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Comparison of the pharmacological properties of GK11 and MK801, two N-methyl-D-aspartate receptor antagonists: towards an explanation for the lack of intrinsic neurotoxicity of GK11

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Abbreviations

ARAC, cytosine-b-D-arabinofuranoside

D-APV, D,L-amino-5-phosphovalerate

DIV, day *in vitro*

ERK, extracellular signal-regulated kinase

ExNMDAR, extrasynaptic NMDA receptor

NMDAR, NMDA receptor

PCP, phencyclidine

SCI, spinal cord injury

sEPSC, spontaneous excitatory postsynaptic current

SynNMDAR, synaptic NMDA receptor

TTX, tetrodotoxin

Abstract (200 words)

Over-stimulation of NMDARs is involved in many neurodegenerative disorders. Thus, developing safe NMDAR antagonists is of high therapeutic interest. GK11 is a high affinity uncompetitive NMDAR antagonist with low intrinsic neurotoxicity, shown to be promising for treating CNS trauma. In the present study, we investigated the molecular basis of its interaction with NMDARs and compared this with the reference molecule MK801. We show, on primary cultures of hippocampal neurons, that GK11 exhibits neuroprotection properties similar to those of MK801, but in contrast with MK801, GK11 is not toxic to neurons. Using patch-clamp techniques, we also show that on NR1a/NR2B receptors, GK11 totally blocks the NMDA-mediated currents but has a 6 fold lower IC_{50} than MK801. On NR1a/NR2A receptors, it displays similar affinity but fails to totally prevent the currents. Since NR2A is preferentially localized at synapses and NR2B at extrasynaptic sites, we investigated, using calcium imaging and patch-clamp approaches, the effects of GK11 on either synaptic or extrasynaptic NMDA mediated responses. Here we demonstrate that in contrast with MK801, GK11 better preserve the synaptic NMDA mediated currents. Our study supports that the selectivity of GK11 for NR2B containing receptors accounts contributes, at least partially, for its safer pharmacological profile.

Key Words: Excitotoxicity, NMDA antagonist, recombinant NMDA receptor, whole cell recording, Gacyclidine (GK11).

Running title: GK11 effects on N-methyl-D-aspartate receptors

INTRODUCTION

Two decades of pharmacological, physiological, and genetic studies have established that NMDA receptors (NMDARs), a subtype of ionotropic glutamate receptors, play a key role in physiological and pathological processes (Dingledine et al. 1999). Activation of NMDARs is thus required for numerous fundamental physiological processes related to synaptic plasticity including learning, memory, long-term potentiation and long-term depression (Collingridge and Bliss 1995). However, excessive activation of NMDARs can generate an uncontrolled influx of calcium and start off the excitotoxic cascade that is thought to contribute to neuronal cell injury and death in many different acute and chronic neurologic diseases including stroke, traumas, Parkinson's and Alzheimer's diseases (Doble 1995; Arundine and Tymianski 2003). Several studies based on *in vitro* and *in vivo* models of ischemic and traumatic brain injury have demonstrated that neuronal cell death is primarily mediated through NMDARs and that blocking NMDARs during or shortly after an excitotoxic event can prevent much of the neuronal death that would have otherwise occurred (Rothman and Olney 1986; Clark and Rothman 1987; Choi 1988; Olney et al. 1991). Accordingly, both competitive and non-competitive NMDAR antagonists have been investigated as potential neuroprotective agents in clinical trials for different CNS pathologies (Davies and Watkins 1982; Davies et al. 1986; Priestley et al. 1996). However, these trials did not live up to the pre-clinical expectations as they were inefficient and/or had deleterious side effects (Muir and Lees 1995; Lees 2000; Sacco et al. 2001). Indeed administration of these compounds in man classically induced cardiovascular as well as psychotropic adverse effects (Leppik and Schmidt 1988). Additionally, further pre-clinical studies demonstrated that single dose administration of NMDAR antagonists in rats leads to neuronal degeneration in the cingulate cortex (Olney et al. 1989; Olney 1990; Olney et al. 1991).

NMDARs are proposed to be tetrameric protein complexes comprised a NR1 subunit with at least one type of NR2 subunit. Different NR2 subunits confer distinct electrophysiological and pharmacological properties on the receptors and couple them with different signalling machineries involved in specific physiological and pathophysiological pathways (Monyer et

al. 1994; Cull-Candy et al. 2001; Vanhoutte and Bading 2003; Liu et al. 2004). Moreover recent experiments suggest that synaptic and extrasynaptic NMDA receptors may be enriched in respectively the NR2A and NR2B subunits (Liu et al. 2007), and present distinct roles in synaptic plasticity, coupling to intracellular pathways, and apoptotic or necrotic cell death (Rumbaugh and Vicini 1999; Tovar and Westbrook 1999; Hardingham and Bading 2002; Hardingham et al. 2002; Sinagra et al. 2005; Ivanov et al. 2006). Because forebrain NMDARs principally include NR2A and/or NR2B subunits (Sheng et al. 1994; Takai et al. 2003), the development of antagonists selective for the NR2B subunit has also been suggested (Gill et al. 2002).

In the early eighties', Kamenka *et al.* (1982) reported the synthesis of a new NMDAR antagonist derived from the phencyclidine (PCP) structure: GK11. Pharmacological studies then revealed that this compound is a channel blocker the binding site of which overlaps that of the classical NMDAR uncompetitive antagonist MK801 (Hamon et al. 1999; Hirbec et al. 2000a; Hirbec et al. 2000b). GK11 was shown to display a relative high affinity for telencephalic NMDARs with K_d in the nanomolar range (Hirbec et al. 2000a; Hirbec et al. 2000b). In agreement with these data, GK11 has potent neuroprotective properties both *in vitro* (Drian et al. 1999) and *in vivo* (Hirbec et al. 2001a). In either contusive or photochemical rat models of spinal cord injury (SCI), GK11 efficiently prevented both the functional and morphological impairments associated with SCI (Gaviria et al. 2000a; Gaviria et al. 2000b). GK11 also displayed beneficial effects in brain trauma (Smith et al. 2000). Very interestingly and unlike the other high affinity NMDAR antagonists, preliminary experiments have shown that GK11 is nearly devoid of any intrinsic neurotoxicity even at extremely high doses (20mg/kg i.v.) (Hirbec et al. 2001b). This was further confirmed in a much more extensive study aimed at comparing the intrinsic neurotoxicity of GK11 and MK801 using a wide range of behavioural and histological techniques (Hirbec *et al.* unpublished results). The safe pharmacological profile of GK11 was also confirmed by the results from phase I & IIb clinical trials in which GK11 was assessed for SCI treatment (Lepeintre et al. 2004).

The present study was designed to determine whether the special properties of GK11, in particular its safer pharmacological profile compared with MK801, could be explained by different molecular mode of action of these two compounds at NMDARs. As a first step we therefore compared, on sister hippocampal cultures, both the neuroprotective and intrinsic neurotoxic effects of both drugs. By using patch-clamp techniques, we also compared their respective effects on native NMDARs expressed in hippocampal neurons. Then, to probe the selectivity of GK11 and MK801, we measured their effects on recombinant NR1a/NR2A and NR1a/NR2B receptors expressed in HEK293 cells and showed that in contrast with MK801, GK11 did not totally block the currents mediated through the NR2A-containing receptors. Finally, we used specific electrophysiological approaches and calcium imaging on native neurons to understand the significance of GK11 subtype selectivity. Here we demonstrate that GK11 preferentially blocks extrasynaptic NMDARs while partially preserving synaptic communication. Our results show that although at first sight both drugs seem to have comparable effects on native NMDARs, their subunit selectivity may account, at least partly, for their differences in terms of pharmacological tolerance.

MATERIALS AND METHODS

PRIMARY CULTURE OF NEURONAL CELLS

Hippocampal culture

Hippocampal cultures were prepared as previously described (Noel et al. 1999). Briefly, hippocampal neuronal cells were isolated from embryonic rats (E18) and plated on either poly-D-Lysine (Sigma-Aldrich, Saint Louis, MO, USA) and laminin (Invitrogen, Cergy Pontoise, France) treated plastic plates (electrophysiological studies) or poly-D-lysine and laminin treated glass coverslips (neuroprotection and neurotoxicity studies) using Neurobasal medium (Invitrogen) supplemented with 1% L-Glutamine (Invitrogen), 2% B27 (Invitrogen) and 3% foetal bovine serum (Invitrogen). Cultures were grown at 35°C in a humidified

atmosphere containing 5% CO₂, and from the third day in culture, the medium was supplemented with 5 µM cytosine-b-D-arabinofuranoside (ARAC; Sigma-Aldrich). Hippocampal cultures were prepared either at low density (125 cell/mm²) for electrophysiological studies or high density (1000 cell/mm²) for neuroprotection, intrinsic neurotoxicity studies and spontaneous activity recordings. The cultures were used for experiments 12-14 days after plating for all experiments except for spontaneous activity. Indeed, for such experiment we observed that when cultures were kept for 19-21 days *in vitro* (DIV), the frequency of the spontaneous events was increased a fact that is likely to be associated with a better developed neuritic network.

Cortex culture

Primary cortical cultures were prepared from foetal mice at 15-16 days gestation as previously described (Rose K 1993). Cerebral cortices were then dissected in DMEM (Sigma-Aldrich). Dissociated cortical cells were re-suspended in DMEM supplemented with 5% foetal bovine serum, 5% horse serum and 2 mM glutamine and plated in glass Petri dishes coated with poly-D-lysine and laminin. Cultures were kept at 37°C in a humidified atmosphere containing 5% CO₂. Experiments of calcium recordings were performed after 14 DIV.

PHARMACOLOGICAL TREATMENTS

Neuroprotection studies

To assess the neuroprotective properties of the drugs, 12-14 DIV hippocampal cultures were challenged with Neurobasal medium devoid of serum and containing 500 µM glutamate (adapted from (Drian et al. 1999). After 5 min of incubation, the culture medium was replaced with Neurobasal containing the neuroprotective drug at the appropriate concentration. Cultures were then returned to the incubator. The two drugs used in the present study were GK11 (gacyclidine) and MK801, were tested at concentrations ranging between 100 nM and 10 µM. After 24h, the cells were fixed for immunocytochemistry by immersion of the coverslips for 45 min at room temperature in 4% paraformaldehyde in 0.1 M phosphate buffer. Labelling of the surviving neurones was performed as followed: after 10 min treatment

with 1% H₂O₂, cells were incubated overnight at 4°C with monoclonal anti-Map2 (clone HM-2, 1:1000; Sigma-Aldrich) antibody prepared in PBS containing 2% BSA and 0.1% Triton X-100. The secondary antibody was peroxidase-coupled anti-mouse antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), revelation was performed with 0.2% DAB in 0.1 M Tris Buffer.

The number of surviving neurones, as assessed by counting the density of Map₂ positive cells, was compared to both sham cultures (i.e. those submitted to the same number of medium changes as the experimental protocol), and to control cultures that were kept untouched. Counts were performed on at least 3 wells per condition and in 3 independent series of experiments, using the Samba image analysis software (Samba technologies, France). Results are means ± SEM and statistical analyses were performed using the one-way ANOVA statistical test.

Intrinsic neurotoxicity studies

To assess the potential adverse effects of GK11 and MK801 on hippocampal neuron viability, 12-14 DIV hippocampal cultures were challenged with 10 µM and 100 µM GK11 or MK801 by directly adding the compounds to the culture medium. Control cultures received an equivalent volume (50 µl) of water. Cultures were then returned to the incubator. After 24h, they were fixed for immunocytochemistry and further processed for Map₂ labelling as previously described. Neuronal viability was assessed by measuring the percentage of Map₂ immunopositive surface area with Samba image analysis software. Compared to neurone counting, this parametre allowed us to quantify not only neuronal survival but also to evaluate their health. A decrease in the percentage of immunolabelled surface may reveal either neuronal death (loss of neurones) or shrinkage and reorganization of the neuritic arborization which can be interpreted as a sign of neuronal suffering. Quantification was performed on at least 3 wells per condition and in 4 series of independent experiments. Results are means ± SEM and statistical analyses were performed using the one-way ANOVA statistical test.

ELECTROPHYSIOLOGICAL RECORDINGS

NMDA- induced current recordings

Whole cell patch recordings were performed at a -70 mV holding potential, using an Axopatch 200B amplifier (Molecular Devices Corp., Sunnyvale, CA, USA). Ionic currents were recorded using the following extracellular solution maintained at room temperature (mM): NaCl (140), KCl (5), CaCl₂ (2), Hepes (10), Glucose (20), glycine (0.01), pH = 7.35 (adjusted with NaOH). Mg²⁺ was omitted to prevent voltage-dependent block of NMDAR operated channels (Mayer et al. 1984). Voltage-activated Na⁺ channels were blocked by adding to this solution 0.5 μM tetrodotoxin (TTX; Latoxan, Valence, France), AMPA/kainate glutamate receptors were inhibited by adding 1 μM NBQX (Sigma-Aldrich) and glycine receptors were inhibited by adding 1 μM strychnine (Sigma-Aldrich). Recording pipettes (2-4 MΩ) were pulled from microhematocrit tubes (Modulohm A/S, Herlev, Denmark) and filled with the following solution (mM): CsCl (136), Hepes (25), Glucose (10), EGTA (10), Mg-ATP (3), Na-GTP (1), pH = 7.35 (adjusted with CsOH). The osmolarity of all buffers used in this study was 310 mOsm. The recording chamber was continuously superfused with the extracellular solution using a custom-made perfusion system with an outflow of 250 μl/min. NMDA-induced currents were elicited every 2 min by a standard 5 second application of 100 μM NMDA with an outflow of 500 μl/min. Tested substances (GK11, MK801 and DAPV) were diluted in the perfusion medium. They were applied at the appropriate concentrations in presence of 100 μM NMDA by switching channels of the perfusion system.

Signals were filtered at 5 kHz, digitized at 10 kHz, and stored on-line using pCLAMP 6 (Molecular Devices Corporation, Sunnyvale, CA, USA). Inhibition of NMDA-induced current by the antagonist at a given concentration was measured either as the total area (recordings on hippocampal neurones) or at the maximum value of the peak (recordings on HEK293 cells). It was expressed as percentage of the current elicited by NMDA only (mean of 3 successive evoked responses). Data reported are means ± SEM of the indicated number of individual determinations. IC_{50s} were calculated by fitting the data to either formula a (single binding site) or formula b (two binding sites) using SigmaPlot 10.0 software (Formula a: $I(x) =$

$Ax/(IC_{50}+x)$; Formula b: $I(x) = (A1x/(IC_{50_1}+x)) + (A2x/(IC_{50_2}+x))$; where: I is the inhibition percentage of the NMDA-induced current, x is the concentration of the antagonist (mol.l^{-1}), A is the maximum response and IC_{50} is the half-maximal inhibition concentration. IC_{50} were reported as means \pm SE. To estimate whether the double sigmoidal fitting (two binding sites) was more probable than the single sigmoidal fitting (single binding site), we compared the square residues using a t-test.

Spontaneous excitatory postsynaptic current (sEPSC) recording

The compositions of the extracellular and intracellular medium used were the same as described above. Hippocampal neurones were voltage-clamped at -70mV and sEPSC recorded in the presence of 2 μM NBQX (Sigma-Aldrich) and 2 μM Gabazine (SR-95531; Sigma-Aldrich) to eliminate the AMPA-Kainate and GABA_A -receptor mediated spontaneous currents respectively. EPSC were filtered at 5 kHz, sampled at 10 kHz. Drugs were applied for 80s via the perfusion system. For the calculation of the frequency and the amplitude of bursts, events were analysed for 40s before and for the last 40 seconds of the drug application.

Calcium Imaging

Primary cortical neurone cultures were loaded for 45 min at 37°C with 10 μM fura-2/AM and 0.2% pluronic acid (F-127; Invitrogen) and incubated for an additional 15 min at room temperature in HEPES and Bicarbonate – Buffered Saline Solution (HBBSS; Invitrogen) containing (in mM) 116 NaCl, 5.4 KCl, 1.8 CaCl_2 , 0.8 MgSO_4 , 1.3 NaH_2PO_4 , 12 HEPES, 5.5 Glucose, 25 bicarbonates and 10 μM glycine at pH= 7.45. Experiments were performed at room temperature with continuous perfusion at 2 ml/min with a peristaltic pump, on the stage of an inverted Nikon Eclipse microscope equipped with a 100 W mercury lamp and oil immersion Nikon 40X objective with 1.4 numerical aperture. Fura-2 (excitation: 340/380nm, emission: 510 nm) ratio images were acquired every 2 seconds with a CCD camera (Princeton Instruments, Trenton, NJ, USA) and digitized (512 x 512 pixels) using Metafluor 6.3 (Universal Imaging Corporation, Chester, USA). Fluorescence ratio (340/380 nm) were

converted to intracellular Ca^{2+} concentrations using the following formula: $[\text{Ca}^{2+}]_i = K_d[(R - R_{\min}) / (R_{\max} - R)] F_0 / F_S$, where R is the observed 340/380 fluorescence ratio, R_{\min} is the ratio for a Ca^{2+} -free solution, R_{\max} is the ratio for a saturated Ca^{2+} solution, $K_d = 135 \text{ nmol/l}$ (the dissociation constant for fura-2), F_0 is the intensity of a Ca^{2+} -free solution at 380 nm, and F_S is the intensity of a saturated Ca^{2+} solution at 380 nm. The recordings of selective synaptic or extrasynaptic activities are well described by Hardingham et al. (Hardingham et al. 2001; Hardingham et al. 2002). In brief, the synaptic activity was induced by blocking GABA_A receptors with 50 μM bicuculline (Sigma-Aldrich) and the presence of 2.5 mM 4-AP (Sigma-Aldrich). Under these conditions, cortical neurones fired bursts of action potential which resulted in a calcium plateau visualized by videomicroscopy. This calcium increase was fully blocked by the co-application of 10 μM MK801, 100 μM D,L-amino-5-phosphovalerate (D-APV, Sigma-Aldrich) or 0.5 μM TTX (data not shown). To selectively activate extrasynaptic NMDARs (ExNMDARs), synaptic NMDAR (SynNMDAR) responses were first inactivated by exposing neurones to 10 μM MK801 during a bicuculline and 4-AP treatment. Then, the extrasynaptic activity was activated by an application of 50 μM NMDA. At least 3 independent experiments and 120 to 130 individual neurones were analyzed. Results are expressed as means \pm SEM. Statistical analysis was performed with StatView (Abacus, Berkeley, CA, USA) by one-way ANOVA followed by a PLSD Fisher test (*: $p < 0.05$).

RECOMBINANT NMDARS IN HEK293 CELLS

Expression of NR1a, NR2A and NR2B subunits

cDNAs encoding the rat full length sequences of NR1a, NR2A and NR2B cloned in pcDNA I were a generous gift from Dr. Jeremy Henley (University of Bristol, UK). The peGFP(C2) plasmid was obtained from Clontech.

Human embryonic kidney 293 cells (HEK293) were grown in Duplecco's medium (Invitrogen) supplemented with 1% L-glutamine (Invitrogen), 1% penicillin/streptomycin (Sigma-Aldrich) and 10% foetal bovine serum (Invitrogen). Exponentially growing cells were plated on 12-mm

glass coverslips (Marienfeld GmbH, Lauda-Königshofen, Germany) coated with poly-D-Lysine and grown at 37°C in a humidified atmosphere containing 5% CO₂. 24 hours after plating, cells were transiently transfected with a total of 0.5 µg DNA using 1.5 µl of FuGENE 6 transfection reagent (Roche, Meylan, France). DNA ratios were NR1a:NR2X_(X=A or B):GFP = 10:10:1. Co-transfection with GFP allowed ready recognition of the transfected cells. Additionally, after transfection, cells were treated with 100 µM D-APV to prevent cell death. Studies on the recombinant receptors were performed 24 hours after transfection.

Characterization of the recombinant NMDAR subunits

Characterization of our recombinant model showed that transfection efficiency for the three DNAs was about 11% (data not shown), and that 81% of the GFP-expressing cells mediated NMDA evoked responses indicating that functional ion channels had been expressed. When HEK293 cells were transfected with GFP or GFP/NR1a only, NMDA application did not elicit any current. In contrast, the maximum current peaks recorded on the cells expressing NR1a/NR2A and NR1a/NR2B recombinant receptors were 696±53 pA (n= 70) and 786±145 pA (n=16) respectively. These currents were totally blocked after addition of 500 µM D-APV to the extracellular medium (data not shown).

MATERIALS (drugs)

GK11 (cis(pip/me)-1-[1-(2-Thienyl)-2-methylcyclohexyl]piperidine, Gacyclidine) was a generous gift from Expansia (France). All other chemicals were obtained from commercial sources and were of the highest purity available (Sigma-Aldrich, Saint-Louis, MO, USA).

RESULTS

Pharmacological properties of MK801 and GK11 on native NMDA receptors

In vitro neuroprotective properties of GK11 and MK801

Previous studies from our laboratory have demonstrated that GK11 and MK801 displayed very similar neuroprotective properties on cultured cortical neurones challenged with a toxic dose of glutamate (Drian et al. 1999). To better interpret our functional experiments (see

below), here we investigated whether it was reproduced on primary hippocampal cultures. Sister control cultures only submitted to medium changes showed cell death which was much more pronounced than on cortical cultures reaching about 90% ($p < 0.001$; Fig. 1A). Treatment with 500 μM glutamate induced additional cell death with only $1.2 \pm 0.1\%$ of surviving neurones ($p < 0.001$ compared to both sham and control cultures). Treatment of the glutamate-challenged cultures with either MK801 or GK11 improved the survival rate of the neurones in a concentration dependant manner. The neuroprotective effects were significant from the lowest concentration tested ($p < 0.01$ for both GK11 and MK801 treatments at 100nM). At the highest concentrations (10 and 100 μM), treatment with NMDAR antagonists exhibited strong neuroprotective effects with increased density of surviving neurones compared to sham cultures, and thus suggesting that at these concentrations, GK11 and MK801 protected both against the specific effects of glutamate treatment and the toxic consequences associated with medium changes. At 1 μM , the proportions of surviving neurones were $30.8 \pm 2.1\%$ for GK11 and $38.9 \pm 1.7\%$ for MK801 compared to control cultures ($p < 0.001$), whereas at 10 μM they were $54.6 \pm 2.3\%$ and $44.3 \pm 2.0\%$ respectively ($p < 0.001$). As expected, our results show that both compounds had similar neuroprotective effects on hippocampal neurones.

In vitro intrinsic neurotoxicity of MK801 and GK11

To optimize the evaluation of GK11 and MK801 intrinsic neurotoxicity, we developed an *in vitro* pharmacological approach based on the measurement of the Map₂ immunopositive surface area (see materials and methods) to detect neuronal suffering after incubation in the presence of the drugs. Fig. 1B shows that after 24h, treatment with 10 μM MK801 led to a very significant reduction in the Map₂ immunopositive surface ($82.4 \pm 2.3\%$ compared to control cultures, $p < 0.001$). This decrease may reveal both neuronal cell death and adverse effects on the cell viability. MK801 deleterious effects were further increased at 100 μM with a percentage of Map₂ immunopositive surface reduced to $77.9 \pm 3.1\%$ compared to control cultures ($p < 0.001$). In contrast, treatment of the cultures with equivalent doses of GK11 had no significant effect on neuronal viability. Indeed, treatment with GK11 did not significantly

affect the percentage of surface labeled with Map₂ (compared to control cultures: 96.3±3.0% at 10 μM and 90.6±2.4% at 100 μM, $p<0.001$). When the cultures were challenged for longer time (i.e. 48h) with the drugs, the intrinsic neurotoxicity of MK801 was markedly increased. Under these conditions and compared to control cultures, the percentage of surface area labeled with Map₂ was reduced to 56.4±2.4% at 10 μM and 44.6±1.7% at 100 μM (Fig. 1C, $p<0.001$). Under light microscopy, examinations of the MK801-treated cultures revealed alterations in the neuritic field organization, with neurites either packed in coarse cables extending in between cell clumps (10 μM) or retracted around clumps (100 μM) (Fig. 1D). Additionally, some strongly stained tightly packed pericarya revealed alterations in the neurons morphology. In comparison, extending the exposure time to GK11 had only minor effect on the neuritic network. Compared to control cultures, the surface area labeled with Map₂ was only reduced to 83.0±2.7% at 10 μM and 70.8±2.1% at 100 μM, $p<0.001$). Additionally, the effects of GK11 were always significantly lower than those of MK801 ($p<0.01$ whatever the concentration and the duration of exposure to the drug). GK11-treated cultures also appeared to be much healthier (Fig. 1D), with neurites being either well dispersed on the dish with a few lightly stained pericarya (10 μM) or slightly bundled inbetween flattened groups of moderately stained pericarya (100 μM). Interestingly, at 100 μM GK11 had significantly less adverse effects than 10 μM MK801 ($p<0.05$ and $p<0.001$ after 24h and 48h treatment respectively). The present data thus reveal that at the optimal neuroprotective concentration, MK801 exhibits significant intrinsic neurotoxicity while GK11 only weakly affected the cell viability.

Effects of MK801 and GK11 on NMDA-induced currents in hippocampal neurones

Using the patch-clamp technique in the whole-cell configuration, we recorded NMDA-evoked currents elicited by application of 100 μM NMDA and 10 μM glycine on 12-14 DIV hippocampal neurones. In the absence of NMDAR antagonists, the recordings displayed the typical pattern of NMDA-induced responses (Fig. 2), the maximum peak currents measured were 1146±49 pA (n=80). Responses were stable for at least 15 min (Fig. 3a). They were

completely blocked in the presence of 500 μM D-APV (data not shown) and partially blocked in the presence of either 10 μM GK11 or MK801 (Figs 2a and b).

MK801 is known to act as an irreversible blocker of the NMDAR associated channel. To test whether GK11 displayed the same properties, we evaluated the recovery rate of the NMDA-induced responses after blockade with 10 μM GK11. As for MK801, GK11 mediated blockade persisted when the cell was washed with control solution for 5 min and then tested with subsequent applications of NMDA (Figs 2a and b). Under our experimental conditions, recovery of either MK801 or GK11 blockade was not faster when NMDA was applied continuously to the cell (Figs 2c and d). As compared to -70mV, when the cells were held at +30 mV, the blockade was decreased by about 2 fold, demonstrating some voltage-dependant block for both compounds. Indeed, at -70 mV the NMDA-induced currents were blocked by $65.6\pm 8.5\%$ with MK801 and $45.1\pm 4.5\%$ with GK11 whereas at +30 mV they were blocked by $36.0\pm 5.7\%$ and $22.2\pm 5.3\%$ respectively (Figs 2e and f). However, whatever the holding potential or the antagonist, the blockade remained stable throughout the NMDA application (continuous perfusion). Thus our results show that as for MK801, GK11 remained trapped inside the channel and could be considered as a permanent channel blocker. As a result of this property, we used only one cell per concentration to build up the dose-response curves of GK11 and MK801.

As shown in Fig. 3b, increasing MK801 and GK11 concentrations reduced NMDA steady state currents in a dose-dependent manner. Both MK801 and GK11 presented dose-response curves (Fig. 3c) that were statistically best fitted using a two site interaction model ($p < 0.0001$, t-test). This suggested the presence of at least two different NMDAR populations on 2 week cultured hippocampal neurones: a high affinity (site 1) and a low affinity site (site 2) (Table 1). Analysis of the fitting parameters revealed that GK11 had a 5 fold lower affinity than MK801 on the low affinity site and only a two-fold weaker affinity on the high affinity site (Table 1). On the low affinity site, IC_{50} values were $55.9\pm 3.9 \mu\text{M}$ and $197\pm 31 \mu\text{M}$ for MK801 and GK11 respectively, whereas on the high affinity site, they were respectively of $105\pm 54 \text{ nM}$ and $262\pm 23 \text{ nM}$. In addition, MK801 and GK11 blocks were complete at concentrations

higher than 1mM. The present results thus confirm that both MK801 and GK11 are potent uncompetitive blockers of the NMDAR associated channel.

Pharmacological properties of MK801 and GK11 on recombinant NMDARs

Effects of MK801 and GK11 on NMDA-induced currents in recombinant NMDARs

To investigate whether MK801 and GK11 differences in terms of intrinsic neurotoxicity could be explained by subunit selectivity of these two compounds, we tested the effect of GK11 on NMDA-induced currents elicited on HEK293 cells transiently transfected with the NR1a/NR2A or the NR1a/NR2B NMDAR subtypes, and compared its dose-response curves to those of MK801. Whatever the subunit composition, increasing the concentrations of both NMDAR antagonists blocked NMDA-induced currents in a dose dependent manner. Dose-response curves were statistically best fitted using a single site interaction model. However, Fig. 4 shows that the effects of two compounds differed according to the subunit composition. On NR1a/NR2B containing receptors, for concentrations above 1mM, both antagonists were able to totally inhibit NMDA-induced currents (Fig. 4b). On this receptor subtype, GK11 had a 6 fold weaker affinity than MK801 and IC_{50s} were $5.8 \pm 1.3 \mu M$ for MK801 and $37.4 \pm 8.7 \mu M$ for GK11 (Fig. 4b). On NR1a/NR2A containing receptors, both antagonists presented close affinities with IC_{50} equal to $2.23 \pm 0.30 \mu M$ and $5.25 \pm 1.4 \mu M$ for MK801 and GK11 respectively (Fig. 4d). However, very interestingly on this type of recombinant NMDAR, the two compounds greatly differ in terms of maximal inhibitory effect. Indeed, for the two NMDAR antagonists, the maximal inhibitory effect was achieved for concentration above $50 \mu M$. However, at these concentrations, MK801 totally blocked NMDA-induced currents, whereas GK11 inhibited them only by $52.3 \pm 2.6\%$. Taken together, these results show that MK801 and GK11 are significantly different in terms of blockade of the different NMDAR subtypes.

Functional consequences of MK801 and GK11 blockade

Taking into account the proposed subcellular localization of both NMDAR subunits, namely NR2A preferentially synaptic and NR2B preferentially extrasynaptic and our results on

recombinant NMDARs, we speculated that GK11 in contrast with MK801 will at least partially preserve the normal synaptic transmission.

Effects of MK801 and GK11 on synaptic & extrasynaptic evoked NMDA-mediated responses

To test this hypothesis, we used the experimental protocol developed by Hardingham et al. (Hardingham et al. 2001) to specifically activate either the SynNMDARs or the ExNMDARs and compared the blocking effects of GK11 and MK801. As shown in Fig. 5, at 10 μM GK11 prevented $34.5 \pm 7.1\%$ of the calcium load mediated through the SynNMDARs and $52.6 \pm 5.5\%$ of that mediated through the ExNMDARs (Figs 5b and c). At 100 μM GK11, the blockade of ExNMDARs was nearly total ($94.3 \pm 1.6\%$) whereas the calcium load through SynNMDARs was only blocked by $56.4 \pm 4.3\%$. Similar experiments performed with MK801 showed that it was more potent than GK11 at SynNMDARs. Indeed, at 1 μM , it was as potent as 10 μM GK11 at preventing calcium load (about 35% of blockade), and at 10 μM it was more potent than 100 μM GK11 ($61.3 \pm 5.3\%$ and $56.4 \pm 4.3\%$ blockade with MK801 and GK11 respectively). Interestingly, this is not the case for ExNMDARs. Indeed, at 10 μM MK801 was only slightly more potent than 10 μM GK11, preventing 62.9% of the calcium load compared to 51.6% for the latter compound.

Effects of MK801 and GK11 on spontaneous activity

To obtain further insight on the physiological consequences of treatment with either GK11 or MK801, we compared the effects of both drugs on spontaneous neuronal activity (Fig. 6). To obtain sufficient baseline events, recordings were performed on more mature (19-21 DIV) dense hippocampal neuronal cultures. As indicated in materials and methods, sEPSCs were isolated in the presence of AMPA- and GABA_A-receptor antagonists and in the absence Mg²⁺. Under these conditions, sEPSC occur as bursts with frequency ranging from 1.9 to 7.8 Hz. These bursts were fully blocked after application of the competitive NMDAR antagonist D-APV (data not shown). Perfusion of 10 μM MK801 significantly reduced the spontaneous activity, decreasing by about 50% the frequency and the amplitude of the sEPSCs. At 100 μM , these effects were further increased with frequency and amplitude blocked by about 80% (Fig. 6a). In contrast, application of 10 μM GK11 had no specific effect on either the

frequency or the amplitude of the sEPSCs. At 100 μ M, there was a small reduction (37%) in the amplitude of the NMDAR-mediated events (Fig. 6b). Notably, the effect of 100 μ M GK11 perfusion on sEPSCs tended to be smaller than that measured with 10 μ M MK801.

DISCUSSION

Over-activation of NMDARs occurs in many different pathological situations and can lead to cell death and severe impairment of important neurological functions (Arundine and Tymianski 2003). Thus, blocking excitotoxicity with NMDAR antagonists would be a rational approach for treatment. Despite considerable efforts to develop NMDA antagonists for the treatment of acute or chronic disorders, very few molecules with a good therapeutic index have been produced. Several potent and selective NMDA antagonists failed in clinical trials principally because of their side effects (Ikonomidou and Turski 2002; Hoyte et al. 2004).

Over the last decade, our laboratory has developed therapeutic strategies for acute degenerative diseases and in particular SCI (Gimenez y Ribotta and Privat 1998; Gimenez y Ribotta et al. 2002; Privat 2005). One approach, which consisted in developing neuroprotective molecules based on the structure of the PCP, led us to identify GK11 as a potential new drug candidate (Hirbec et al. 2001a). Initial pharmacological studies showed that GK11 is a potent NMDAR antagonist, with affinity for NMDARs close to that of MK801, the reference antagonist (Hirbec et al. 2000b). Interestingly, further experimental studies revealed that, whether in animal models (Hirbec et al. 2001a) or in man (Tadie 2003; Lepeintre et al. 2004), GK11 did not exhibit the adverse side effects that are normally observed with classical high affinity NMDAR antagonists and led us to investigate whether the safer profile of GK11 could be ascribed to specific pharmacological properties on NMDARs.

Our study shows that, as already demonstrated for MK801 (Huettnner and Bean 1988), GK11 reduces NMDA-activated currents in primary hippocampal neurones and acts as a permanent blocker of the NMDAR associated channels. Consistent with the binding of both

MK801 and GK11 inside the channel, we found that blockade of the NMDA-induced currents were voltage dependent. The voltage dependence of GK11 blockade is consistent with previous results obtained for ketamine and phencyclidine, two other PCP related compounds (Honey CR 1985; MacDonald et al. 1987). The antagonistic effects elicited by the two compounds were dose dependent and computations of the dose-response curves revealed that both compounds interacted with two independent sites. Affinities on the high and low affinity sites were respectively in the low nanomolar and micromolar range. GK11 appeared less potent at preventing NMDAR induced currents, with IC_{50} values for both sites 2-5 times higher than those calculated for MK801. These results differ slightly from our previous experiments on tissue homogenates, which showed that GK11 and MK801 exhibited similar K_d values for NMDARs (Hirbec et al. 2000a; Hirbec et al. 2000b). However the divergence between the two studies is not considerable and is probably due to the experimental conditions. Indeed electrophysiological recordings in neurones represent a condition closer to the physiological functioning of the receptor. Thus, better integrity of the cell membranes as well as preserved downstream scaffolding signalling molecules may affect the conformation of the channel and are likely to influence the binding parameters. In slight contrast with the electrophysiological recordings but in agreement with the binding results, GK11 and MK801 displayed similar neuroprotective effects on glutamate-induced neuronal death. Notably, at low concentrations, MK801 was slightly more protective than GK11, a result which is in agreement with its higher affinity for NMDARs. Compared to the initial study from our laboratory (Drian et al. 1999), this effect could be measured even when the neuroprotective treatment was administered after the insult.

In vitro determination of the intrinsic neurotoxicity of the tested compounds revealed that the decrease of the percentage of Map₂ immunopositive surface area measured was highly significant, with maximal effect reaching about 60% decrease after 48h exposure to the drug. This relatively low apparent toxic effect may be attributed to different factors: (i) complexing of the drug with the serum contained in the medium; (ii) presence of unknown neuroprotective substances in Neurobasal (commercial product with undisclosed detailed

formula); (iii) lastly, as compared to the *in vivo* situation, it should be remembered that neuronal cultures consist of isolated cells the connections of which *in vitro* are not identical to their *in situ* counterparts. Nevertheless, using the quantitative parameters employed here, neuronal cultures were shown to represent an adequate model to assess the potential intrinsic neurotoxicity of the molecules. In agreement with published *in vivo* data (Olney et al. 1989), our results reveal that cultures treated with neuroprotective concentrations of MK801 (i.e. 10 μ M) exhibit neuronal suffering. In cultures, this was characterized by disruption in the neuritic network organization and increase in the Map₂ labelling in the cell bodies. Neuron viability was further reduced when the concentration or the duration of exposure to the drug was further increased. In contrast with MK801, our results show that at optimal concentrations for neuroprotection, GK11 did not elicit any neuronal suffering after 24h exposure and only very low intrinsic neurotoxicity after 48h. At 10-fold higher concentrations signs of neuronal suffering slightly increased. Interestingly, the neurotoxic effect of GK11 was at least 10 times lower than that of MK801, a fact that should be compared with our neuroprotection data, which show that both compounds displayed very similar profiles. The reduced intrinsic neurotoxicity of GK11 confirms our preliminary *in vivo* findings (Hirbec et al. 2001b) and is supported by *ex-vivo* experiments performed in Dr A. Ring's laboratory (Norwegian Defence Research Establishment). The latter have shown that, in contrast with other NMDAR antagonists, no significant increase in propidium iodide incorporation was detected in acute hippocampal slices treated for up-to 96h with GK11 (personal communication). Together, these results suggested that GK11 is likely to have an original mode of action.

The subcellular localization of the different NMDAR subunits has been a matter of debate for several years, but recent *in-vitro* studies have demonstrated that both NR2A- and NR2B-containing NMDARs can be located in either synaptic or extrasynaptic compartments (Thomas et al. 2006; Liu et al. 2007). Recordings on hippocampal slices have also evidenced that although the vast majority of synaptic receptors are NR2A-containing, a small proportion of functional NR1/NR2B receptors are expressed at the synapse (Liu et al. 2004; Wong et al.

2005)). Interestingly using cortical neuronal cultures, Liu et al. (2007) have demonstrated that NR2B-containing receptors account for about 1/3 of the synaptic currents and that about 25% of the total currents gated by extrasynaptic NMDARs. Our work on recombinant NMDARs shows that MK801 is a potent inhibitor of both NR2A- and NR2B-containing receptors and does not display significant subunit specificity. GK11 is also able to totally prevent NMDA-induced currents at NR2B-containing NMDARs but appeared about 6-7 times less potent than MK801. Very interestingly, we also demonstrated that GK11, even at very high concentrations (up to 1 mM), fails to totally block the NMDA-elicited currents at the NR2A-containing receptors. The molecular mechanism involved in this unique property is currently not known and needs further investigation. However, taking into account the results from Liu et al. (2007), this finding suggests that both compounds would block the activation of the extrasynaptic receptors that contains majority of NR2B-containing NMDARs whereas, MK801, but not GK11, will be an efficient blocker at synaptic NR2A enriched receptors.

Using a similar protocol to that developed by Hardingham et al. (2002) to selectively activate SynNMDARs or ExNMDARs; we have shown that, as expected, MK801 is able to efficiently block both receptor subtypes. It even appears slightly more potent at SynNMDARs. Interestingly, and in agreement with our data on recombinant receptors, here we show that GK11 is much more potent at blocking ExNMDARs versus SynNMDARs. The maximal blocking effect of GK11 at SynNMDARs was 56%, a value that is close to the maximal blocking effect observed on NR2A-containing recombinant receptors. Moreover at this concentration, GK11 blocked activation of the ExNMDARs by about 95 %. In agreement with these results on evoked NMDA-mediated responses we have shown that, compared to MK801, GK11 better preserves the spontaneous activity of the neurones mediated through NMDARs. These latter results are in good agreement with the reported *in vitro* (i.e. present study) and *in vivo* (Olney et al. 1989) and Hirbec et al., in preparation) intrinsic neurotoxicity of GK11 and MK801 respectively. Indeed *in vivo* blockade of NMDARs induces neuronal apoptosis in many regions of the developing brain (Olney et al. 1991; Sharp et al. 1991), and studies on cultured neurones suggest a neuroprotective role for SynNMDARs (Brenneman et

al. 1990) that may be attributed to specific activation of signalling cascades such as the ERK signalling pathways (Ivanov et al. 2006). Thus, better preservation of normal synaptic function under GK11 treatment would prevent NMDAR hypofunction mediated neuronal death.

In their study Liu et al. (2007) reported that activation of either synaptic or extrasynaptic NR2B-containing receptors results in excitotoxicity, whereas activation of either synaptic or extrasynaptic NR2A-containing receptors promotes neuronal survival. In contrast, in their work Ivanov et al. (2006) concluded that the NR2B subunit exerts a dual role in the regulation of the ERK signalling cascade with synaptic NR2B activating survival pathways and extrasynaptic NR2B leading to ERK inactivation. These findings suggest that the safer profile of GK11 may either be related to (i) its lower blocking effect at NR2A-containing NMDARs and in that case irrespective of its subcellular localization, or (ii) its lower blocking effects at synaptic NMDARs.

In the past few years, different NMDAR antagonists with improved therapeutic safety have been developed. A first class of compounds includes Memantine, and the better safety profile of these types of compounds has been ascribed to either their moderate potency, strong voltage dependence and fast blocking/unblocking kinetics (for review see Lipton 2006). Over these compounds GK11 has the advantage of presenting a greater safety margin (Hirbec et al., unpublished results). Another class of interesting compounds are those which are selective for NR2B-containing receptors, such as Ifenprodil (Kemp and McKernan 2002). Compared to this latter compound, GK11 appeared about 50 times less potent at NR1a/NR2B recombinant receptors expressed in HEK293 cells ($0.59 \pm 0.06 \mu\text{M}$, Harvey-Girard and Dunn 2003) and can prevent 50% of the current mediated through NR1a/NR2A recombinant receptors with a relative high affinity. On the contrary, Ifenprodil presents an about 400-fold lower affinity for NR2A-containing receptors (Williams et al. 1993). These differences are likely to be due to differences in mechanisms mode of action. Indeed, Ifenprodil binds to the extracellular domain of the NR2B subunit (for review see Williams 2001) whereas GK11 binding site is located inside the channel (Hirbec et al. 2000a). When

compared with Ifenprodil and “Ifenprodil-like” compounds, GK11 seems to present a better pharmacokinetic behaviour and less off-target activities. In particular, it was shown that GK11 has no potent interaction with adrenergic α_1 receptors (IPSEN-Beaufour, personal communication). Thus the specific features of GK11 will allow the use of lower doses and will thus minimize the risk of adverse side effects by interaction with other less-specific molecular targets.

All together the results from the present study provide the first link between the molecular action of GK11 and its safer pharmacological profile. Indeed, one can speculate that as for the other high affinity NMDAR antagonists, GK11 can exert good neuroprotective properties by efficiently blocking the extrasynaptic receptors that are activated by the glutamate overload. However, it will at least partially preserve the normal synaptic transmission principally mediated through NR2A-containing receptors and will thus exhibit a much safer profile. This is particularly relevant in regions that are not affected by the glutamate overload. Nevertheless, the unique mode of action of GK11 at NMDARs might not be the only explanation for its original pharmacological profile. Indeed, we have previously shown that, in addition to NMDARs, GK11 also interacts at relative high affinity with another molecular target, the so-called “non-NMDA” binding sites which we speculated may play a role in the modulation of the NMDAR complex (Hirbec et al. 2001b).

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REFERENCES

Arundine M. and Tymianski M. (2003) Molecular mechanisms of calcium-dependent neurodegeneration in excitotoxicity. *Cell Calcium* **34**, 325-337.

Brenneman D. E., Yu C. and Nelson P. G. (1990) Multi-determinate regulation of neuronal survival: neuropeptides, excitatory amino acids and bioelectric activity. *Int J Dev Neurosci* **8**, 371-378.

Choi D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**, 623-634.

Clark G. D. and Rothman S. M. (1987) Blockade of excitatory amino acid receptors protects anoxic hippocampal slices. *Neuroscience* **21**, 665-671.

Collingridge G. L. and Bliss T. V. (1995) Memories of NMDA receptors and LTP. *Trends Neurosci* **18**, 54-56.

Cull-Candy S., Brickley S. and Farrant M. (2001) NMDA receptor subunits: diversity, development and disease. *Curr Opin Neurobiol* **11**, 327-335.

Davies J. and Watkins J. C. (1982) Actions of D and L forms of 2-amino-5-phosphonovalerate and 2-amino-4-phosphonobutyrate in the cat spinal cord. *Brain Res* **235**, 378-386.

Davies J., Evans R. H., Herrling P. L., Jones A. W., Olverman H. J., Pook P. and Watkins J. C. (1986) CPP, a new potent and selective NMDA antagonist. Depression of central neuron responses, affinity for [3H]D-AP5 binding sites on brain membranes and anticonvulsant activity. *Brain Res* **382**, 169-173.

Dingledine R., Borges K., Bowie D. and Traynelis S. F. (1999) The glutamate receptor ion channels. *Pharmacol Rev* **51**, 7-61.

Doble A. (1995) Excitatory amino acid receptors and neurodegeneration. *Therapie* **50**, 319-337.

Drian M. J., Kamenka J. M. and Privat A. (1999) In vitro neuroprotection against glutamate toxicity provided by novel non-competitive N-methyl-D-aspartate antagonists [In Process Citation]. *J Neurosci Res* **57**, 927-934.

Gaviria M., Privat A., d'Arbigny P., Kamenka J., Haton H. and Ohanna F. (2000a) Neuroprotective effects of a novel NMDA antagonist, Gacyclidine, after experimental contusive spinal cord injury in adult rats. *Brain Res* **874**, 200-209.

Gaviria M., Privat A., d'Arbigny P., Kamenka J. M., Haton H. and Ohanna F. (2000b) Neuroprotective effects of gacyclidine after experimental photochemical spinal cord lesion in adult rats: dose-window and time-window effects. *J Neurotrauma* **17**, 19-30.

Gill R., Alanine A., Bourson A., Buttelmann B., Fischer G., Heitz M. P., Kew J. N., Levet-Trafit B., Lorez H. P., Malherbe P., Miss M. T., Mutel V., Pinard E., Roever S., Schmitt M., Trube G., Wybrecht R., Wyler R. and Kemp J. A. (2002) Pharmacological characterization of Ro 63-1908 (1-[2-(4-hydroxy-phenoxy)-ethyl]-4-(4-methyl-benzyl)-piperidin-4-ol), a novel subtype-selective N-methyl-D-aspartate antagonist. *J Pharmacol Exp Ther* **302**, 940-948.

Gimenez y Ribotta M. and Privat A. (1998) Biological interventions for spinal cord injury. *Curr Opin Neurol* **11**, 647-654.

Gimenez y Ribotta M., Gaviria M., Menet V. and Privat A. (2002) Strategies for regeneration and repair in spinal cord traumatic injury. *Prog Brain Res* **137**, 191-212.

Hamon J., Espaze F., Vignon J. and Kamenka J. M. (1999) The search for TCP analogs binding to the low affinity PCP receptor sites in the rat cerebellum. *Eur. J. Med. Chem.* **34**, 125-135.

Hardingham G. E. and Bading H. (2002) Coupling of extrasynaptic NMDA receptors to a CREB shut-off pathway is developmentally regulated. *Biochim Biophys Acta* **1600**, 148-153.

Hardingham G. E., Arnold F. J. and Bading H. (2001) Nuclear calcium signaling controls CREB-mediated gene expression triggered by synaptic activity. *Nat Neurosci* **4**, 261-267.

Hardingham G. E., Fukunaga Y. and Bading H. (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. *Nat Neurosci* **5**, 405-414.

Harvey-Girard E. and Dunn R. J. (2003) Excitatory amino acid receptors of the electrosensory system: the NR1/NR2B N-methyl-D-aspartate receptor. *J Neurophysiol* **89**, 822-832.

Hirbec H., Gaviria M. and Vignon J. (2001a) Gacyclidine: a new neuroprotective agent acting at the N-methyl-D-aspartate receptor. *CNS Drug Rev* **7**, 172-198.

Hirbec H., Kamenka J. M., Privat A. and Vignon J. (2000a) Binding properties of [³H]gacyclidine in the rat central nervous system, *European Journal of Pharmacology* 388(3) 235-239. *Eur J Pharmacol* **388**, 235-239.

Hirbec H., Kamenka J. M., Privat A. and Vignon J. (2001b) Interaction of gacyclidine enantiomers with 'non-NMDA' binding sites in the rat central nervous system. *Brain Res* **894**, 189-192.

Hirbec H., Teilhac J. R., Kamenka J. M., Privat A. and Vignon J. (2000b) Binding properties of [³H]gacyclidine (cis(pip/me)-1-[1-(2-thienyl)-2-methylcyclohexyl]piperidine) enantiomers in the rat central nervous system. *Brain Res* **859**, 177-192.

Honey CR M. Z., MacDonald JF. (1985) Ketamine and phencyclidine cause a voltage-dependent block of responses to L-aspartic acid. *Neuroscience letters* **61**, 135-139.

Hoyte L., Barber P. A., Buchan A. M. and Hill M. D. (2004) The rise and fall of NMDA antagonists for ischemic stroke. *Curr Mol Med* **4**, 131-136.

Huettner J. E. and Bean B. P. (1988) Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. *Proc Natl Acad Sci U S A* **85**, 1307-1311.

Ikonomidou C. and Turski L. (2002) Why did NMDA receptor antagonists fail clinical trials for stroke and traumatic brain injury? *Lancet Neurol* **1**, 383-386.

Ivanov A., Pellegrino C., Rama S., Dumalska I., Salyha Y., Ben-Ari Y. and Medina I. (2006) Opposing role of synaptic and extrasynaptic NMDA receptors in regulation of the extracellular signal-regulated kinases (ERK) activity in cultured rat hippocampal neurons. *J Physiol* **572**, 789-798.

Kamenka J. M., Chiche B., Goudal R., Geneste P., Vignon J., Vincent J. P. and Lazdunski M. (1982) Chemical synthesis and molecular pharmacology of hydroxylated 1-(1-phenylcyclohexyl)-piperidine derivatives. *J Med Chem* **25**, 431-435.

Kemp J. A. and McKernan R. M. (2002) NMDA receptor pathways as drug targets. *Nat Neurosci* **5 Suppl**, 1039-1042.

Lees K. R. (2000) Neuroprotection. *Br Med Bull* **56**, 401-412.

Lepeintre J. F., D'Arbigny P., Mathe J. F., Vigue B., Loubert G., Delcour J., Kempf C. and Tadie M. (2004) Neuroprotective effect of gacyclidine. A multicenter double-blind pilot trial in patients with acute traumatic brain injury. *Neurochirurgie* **50**, 83-95.

Leppik I. E. and Schmidt D. (1988) Consensus statement on compliance in epilepsy. *Epilepsy Res Suppl* **1**, 179-182.

Lipton S. A. (2006) Paradigm shift in neuroprotection by NMDA receptor blockade: memantine and beyond. *Nat Rev Drug Discov* **5**, 160-170.

Liu X. B., Murray K. D. and Jones E. G. (2004) Switching of NMDA receptor 2A and 2B subunits at thalamic and cortical synapses during early postnatal development. *J Neurosci* **24**, 8885-8895.

Liu Y., Wong T. P., Aarts M., Rooyackers A., Liu L., Lai T. W., Wu D. C., Lu J., Tymianski M., Craig A. M. and Wang Y. T. (2007) NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. *J Neurosci* **27**, 2846-2857.

MacDonald J. F., Miljkovic Z. and Pennefather P. (1987) Use-dependent block of excitatory amino acid currents in cultured neurons by ketamine. *J Neurophysiol* **58**, 251-266.

Mayer M. L., Westbrook G. L. and Guthrie P. B. (1984) Voltage-dependent block by Mg²⁺ of NMDA responses in spinal cord neurones. *Nature* **309**, 261-263.

Monyer H., Burnashev N., Laurie D. J., Sakmann B. and Seeburg P. H. (1994) Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* **12**, 529-540.

Muir K. W. and Lees K. R. (1995) Clinical experience with excitatory amino acid antagonist drugs. *Stroke* **26**, 503-513.

Noel J., Ralph G. S., Pickard L., Williams J., Molnar E., Uney J. B., Collingridge G. L. and Henley J. M. (1999) Surface expression of AMPA receptors in hippocampal neurons is regulated by an NSF-dependent mechanism. *Neuron* **23**, 365-376.

Olney J. W. (1990) Excitotoxic amino acids and neuropsychiatric disorders. *Annu Rev Pharmacol Toxicol* **30**, 47-71.

Olney J. W., Labruyere J. and Price M. T. (1989) Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science* **244**, 1360-1362.

Olney J. W., Labruyere J., Wang G., Wozniak D. F., Price M. T. and Sesma M. A. (1991) NMDA antagonist neurotoxicity: mechanism and prevention. *Science* **254**, 1515-1518.

Priestley T., Laughton P., Macaulay A. J., Hill R. G. and Kemp J. A. (1996) Electrophysiological characterisation of the antagonist properties of two novel NMDA receptor glycine site antagonists, L-695,902 and L-701,324. *Neuropharmacology* **35**, 1573-1581.

Privat A. (2005) [Pathophysiology and treatment of spinal cord injury]. *Bull Acad Natl Med* **189**, 1109-1117; discussion 1117-1108.

Rose K G. M., Choi DW (1993) Cytotoxicity in murine neocortical cell culture., in *In vitro biological methods. Methods in toxicology* (Tyson CA F. J., ed.), pp 46-60. San Diego: Academic.

Rothman S. M. and Olney J. W. (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann Neurol* **19**, 105-111.

Rumbaugh G. and Vicini S. (1999) Distinct synaptic and extrasynaptic NMDA receptors in developing cerebellar granule neurons. *J Neurosci* **19**, 10603-10610.

Sacco R. L., DeRosa J. T., Haley E. C., Jr., Levin B., Ordroneau P., Phillips S. J., Rundek T., Snipes R. G. and Thompson J. L. (2001) Glycine antagonist in neuroprotection for patients with acute stroke: GAIN Americas: a randomized controlled trial. *Jama* **285**, 1719-1728.

Sharp F. R., Jasper P., Hall J., Noble L. and Sagar S. M. (1991) MK-801 and ketamine induce heat shock protein HSP72 in injured neurons in posterior cingulate and retrosplenial cortex. *Ann Neurol* **30**, 801-809.

Sheng M., Cummings J., Roldan L. A., Jan Y. N. and Jan L. Y. (1994) Changing subunit composition of heteromeric NMDA receptors during development of rat cortex. *Nature* **368**, 144-147.

Sinagra M., Verrier D., Frankova D., Korwek K. M., Blahos J., Weeber E. J., Manzoni O. J. and Chavis P. (2005) Reelin, very-low-density lipoprotein receptor, and apolipoprotein E receptor 2 control somatic NMDA receptor composition during hippocampal maturation in vitro. *J Neurosci* **25**, 6127-6136.

Smith J. S., Fulop Z. L., Levinsohn S. A., Darrell R. S. and Stein D. G. (2000) Effects of the novel NMDA receptor antagonist gacyclidine on recovery from medial frontal cortex contusion injury in rats. *Neural Plast* **7**, 73-91.

Tadie M., Gaviria, M., Mathe, J-F., Menthonenex, Ph., Loubert G., Lagarrigue J., Saint-Marc C., Argenson C., Kempf Ch., D'Arbigny P., Kamenka J-M., Privat A., Carli P. (2003) early care and treatment with a neuroprotective drug, gacyclidine, in patients with acute spinal cord injury. *rachis* **15**, 363-376.

Takai H., Katayama K., Uetsuka K., Nakayama H. and Doi K. (2003) Distribution of N-methyl-D-aspartate receptors (NMDARs) in the developing rat brain. *Exp Mol Pathol* **75**, 89-94.

Thomas C. G., Miller A. J. and Westbrook G. L. (2006) Synaptic and extrasynaptic NMDA receptor NR2 subunits in cultured hippocampal neurons. *J Neurophysiol* **95**, 1727-1734.

Tovar K. R. and Westbrook G. L. (1999) The incorporation of NMDA receptors with a distinct subunit composition at nascent hippocampal synapses in vitro. *J Neurosci* **19**, 4180-4188.

Vanhoutte P. and Bading H. (2003) Opposing roles of synaptic and extrasynaptic NMDA receptors in neuronal calcium signalling and BDNF gene regulation. *Curr Opin Neurobiol* **13**, 366-371.

Williams K. (2001) Ifenprodil, a novel NMDA receptor antagonist: site and mechanism of action. *Curr Drug Targets* **2**, 285-298.

Williams K., Russell S. L., Shen Y. M. and Molinoff P. B. (1993) Developmental switch in the expression of NMDA receptors occurs in vivo and in vitro. *Neuron* **10**, 267-278.

Wong E., Ng F. M., Yu C. Y., Lim P., Lim L. H., Traynelis S. F. and Low C. M. (2005) Expression and characterization of soluble amino-terminal domain of NR2B subunit of N-methyl-D-aspartate receptor. *Protein Sci* **14**, 2275-2283.

Table 1

Summary of the fitting parameters of the dose-response curves which illustrate the inhibitory properties of MK801 and GK11 on cultured hippocampal neurones and HEK cells transfected with either NR1a/NR2A or NR1a/NR2B subunits.

	MK801		GK11	
	max ± SE	IC50 ± SE	max ± SE	IC50 ± SE
Neurones				
Site 1	54.1 ± 8.9 %	105 ± 54 nM	26.9 ± 9.7 %	262 ± 23 nM
Site 2	103 ± 4.6 %	55.9 ± 3.9 µM	111 ± 5.6 %	197 ± 31 µM
HEK				
NR1a/NR2A	104 ± 2.4 %	2.23 ± 0.3 µM	52.3 ± 2.6 %	5.25 ± 1.4 µM
NR1a/NR2B	95.9 ± 4.6 %	5.80 ± 1.3 µM	96.6 ± 5.4 %	37.4 ± 8.7 µM

FIGURE LEGENDS

Fig. 1 Pharmacological properties of MK801 and GK11 on native NMDA receptors. (A) Comparison of the neuroprotective properties of GK11 and MK801 on mature hippocampal cultures submitted to a glutamatergic stress. The survival rate of neurones was expressed as percentages of the number of neurones in control cultures (control -chg). Treated cultures were submitted to the glutamate insult followed by application of the neuroprotective drug at the appropriate concentration. Control -chg conditions represented sister cultures that were left untouched (no medium change); Control +chg conditions represented sister cultures that were submitted to the same number of medium changes as the treated cultures. (B-C) Comparison of the intrinsic neurotoxicity of GK11 and MK801 on mature hippocampal cultures 24h (B) or 48h (C) after challenging with the drugs. Viability of the neurones was assessed by determining the percentage of surface labelled with Map2 and is expressed as percentages of those measured in the control cultures. Treated cultures were challenged with different concentrations of the drugs, added directly to the culture medium. (D) Immunocytochemical detection of Map2 in cultured hippocampal cells, 48 hours after challenging with NMDA antagonists. In control cultures (1), thin neurites are dispersed on the surface of the dish, and pericarya are rarely stained. In MK 801 treated cultures, neurites are either packed in coarse cables extending in between cell clumps (2, 10 μ M) or retracted around clumps (3, 100 μ M) and some tightly packed pericarya are strongly stained. In GK11 treated cultures, neurites are either dispersed on the dish, with a few lightly stained pericarya (4, 10 μ M) or slightly bundled in between flattened groups of moderately stained pericarya (5, 100 μ M). Scale bar is 100 μ m.

Data are expressed as means \pm SEM of at least three independent experiments. Statistical analysis: *** $p < 0.001$ versus control -chg; °° $p < 0.01$ and °°° $p < 0.001$ versus glutamate; + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ MK801 versus GK11 (One-way ANOVA).

Fig. 2 Recovery from blockade by 10 μ M GK11 or 10 μ M MK801 on NMDA-induced currents in rat hippocampal neurones (DIV 14). (a) and (b) After blocking the response to 100 μ M NMDA with GK11 (a) or MK801 (b), the recovery of the NMDA-induced current was monitored at -70 mV by applying short pulses of 100 μ M NMDA at 5 min intervals. (c) and (d) After blocking the response to 100 μ M NMDA with GK11 (c) or MK801 (d), 100 μ M NMDA was applied continuously for 5 min at -70 mV. After 2 min washing, a short pulse of 100 μ M NMDA was then applied. (e) and (f) Following blocking with by 100 μ M NMDA and 10 μ M GK11 (e) or MK801 (f), 100 μ M NMDA was applied continuously for 5 min at +30 mV. After 2 min washing, a short pulse of 100 μ M NMDA was then applied. Data are expressed as percentages of the integrated area under the peak corresponding to the NMDA-induced current. At least 5 cells were used to quantify the effects (the actual number of cells used in the various conditions are indicated on the histograms).

Fig. 3 Inhibition effects of MK801 and GK11 on NMDA-induced currents in rat hippocampal neurons (DIV 14). (a) Representative whole cell NMDA-mediated currents recorded at -70mV. Evoked responses were elicited by short pulses of 100 μ M NMDA every 2 min and for a total duration of 15 min. (b) Whole cell NMDA-mediated currents (-70mV) evoked by 100 μ M NMDA (control) or by 100 μ M NMDA in the presence of either 1 μ M MK801 or 1 μ M GK11 (c) Sigmoidal fitting of the dose–response curve of the effects of MK801 (●) and GK11 (○) on the NMDA induced responses. Data are expressed as the percentages of the integrated areas under peaks corresponding to the NMDA-induced current. Each point is the means \pm SEM of n independent determinations, with n being indicated either above (MK801) or below (GK11) the points.

Fig. 4 Inhibition effects of MK801 and GK11 on NMDA-induced currents recorded on recombinant NMDA receptors expressed in HEK293 cells (-70mV). (a) On NR1a/NR2B,

whole cell NMDA-mediated currents evoked by 100 μ M NMDA (control) or by 100 μ M NMDA in the presence of either 30 μ M MK801 or 100 μ M GK11. (b) Sigmoidal fitting of the dose–response curve of the effects of MK801 (●) and GK11 (○) on the NMDA-induced currents measured on NR1a/NR2B recombinant receptor. (c) On NR1a/NR2A, whole cell NMDA-mediated currents evoked by 100 μ M NMDA (control) or by 100 μ M NMDA in the presence of either and 100 μ M NMDA and 30 μ M MK801 or 1 mM GK11. (d) Sigmoidal fitting of the dose–response curve of the effects of MK801 (●) and GK11 (○) on the NMDA-induced currents measured on NR1a/NR2A. Data are expressed as the percentage of the maximum peak value corresponding to the NMDA-induced current. Each point is the mean \pm SEM of n independent determinations, with n being indicated either above (MK801) or below (GK11) the points.

Fig. 5 Effects of MK801 and GK11 on synaptic versus extrasynaptic NMDARs on mouse cortical neurones in culture (DIV 14). (a) Typical imaging of intracellular calcium influx exposed to a stimulus activating synaptic NMDARs (by the co-application of 50 μ M bicuculline and 2.5 mM 4AP) and then blocked by 100 μ M APV. Effects of GK11 10 μ M and 100 μ M were tested after activation of synaptic NMDARs. The curve represents the mean ratio value of 40 neurones. (b) Histograms representing the effects of GK11 (at 10 and 100 μ M) on normalized synaptic and extrasynaptic NMDARs mediated calcium influx (in arbitrary units in %). Each bar represents the mean \pm SEM of 3 independent experiments and 120-130 neurones measured. (c) Typical calcium profile evoked by selective extrasynaptic NMDARs activation. Cortical neurones were sequentially exposed to stimuli activating synaptic NMDA receptors (50 μ M bicuculline and 2.5 mM 4AP) and after blocking synaptic NMDARs with 10 μ M MK801, to stimulate activating extrasynaptic NMDARs (50 μ M NMDA). Effects of GK11 10 μ M and 100 μ M were tested after a typical activation of extrasynaptic NMDARs. The curve represents the mean ratio value of 40 neurones. (d) Histograms representing the effects of MK 801 (at 1 and 10 μ M) on normalized synaptic and extrasynaptic NMDARs

mediated calcium influx (in arbitrary units in %). Each bar represents the mean \pm SEM of 3 independent experiments and 120-130 neurones measured.

Fig. 6 Modulation of spontaneous EPSCs on rat hippocampal neurones (DIV 19-21). (a) and (b) Representative examples of individual experiment showing the reduction of spontaneous activity by application of 100 μ M MK801 (a) or 100 μ M GK11 (b). Spontaneous EPSCs were recorded at -70 mV in the absence of Mg^{2+} and the presence of 2 μ M NBQX and 5 μ M Gabazine. (c) Effects of GK11 and MK801 on the frequency and the amplitude of the spontaneous EPSCs. Each bar is the mean \pm SEM of at least 6 independent determinations.

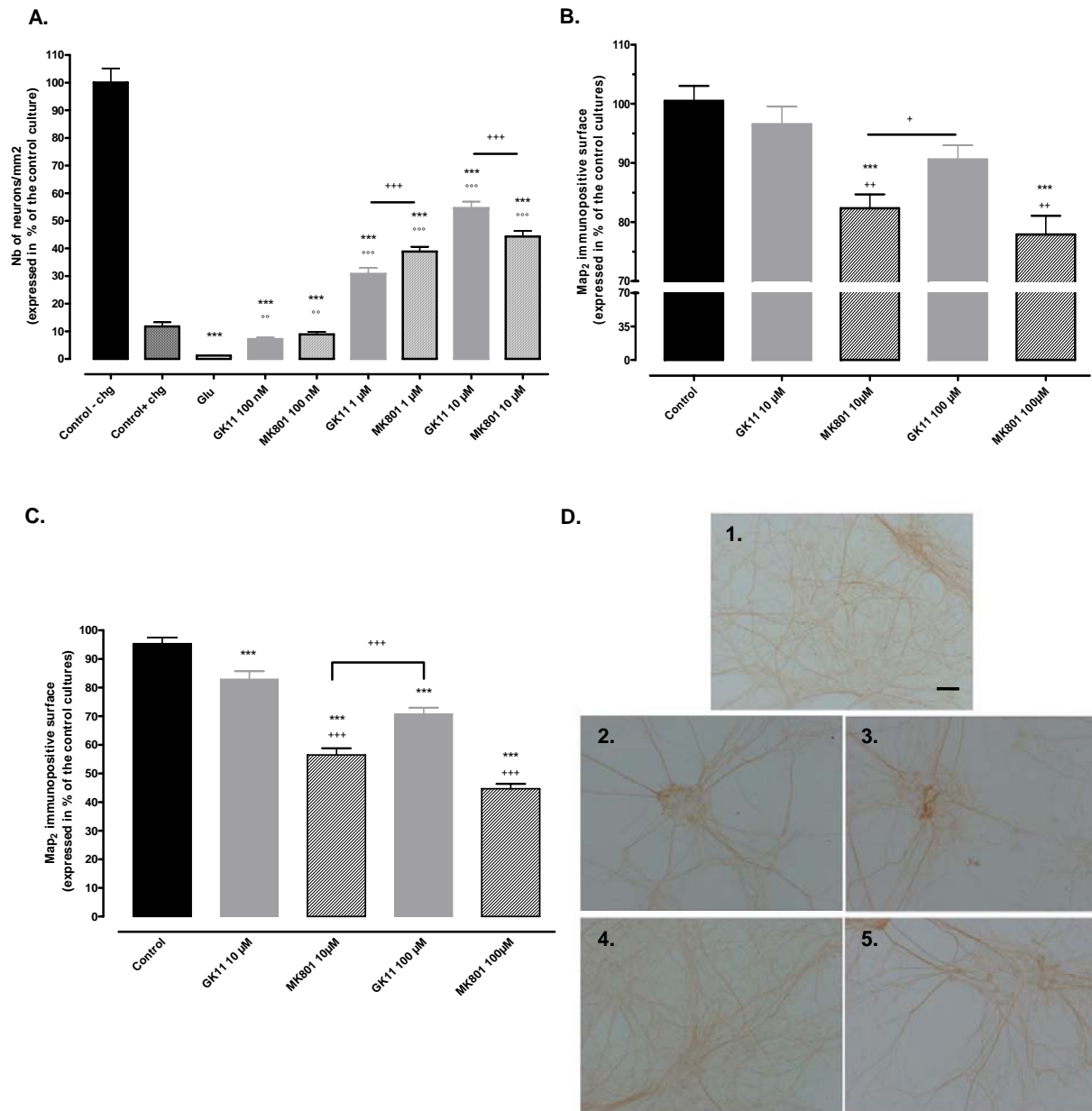


Figure 1
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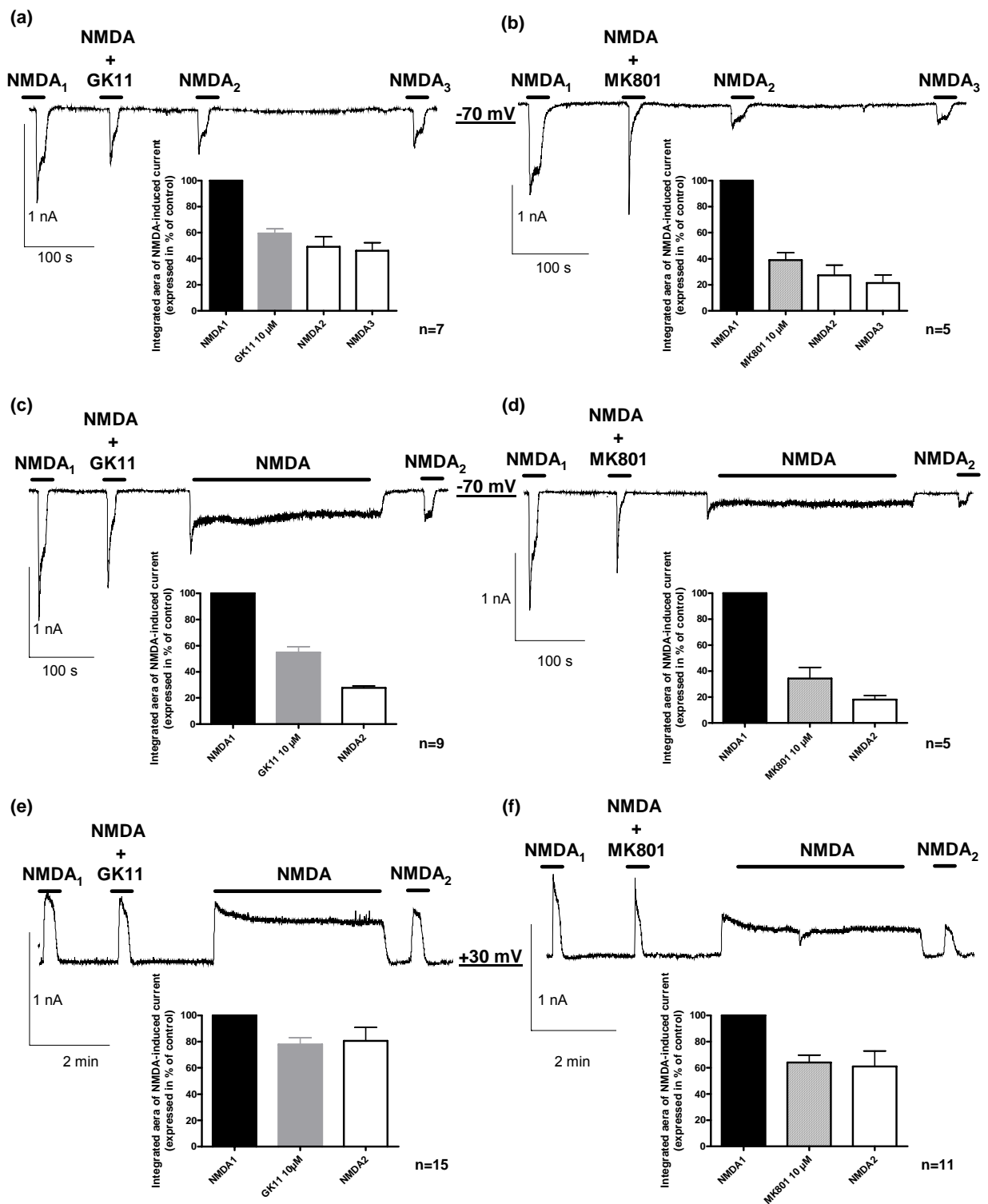


Figure 2

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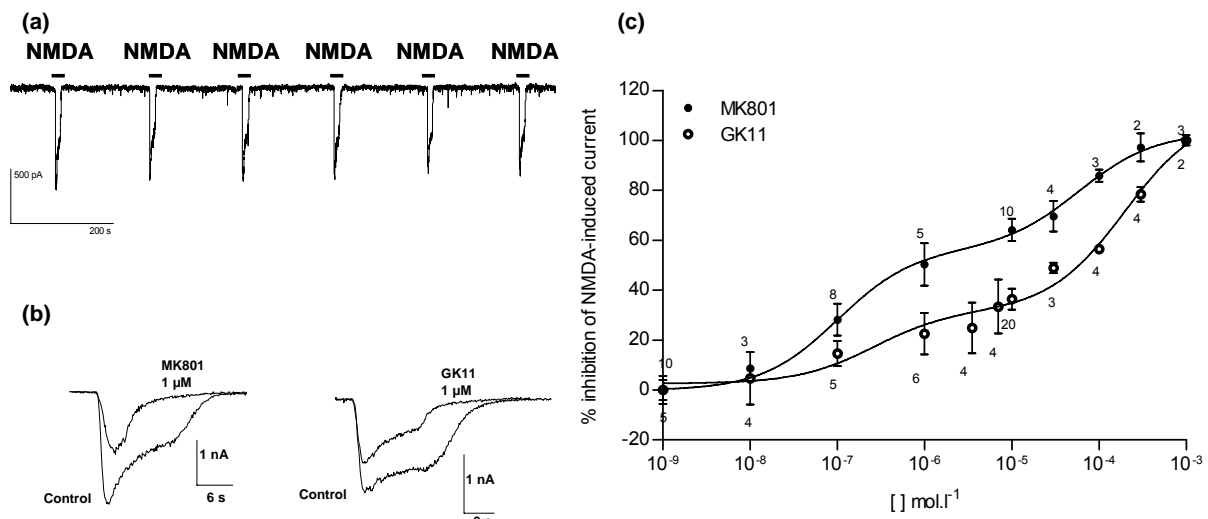


Figure 3

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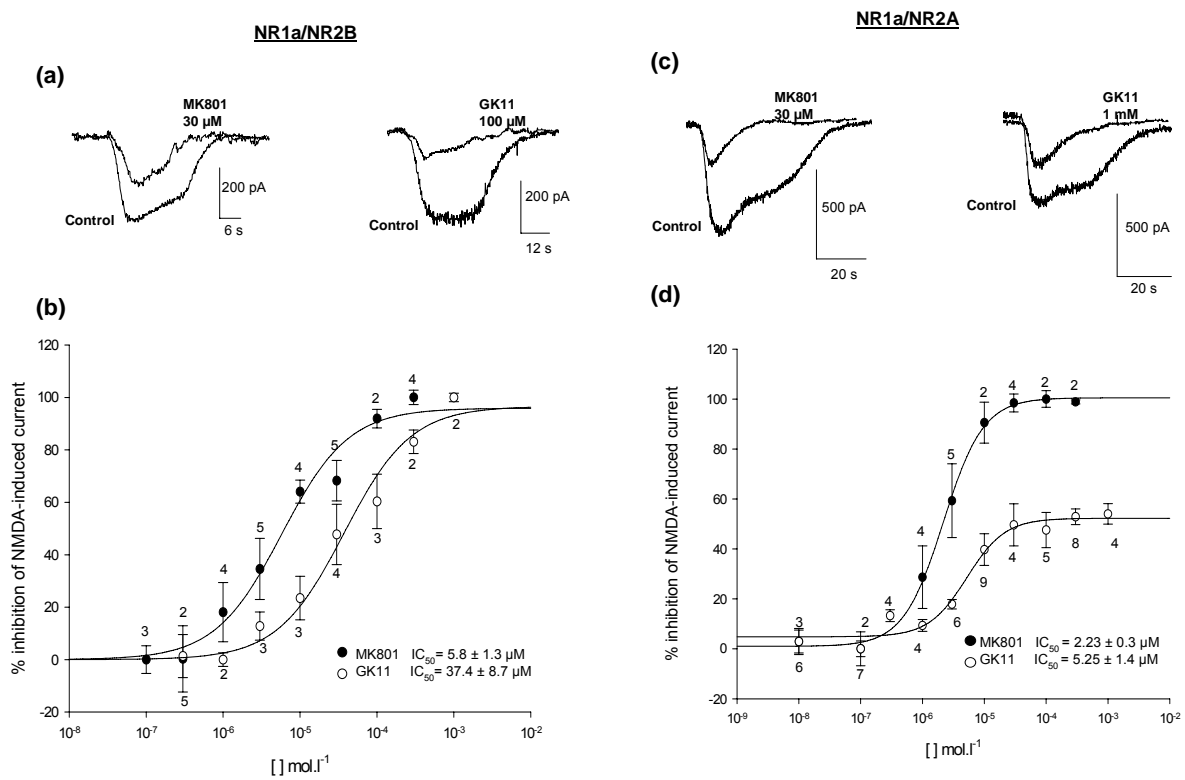


Figure 4

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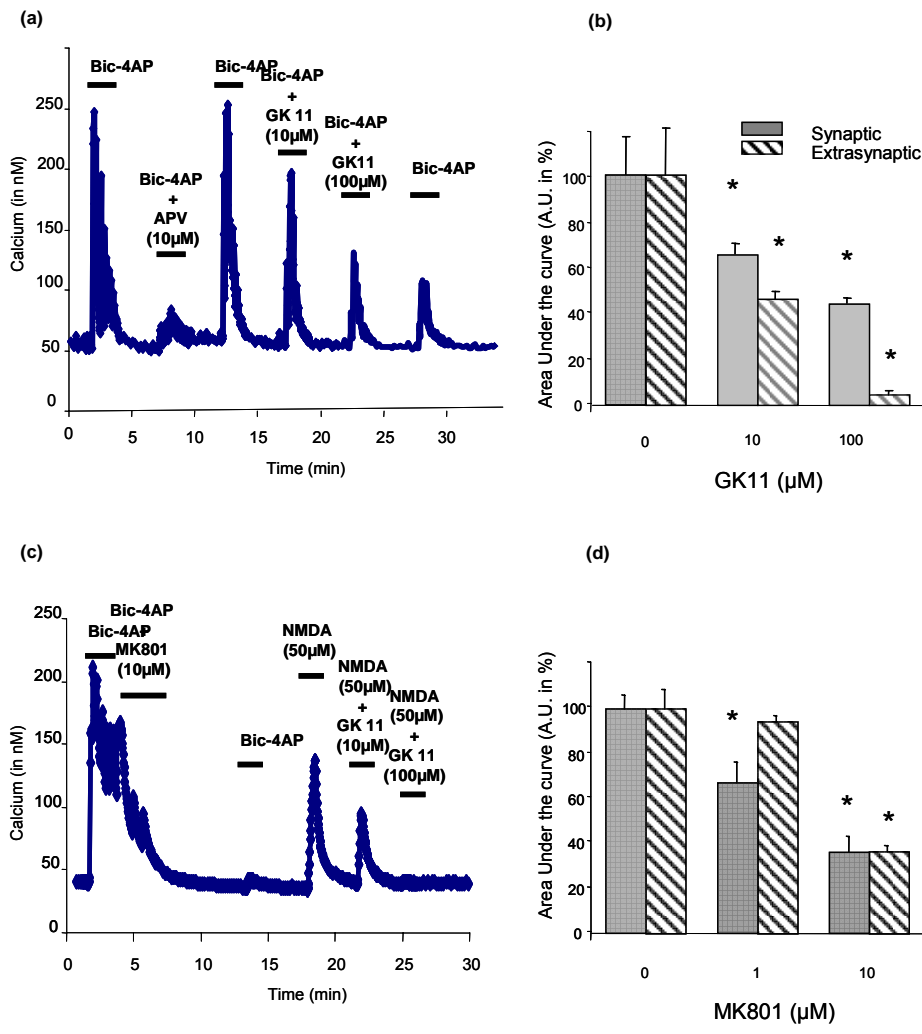


Figure 5
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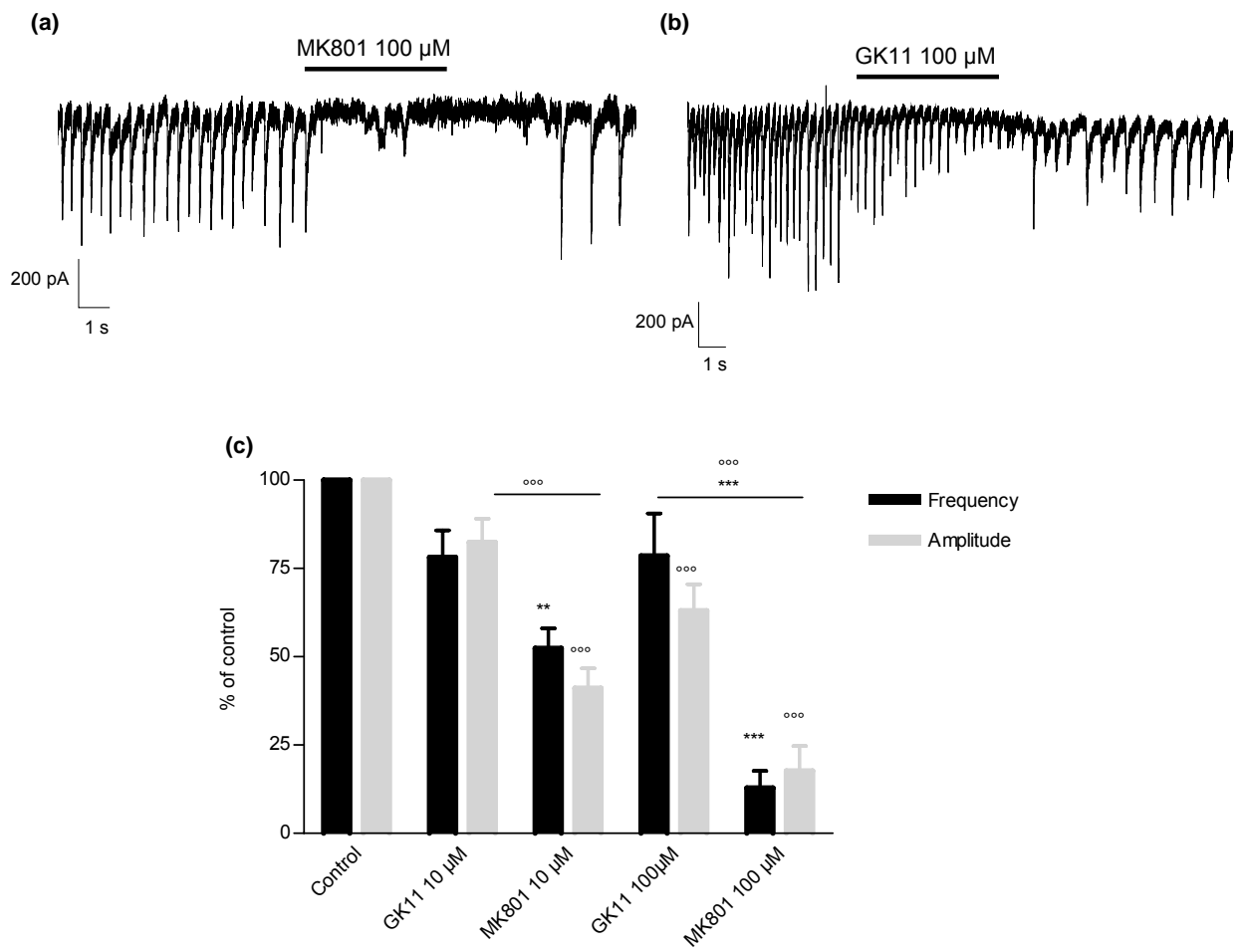


Figure 6

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