4) did not result in activity increase at NR2A FLIPR/ Ca\(^{2+}\) assay (table 2). Loss of activity was observed with the bis-methylated analogue 5. In the shorter sub-series (compounds 6-11) a drop of activity of at least 10-fold was observed, with the 3-pyridinyl derivative 6 and tetrahydro-2-furanyl derivative 7 being the most active. In particular when R\(_5\) is a pyridinyl residue (compounds 6, 8 and 10), the 3-substitution was the preferred one. Further modification (compound 12) was detrimental for activity.

This is the first evidence describing compounds with such a high degree of selectivity for NR1/NR2A compared to NR1/NR2B receptor, to the best of our knowledge. NR1/NR2A selective molecules were described in the literature, including the competitive NMDAR antagonists perzinfotel (EAA-090) or NVP-AAM077. Perzinfotel was shown to be 10 times more potent at blocking NR2A- versus NR2B- or NR2C-containing NMDARs (Sun et al., 2004). NVP-AAM077 was originally described to displace [\(^{3}\)H]CGP 39653 with nanomolar
affinity, displaying greater than 100-fold preferential blockade for NR2A- compared to NR2B-containing receptors in functional assays (Auberson et al., 2002). More detailed studies have shown that NVP-AAM077 only displays about 10-fold preference for NR2A over NR2B containing receptors (Neyton and Paoletti, 2006), and the latter is the degree of selectivity observed for NVP-AAM077 in our FLIPR/Ca\textsuperscript{2+} experimental conditions (table 1).

To confirm affinity and functional activity at native NMDARs, studies were performed using membranes and cell cultures, respectively, from rat brain cortex. Rat brain cortex and derived cells in culture are known to express both NR2A and NR2B subunits (Takay et al., 2003; Zhong et al., 1994). \[^3\text{H}]\text{CGP39653}\) binding data showed that NVP-AAM077 fully displaced \[^3\text{H}]\text{CGP 39653}\) fitting a two-site binding equation, as expected for a molecule able to interact with both NR2A- and NR2B-containing receptors (figure 5 and table 5). On the other side, compounds 1, 6 and 13 only partially displaced \[^3\text{H}]\text{CGP 39653}\) with micromolar affinities (figure 5 and table 5). Partial inhibition of \[^3\text{H}]\text{CGP 39653}\) binding, partial efficacy in rat native tissue FLIPR/Ca\textsuperscript{2+} assay (figure 3), together with the full efficacy profile at NR1/NR2A recombinant receptor (table 2), suggest that compounds 1, 6, and 13 interact selectively with a subpopulation of NMDARs, presumably the NR2A-containing receptors, at a \[^3\text{H}]\text{CGP 39653}\) binding overlapping site or at an allosteric site modulating \[^3\text{H}]\text{CGP 39653}\) binding.

However, the novel hits apparently do not bind to the classical glutamate site present in all NR2 subunits and recognised by \[^3\text{H}]\text{CGP 39653}\) (Laurie and Seeburg, 1994; Kendrick et al., 1996), given their NR1/NR2A selective profile at recombinant NMDAR, and the inability of glutamate to surmount their inhibitory effect at recombinant NR1/NR2A (table 3).

Literature reports described that glycine site antagonist 5,7-dichlorokynurenate produced partial inhibition of \[^3\text{H}]\text{CGP 39653}\) binding (Reynolds, 1994), and GV150526A competitively reversed the high affinity component of \[^3\text{H}]\text{-CGP 39653}\) binding inhibition by
glycine (Mugnaini et al., 2001). Interestingly, our FLIPR/Ca$^{2+}$ mode of action experiments showed that glycine could surmount the inhibitory effect of compounds 1-3, 6 and 13, but not of NVP-AAM077, which was surmounted by 1 mM L-glutamate instead (table 3). For the above reasons, it could be derived that the hits 1-3, 6, 13 might be glycine site antagonists; however, the tested compounds 1, 6 and 13 at 10 µM only induced 24, 13 and 14% displacement in rat native receptors, respectively, of glycine site antagonist [3H]MDL 105,519 binding (table 4). Although [3H]MDL 105,519 displacement binding assay remains to be performed with recombinant human NR1/NR2A receptor, data obtained in rat brain cortical membranes suggest that compounds 1, 6 and 13 are not acting at the glycine site, but rather at a site allosterically modulated by glycine. In addition, no glycine site antagonist has been reported in literature showing a complete selectivity for NR1/NR2A receptor over NR1/NR2B. Indeed, glycine site antagonists generally display only slight preference for NR2A containing receptors, as it is observed for GV196771A in our FLIPR/Ca$^{2+}$ assay (table1), due to the about 10-fold lower affinity for glycine of receptors containing the NR2A subunit than other NR2 subunits (Dingledine et al., 1999).

It is therefore tempting to speculate that the discovered hits bind to a region of NR1/NR2A receptor in close proximity of both glutamate and glycine binding sites. Novel hits were surmounted by glycine but not by L-glutamate in functional assay (figure 2 and table 3), and displaced the glutamate site antagonist [3H]CGP 39653 but not the glycine site antagonist [3H]MDL 105,519 in binding assays (tables 5 and 6). Therefore, these compounds might preferentially interact with or at the interface between both agonist binding domains (ABDs) of NR1/NR2A receptor, central to agonist/competitive antagonist binding and to activation gating. Glycine and glutamate binding sites are located within NR1 and NR2 subunit ABDs, respectively, and not between subunits (Mayer, 2006). Agonist binding results in closure of the ‘clam shell’ like ABD, while antagonist binding stabilizes the binding domain clamshell
in an open conformation, and mechanisms for allosteric coupling between glycine and glutamate ABDs have been proposed (Mayer, 2006). In addition, the existence of independent NR1 and NR2 subunit activating transitions has been postulated according to single channel recordings (Erreger et al., 2005), and novel hits might preferentially interact with NR1/NR2A having glycine and glutamate clam shell ABDs open and closed, respectively (figure 6).

It will be necessary to perform X-rays crystal studies on purified recombinant receptor, or part of it, to clarify site of action of discovered hits (such as compound 1, 6, or 13) and to gain an insight on structural determinants of its NR1/NR2A selectivity; binding studies on both recombinant receptors and isolated soluble ligand binding domains, site directed mutagenesis, chimeric receptor studies might also be useful in clarifying the novel hits interactions with NR2A and/or NR1 domains.

Compound 1 and other described hits may constitute useful tools or chemical starting points to investigate the role of NR1/NR2A receptor in physiological and pathological conditions.
ACKNOWLEDGMENTS

We thank: Guadalupe del Real for her work supplying cells for the HTS and all SMTech team in Tres Cantos for delivering plates to screen 2M compounds; Irene Areri, Sylvie Gehanne, Jens Klein, Carla Marchioro of GSK Analytical Chemistry for structure elucidation or HPLC separation of the described compounds; Emma Ward, Tina Sawhney and the whole GSK BioReagents team for the preparation of BacMam and plasmid reagents; Luisa Mengatto, Irene Lago from Screening and Compound Profiling department Verona for FLIPR assay development; Charles Large, Ceri Davies, Davide Quarta, Michela Bettati, Massimo Manicardi, Paolo Cavanni, Roberto Arban, Wolfgang Jarolimek for their support to the work.
REFERENCES


in extrasynaptic NMDA receptor signaling and expression contributes to phenotype onset in Huntington's disease mice. Neuron 65:178-90.


Reynolds IJ (1994) [3H]CGP 39653 binding to the agonist site of the N-methyl-D-aspartate receptor is modulated by Mg2+ and polyamines independently of the arcaine-sensitive polyamine site. *J Neurochem* 62:54-62.


FOOTNOTES

Current address for Ezio Bettini, Anna Sava, Cristiana Griffante, Corrado Carignani, Alberto Buson, Anna Maria Capelli, Michele Negri, Filippo Andreetta, and Francesca Cardullo:

Aptuit, via Fleming 4, 37135 Verona, Italy.
LEGENDS FOR FIGURES

Figure 1
Structures of selected HTS hits discovered through FLIPR/Ca^{2+} NR1/NR2A assay

Figure 2
Graph of NR1/NR2A FLIPR/Ca^{2+} mode of action experiments relative to compound 6, performed as described in table 3.

Figure 3
Representative FLIPR experiments showing inhibition of NMDA-triggered intracellular Ca^{2+} response in rat cortical cultures. In (A), cells were first added with different concentrations of the antagonists memantine or Ro 25-6981 for 10 min, then exposed to 15 µM NMDA. In (B) cells were first added with different concentrations of compound 1, 6 or 13 (in presence of 1µM Ro 25-6981) for 10 min, then exposed to 15 µM NMDA. Data are expressed as percentage of 15µM NMDA-evoked response.

Figure 4
NR1/NR2A receptor current inhibition by compound 1. (A) Representative current traces recorded from a HEK 293T cells transfected with NR1/NR2A receptor, stimulated with 30 µM NMDA (filled bars) in the continuous presence of 3 µM glycine and compound 1 (0.03, 0.3 and 3 µM, empty bars). Calibration bars: 500 pA, 1 s. (B) Concentration-response curve of the NMDA current normalized to control and plotted as a function of the concentration of compound 1. An IC_{50} of 109 nM was obtained by fitting of data to the Hill equation. Data are expressed as means ± S.E.M. (n = 3-9).
Figure 5

[3H]CGP 39653 displacement curves for compounds 1, 6, 13, and NVP-AAM077. Sample graphs of experiments performed as described in table 5.

Figure 6

Hypothetical interaction model of novel hits with NR1/NR2A receptor. Clam shell like agonist binding domains (ABD) and transmembrane domains (TMD) of one NR1 (filled figure) and one NR2A (open figure) subunit are graphically represented. The existence of four open/closed clam shell ABDs combination is postulated (states 1 to 4). Glycine (filled triangle) and L-glutamate (open triangle) stabilize the clam shell ABD of NR1 and NR2A, respectively, in closed conformation (state 4). Novel hits might interact specifically with an open/closed ABDs combination (state 2).
Table 1

Inhibitory effect (pIC$_{50}$) in NR1/NR2A and NR1/NR2B FLIPR/Ca$^{2+}$ assays for reference compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>NR1/NR2A pIC$_{50}$ mean ± S.D. (no.)</th>
<th>NR1/NR2B pIC$_{50}$ mean ± S.D. (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NVP-AAM077</td>
<td>7.8 ± 0.4 (49)</td>
<td>6.9 ± 0.4 (50)</td>
</tr>
<tr>
<td>GV196771A</td>
<td>8.0 ± 0.1 (6)</td>
<td>7.3 ± 0.2 (6)</td>
</tr>
<tr>
<td>Ro 25-6981</td>
<td>&lt; 5.3 (8)</td>
<td>7.2 ± 0.1 (8)</td>
</tr>
<tr>
<td>CP-101,606</td>
<td>&lt; 4.3 (12)</td>
<td>7.0 ± 0.4 (12)</td>
</tr>
<tr>
<td>(+)-MK-801</td>
<td>6.9 ± 0.2 (52)</td>
<td>7.1 ± 0.2 (53)</td>
</tr>
<tr>
<td>PCP</td>
<td>5.9 ± 0.4 (19)</td>
<td>6.4 ± 0.2 (19)</td>
</tr>
<tr>
<td>Memantine</td>
<td>4.8 ± 0.3 (55)</td>
<td>5.5 ± 0.4 (61)</td>
</tr>
</tbody>
</table>

Values are pIC$_{50}$ means ± S.D. from human recombinant NR1/NR2A and NR1/NR2B FLIPR/Ca$^{2+}$ assays. Number of independent experiments is indicated in parenthesis. Maximal inhibition was 95-103% for all listed compounds with measurable pIC$_{50}$, unless for Ro 25-6981 and CP-101,606, which had maximal inhibition in NR1/NR2B assay of 85% and 75%, respectively.
Table 2

Inhibitory effect (pIC$_{50}$) in NR1/NR2A and NR1/NR2B FLIR/Ca$^{2+}$ assays for compounds identified through HTS and similarity searches.

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>R$_3$</th>
<th>R$_4$</th>
<th>R$_5$</th>
<th>-X-</th>
<th>NR1/NR2A pIC$_{50}$ mean (no.)</th>
<th>NR1/NR2B pIC$_{50}$ mean (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>Phenyl</td>
<td>-N</td>
<td>6.8 (21)</td>
<td>&lt; 4.3 (21)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>2,5-Dimethyl-3-furanyl</td>
<td>-N</td>
<td>6.3 (14)</td>
<td>&lt; 4.3 (12)</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>3-Pyridinyl</td>
<td>-N</td>
<td>6.2 (13)</td>
<td>&lt; 4.3 (11)</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>Cyclohexyl</td>
<td>-N</td>
<td>6.4 (4)</td>
<td>&lt; 4.3 (6)</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>Me</td>
<td>H</td>
<td>Me</td>
<td>H</td>
<td>2-F-Phenyl</td>
<td>-N</td>
<td>&lt; 4.3 (6)</td>
<td>&lt; 4.3 (6)</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>3-Pyridinyl</td>
<td>-CH$_2$-</td>
<td>5.4 (14)</td>
<td>&lt; 4.3 (12)</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>Tetrahydro-2-furanyl</td>
<td>-CH$_2$-</td>
<td>5.5 (5)</td>
<td>&lt; 4.3 (5)</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>4-Pyridinyl</td>
<td>-CH$_2$-</td>
<td>4.9 (6)</td>
<td>&lt; 4.3 (7)</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>4-Cl-Phenyl</td>
<td>-CH$_2$-</td>
<td>&lt; 4.3 (6)</td>
<td>&lt; 4.3 (6)</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>F</td>
<td>Cl</td>
<td>H</td>
<td>H</td>
<td>2-Pyridinyl</td>
<td>-CH$_2$-</td>
<td>4.6 (3)</td>
<td>&lt; 4.3 (4)</td>
</tr>
<tr>
<td>11</td>
<td>1</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>Me</td>
<td>Phenyl</td>
<td>-CH$_2$-</td>
<td>&lt; 4.3 (4)</td>
<td>&lt; 4.3 (4)</td>
</tr>
<tr>
<td>12</td>
<td>0</td>
<td>H</td>
<td>H</td>
<td>Me</td>
<td>H</td>
<td>3-Pyridinyl</td>
<td>-CH$_2$-</td>
<td>&lt; 4.3 (4)</td>
<td>&lt; 4.3 (4)</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.4 (15)</td>
<td>&lt; 4.3 (13)</td>
</tr>
</tbody>
</table>

Values are pIC$_{50}$ means from human recombinant NR1/NR2A and NR1/NR2B FLIR/Ca$^{2+}$ assays. Standard deviations were $\leq 0.3$ for all tested compounds, unless compound 7 and 13.
which had 0.4 and 0.5, respectively, as S.D. value. Maximal inhibition was 92-100% for all listed compounds in NR1/NR2A assays. Number of independent experiments is indicated in parenthesis.
Table 3

1mM glycine, but not 1mM glutamate, ability to reverse selected HTS hits inhibitory effect in NR1/NR2A FLIPR/Ca\(^{2+}\) assays

<table>
<thead>
<tr>
<th>Compound</th>
<th>+ 1 mM Gly pIC(_{50}) mean (no.)</th>
<th>+ 1 mM Glu pIC(_{50}) mean ± S.D. (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt; 4.3 (3)</td>
<td>6.5 ± 0.04 (3)</td>
</tr>
<tr>
<td>2</td>
<td>&lt; 4.3 (3)</td>
<td>6.2 ± 0.3 (3)</td>
</tr>
<tr>
<td>3</td>
<td>&lt; 4.3 (3)</td>
<td>6.1 ± 0.1 (3)</td>
</tr>
<tr>
<td>6</td>
<td>&lt; 4.3 (3)</td>
<td>5.3 ± 0.1 (3)</td>
</tr>
<tr>
<td>13</td>
<td>&lt; 4.3 (3)</td>
<td>5.3 ± 0.2 (3)</td>
</tr>
<tr>
<td>NVP-AAM077</td>
<td>7.2 ± 0.3 (5)</td>
<td>5.1 ± 0.1 (5)</td>
</tr>
<tr>
<td>(+)-MK-801</td>
<td>7.3 ± 0.3 (4)</td>
<td>7.4 ± 0.3 (4)</td>
</tr>
</tbody>
</table>

Values are pIC\(_{50}\) mean ± S.D. from NR1/NR2A FLIPR/Ca\(^{2+}\) assay in presence of 1 mM glycine (Gly) or 1mM L-glutamate (Glu), added 5 minutes after compound addition. Number of independent experiments is indicated in parenthesis. Maximal inhibition was 92-100% for all listed compounds. The ability of 1mM glycine to surmount inhibitory effect of compounds 1-3, 6, and 13 is reflected in pIC\(_{50}\) values <4.3, measured 5 minutes after glycine addition. 1mM glycine is not able to surmount NVP-AAM077 inhibitory effect. (+)-MK-801 inhibitory effect was not surmounted by any of the two agonists.
Table 4

Displacement binding assay in rat brain cortex membranes

<table>
<thead>
<tr>
<th>Compound</th>
<th>[(^3)H]CGP 39653</th>
<th>[(^3)H]MDL 105,519</th>
<th>[(^3)H]TCP</th>
<th>[(^3)H]ifenprodil</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>36%</td>
<td>24%</td>
<td>1%</td>
<td>11%</td>
</tr>
<tr>
<td>6</td>
<td>57%</td>
<td>13%</td>
<td>7%</td>
<td>- 20%</td>
</tr>
<tr>
<td>13</td>
<td>13%</td>
<td>14%</td>
<td>8%</td>
<td>- 1%</td>
</tr>
</tbody>
</table>

Displacement binding data obtained with 10\(\mu\)M compound in rat brain cortex membranes (MDS Pharma Services) using radioligands specific for different sites of NMDA receptor: [\(^3\)H]CGP 39653, [\(^3\)H]MDL 105,519, [\(^3\)H]TCP, and [\(^3\)H]ifenprodil for glutamate site, glycine site, pore, and NR2B NTD, respectively. Results are presented as the percentage of inhibition of specific binding obtained from two determinations.
Table 5

Inhibition of [3H]CGP 39653 binding in rat brain cortex membranes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hill Slope</th>
<th>pIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>% Inhibition</th>
<th>no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.8 ± 0.1</td>
<td>6.5 ± 0.1</td>
<td>44 ± 3</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>1.0 ± 0.1</td>
<td>5.6 ± 0.1</td>
<td>31 ± 3</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>1.1 ± 0.5</td>
<td>4.8 ± 0.4</td>
<td>24 ± 4</td>
<td>4</td>
</tr>
<tr>
<td>NVP-AAM077</td>
<td>-</td>
<td>8.5 ± 0.2 (high)</td>
<td>54 ± 9 (high)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.3 ± 0.2 (low)</td>
<td>46 ± 9 (low)</td>
<td></td>
</tr>
</tbody>
</table>

Binding inhibition CRCs of the non selective NMDAR antagonist [3H]CGP 39653 were generated for selected hits. Curves were fit to a single-component Hill equation excepted for NVP-AAM077 for which a two-site binding equation fitted better (p < 0.05, F-test, GraphPad Prism software). Values represent mean ± S.E.M. of no. independent experiments performed in duplicate or triplicate.
Figure 2

FLIPR counts (%)

compound 6
compound 6 + 1mM glycine
compound 6 + 1mM glutamate
Figure 5

![Graph showing specific bound (%) of [3H]CGP 39653 against displacer (log M) for different compounds: compound 1, compound 6, compound 13, and NVP-AAM077.](image-url)
Figure 6