Effects of Pan- and Subtype-Selective N-Methyl-d-aspartate Receptor Antagonists on Cortical Spreading Depression in the Rat: Therapeutic Potential for Migraine

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ABSTRACT

Spreading depression (SD) has long been associated with the underlying pathophysiology of migraine. Evidence that the N-methyl-d-aspartate (NMDA) glutamate receptor (NMDA-R) is implicated in the generation and propagation of SD has itself been available for more than 15 years. However, to date, there are no reports of NMDA-R antagonists being developed for migraine therapy. In this study, an uncompetitive, pan-NMDA-R blocker, memantine, approved for clinical use, and two antagonists with selectivity for NMDA-R containing the NR2B subunit, (1S,2S)-1-(4-hydroxyphenyl)-2-(4-hydroxy-4-phenylpiperidino)-1-propanol (CP-101,606) and (±)-(R',S')-α-(4-hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidine propanol (Ro 25-6981), were investigated to assess their protective effects against SD in the rat. Under isoflurane anesthesia, d.c. potential and the related cortical blood flow and partial pressure of O2 (PO2) were recorded simultaneously at separate cortical sites. Drugs (1, 3, and 10 mg/kg i.p.) were given 1 h or 30 min before KCl application to the brain surface. Core temperature and arterial PCO2, PO2, and pH measurements confirmed physiological stability. KCl induced 7.7 ± 1.8 (mean ± S.D.) SD events with d.c. amplitude of 14.9 ± 2.8 mV. Memantine and CP-101,606 dose-dependently decreased SD event number (to 2.0 ± 1.8 and 2.3 ± 2.9, respectively) and SD amplitude at doses relevant for therapeutic use. Ro 25-6981 also decreased SD events significantly, but less effectively (to 4.5 ± 1.6), without affecting amplitude. These results indicate that NR2B-containing NMDA receptors are key mediators of SD, and as such, memantine- and NR2B-selective antagonists may be useful new therapeutic agents for the treatment of migraine and other SD-related disorders (e.g., stroke and brain injury). Whether chronic, rather than acute, treatment may improve their efficacy remains to be determined.

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Spreading depression (SD) is a slowly (2–6 mm/min) propagating wave of transiently increased cerebral blood flow, suppressed cortical activity, and loss of membrane potential.
diffusion, and blood flow during SD propagation can be detected using magnetic resonance imaging. SD has been associated with the underlying pathophysiological mechanisms of migraine aura for almost 50 years. Functional magnetic resonance imaging and magnetoencephalography studies indicate that cerebrovascular and magnetic field changes that may be related to SD occur in the visual cortex of migraineurs (for review, see Sanchez-del-rio et al., 2006). The models used to study SD can predict migraine therapeutic activity; for example, chronic, but not acute, treatment with sodium valproate or propranolol reduces migraine severity clinically and also inhibits SD generation in the rat (Bowyer et al., 2005; Ayata et al., 2006).

The precise mechanistic links between SD and migraine still require elucidation. But there is increasing evidence that SD can induce migraine headache (Moskowitz et al., 1993). Thus, cortical depolarization, equivalent to the aura phase of migraine, activates the trigeminal nociceptive pathway in the brainstem, thereby putatively precipitating the headache phase (Bolay et al., 2002; Kunkler and Kraig, 2003). In subjects with headache induced using glyceryl trinitrate, a brainstem region commensurate with the animal studies is activated in migraineurs but not in nonmigraineurs, suggesting a specific migraineur-related sensitivity (Afridi et al., 2005): this sensitivity could make migraineurs more susceptible to the effects of SD.

Nevertheless, the cellular mechanisms underlying the initiation and the propagation of SD remain enigmatic (Smith et al., 2006). Two main theories of SD propagation have been forged. The first is Grafstein's potassium hypothesis, in which K+ is released during neuronal depolarization and accumulates in the interstitial spaces; this excessive amount of K+ further depolarizes the surrounding cells, leading to a regenerative wave of SD. The second hypothesis, proposed by Van Harreveld, is based on glutamate release during SD and on the fact that glutamate itself has been shown to induce SD when applied to the cortex. However, others have questioned the ability of glutamate to trigger SD on its own (Obrenovitch and Zilkha, 1995); it seems likely that both mechanisms could contribute.

The native NMDA-R is composed of at least one NR1 subunit and one or more NR2 (A, B, C, and D) subunits. Expression of both NR1 and NR2 subunits is required to form functional channels. Numerous studies demonstrate that NR2B isoforms, present in the receptor, play a key role in the pharmacological and functional properties of NMDA-R (Dingledine et al., 1999; Loftis and Janowsky, 2003). NMDA-R containing the NR2B subunit are mainly expressed, in a synapse-selective manner, in the adult mammalian forebrain, including cortex and hippocampus (Loftis and Janowsky, 2003), tissues that support SD. Since Marrannes et al. (1988) confirmed that NMDA-R is an important component in the generation and propagation of SD and associated inward currents, a variety of NMDA-R antagonists have been shown to block cortical SD (Marrannes et al., 1988; Lauritzen and Hansen, 1992; Anderson and Andrew, 2002). The role of NMDA [but not kainate or AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate) receptors] in SD is suggested by the abilities of MK-801 (dizocilpine) and SKF-10047 (noncompetitive NMDA-R ion channel blockers) (Willette et al., 1994) and L-701,324 and ZD9379 (antagonists of the glycine site of the NMDA-R complex) (Obrenovitch and Zilkha, 1996; Tutlisumak et al., 1998), and CP-101,606 (traxoprodil) and ifenprodil (NR2B subunit-selective antagonists) (Menniti et al., 2000; Faria and Mody, 2004) to inhibit SD initiation and propagation.

NMDA-R antagonists block neurotoxicity induced by excessive glutamate release into the synaptic cleft. However, clinical trials of a large number of NMDA-R antagonists (mainly for stroke) have failed due to the side effects resulting from the blockade of normal neuronal function (James and Lunnon, 2005; Chen and Lipton, 2006). Contrasting with the more potent NMDA-R antagonists, memantine, an uncompetitive, low-affinity, open channel blocker, is clinically well tolerated (Kornhuber and Weller, 1997; Bullock, 2006). This safe clinical profile seems to result from its “use-dependent” prevention of hyperactivity of the NMDA receptor channel complex without disrupting normal activity (Kornhuber and Weller, 1997; Chen and Lipton, 2006).

The present study was therefore performed in a rat model of SD to further evaluate the role of the NMDA-R in vivo by investigating the effects of memantine and the NR2B-selective antagonists Ro 25-6981 and CP-101,606 on the initiation and propagation of SD events, determined using cortical d.c. potential recordings and the related changes in cortical blood flow (CBF) and cortical partial pressure of O2 (pO2). We show that these three compounds inhibit KCl-induced SD in a dose-related manner at concentrations that are therapeutically relevant, indicating that memantine, a drug currently licensed for human use, and inhibitors selective for NR2B subunits, are likely to be of therapeutic value for migraine and other SD-related disorders.

Materials and Methods

All work was conducted in compliance with United Kingdom Home Office guidelines and the Animals (Scientific Procedures) Act 1986, and all work was reviewed and approved by the GlaxoSmithKline Ethical Review Panel.

Rat Surgical Preparation. Male Sprague-Dawley rats weighing 270 to 330 g (Harlan UK Limited, Bicester, UK) were housed in the animal unit at GlaxoSmithKline (Harlow, Essex, UK) at 24°C on a 12-h light/dark cycle and fed with a pellet diet and tap water ad libitum.

Rats were anesthetized throughout the experiment with isoflurane (5% for induction, 2–3% during surgery, and 1% during the recordings) in a mixture of N2O and O2 (2:1). After induction, the trachea was intubated for connection to a pressure-cycled small animal ventilator (SAR-830/P; CWE Inc., Ardmore, PA). The femoral artery was cannulated to permit sampling for blood gas analysis and continuous blood pressure monitoring. The femoral vein was cannulated to permit the administration of compounds. Before KCl application and every 30 min until the end of the experiment arterial blood samples were obtained for measurement of pO2 and pCO2 and of pH.

Throughout the experiment, the core temperature of the animals was monitored using a rectal probe and maintained at 37.5 ± 0.4°C (mean ± S.D.) using a homeothermic blanket control unit. In addition, the physiological parameters of the animals were maintained at normal levels (mean arterial blood pressure 110 ± 30, pH 7.39 ± 0.06, pO2 120 ± 20, pCO2 40 ± 5; all means ± S.D.) by adapting the ventilation parameters.

The rat's head was placed in a stereotactic frame. The skull was exposed by a midline incision. Using a stereomicroscope for guidance, three holes were carefully drilled on the right side using a saline-cooled drill: one hole for KCl application (2.0 mm anterior and 1.0 mm lateral to bregma), one hole for d.c. potential recordings (3.0
mm posterior and 1.0 mm lateral to bregma), and one hole for CFBLDF and pO2 recordings (3.0 mm posterior and 2.0 mm lateral to bregma). The dura in each craniotomy was carefully excised so as not to injure the underlying brain. The craniotomies were then filled with mineral oil to prevent dehydration.

**Extracranial Field Potential Recordings.** Extracranial d.c. field potential recordings were made using Teflon-insulated, 0.25-mm-diameter Ag/AgCl microelectrodes (Harvard Apparatus Ltd., Edenbridge, Kent, UK). The final ~5-mm portion of the recording electrode was exposed and chlorinated with hypochlorite sodium. The insulated electrode was bent over (to prevent cortical damage) and then placed, using a micromanipulator, on the surface of the cortical parenchyma of the right parietal lobe; a Ag/AgCl reference electrode was inserted under the skin of the neck. Electrodes were connected to a d.c.-coupled field-effect input stage and amplified using a Neurolog NL834 four-channel d.c. preamplifier; d.c. signals were filtered with a 50-Hz low-pass filter.

**Laser Doppler Flux and Tissue Oxygen Partial Pressure Measurements.** Continuous and simultaneous recordings of cortical blood flow (CFBLDF), and oxygenation (pO2) and temperature were provided using a triple-sensor device (250 μm diameter; Oxy- lite/Oxynol; Oxford Optronix Ltd., Milton Park, Oxford, UK), inserted 800 μm into the parietal cortex. The careful insertion of the triple-sensor device into the parietal cortex did not trigger SD.

**Experimental Protocol.** Following surgery, animals were pre-dosed with test compounds as follows: 1 h before SD initiation, with 0.9% (w/v) saline (2 ml/kg i.p.), propranolol hydrochloride (20 mg/kg i.p.), sodium valproate (200 mg/kg i.p.), MK-801 hydrogen maleate (2 mg/kg i.v.), or memantine hydrochloride (1, 3, or 10 mg/kg i.p.); or 30 min before SD initiation with 0.9% (w/v) saline containing 2% (v/v) dimethyl sulfoxide and 10% (w/v) Kleptose HPB (designated “Klepto- tose”), Ro 25-6981 (1, 3, or 10 mg/kg i.p.), or CP-101,606 (1, 3, or 10 mg/kg i.p.). SD was then initiated by application of an ~3 mg KCl crystal to the parietal cortex. Recordings were acquired for at least 10-min pre-KCl (baseline) and for 1 h afterward. The experiment was terminated with an i.p. injection of Euthatal (150 mg/kg pentobar- bital sodium).

**Blood and Brain Sample Analysis.** To determine the concentra- tions of memantine, CP-101,606, and Ro 25-6981, one third of the animals had a blood sample taken from the femoral vein cannula (50 μl diluted 1:1 in sterile water) immediately before SD initiation and again immediately after the death of the animal, together with the brain. The tissue samples were frozen on dry ice, and they were stored at ~80°C before subsequent analysis. Blood and brain samples were extracted using protein precipitation. Rat brains were homogenized with sterile water [50/50 (v/v)]. Diluted blood (100 μl) and brain homogenate (50 μl) samples were extracted using 80:20 (v/v) acetonitrile/10 mM ammonium acetate containing, as internal standard, 0.05 μg/ml donepezil (250 μl). Samples were then mixed and centrifuged at 2465g for 20 min, and an aliquot of the superna- tant was analyzed by high-performance liquid chromatography/tan- dem mass spectrometry. Memantine, CP-101,606, Ro 25-6981, and the internal standard were separated using reverse phase chroma- tography-high-performance liquid chromatography (Agilent 1100; Agilent Technologies, Palo Alto, CA) with a mobile phase of 70% (v/v) 10 mM ammonium acetate containing 0.1% Formic acid and 30% (v/v) acetonitrile pumped at a flow rate of 1.0 ml/min. The stationary phase was a Cyano Discovery column (50 × 4.6 mm i.d., 5 μm; Supelco, Bellefonte, PA) in each detection. The Analyses of the analytes was determined using a heat-assisted electrospray interface in positive ion mode using a Sciex API4000 mass spectrometer (Applied Biosys- tems, Toronto, ON, Canada). Nominal multiple reaction monitoring transitions (m/z) for memantine, CP-101,606, Ro 25-6981, and donepezil were 180.1 to 106.8, 340.0 to 187.8, 540.0 to 187.8, and 380.1 to 90.8, respectively. Samples were assayed against calibration curves prepared in the appropriate matrix in the range 0.005 to 5.0 μg/ml. The lower limit of quantification for memantine, CP-101,606, and Ro 25-6981 was 0.005 μg/ml in the blood samples and 0.010 μg/ml for CP-101,606 and Ro 25-6981 and 0.1 μg/g for memantine in the brain samples. Brain concentrations of memantine, CP-101,606, and Ro 25-6981 were corrected for the residual blood contamination of the brain, which was estimated to be 15 μg/g brain tissue (Brown et al., 1986).

**Chemicals.** Chemicals were obtained from the following sources. KCl, propranolol hydrochloride, sodium valproate, MK-801 hydrogen maleate, memantine hydrochloride, Ro 25-6981, and dimethyl sulfoxide were from Sigma Chemical (Poole, Dorset, UK). Kleptose HPB was from Roquette UK Ltd. (Corby, Northamptonshire, UK). CP-101,606 was synthesized, and its chemical purity was ascertained by Medicinal Chemistry, GlaxoSmithKline.

**Data Analysis.** Data were recorded for offline analysis (Biopac MP100 data acquisition system; Linton Instruments, Norfolk, UK). Changes in extracellular d.c. field potential were analyzed for am- plitude of SD depolarization (millivolts) and number of SD depolar- izations. Changes in laser Doppler flow (relative blood perfusion units; rBPU) and pO2 (mm Hg; automatically corrected for brain temperature) associated with each cortical spreading depression event were assessed as percentage of increase, compared with the baseline, and number of events. Baseline represents the mean of all baseline values recorded during 120 s before any KCl-induced SD event, and it is expressed as 100%. Data were analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). Statistical comparisons were performed using a one-way analysis of variance (ANOVA) followed by post hoc analysis using Tukey’s multiple compari- son test. A Pearson correlation coefficient test was used to de- termine the degree of association between dose and effect. A signifi- cance level of p < 0.05 was adopted throughout. Data are presented as means ± S.D.

**Results**

**Initial Experiments and Model Validation.** Since the generation of SD events can be facilitated in animals with poorly controlled physiological parameters, we conducted a series of control experiments to exclude unwanted contribu- tions of neuronal hyperexcitability and/or changes in vasoconstriction or oligemia to our data. In addition to vehicle controls (Table 1), we studied the effects of the antiepileptic compound sodium valproate (200 mg/kg i.p.) and the β-blocker propranolol (20 mg/kg i.p.) administered acutely. Table 1 shows that neither control compound had any effect on the number (Table 1, top) or the amplitude (Table 1, bottom) of SD events generated, indicating, together with the stability of the monitored physiological parameters, that the operative procedures had minimal effect on cortical excitabil- ity. Next, we characterized the effects of the potent, nonse- lective NMDA receptor antagonist MK-801, which is known to powerfully inhibit SD, as a positive control. Consistent with reports in the literature, we found that MK-801 (2 mg/kg i.v.) blocked almost all SD events compared with ve- hicle (Table 1). Comparing effects on the CFBLDF and the pO2 changes related to the SD events, no significant difference was observed between the sodium valproate- and the propranolol-treated rats compared with the controls, except that the blood flow increase was slightly enhanced with sodium valproate to 236.5% of baseline; p > 0.05. By comparison, MK-801 was not associated with CFBLDF or pO2 changes in the very few events that remained in this group (Table 1). Two different vehicles were used in this study: 0.9% (w/v) saline (for memantine and Ro 25-6981) and Kleptose (for CP-101,606). Rats injected with these vehicles were not dif- ferent in terms of the number (Table 1, top) or amplitude (Table 1, bottom) of SD events. Using d.c. potential as the SD
The table shows the results of an experiment on the inhibition of SD by various compounds. The table includes the treatment groups (Saline, Kleptose, Sodium valproate, Propranolol, MK-801), the SD event number and amplitude, and the SD propagation velocities. The results indicate that certain compounds, such as MK-801 and Kleptose, significantly decreased the number of SD events compared with their respective vehicle controls. The dose of 3 mg/kg of MK-801 was sufficient to cause a statistically significant reduction in event amplitude.

**Cortical Blood Flow and pO2 Recordings.** Comparably with the d.c. potential recordings, SD propagation was associated with obvious and almost contemporaneous deflections in cortical CBFp and pO2 from baseline levels. As for d.c. potential-determined SD events, DMT, CP-101,606, and Ro 25-6981 at the highest dose all significantly decreased the number of SD events (9.03 ± 2.66 mV) (p < 0.05 versus vehicle). Comparing the results from all three doses, DMT and CP-101,606 at 10 mg/kg significantly reduced SD event number compared with the 1-mg/kg dose (p < 0.01).

**Inhibition of d.c. Potential-Determined Cortical SD by NMDA Antagonists.** Figure 2 shows that all three NMDA-related compounds inhibited SD generation measured using d.c. potential recording. The results indicate that these compounds, including memantine, Ro 25-6981, and CP-101,606, were effective at inhibiting SD propagation.

**Determinant, Topical KCl Application.** The application of 17.6 ± 1.75 SD events with amplitude 14.9 ± 2.8 mV per 60-min experiment in saline-predosed rats and 7.17 ± 1.17 SD events with amplitude 17.0 ± 1.7 mV following Kleptose. Using CBFp and pO2 changes as the SD determinant, 7.00 ± 2.00 events were observed following saline and, respectively, 7.00 ± 2.13 and 7.17 ± 1.17 following Kleptose. As a percentage of baseline (calculated as 100%), in saline-injected rats CBFp increased during SD by 180.2 ± 24.2% and pO2 by 203.2 ± 76.5%; Kleptose-injected rats showed an increase in CBFp of 205.3 ± 18.5% and an increase in pO2 of 139.3 ± 18.0% (Table 1). These results also indicate that SD, detected using d.c. potential electrodes, cortical CBFp, or pO2, was essentially identical in number, although the respective amplitudes could vary.

Figure 1 shows a representative original recording of the d.c. potential, CBFp, and pO2 during SD events induced by KCl application in a saline-injected rat. The traces clearly demonstrate the coincidence between SD events detected using the three methods, indicating that SD events could be detected reliably. The temporal offset in the d.c. potential and LDF peaks indicates the difference in time for events to arrive at the detection site from the site of initiation: based on their linear separation, SD velocities of 2 to 5 mm/min were calculated as expected. However, because the linear separation of the detectors at the brain surface was uncertain, and the actual SD trajectory could not be ascertained, SD propagation velocities were not quantified in detail. That events were usually detected at both sites indicates that SD events were relatively unconfined and probably spread right across the ipsilateral cortex. Attempts to evoke SD events using crystalline NaCl placed on the cortical surface instead of KCl were unsuccessful, indicating that SD events were KCl-specific and not mechanically induced.

**Inhibition of d.c. Potential-Determined Cortical SD by NMDA Antagonists.** Figure 2 shows that all three NMDA-related compounds inhibited SD generation measured using d.c. potential recording. Preloading the animals with memantine, Ro 25-6981, or CP-101,606 at the highest dose significantly decreased the number of SD events compared with their respective vehicles to 2.00 ± 1.79 after memantine, to 2.33 ± 1.97 after CP-101,606 (p < 0.001 versus saline and Kleptose, respectively), and to 4.50 ± 1.64 after Ro 25-6981 (p < 0.01 versus saline) (Table 2, top). Table 2, bottom, shows that the d.c. amplitude of the SD events that remained was also significantly decreased following memantine (7.52 ± 4.39 mV) and CP-101,606 (7.29 ± 5.42 mV) (p < 0.01 versus vehicle) but not after Ro 25-6981 (9.21 ± 5.82 mV).

At 3 mg/kg, these compounds also induced a significant decrease in the number of cortical spreading depression events to 4.17 ± 1.94 after memantine (p < 0.01 versus vehicle), to 4.67 ± 1.21 after CP-101,606, and to 5.00 ± 1.41 after Ro 25-6981 (p < 0.05 versus vehicle). But at this dose, only CP-101,606 was sufficiently efficacious to cause a statistically significant reduction in event amplitude (9.67 ± 4.48 mV) (p < 0.05 versus vehicle).

The lowest doses of memantine and Ro 25-6981 had little effect on SD amplitude and number, although memantine seemed to inhibit SD event number as detected with pO2 (2.75 ± 1.71 events compared with 7.00 ± 2.00 events in controls; p < 0.05). However, CP-101,606 significantly decreased the amplitude of SD events (9.03 ± 2.66 mV) (p < 0.05 versus vehicle). Comparing the results from all three doses, memantine and CP-101,606 at 10 mg/kg significantly reduced SD event number compared with the 1-mg/kg dose (p < 0.01).
respectively (p/H9262
bers of CBFLDF events to 3.50
CP-101,606 were associated with significantly reduced num-
2.50
ificant effect on the number of CBFLDF events. However, for
potential recordings, treatment at 1 mg/kg was without sig-
0.01 versus vehicle), respectively.

The main findings of this study are, first, that the NR2B-
selective antagonists CP-101,606 and Ro 25-6981 (at 3 and 10
mg/kg i.p. administered 0.5 h pre-SD initiation with KCl) and
the “use-dependent” pan-NMDA-R ion channel blocker me-
mantine (at 3 and 10 mg/kg i.p. administered 1 h before SD
initiation) inhibit, in a dose-related manner, SD observed in
the rat (Fig. 2; Table 2). At 1 mg/kg, none of the compounds was
active. These results add further experimental evidence im-
and to 4.17 ± 1.84 and 3.00 ± 2.97, respectively, after Ro
25-6981 (p < 0.05 versus vehicle).

At 3 mg/kg, whereas Ro 25-6981 (3 mg/kg) did not affect
the number of CBF_{LD} and pO_{2} events, both memantine and
CP-101,606 were associated with significantly reduced num-
bers of CBF_{LD} events to 3.50 ± 2.07 and 4.33 ± 1.86,
respectively (p < 0.05 versus vehicle), and pO_{2} events to
2.50 ± 2.38 (p < 0.05 versus vehicle) and 3.00 ± 2.28 (p <
0.01 versus vehicle), respectively.

Reflecting the number of SD events detected using d.c.
potential recordings, treatment at 1 mg/kg was without sig-
nificant effect on the number of CBF_{LD} events. However, for
pO_{2}, a decrease to 2.75 ± 1.71 (p < 0.05 versus vehicle) was
observed after memantine.

In all treated groups, CBF_{LD} and pO_{2} increases were
rather variable during SD events, and the amplitudes of the
SD events that remained after test compound treatment
were not significantly different from those after saline or
Kleptose. However, comparing the highest and lowest doses,
event amplitudes tended toward reduction at the higher
dose (e.g., memantine for d.c. potential and pO_{2}); for Ro
25-6981, this tendency was less obvious. The amplitudes of
pO_{2}-detected events following 10 mg/kg CP-101,606 were
significantly lower than those after 1 mg/kg CP-101,606
(Table 2).

Comparing the three doses, CP-101,606 tended to have
more profound, less variable effects. At the highest dose
CP-101,606 significantly reduced the number of CBF_{LD} events compared with the 3- and 1-mg/kg doses (p < 0.01 and
p < 0.001, respectively), and it decreased the number of pO_{2}
events compared with the 1-mg/kg dose (p < 0.01). A corre-
lation analysis indicated a statistically significant dose-de-
pendent effect on event number for memantine (r = −0.92)
and CP-101,606 (r = −0.97) (Table 2); although the corre-
lation coefficient for Ro 25-6981 was not significant, at −0.76 it
was still relatively strong, and SD event number was reduced
in relation to the dose.

Concentrations of Memantine, CP-101,606, and Ro
25-6981. To determine the concentrations of CP-101,606 and
Ro 25-6981 during the experiment, blood samples were taken
at 0.5 and 1.5 h and, for memantine, 1 and 2 h postdose.
Brain samples were taken at the last time point in each case.

Table 3 shows that, for all compounds, brain concentra-
tions were above the levels thought to be required to achieve
full efficacy (see Discussion). CP-101,606 and Ro 25-6981
blood concentrations increased in a dose-related manner, but
they declined rapidly between the 0.5- and 1.5-h sampling
time points following 1-, 3-, and 10-mg/kg i.p doses. CP-
101,606 blood and brain concentrations 1.5 h after the 10-
mg/kg i.p dose were 0.740 ± 0.635 and 1.115 ± 0.892 µM,
respectively, resulting in a mean brain/blood concentration
ratio of 21.895:1, indicating CP-101,606 to be brain-penetrant.
Likewise, Ro 25-6981 blood and brain concentrations at 1.5 h
following 10 mg/kg i.p were 0.442 ± 0.653 and 3.211 ± 4.232
µM, resulting in a mean brain-to-blood concentration ratio of
10.0:1. Memantine blood concentrations also increased in a
dose-related manner, but they seemed not to decline between
the 1- and 2-h sampling time points. Memantine blood and
brain concentrations 2 h after the 10-mg/kg i.p dose were
4.485 ± 1.632 and 95.879 ± 21.895 µM, resulting in a mean
brain-to-blood concentration ratio of 22.4:1.

Discussion
The main findings of this study are, first, that the NR2B-
selective antagonists CP-101,606 and Ro 25-6981 (at 3 and 10
mg/kg i.p. administered 0.5 h pre-SD initiation with KCl) and
the “use-dependent” pan-NMDA-R ion channel blocker me-
mantine (at 3 and 10 mg/kg i.p. administered 1 h before SD
initiation) inhibit, in a dose-related manner, SD observed in
vivo using d.c. potential and cortical CBF_{LD} and pO_{2} in the
rat (Fig. 2; Table 2). At 1 mg/kg, none of the compounds was
active. These results add further experimental evidence im-
Fig. 1. Representative tracings of direct current potential (millivolts) (A),
cortical blood flow (rBPU) (B), and cortical oxygenation (pO_{2}, mm Hg) (C)
recorded simultaneously during 1 h after KCl application at 0 min. KCl
application generated five depolarizations that corresponded with hyper-
emic and hyperoxygenation responses.
plicating the NMDA-R channel complex and, especially, the NR2B subunit in the mediation of SD.

Our results go further than those of other studies in that we directly and dose-responsively compared these compounds using a fully characterized in vivo rat model. SD generation with KCl was confirmed using recordings of d.c. potential, cortical CBFLDF, and cortical pO2. Animals were ventilated, and physiological monitoring confirmed arterial blood gases remained stable and in the normal range. The numbers of SD events generated in control animals were similar to those of other groups (Ayata et al., 2006). MK-801 (2 mg/kg i.v.), at a fully neuroprotective dose (Massieu et al., 1993), blocked SD triggering almost completely (Table 1) (Willette et al., 1994; Obrenovitch and Zilkha, 1996). Furthermore, compounds known not to inhibit SD generation when administered acutely in normal animals (sodium valproate and propranolol) (Ayata et al., 2006) were inactive. In addition, measured exposures of the NMDA-R blockers (Table 3) were pharmacologically relevant (see below).

Our results also show, for the first time, the inhibitory effect of memantine in SD. Interestingly, they support preliminary reports that memantine has efficacy in migraine (Cammarata and Krusz, 2005; Sorensen and Jenson, 2005). Memantine has for several years been used in the treatment of dementia without serious adverse effects (Bullock, 2006), and it is currently in trials for additional neurological disorders, including other forms of dementia, depression, glaucoma, and severe neuropathic pain (Chen and Lipton, 2006). Although many NMDA receptor antagonists have shown efficacy in numerous animal models of neurodegenerative diseases, the use of these compounds in clinical situations has been seriously limited by side effects (James and Lunnon, 2005); CP-101,606 has proven acceptable, although inactive (Yurkewicz et al., 2005). Memantine, uniquely, is an uncompetitive, open channel inhibitor that is claimed to better preserve the critical balance between blocking excessive “activity- (or use-) dependent” NMDA neurotransmission and permitting normal physiological function. Although the cause of the migraine aura itself remains unknown, there is increasing evidence that SD mediates the migraine attack (Sanchez-del-rio et al., 2006). Interestingly, aura and some neurological symptoms, including headache, in some patients with familial hemiplegic migraine, can be stopped with the noncompetitive NMDA-R antagonist ketamine (Kaube et al., 2000). Because memantine seems to have efficacy in migraine (Cammarata and Krusz, 2005; Sorensen and Jenson, 2005), our findings seem to provide further evidence that SD is involved. SD is also implicated in head injury (Strong et al., 2002). Our results suggesting that memantine may have
Memantine, Ro 25-6981, and CP-101,606 inhibit SD generation and amplitude

To indicate dose dependency, Pearson correlation coefficients were as follows: memantine, $r = -0.92$ ($p = 0.04$); CP-101,606, $r = -0.97$ ($p = 0.01$); Ro 25-6981, $r = -0.76$ ($p = 0.12$). Control values were 0.95% (w/v) saline for memantine and Ro 25-6981 and Kleptose for CP-101,606. Values are means ± S.D., and they are expressed as event number in millivols (SD events) and as percentage increase compared with the baseline (cortical CBFmax and pO2, calculated as 100%). For other details, see Table 1.

**TABLE 2**
Memantine, Ro 25-6981, and CP-101,606 inhibit SD generation and amplitude

In Table 2, SD event amplitudes were measured as the free base.

Drug concentrations and brain penetration in vivo

Timepoints were 0.5 and 1.5 h for CP-101,606 and Ro 25-6981, and 1 and 2 h for memantine. Lowest limits of quantification for blood and brain (corrected for residual blood contamination) were, respectively, 0.015 and 0.031 ppm for CP-101,606 and Ro 25-6981, and 1.64 ppm for memantine. Lowest limits of quantification for blood and brain (corrected for residual blood contamination) were, respectively, 0.015 and 0.031 ppm for CP-101,606 and Ro 25-6981, and 1.64 ppm for memantine.

**TABLE 3**
Drug concentrations and brain penetration in vivo

Timepoints were 0.5 and 1.5 h for CP-101,606 and Ro 25-6981, and 1 and 2 h for memantine. Lowest limits of quantification for blood and brain (corrected for residual blood contamination) were, respectively, 0.015 and 0.031 μM (CP-101,606), 0.015 and 0.029 μM (Ro 25-6981), and 0.028 and 5.58 μM (memantine), measured as the free base. n = 2 to 3 rats per time point; where S.D. is not given, n = 2.

**Additional therapeutic benefit in migraine and other SD-associated neurological disorders are therefore important.**

However, the compounds under investigation failed to block SD generation completely (Table 2; Fig. 2), unlike the noncompetitive NMDA-R ion channel antagonist MK-801 (at 2 mg/kg i.v. administered 1 h before SD initiation), even though they achieved brain exposures consistent with full efficacy.

For example, the concentrations of Ro 25-6981 and CP-101,606 achieved in these studies (Table 3) are $>$150-fold higher than their respective IC50 values measured in receptor binding studies in vitro (IC50 = 3.4 and 6.1 nM, respectively), and they are consistent with good in vivo receptor occupancy (≥50% receptor occupancy achieved at 0.98 and 1.91 mg/kg s.c., respectively) (Grimwood et al., 2000; Murray et al., 2000). Likewise, the concentrations of memantine achieved (1.1 and 5.0 μM at the 3- and 10-mg/kg dose, respectively; Table 3) are similar to those claimed to be therapeutically relevant from previous studies in vivo (0.4–1.0 μM) (Danyasz et al., 1997; Chen and Lipton, 2006). Moreover, a brain exposure of approximately 96 μM (at 10 mg/kg; Table 3) greatly exceeds published IC50 values of 6.7 μM in rat cortex (Porter and Greenamyre, 1995). Menniti et al. (2000) observed complete inhibition of electrically stimulated SD with CP-101,606 at a plasma concentration of 250 ng/ml (0.76 μM). Table 3 shows that with the highest dose of CP-101,606, blood concentrations were 2.79 μM at 0.5 h (0.75 μM at 1.5 h), suggesting that comparable levels were achieved. That...
Menniti et al. (2000) blocked SD completely with CP-101,606, whereas the blockade we observed was incomplete, may be due to the precise methods of SD initiation. Our results also complement literature findings with the NR2B-selective antagonist ifenprodil in spontaneous SD generated in mouse entorhinal cortex slice preparations (Faria and Mody, 2004). Given the significant brain concentrations achieved, as discussed above, why do memantine, CP-101,606, and Ro 25-6981 not block SD completely? First, NMDA ion channel blockers displac elastotolin 2[H]MK-801 fully from the NMDA-R site in vitro and in vivo, whereas NR2B-selective compounds do not (Murray et al., 2000). This reflects the fact that NR2B receptors represent only a proportion of the total NMDA receptor population; indeed, maximum efficacy for NR2B “ifenprodil site” ligands was noted to be 60 to 70% of the maximum in the study by Murray et al. (2000). Furthermore, NR2B antagonists such as ifenprodil and Ro 25-6981 decrease open channel probability via an allosteric site on an extracellular domain of the receptor (Kew et al., 1996; Fischer et al., 1997; Perin-Dureau et al., 2002); whereas allosteric regulation causes robust receptor inhibition or “shutdown”, it is unlikely to reach completion. Second, like CP-101,606 and Ro 25-6981, memantine favors certain NMDA-R subtypes (Parsons et al., 2000), and, due to its lower potency and unique uncompetitive and voltage-dependent mechanism, may be sparing of physiological levels of NMDA-R activity (Chen and Lipton, 2006). Again, this is consistent with a lesser effect on SD due to a reduced level of NMDA-R inhibition (relative to that achieved by MK-801) in our experiments and to the superior tolerability of this compound noted in the clinic. Only a small proportion of the total ion fluxes underlying SD are likely to be mediated by activated NMDA-R; if these are not maximal (and SD is not thought to be an excitotoxic process per se), this may also explain why memantine is not fully inhibitory.

Ro 25-6981 is thought, unlike CP-101,606, to bind NMDA-R containing NR1/NR2A/NR2B (Chazot et al., 2002) in addition to NR1/NR2B. Direct comparison of Ro 25-6981 and CP-101,606 (Fig. 2) suggests that Ro 25-6981 is actually inferior to CP-101,606 (and memantine) in blocking SD; Ro 25-6981 also had relatively little impact on SD amplitude compared with memantine, or CP-101,606 especially. Thus, any activity of Ro 25-6981 versus NR1/NR2A/NR2B receptors yields no additional discernible efficacy here. Perhaps the apparently reduced activity of Ro 25-6981 may reflect differences in the precise pharmacology or interaction of these compounds at NMDA receptors or a wider range of uncharacterized targets. Because some antimigraine compounds (e.g., sodium valproate and propranolol) are known to be active only when taken prophylactically (Bowyer et al., 2005; Ayata et al., 2006, and references therein) and NMDA receptors may play a key role in the initiation as well as the propagation of this event, it seems worth investigating whether chronic administration yields improved activity or therapeutic differences between the compounds or an improved therapeutic index.

The inhibitory action of MK-801, Ro 25-6981, CP-101,606, and memantine on SD generation emphasizes an active neurotransmitter role for NMDA receptors in SD. The fact that NR2B localization seems overwhelmingly neuronal (Lofts and Janowsky, 2003) does, however, seem at odds with early research, suggesting that SD was not action potential-mediated because SD propagation seemed possible in the presence of tetrodotorphin (TTX), the Na+ channel inhibitor (although TTX-resistant Na+ channels are also known) (Smith et al., 2006, and references therein). Nevertheless, glial cells also express NMDA-R (Gallo and Ghiani, 2000), and glial cells are also known to be actively involved in modulating SD propagation (Smith et al., 2006). Thus, the TTX paradox deserves further investigation.

Finally, that nonsynaptic-selective NMDA-R channel blockers such as MK-801 inhibit SD propagation almost completely, whereas NR2B-selective antagonists are only partially inhibitory, also suggests, most simply, that other NMDA-R subtypes not blocked by CP-101,606 and Ro 25-6981 also mediate SD. Their cellular location could also be influential, since both neurons and glia participate in SD propagation. Thus, the role of NMDA-R subtypes, metabolic glutamate, and other receptor types in SD propagation could be illuminated by the use of prophylactic antimigraine and other compounds, such as topiramate (Czapinski et al., 2005) and the partial NMDA-R agonist 1-aminoacyclopropanecarboxylic acid (Sheinin et al., 2002). That chronic, but not acute, administration of topiramate, sodium valproate, and propranolol can inhibit SD generation (Ayata et al., 2006) suggests a gene regulatory effect, the understanding of which could also clarify the mechanisms and possibly the pathophysiology of SD propagation, which still remain largely obscure.

Different types of SD have been described based on their properties. For example, in the cat cerebral cortex, the novel antimigraine agent tonabersat blocks the train of secondary SD events that occur subsequent to the primary KCl-evoked event but not the primary event itself (Smith et al., 2006). That memantine and CP-101,606 decrease SD amplitude may also reflect SD modulation: in the cat SD amplitude, velocity and cortical distribution measured using diffusion-weighted magnetic resonance imaging can co-vary, suggesting that although in the present study these compounds may not have blocked SD completely, the SD events remaining may be significantly less influential.

In summary, our data show that an uncompetitive, use-dependent NMDA-R ion channel blocker (memantine) and two NR2B-selective antagonists (CP-101,606 and Ro 25-6981) inhibit, in a dose-related manner, KCI-induced spreading depression and the related increases in CBF_{LDF} and pO2 in the rat. The data support interest in memantine as a new therapeutic strategy for migraine and other SD-associated diseases. Our data also suggest the value of NR2B-selective antagonists for antimigraine therapy: this compound class has shown a better efficacy and safety profile compared with nonsynaptic-selective drugs. The development of a new generation of NR2B antagonists, perhaps with improved pharmacokinetic characteristics, seems to be key for future progress in neurological disease (Chazot, 2004). Whether chronic administration of these compounds before SD initiation can improve efficacy (Bowyer et al., 2005; Ayata et al., 2006), and possibly tolerability, remains to be determined.

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References


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