The effect of (±)-CP-101,606, an NMDA receptor NR2B subunit selective antagonist, in the Morris watermaze

Martin R. Guscott[a],[*], Hannah F. Clarke[b], Fraser Murray[a], Sarah Grimwood[c], Linda J. Bristow[d], Peter H. Hutson[a]

[a] Merck, Sharp and Dohme, The Neuroscience Research Centre, Behavioural Neuroscience, Terlings Park, Harlow CM20 2QR, UK
[b] Department of Experimental Psychology, University of Cambridge, Cambridge CB2 3EB, UK
[c] Pfizer Global Research and Development, Ann Arbor, MI, USA
[d] Merck & Co., Inc., MRLSDB1, 3535 General Atomics Court, San Diego, CA 92121, USA

Abstract

It is well established that the NMDA receptor antagonists block hippocampal long-term potentiation and impair acquisition in the Morris watermaze task, although the role of individual NMDA receptor subtypes is largely unknown. In the present study, we compared the effects of (±)-CP-101,606, an antagonist selective for NMDA receptor NR1/NR2B subunit-containing receptors and the nonselective NMDA receptor antagonist MK-801, on acquisition in the Morris watermaze. Male hooded Lister rats were given 4 trials/day to find a fixed hidden platform submerged beneath the opaque water of the Morris watermaze. Twenty-four hours after the last acquisition trial, a 'probe trial' was conducted to assess the rat's spatial memory for the location of the hidden platform. Those rats treated with MK-801 (0.1 mg/kg, i.p.) 60 min prior to the acquisition and probe trials took significantly longer to find the hidden platform during training and spent significantly less time searching the platform's location during the probe trial than vehicle-treated rats. In contrast, 60-min pretreatment with (±)-CP-101,606 (60 mg/kg, p.o.), a dose that fully occupied hippocampal NR1/NR2B subunit-containing receptors, as determined by ex vivo NMDA receptor-specific [^{3}H]ifenprodil binding immediately following watermaze experiments, had no effect on acquisition or the probe trial. These results suggest that antagonists selective for NR1/NR2B subunit-containing receptors may not impair spatial memory in rats in the Morris watermaze.
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1. Introduction

The NMDA receptor is one of three classes of ionotropic glutamate receptors that mediate the effects of glutamate, the primary excitatory amino acid transmitter, in the central nervous system (CNS). The cDNAs encoding various NMDA receptor subunits have been cloned and characterised (Monyer et al., 1992; Kutsuwada et al., 1992). The NMDA receptor is a ligand-gated ion channel composed of multiple protein subunits grouped into three families; the NR1 subunit, of which there are at least eight splice variants (NR1a–h) and the NR2 subunit which has four subtypes (NR2A–D) each encoded by a separate gene. A third family

of NMDA receptor subtypes has also been described (Das et al., 1998; Chatterton et al., 2000). While the composition and stoichiometry of native NMDA receptor subunit combinations is unclear, it is necessary for at least one NR1 subunit to be combined with one or more NR2 subunits to form functional NMDA receptors with different pharmacological properties (Laurie and Seeburg, 1994; Buller et al., 1994; Priestley et al., 1995). The NR1 subunit is widely expressed throughout the CNS, whereas the NR2 subunits display regional heterogeneity in their expression. NR2B subunit-containing NMDA receptors are restricted to the adult mammalian forebrain and spinal cord with barely detectable levels in the cerebellum. In contrast, NR2C containing NMDA receptors are confined mainly to the cerebellum thalamus and olfactory bulb while the NR2D subunit is found in the diencephalon, mesencephalon and brain stem (Wenzel et al., 1995). NMDA receptors may be involved in the aetiology of neurodegenerative and psychi-

* Corresponding author. Tel.: +44-1279-440566; fax: +44-1279-440369.
E-mail address: martin_guscott@Merck.com (M.R. Guscott).

atric disorders including stroke, head injury (Menniti et al., 1997), epilepsy (Tzschentke, 2002; Koek and Colpaert, 1990; Mintz et al., 1990; Schmutz et al., 1990), Parkinson's disease (Shuto et al., 2001; Blandini et al., 2001) psychoses and analgesia (Boyce et al., 1999; Parsons, 2001). Consequently, there has been considerable interest in the clinical potential of NMDA receptor antagonists (see Kemp and McKernan, 2002 for recent review). However, the therapeutic value of nonselective antagonists has been limited by their extensive side effects, which include psychotomimetic symptoms and effects on learning and memory. Numerous experiments have demonstrated a link between the NMDA receptor and learning and memory processes (for review, see Castellano et al., 2001). Both pre-training and post-training treatment with NMDA receptor antagonists have been shown to impair memory in animals tested in various different tasks (e.g. Morris watermaze, contextual conditioning). Memory impairments are also evident when non-subtype selective NMDA receptor antagonists (e.g. the glutamate site antagonist, (±)-2-amino-5-phosphonopentanoic acid (AP5) are injected into different brain structures, including amygdala and hippocampus. Most notably learning deficits produced by post-training administration of NMDA receptor antagonists and the lack of effect on retrieval of spatial information suggest that they directly affect memory consolidation without affecting nonspecific performance related impairments (Steele and Morris, 1999). It is thought that NMDA receptor antagonists may disrupt memory processes through blockade of NMDA receptor-dependent synaptic plasticity including the formation and maintenance of long-term potentiation.

The heterogeneity of NMDA receptor subtype distribution in the CNS has encouraged the development of subtype selective compounds with the possibility that such compounds may lack the behavioural side effects seen with nonselective NMDA receptor antagonists such as MK-801. Ifenprodil selectively blocks NR2B-containing NMDA receptors (Williams, 1993; Grimwood et al., 1996) and has been shown to have antinociceptive effects at doses that do not impair motor performance (Boyce et al., 1999), suggesting that this class of NMDA receptor antagonist may have a wider therapeutic window compared with the nonselective NMDA receptor antagonists. Recently, more potent and selective NMDA NR2B subunit antagonists have been developed including CP-101,606 (for review, see Chazot, 2000). This compound is reported to demonstrate a greater therapeutic window than ifenprodil in suppressing hyperalgesia in animals models of chronic pain (Taniguchi et al., 1997; Boyce et al., 1999) and to lack the cognitive side effects of other NMDA receptor antagonists, (Weber et al., 1999; Okiyama et al., 1997). In the present study, we compared the effects of the nonselective NMDA receptor antagonist, MK-801 with (±)-CP-101,606 in the Morris watermaze, a hippocampal-dependent test previously shown to be sensitive to nonselective NMDA receptor antagonists.

2. Methods

2.1. Animals

Male hooded Lister rats were used (220–250 g, Harlan, UK). They were housed in groups of five for at least 1 week prior to testing. Animals were maintained on a 12-h light/dark cycle (lights on at 0700 h) with food and water freely available. They were kept in a humidity and temperature controlled room (55% ± 10; 21 °C ± 2) with sawdust bedding. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act (1986) and its associated guidelines.

2.2. Behavioural studies

The Morris watermaze used consisted of a white fibre-glass 2 m-diameter circular pool, filled with an opaque mixture of water and white non-toxic dye (E308, Morton) maintained at 26–28 °C. The pool was located in the centre of a sound attenuated room. Around the outside of the pool 3D 'extra maze cues' were displayed. 'North' was arbitrarily determined and the pool was divided up into four equal quadrants: 'Northeast', 'Southeast', Southwest', and 'Northwest'. A hidden platform (14 × 14 cm) submerged 1 cm below the water surface was placed in the middle of one of the quadrants. A closed-circuit video camera, fitted with a wide-angle lens, was mounted directly above the centre of the pool and connected to an image analyser, which digitised the image. The digital information was relayed to a PC running 'HVS Water32', a software package supplied by HVS (UK). The software package provided the following measures: latency to reach the platform, length of path taken, average swimming speed, the time spent and the total distance travelled in the target quadrant.

During the acquisition phase of the experiments, each rat was given four trials each day until they took approximately 15 s to find the platform. During this time, the hidden platform was submerged in the watermaze. For each daily trial, the rat was taken from the home cage and placed into the watermaze at one of four quasi-randomly determined locations ('North', 'East', 'South', 'West') with its head facing, and almost touching, the pool wall. Trials began when the rat was released by the experimenter and ended when it climbed onto the platform and the mean escape latency was recorded. The maximum trial length was 60 s. If by that time, the rat had not climbed onto the platform the trial ended automatically, the experimenter placed the rat on the platform and an escape latency of 60 s was recorded. The rat remained on the platform for 30 s and was then removed to a high sided opaque plastic container for a further 30 s (intertrial interval). At the end of the intertrial interval, the rat was placed in the pool again, but at a different location and upon release the next trial began. Normally, the escape latency declines during acquisition from 60 s to around 15 s as the animal learns the location of

the hidden platform. Twenty-four hours after the last acquisition trial, a 'probe trial' was used to assess the rat's spatial retention of the location of the hidden platform. During this trial, the platform was removed from the maze and the rat was allowed to search the pool for 60 s before being removed. The animals were pretreated with test compound exactly as during the acquisition phase of the task. During this time, animals should spend more time searching the quadrant that previously contained the hidden platform than the other three quadrants.

Attenuation in performance that may be due to nonspecific effects (such as visual or motor impairment resulting in a reduced ability to swim to the platform) rather than deficits in cognitive processes per se was determined using a visual platform trial. Animals were tested in a visible platform trial on the day immediately following the probe trial. During the visual trial, the platform was placed in a different quadrant to that used during the acquisition phase, and raised 1 cm above the water level such that it was visible. Animals were dosed with test compound then tested at the same time after dosing as during the acquisition of the task, and in the probe trial. Absence of any difference from the latency of rats to reach the visible platform is required to attribute any difference in the escape latency for the hidden platform trials to be due to an affect on cognitive processes.

2.3. [³H]MK-801 and [³H]ifenprodil binding to rat forebrain membranes ex vivo

Immediately following the watermaze experiment, animals were humanely killed for binding studies. At the same time, a separate batch of animals was also pretreated with ifenprodil (30 mg/kg, i.p.) or MK-801 (3 mg/kg, i.p.) and humanely killed 60 min later. The forebrain (i.e., whole brain minus cerebellum) was rapidly removed and both the cortex and hippocampus dissected, weighed, and homogenised in 24 volumes of ice-cold 50 mM Tris–acetate buffer (pH 7.0) using a Polytron homogeniser for 10 s. Homogenates were briefly stored on ice before NMDA receptor-specific [³H]ifenprodil or [³H]MK-801 binding was determined. Ex vivo [³H]ifenprodil binding was as described by Grimwood et al., 2000. Brain homogenate of 300 μl was incubated with 150 μl 50 mM Tris–acetate buffer (pH 7.0), 100 μM R(+)-3-(3-hydroxyphenyl)-N-propylpiperidine hydrochloride [R(+)-3-PPP], 1 μM 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-[3-phenylpropyl]-piperazine dihydrochloride (GBR-12909), and 1 μM 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (GBR-12935) (to mask non-NMDA receptor-specific [³H]ifenprodil binding sites) and 50 μl 5 nM [³H]ifenprodil for 2 h on ice. Free radioligand was separated from bound by filtration through Whatman GF/B filters presoaked in ice-cold assay buffer containing 0.05% polyethylenimine, using a Brandel cell harvester. Filters were soaked overnight in 10 ml of Hydrofluor scintillation fluid (National Diagnostics) for quantification of radioactivity

using a β-counter. For ex vivo [³H]MK-801 binding, 300 μl of homogenate was incubated with 150 μl of 5 mM Tris–acetate buffer pH 7.4 containing 100 μM glutamate, 100 μM glycine and 50 μl of 2 nM [³H]MK-801 at room temperature for 2 h. Nonspecific binding levels for [³H]ifenprodil and [³H]MK-801 were defined by injecting groups of animals with either ifenprodil (30 mg/kg, i.p.) or MK-801 (3 mg/kg, i.p.), respectively.

2.4. Drugs

MK-801 and ifenprodil were obtained from Tocris Cookson. (±)-CP-101,606 was synthesised in the Medicinal Chemistry Department, MSD, Harlow, UK. MK-801 (vehicle = 0.9% saline) was administered 60 min before testing in a dosing volume of 1 ml/kg. (±)-CP-101,606 (vehicle = distilled water) was administered 60 min before testing in a dosing volume of 3 ml/kg. [³H]Ifenprodil (40–50.3 Ci/mmol) and [³H]MK-801 (22.3 Ci/mmol) were from NEN Life Science Products (Boston, MA, USA).

2.5. Statistics

Mean data were analysed by between subjects analysis of variance (ANOVA) followed by Dunnett's *t*-test or within subjects repeated measures ANOVA using BMDP statistical software package. $P < 0.05$ was taken as significant. All data are presented as mean ± S.E.M. unless otherwise stated.

3. Results

3.1. Behavioural studies

Preliminary studies (data not shown) suggested that MK-801 at doses higher than 0.1 mg/kg, i.p. caused significant motor impairments and severely affected the animals' ability to complete the watermaze task. Therefore, a relatively low dose of MK-801 producing approximately 25% occupancy of NMDA receptors was used in this study in an effort to assess specific effects on learning and memory without motor effects confounding the experiment. Vehicle-treated and drug-treated animals in both experiments showed a reduced escape latency with repeated acquisition trials (Fig. 1) (Repeated measures ANOVA; MK-801: $F(6168) = 38.42$; $P < 0.01$; (±)-CP-101,606: $F(6132) = 40.39$; $P < 0.01$). Repeated measures ANOVA also revealed an effect of treatment on escape latency in the MK-801 ($F(1,28) = 18.04$; $P = 0.0002$) but not the (±)-CP-101,606 experiment ($F(1,22) = 0.74$; $P = 0.3974$) and no treatment/trial interaction in either experiment (MK-801: $F(6168) = 0.54$; $P = 0.7795$; (±)-CP-101,606: $F(6132) = 0.63$; $P = 0.7053$). Analysis of distance travelled confirmed the results found with escape latency
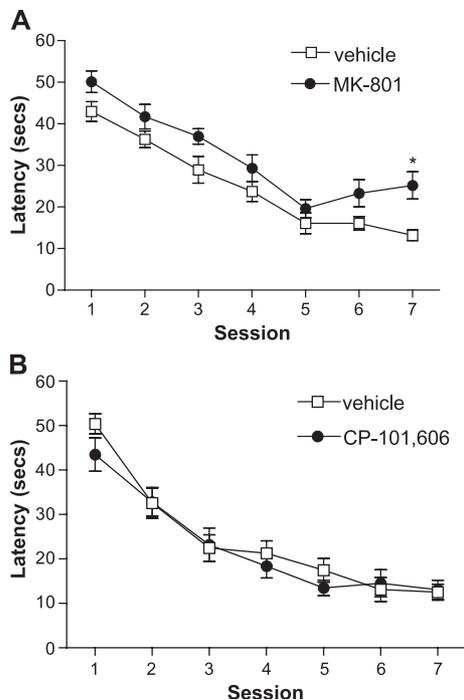
Fig. 1. The effect on MK-801 (0.1 mg/kg, i.p.) (A), and (±)-CP-101,606 (60 mg/kg, p.o.), (B) on latency to find a hidden platform in the watermaze. Lister hooded rats were given 4 trials/day to find a hidden platform. Mean data ± S.E.M. are expressed for $n = 15$ rats/group (A) and $n = 12$ rats/group (B). *$p < 0.05$ vs. vehicle-treated controls.

while analysis of the swim speed revealed no effect of treatment in either study (data not shown).

Twenty-four hours after the last acquisition trial, the hidden platform was removed and the animals' ability to remember the location of the platform was assessed (probe trial). MK-801-treated animals spent 32.4% of the trial searching the target (NE) quadrant of the watermaze compared with 29.5%, 23.3% and 14.8% searching the SE, SW and NW quadrants, respectively. In contrast, the vehicle-treated controls in this experiment spent 46.2% exploring the NE target quadrant compared with 24.8%, 16.0% and 12.8% searching the SE, SW and NW quadrants, respectively (Fig. 2). A within subjects ANOVA showed that the time spent exploring the target quadrant was significantly greater than all three other quadrants for the vehicle-treated rats (NE vs. SE: $F(1,14) = 16.84$; $P < 0.01$; NE vs. SW: $F(1,14) = 26.79$; $P < 0.01$; NE vs. NW ($F(1,14) = 42.79$; $P < 0.01$). However, the MK-801-treated rats time spent searching the target quadrant was only significantly greater than that of one other quadrant (NE vs. SE: $F(1,14) = 0.21$; $P = 0.65$; NE vs. SW: $F(1,14) = 1.52$; $P = 0.24$; NE vs. NW ($F(1,14) = 10.12$; $P = 0.01$). A between subjects ANOVA also showed that MK-801-treated animals spent significantly less time searching the target quadrant compared with their vehicle-treated controls ($F(1,28) = 5.32$; $P = 0.029$).

(±)-CP-101,606-treated animals spent 56.5% of the trial searching the target (NE) quadrant of the watermaze com-

pared with 28.3%, 5.4% and 9.7% searching the SE, SW and NW quadrants, respectively. In contrast, the vehicle-treated controls in this experiment spent 47.4% exploring the NE target quadrant compared with only 27.8%, 12.9% and 11.9% searching the SE, SW and NW quadrants, respectively. A within subjects ANOVA showed that the time spent exploring the target quadrant was significantly greater than all three other quadrants for the vehicle-treated rats (NE vs. SE: $F(1,11) = 7.03$; $P = 0.02$; NE vs. SW: $F(1,11) = 21.44$; $P < 0.01$; NE vs. NW ($F(1,11) = 35.66$; $P < 0.01$). In this experiment, the (±)-CP-101,606-treated rats also spent significantly more time searching the target quadrant compared with the other areas in the watermaze (NE vs. SE: $F(1,11) = 10.68$; $P = 0.01$; NE vs. SW: $F(1,11) = 77.76$; $P < 0.01$; NE vs. NW ($F(1,11) = 78.04$; $P < 0.01$). Although (±)-CP-101,606-treated animals spent more time searching the target quadrant compared with their vehicle-treated controls, a between subjects ANOVA revealed no significant treatment effects ($F(1,22) = 1.76$; $P = 0.198$).

There was no significant difference in distance travelled or speed between MK-801 or (±)-CP-101,606 animals and their vehicle controls during the probe trial (data not shown). The day after the probe trial, the animals were tested for their ability to swim to a visible platform after pretreatment with test compound. Neither MK-801 nor (±)-CP-101,606 significantly affected animals ability to swim to a visible platform (data not shown).
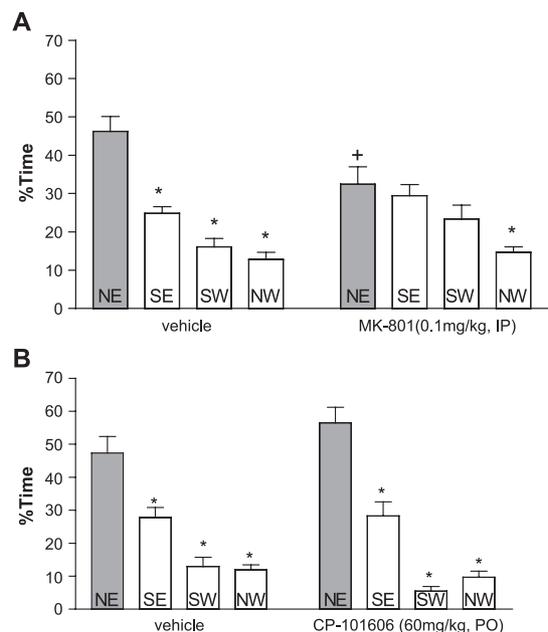

Fig. 2. The effect on MK-801 (0.1 mg/kg, i.p.) (A), and (±)-CP-101,606 (60 mg/kg, p.o.), (B) on %time searching a hidden platform location in the watermaze. Lister hooded rats were given 4 trials/day to find a hidden platform for 7 days and then 24 h after the last trial their %time searching the quadrant containing the hidden platform (NE) was assessed. Mean data ± S.E.M. are expressed for $n = 15$ rats/group (A) and $n = 12$ rats/group (B). *$p < 0.05$ vs. NE (target) quadrant; +$p < 0.05$ vs. corresponding quadrant for vehicle-treated controls.
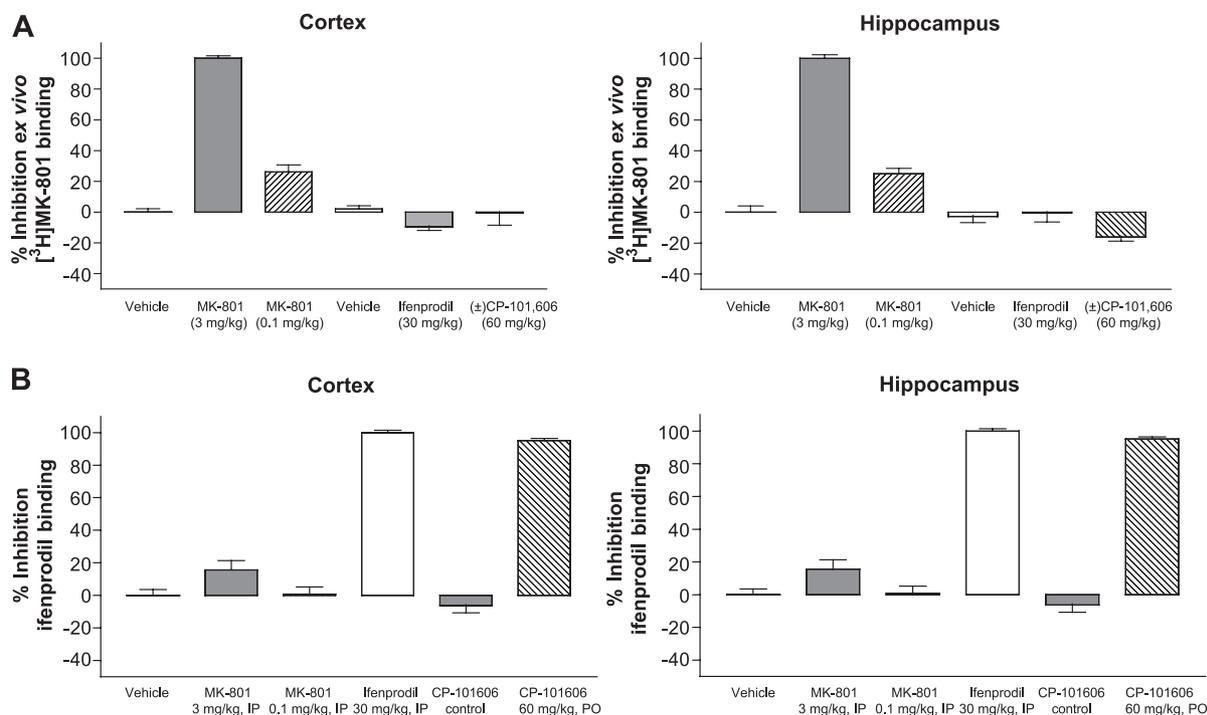
Fig. 3. Modulation of ex vivo [^{3}H]MK-801 (A) and [^{3}H]ifenprodil (B) binding by NMDA receptor ligands. The NMDA receptor antagonist MK-801 (0.1 mg/kg, i.p.) and the NR2B subtype-selective ifenprodil site antagonist ($\pm$)-CP-101,606 (60 mg/kg, p.o.) were shown to occupy 25% and 100% of [^{3}H]MK-801 and NMDA-specific [^{3}H]ifenprodil binding sites, respectively, on the NMDA receptor complex of rat cortex and hippocampus.

3.2. Receptor occupancy

MK-801 (i.p.) of 0.1 mg/kg inhibited ex vivo [^{3}H]MK-801 binding to rat cortex and hippocampus receptors by approximately 25% (Fig. 3A), suggesting that at this dose of MK-801, approximately 25% of NMDA receptors were occupied. ($\pm$)-CP-101,606 (60 mg/kg, p.o.) completely inhibited ex vivo [^{3}H]ifenprodil binding to both rat cortex and hippocampus, suggesting that this dose of ($\pm$)-CP-101,606 fully occupied NR2B subunit-containing NMDA receptors (Fig. 3B). As expected, ($\pm$)-CP-101,606 and MK-801 did not effect [^{3}H]MK-801 and [^{3}H]ifenprodil binding, respectively.

4. Discussion

In this study, we have compared the effects of the NR2B-selective ifenprodil site antagonist ($\pm$)-CP-101,606 to the nonselective antagonist MK-801 using a hippocampal-dependent acquisition task in the Morris watermaze, to determine whether impairment of spatial memory in rats varies with different classes of NMDA receptor antagonist. Ex vivo [^{3}H]MK-801 and [^{3}H]ifenprodil binding has been used to determine that administration of MK-801 (0.1 mg/kg, i.p.) and ($\pm$)-CP-101,606 (60 mg/kg, p.o.) occupied approximately 25% and 100% of NMDA receptors in rat cortex and hippocampus. Consistent with the previous observations,

severe motor impairments and subsequent inability to perform the watermaze task were observed at higher doses of MK-801 (>0.1 mg/kg) confirming the profound side-effect liability of nonselective NMDA receptor antagonists. In contrast, ($\pm$)-CP-101,606 at a dose of 60 mg/kg, p.o. occupied close to 100% of NR2B subunit-containing NMDA receptors without impairing motor function.

In the watermaze, vehicle- and drug-treated animals from both experiments showed a reduced latency to escape onto the hidden platform with increasing number of training sessions. This suggests that all animals regardless of drug treatment were able to learn the watermaze task. However, the significant effect of treatment in the MK-801 study, but not the ($\pm$)-CP-101,606 study, implies that there was still a significant impairment in latency to find the hidden platform for the nonselective NMDA receptor antagonist when compared to vehicle controls. This impairment was not seen with swim speed for either drug-treated rats. Therefore, effects found on latency to escape the hidden platform may be independent of any nonspecific performance related effects.

Twenty-four hours after the last acquisition trial, the hidden platform was removed and the animals' ability to remember the location of the hidden platform was assessed (probe trial). MK-801-treated rats spent significantly less time searching the location of the hidden platform than their vehicle controls. Additionally, although the vehicle-treated rats spent significantly more time searching the target

quadrant compared with other quadrants on the maze, the MK-801 rats did not. This suggests that the vehicle-treated rats used a spatial strategy to find the hidden platform, but that the MK-801-treated rats did not. The acquisition of a spatial map regarding the platform position within the Morris watermaze can be acquired in a number of different ways. The most refined strategy is to use the spatial cues distributed around the room. Spatial learning involves using two or more landmarks to encode the geometric position of the platform. This strategy is hippocampus-dependent and can be disrupted by hippocampal lesions (Pearce et al., 1998; Steele and Morris, 1999). Another strategy of finding the location of a hidden platform is to use heading vectors. Here the location of the platform is determined by remembering its approximate proximity and direction in relation to one cue only. This is not affected by hippocampal lesions (Pearce et al., 1998). Typically, a rat might employ this strategy at the start of a watermaze task and then develop a spatial strategy as it becomes more familiar with the maze. However, rats given hippocampal lesions or treatment with an NMDA receptor antagonist would only be able to use a heading vector strategy to escape onto the hidden platform. This might explain why, during the acquisition stage of this experiment, MK-801-treated rats were still able to learn the watermaze task, i.e. their escape latencies were reduced over repeated sessions. However, although the MK-801-treated rats were able to learn the task, there was still a significant effect of treatment compared with vehicle controls and a between subjects one-way ANOVA was significant at the last time point (Day 7) for the rats latency to find the hidden platform. This confirms previous findings that learning and memory can be disrupted by MK-801 (Gorter and de Bruin, 1992; Filliat and Blanchet, 1995; Ylinen et al., 1995; Pitkanen et al., 1995) and other nonselective NMDA receptor antagonists (Kesner and Dakis, 1993; Castellano et al., 2001; Newcomer and Krystal, 2001; Kawabe et al., 1998; Roullet et al., 2001) and further implicates the NMDA receptor in learning and memory processes. Experiments using different dosing regimes before, during and after acquisition of the watermaze task suggests that NMDA receptor antagonists affect both acquisition and consolidation of the task but not retrieval (Liang et al., 1994; Steele and Morris, 1999).

Animals treated with the NR2B subtype selective antagonist, (±)-CP-101,606 showed no significant impairment in time spent searching the target quadrant during the probe trial, compared with their vehicle-treated controls. Indeed their %time searching the target quadrant was increased compared with controls, although this failed to reach statistical significance. This builds on other findings where NR2B subunit selective NMDA receptor antagonists had no effect on long-term potentiation (Weber et al., 1999) and improved cognitive function after experimental fluid-percussion brain injury (Okiyama et al., 1997). Consistent with our current observations, Okiyama et al. (1997) saw no significant effect of 2 days of treatment with CP-

101,606 (6.5 mg/kg, i.p.) in sham-operated rats in a modified Morris watermaze. However, in the present study, 7-day training was required to detect cognitive deficits caused by MK-801, so it is possible that any impairment caused by CP-101,606 may not have been detected at 2 days. Furthermore, the use of ex vivo binding studies after behavioural testing confirmed that the dose of CP-101,606 (60 mg/kg, p.o.) in the present study occupied close to 100% of NR2B subunit-containing NMDA receptors which may not have been achieved by the dose of CP-101,606 used by Okiyama et al. (1997). These results imply that the NR2B subunit-containing receptor may not play an important role in cognitive function, however, mice over-expressing the NR2B subunit-containing receptor were found to have increased long-term potentiation formation and enhanced performance in several behavioural tasks including novel object recognition and spatial navigation suggestive of improved cognitive ability (Tang et al., 1999). It is therefore surprising that NR2B subunit antagonists do not appear to impair performance in the Morris watermaze. The reasons for this apparent discrepancy are unclear, however, the present data suggest that a selective NR2B subunit antagonist may have therapeutic potential in a number of disorders (Boyce et al., 1999; Menniti et al., 1997; Chizh et al., 2001; Parsons, 2001) without causing deficits of hippocampus-dependent learning and memory characteristic of nonselective NMDA receptor antagonists.

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