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# The neuroprotective glycine receptor antagonist GV150526 does not produce neuronal vacuolization or cognitive deficits in rats

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**Abstract**

The neuroprotective activity of the novel glycine receptor antagonist (*E*)-3[(phenylcarbamoil)ethenil]-4,6-dichloroindole-2-carboxylic acid sodium salt) (GV150526) was recently reported in a model of focal ischemia in the rat. Here it was investigated whether GV150526 treatment results in any of the adverse side effects commonly detected after injection of NMDA (*N*-methyl-D-aspartate) receptor antagonists. First, it was found that neuronal vacuolization in the posterior cingulate/retrosplenial area of the cortex was not induced by GV150526 (200 mg/kg, i.v.), but was evident after injection of the NMDA receptor antagonist dizocilpine (MK801) (1 mg/kg, s.c.). In a second set of experiments, the effects of GV150526 were examined on perforant path-dentate gyrus long-term potentiation in rats. GV150526 (3 mg/kg, i.v.) injected 30 min or 150 min prior to tetanization did not block potentiation of the e.p.s.p. slope and population spike amplitude. In contrast, in animals treated with MK801 (1 mg/kg, i.p.) 150 min before tetanization there was a clear block of long-term potentiation of the e.p.s.p. slope and population spike amplitude. The effects of GV150526 were also examined in the Morris Water Maze. Rats injected with GV150526 (10 mg/kg or 60 mg/kg, p.o.) did not show any impairment in learning when compared to control. MK801 (0.08 mg/kg, i.p.), on the other hand, significantly affected the ability to locate the escape platform in the Water Maze. These findings show that GV150526 is devoid of adverse side effects even at doses well above those producing a neuroprotective effect. This drug has therapeutic potential with a much greater margin of safety than NMDA channel blockers or competitive NMDA receptor antagonists. 

*Keywords:* Neuronal vacuolization; Long-term potentiation; Learning; Memory; GV150526; MK801; Glycine receptor antagonist; NMDA (*N*-methyl-D-aspartate); (Rat)

## 1. Introduction

The discovery of potent glutamate antagonist drugs in the 1980s allowed the excitotoxic hypothesis of cerebral ischemia to be tested experimentally (Kemp et al., 1986). The results have demonstrated the remarkable efficacy of NMDA (*N*-methyl-D-aspartate) receptor antagonists as neuroprotective agents and have suggested a number of possible therapeutic applications. In focal ischemic models such as the middle artery cerebral occlusion, the NMDA receptor antagonists have shown to reduce infarct size (Simon et al., 1984; Ozyurt et al., 1988; Dirnagl et al., 1990; Bielenberg and Beck, 1991; Sauer et al., 1993). However, the side effects profile of these compounds, i.e., cardiovascular effects, neuronal vacuolization, cognitive deficits, has raised doubts about their clinical utility.

Antagonists for the glycine co-agonist site on the NMDA receptor complex have been recently developed to obtain neuroprotective activities without inducing the side effects (see Danysz and Parsons, 1998, for review). In particular, the glycine receptor antagonist GV150526 has been well characterized and its protective action has been demonstrated in the middle artery cerebral occlusion model in rat (Bordi et al., 1997).

In the present report, we investigated, using electrophysiological and behavioral measurements, the effects of GV150526 in standard cognitive tests in the rat to evaluate potential side effects of the drug. (*E*)-3[(phenylcarbamoil)ethenil]-4,6-dichloroindole-2-carboxylic acid sodium salt) (GV150526) was studied in vivo on hippocampus long-term potentiation, a long-lasting enhancement of synaptic efficacy induced by tetanic stimulation of afferent fibers (Bliss and Lømo, 1973; Bliss and Collingridge, 1993), and in the behavioral Morris Water Maze test, a procedure to study deficits in learning and

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memory formation (Morris, 1984). To compare the effects of antagonists of NMDA receptor, the ion channel blocker dizocilpine (MK801) was also studied in these tests. Neuronal vacuolization in the posterior cingulate/retrosplenial cortex of rat brains was evaluated after GV150526 or MK801 administration.

## 2. Materials and methods

### 2.1. Long-term potentiation in the hippocampus

Male Sprague–Dawley rats weighing 250–350 g were anesthetized with urethane (1.5 g/kg body weight) and placed in a Kopf stereotaxic frame adjusted so that the surface of the skull was level between lambda and bregma. Body temperature was regulated at $37 \pm 1°$ by means of a heating pad. A bipolar stimulating electrode was placed in the left perforant path (AP −8.0; ML 4.1) and evoked potentials were recorded extracellularly with a stainless steel electrode (1–2 MΩ impedance) from the hilus of the ipsilateral dentate gyrus (AP −4.0; ML 2.3). Both electrodes were driven ventrally using hydraulic micropositioners to search for the best location and to optimize the amplitude of the population spike obtained with the test pulses. Neural potentials were amplified (×200) by an a.c. amplifier, band-pass filtered (2–2000 Hz) and recorded digitally on a personal computer (Axobasic system, Axon Instruments). Test pulses (0.1 ms duration, 0.033 Hz, 150–300 μA) were applied for at least 30 min prior to drug administration at a level that evoked a population spike with an amplitude of about 1/3 of the maximum. Tetanic stimulation (three trains, 10 s apart, 400 Hz, 33 impulses in each train) was applied at the same intensity of test pulse. Recordings of the evoked potential continued for at least 1 h after tetanization. The population spike amplitude was calculated as the vertical distance from the peak of the negative spike to a tangent drawn between the two positive e.p.s.p. peaks. The e.p.s.p. slope was determined with a least squares fit of a linear portion of the rising phase of the evoked synaptic response. Both the e.p.s.p. slope and the population spike were calculated on-line for each evoked potential with custom-made software and are expressed as percent change from baseline (Bordi and Ugolini, 1995). Vehicle or drug solutions were injected 30 min or 150 min before tetanization. A lateral tail vein was cannulated for intravenous (i.v.) injections. Drugs were tested only once in each animal, at a single dose.

GV150526 (3 mg/kg, i.v.) was dissolved in the minimum amount of dimethyl sulfoxyde (DMSO) and then diluted to a final volume with distilled water. MK801 maleate (1 mg/kg, i.p., Fluka Biochemicals) was dissolved in distilled water. The drugs were injected in a dose volume of 1 ml/kg body weight. Control animals received only vehicle (DMSO and distilled water).

At the end of each experiment, the animals were decapitated and perfused through the heart with 0.9% saline and 10% formalin solutions. The electrode placement was verified in a number of randomly selected animals by light microscopic examination of 40-μm sections stained with thionin. Statistical analysis was done using analysis of variance (ANOVA) plus Newman–Keul's post-hoc tests.

### 2.2. Morris Water Maze test

Male Sprague–Dawley rats weighing 300–350 g were used in the behavioral experiments. The Water Maze consisted of a circular pool, 120 cm diameter, filled with water rendered opaque by milk and maintained at $25 \pm 2°C$. The wall of the pool, 40 cm height, was painted white. A platform, 10 cm in diameter and submerged 2.5 to 3 cm, represented a manner to escape from the water. The day before testing, the animals were given 2 min each of free swimming in the pool to acclimatize them to the water. Behavioral data were acquired with a tracking system (San Diego Inst.) and included path length and escape latency in training trials and differential quadrant search time in a probe test trial in which the platform was removed. All rats received three trials a day, for 4 consecutive days with an intertrial interval of 60 s, during which the rat remained on the platform. The start positions were selected quasirandomly from six equally spaced wall locations. If the animal did not find the platform within 120 s, it was placed on the platform for 60 s (Bordi et al., 1996).

GV150526 (10 mg/kg or 60 mg/kg, p.o.) or MK801 (0.05 or 0.08 mg/kg, s.c.) was given 1 h or 30 min before the test, respectively, for 4 consecutive days. Statistics were performed using the ANOVA followed by Dunnett's test.

### 2.3. Neuronal vacuolization

Female Sprague–Dawley rats were used in this study because of their higher sensitivity to neurovacuole formation, as previously reported (Olney et al., 1989; Fix et al., 1995). Groups of six female rats received a single i.v. injection of 200 mg/kg GV150526 in 5% w/v dextrose solution, administered at the dose volume of 44.5 ml/kg at a rate of 0.25 ml/min. As a positive control group, to confirm the presence of neurovacuoles, six female rats received a single subcutaneous (s.c.) injection of MK 801 at a dosage of 1 mg/kg in saline (0.5 mg/ml). As negative controls, six female rats received 44.5 ml/kg of 5% w/v dextrose solution, at a rate of 0.25 ml/min. Three animals in each group were killed approximately 4 h after injection, and the rest of the rats 12 h after injection.

The dose of 200 mg/kg was chosen as the dose of GV150526 at which the first clinical signs (subdued behavior, increased respiration rate, piloerection) were observed in a previous acute toxicity study (Dorigatti and Terron, unpublished results). The dose of 1 mg/kg MK801

is known to induce neurovacuoles in the posterior cingulate/retrosplenial areas of the brain cortex in animals both at 4 and 12 h after treatment (Olney et al., 1989).

All animals were observed for any signs of ill health or reaction to treatment regularly throughout the day of treatment. Animals were deeply anesthetized (3 ml/kg of 10% chloral hydrate, i.p.), the heart was cannulated via the left ventricle and blood was removed via the right auricle, using a saline perfusion for 5 min followed by a fixative solution (glutaraldehyde 1% with paraformaldehyde 4% in phosphate-buffered solution) for 15 min. After fixation, the brain was removed and after dehydration was embedded in methacrylate resin, sectioned using a rotative microtome, and stained using toluidine blue 0.1%. For each rat, brain tissue was cut oro-aborally in slices of about 1.5 mm thickness, starting from the coronal point +1 and ending at −8 mm from the bregma (Paxinos and Watson, 1986). Each slice, containing both the cerebral hemispheres, was processed and embedded in plastic methacrylate resin. The six slices which were obtained for each rat were successively cut into 12 sections of 2 μm thickness at a distance of 4 μm from each other, yielding 72 sections per rat brain. For each section, histological evaluation was conducted under blind conditions at a magnification of 100×.

## 3. Results

### 3.1. Neuronal vacuolization

On microscopic examination, neurovacuoles in the posterior cingulate/retrosplenial brain cortex were observed in animals injected with 1 mg/kg MK801 (Fig. 1A), in agreement with previous studies using this model (Olney et al., 1989; Fix et al., 1995). No neuronal vacuolation, on the other hand, was observed in the posterior cingulate/retrosplenial brain cortex of animals receiving 200 mg/kg of GV150526 (Fig. 1B). This was true for rats killed 4 or 12 h after administration. Animals injected with 5% dextrose solution (negative controls) also did not show neuronal vacuolization (not shown). These data are also consistent with reports suggesting that compounds acting at glycine and polyamine sites do not induce neuronal vacuolation in the posterior cingulate/retrosplenial brain cortex (Duval et al., 1992; Hargreaves et al., 1993).

### 3.2. Long-term potentiation in the hippocampus

Baseline evoked responses to perforant path stimulation were recorded in the dentate gyrus for at least 30 min before drug administration. Following drug administration, evoked potentials were recorded for 30 min before tetanic stimulation was given. In control animals ($n = 5$), injection of vehicle had no effect on baseline and tetanic stimulation in all cases resulted in a clear potentiation of both the e.p.s.p. slope and population spike amplitude recorded for at least 1-h post-tetanus (Fig. 2A). Neither the population spike amplitude, nor the e.p.s.p. slope was changed by the glycine receptor antagonist GV150526 ($n = 5$) compared to the pre-injection baseline values. The induction of long-term potentiation was not blocked by the drug (Fig. 2B). These results seem to indicate that GV150526 is not involved in the process of long-term potentiation. However, since it has been shown that some

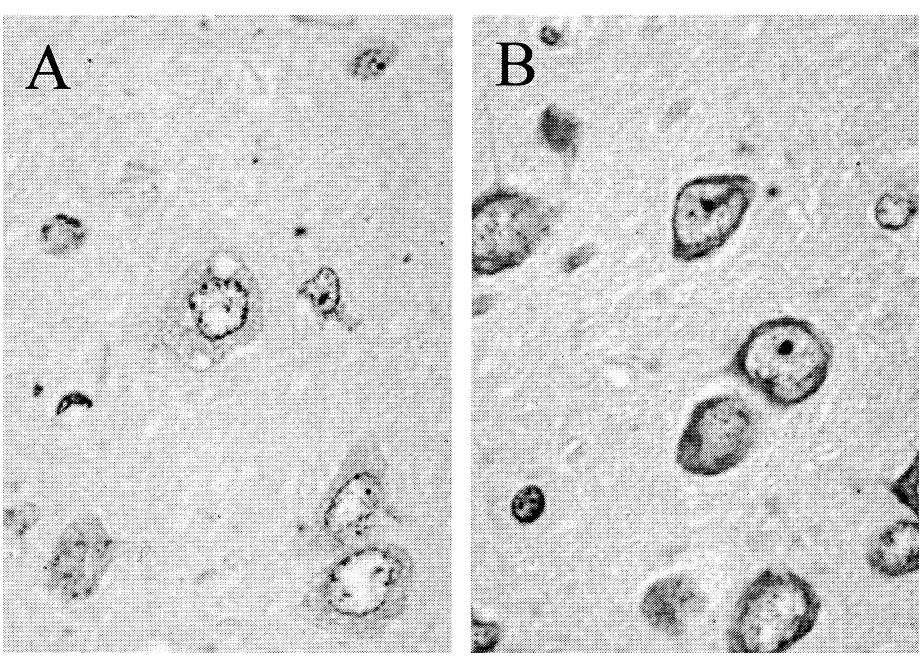


Fig. 1. Micrographs of posterior cingulate neurons in female rats after injection of glycine antagonist GV150526 or NMDA receptor antagonist MK801. (A) Vacuolization of neurons in posterior cingulate cortex 4 h after treatment with MK801 (1 mg/kg, s.c.). (B) Normal appearance of cortical neurons 4 h after treatment with GV150526 (200 mg/kg, i.v.). (100×).

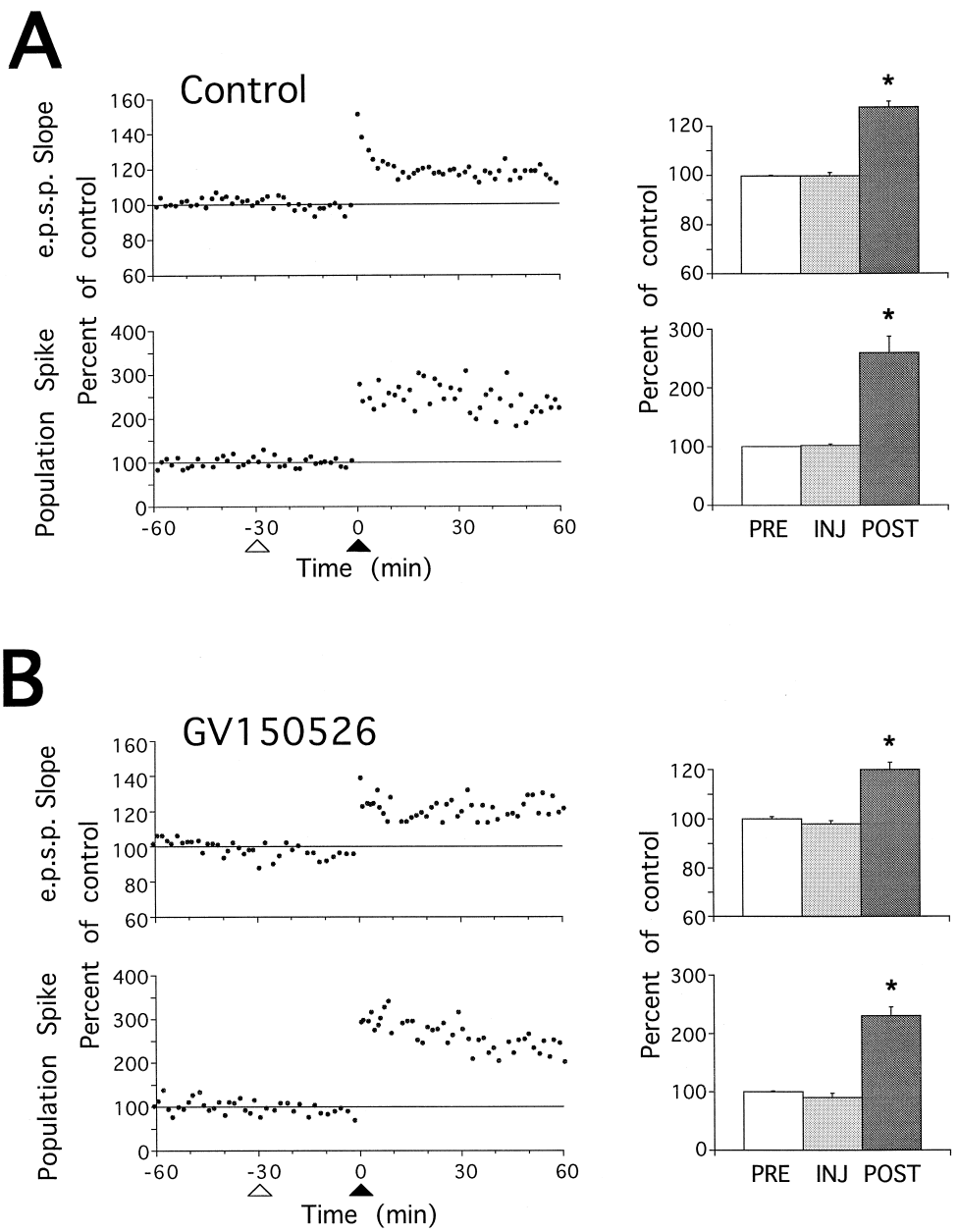


Fig. 2. Effects of vehicle ($n = 5$) or GV150526 ($n = 5$) on perforant path-dentate gyrus long-term potentiation. (A) Consistent long-term potentiation was induced in control animals without affecting the baseline neuronal potentials. (B) GV150526 did not affect the induction of long-term potentiation of both the e.p.s.p. slope and the population spike amplitude. White triangles show the start of the injection. Black triangles show when tetanus was induced (three trains 10 s apart, 400 Hz, 33 impulses each). Time plots show representative single experiments. Each point is the average of three evoked potentials. Histograms show the mean and standard error of e.p.s.p. slope and population spike recorded pre-injection (PRE), post-injection (INJ), and 1 h post-tetanus (POST). Small asterisks denote statistical difference between baseline PRE and INJ or POST ($^{*}P < 0.01$; paired $t$-test).

NMDA receptor antagonists like MK801 are able to block long-term potentiation when administered 150 min, but not 30 min, before tetanus (Abraham and Mason, 1988) due to the reputed ''use dependency'' of the drug (Errington et al., 1987), the effects of GV150526 on long-term potentiation were tested also 150 min after drug administration and compared to the effects of MK801. Fig. 3 shows that the GV150526 ($n = 5$) did not affect the baseline evoked potentials and did not block long-term potentiation. In contrast, the NMDA receptor antagonist MK801 ($n = 5$) effectively blocked the induction of long-term potentiation of the e.p.s.p. slope and population spike amplitude, without changing the baseline responses.

### 3.3. Morris Water Maze test

The effects of GV150526 (10 mg/kg, p.o.,) on Water Maze performance were studied in a group of rats ($n = 10$). No difference was found between the escape latencies over

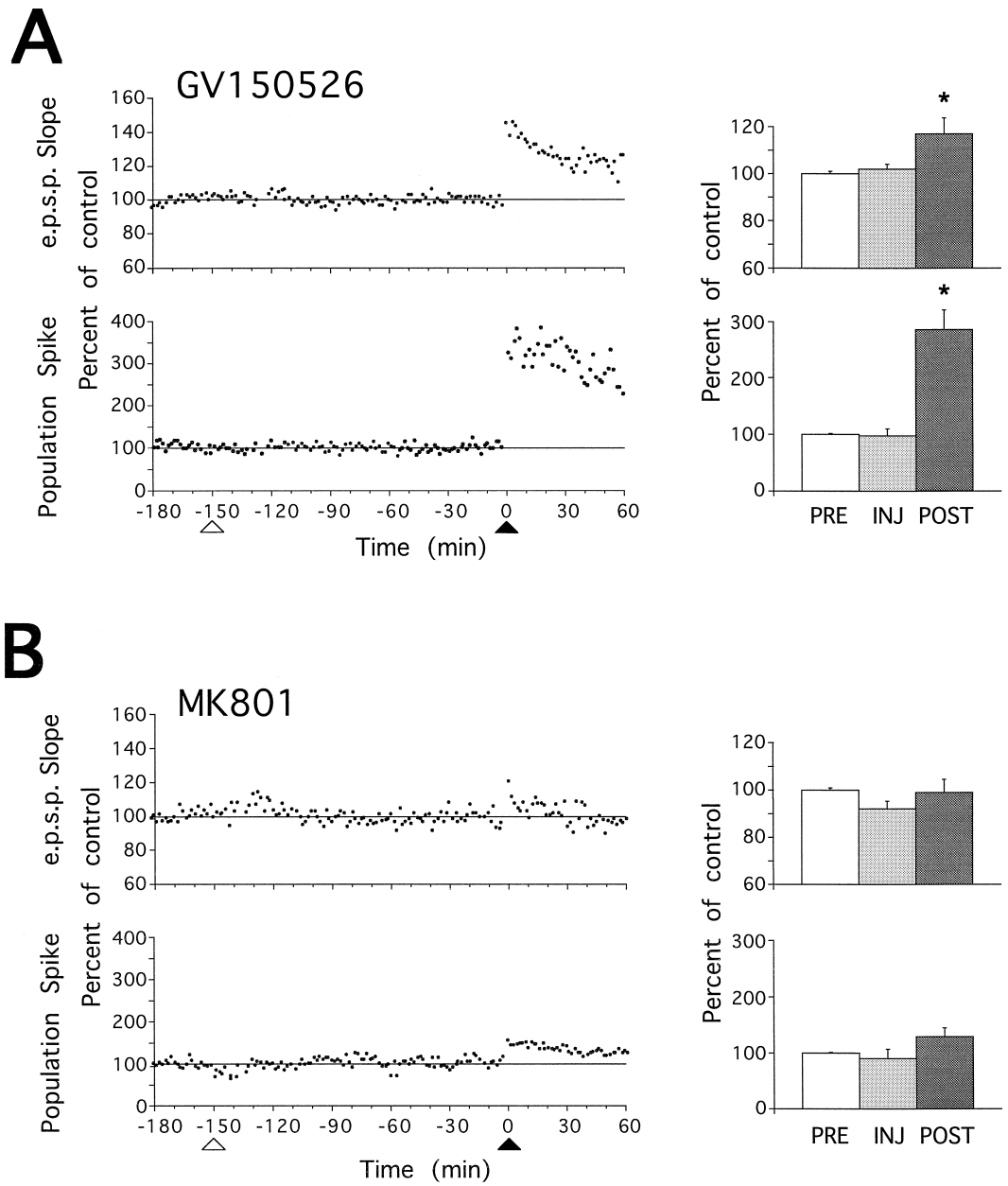


Fig. 3. Representative time plots from individual experiments and average histograms showing the effects on long-term potentiation of the NMDA receptor antagonist MK801 ($n = 5$) or GV150526 ($n = 4$) injected i.p. 150 min before tetanization. Histograms show means and standard errors of all experiments. (A) GV150526 did not affect the baseline response nor the induction of long-term potentiation. (B) MK801 was able to block long-term potentiation without affecting baseline evoked potentials ($^*P < 0.01$; paired $t$-test).

the 4 days of training of the drug-treated group and the control group. Fig. 4A shows the decline in the time to escape over trials. In separate experiments, the effects of MK801 (0.05 mg/kg or 0.08 mg/kg, s.c., $n = 10$ each group) were tested. MK801 produced an impairment in learning, expressed as time to reach the hidden platform, in the group injected with 0.08 mg/kg dose (Fig. 4B). Higher concentrations of the drug, comparable to those effective in neuroprotection models ($\sim 1$ mg/kg), induced clear behavioral stereotypes and could not be tested in our preparation.

Finally, the effects of GV150526 administered at a higher dosage (60 mg/kg, p.o., $n = 7$) were compared to those of vehicle-treated control ($n = 7$). This concentration is 10–20 times the neuroprotective doses (Bordi et al., 1997; Di Fabio et al., 1997). The animals were trained for 4 consecutive days to reach the platform. Both groups showed a significant improvement in performance during training, as indicated by a progressive reduction in the escape latency. The GV150526 and control groups, however, did not differ (data not shown). At day 4, the mean escape latency of the GV150526 group (mean = 11.9 s)

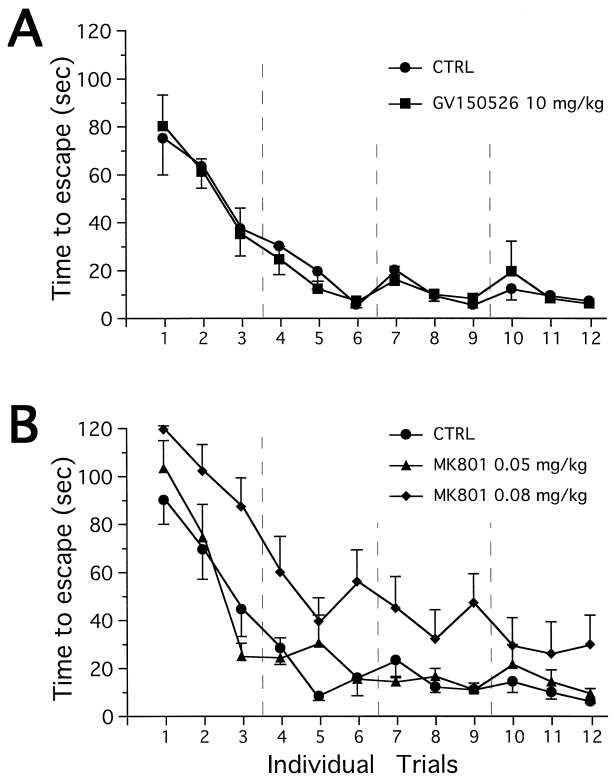


Fig. 4. Mean time to reach the platform in the Morris Water Maze on each of three acquisition trials following drug treatment on days 1 to 4. (A) Injections of GV150526 (10 mg/kg, p.o., $n = 10$) did not affect escape latencies over the 4 days of training. (B) The NMDA antagonist MK801 increased escape latencies when injected at 0.08 mg/kg ($n = 10$) dose (at every trial, $P < 0.01$), but not at 0.05 mg/kg ($n = 10$).

was not significantly different from that of the control group (mean = 13.6 s). None of the treated animals (GV150526 or vehicle) showed any gross behavioral abnormalities.

## 4. Discussion

The results of these experiments indicate that administration of GV150526 does not impair the performance of rats in the Morris Water Maze. Even when high doses of the drug were injected, well above those exerting a neuroprotective effect (10–20 times higher), no deficits were found in this behavioral test of learning and memory. In contrast, the NMDA receptor antagonist MK801 produced a clear deficit in the Morris Water Maze, in agreement with a number of studies showing that MK801 and other NMDA receptor antagonists severely impair learning and memory (Collingridge et al., 1983; Morris et al., 1986; Errington et al., 1987; Robinson et al., 1989; Heale and Harley, 1990; Walker and Gold, 1991).

Long-term potentiation, a synaptic model of memory formation (Bliss and Collingridge, 1993), was also not compromised in rats injected with the glycine receptor antagonist GV150526. MK801, however, when administered 150 min before tetanization, prevented potentiation of both the e.p.s.p. slope (i.e., synaptic long-term potentiation) and the population spike amplitude, as demonstrated by others (Abraham and Mason, 1988).

Taken together, these findings suggest that the cellular mechanisms which generate long-term potentiation are also involved in behavioral tests of learning and memory. The NMDA receptor antagonist MK801 produced amnesia in the Water Maze test and blocked long-term potentiation in the hippocampus. The glycine receptor antagonist GV150526 was not amnesic in the behavioral tests and did not prevent long-term potentiation. A recent report, however, showed that the glycine site receptor antagonist 7-chlorokynurenate injected intraventricularly impaired water maze performance without blocking long-term potentiation in vivo (Bannerman et al., 1997). This dissociation was explained by the observation that levels of 7-chlorokynurenate in the hippocampus were too low to be sufficient to affect long-term potentiation. Intrahippocampal injection of 7-chlorokynurenate in fact blocked the induction of long-term potentiation at CA1 pyramidal cell-commissural synapses in vivo (Thiels et al., 1992).

The present data support the hypothesis that the NMDA receptor is critically involved in the acquisition of at least some forms of learning, which makes the classic NMDA receptor antagonists not suitable for therapeutic applications. In contrast to the effects of 7-chlorokynurenate, a number of glycine site antagonists do not affect behavioral learning tasks or long-term potentiation (Kretschmer et al., 1997; Danysz and Parsons, 1998; Priestley et al., 1998). In line with these findings, the modulator glycine-site NMDA receptor antagonist GV150526 does not cause learning impairment, suggesting that this drug could be safe in humans. This effect is specific because GV150526 has shown a 1000- to 10,000-fold selectivity for the strychnine-insensitive glycine binding site with respect to all major neurotransmitter and peptide receptors, channels and receptor regulatory sites (Di Fabio et al., 1997; Mugnaini et al., 1998).

Another adverse effect reported with NMDA receptor antagonists, neuronal vacuolization in the posterior cingulate/retrosplenial cortex (Olney et al., 1989; Auer and Coulter, 1994; Fix et al., 1995; Wozniak et al., 1996), was not observed in animals injected with 200 mg/kg GV150526, a concentration over 50 times higher the neuroprotective concentration (Bordi et al., 1997; Di Fabio et al., 1997). This result confirms earlier findings that showed an absence of neuropathological changes in rat brains following injection of glycine site antagonist (Duval et al., 1992; Hargreaves et al., 1993; Hawkinson et al., 1997). Injection of MK801 at a neuroprotective dose (1 mg/kg) produced, however, clear neurovacuoles in the retrosplenium and cingulate cortex.

In general, glycine site antagonists are attractive as potential therapeutic agents because they have few known

side effects. The paucity of side effects is at first difficult to understand, because the functional characteristics of these antagonists resemble those of NMDA receptor antagonists. Occupancy of the glycine site is thought to be required for efficient activation of the NMDA channel by glutamate (Kleckner and Dingledine, 1988; Corsi et al., 1996). One possibility, suggested by Danysz and Parsons (1998), is that glycine and NMDA receptor antagonists (or channel blockers, such as MK801) may have different selectivity for NMDA receptor subtypes. Alternatively, glycine antagonists may induce receptor desensitization (Parsons et al., 1993), which could differentiate between various forms of NMDA receptor activation. According to this hypothesis, NMDA receptor desensitization by glycine antagonists could block the transient physiological activation of glutamate receptors without activation of their long-term neurotoxic effects. These possibilities are currently under investigation using GV150526.

In conclusion, the present findings suggest that GV150526 is devoid of the side effects usually reported with NMDA receptor antagonists and, given its potent neuroprotective efficacy (Bordi et al., 1997; Di Fabio et al., 1997), this drug has therapeutic potential with a much greater margin of safety than NMDA channel blockers or competitive NMDA receptor antagonists.

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