



Chronic Memantine Does Not Block 3-Nitropropionic Acid-Delayed Ischaemic Tolerance in Rat Hippocampal Slices *Ex Vivo*

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(Received 20 November 2003; Revised 19 December 2003; In final form 12 January 2004)

The moderate affinity uncompetitive NMDA receptor antagonist memantine, at concentrations found to be neuroprotective in animal models of chronic excitotoxicity, did not reduce ischaemic tolerance induced chemically with 3 nitropropionic acid (3-NP), but actually tended to enhance this effect *ex vivo*. Injection of 3-NP (20 mg/kg i.p.) - 24 h prior to the *in vitro* experiment - significantly protected against hypoxia/hypoglycaemia-induced suppression of extracellular field excitatory postsynaptic potentials (fEPSPs) in rat hippocampal slices (62.2% vs. control of 16.8 %), whereas 3 days pre-treatment with memantine (20 mg/kg/day -Alzet minipumps) tended to enhance recovery further following 3-NP preconditioning (89.7%). This low dose of memantine had no effect on fEPSPs in the absence of preconditioning. As expected, 3 days pre-treatment with a high dose of (+)MK-801 (dizocilpine; 2 mg/kg/day -Alzet minipumps) tended to reduce ischaemic tolerance following 3-NP preconditioning (45.3%). We conclude that although NMDA receptors do seem to be involved in chemically-induced ischaemic tolerance, semi-chronic pre-treatment with therapeutically-relevant doses of memantine does not block ischaemic tolerance.

Keywords: 3-Nitropropionic acid; Hippocampus; Ischaemic tolerance; NMDA receptor antagonist; Memantine; Rat

INTRODUCTION

It has been known for several years that under certain conditions a short ischaemic/hypoxic episode or pharmacological blockade of mitochondrial oxidation (Riepe *et al.*, 1997) can decrease the susceptibility of

neuronal tissue to subsequent severe insult - a phenomenon called ischaemic tolerance. This fact might be of particular clinical importance in situations where danger of succeeding episodes after moderate ischaemic insult can be anticipated such as in vascular dementia. It was also previously shown that induction of ischaemic tolerance can be prevented by the *N*-methyl-D-aspartate (NMDA) receptor antagonist (+)MK-801 (dizocilpine) (Kato *et al.*, 1992), suggesting that a certain form of neuronal plasticity is involved in these processes. The fact that NMDA receptor antagonists can block the development of ischaemic tolerance is potentially disconcerting, as many NMDA receptor antagonists are at different stages of development as neuroprotectants. However it should be pointed out that the results obtained by Kato *et al.* (1992) cannot be applied to all classes of NMDA-receptor antagonists. Thus (+)MK-801 is a high affinity channel blocker with slow on-off kinetics and very weak voltage-dependence (Parsons *et al.*, 1993), and a very high dose of 3 mg/kg was administered i.e. 10 - 20 times higher than that producing effects in behavioural models. These features make the blockade of NMDA receptors by this compound at this dose essentially irreversible and independent of the intensity or temporal pattern of receptor stimulation (Frankiewicz and Parsons, 1999; Frankiewicz *et al.*, 2000). In contrast, moderate affinity uncompetitive NMDA receptor antagonists like memantine, show fast on-off kinetics and strong voltage-dependence and thereby offer much better separation between physiological and pathological activation of NMDA receptors (Frankiewicz *et al.*, 1996; 2000; Frankiewicz and Parsons, 1999). Memantine was recently approved in Europe and the USA for the treatment of moderate to severe Alzheimer's disease but has

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been in use in Germany for over a decade in the treatment of dementia, and is essentially devoid of side effects at therapeutic doses (Ditzler, 1991; Misztal *et al.*, 1996; Zajaczkowski *et al.*, 1996; Winblad and Poritis, 1999; Reisberg, 2000). We wanted to evaluate if the concentrations of memantine used in patients and found to be neuroprotective in animal models of chronic neurodegeneration also interfere with ischaemic tolerance.

Excitotoxicity, mitochondrial dysfunction and free radical-induced oxidative damage have been implicated in the pathogenesis of several different neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease, Alzheimer's disease, and Huntington's disease (Schulz *et al.*, 1997). The mitochondrial toxin 3-nitropropionic acid (3-NP) has often been used to model these diseases but has also been shown to induce chemical ischaemic tolerance (e.g., Kasischke *et al.*, 1996; Riepe *et al.*, 1997). Therapeutically relevant concentrations of memantine are neuroprotective against acute 3-NP toxicity *in vivo* (Wenk *et al.*, 1996) and semi-chronic 3-NP toxicity *in vitro* (Brown *et al.*, 2003). As such, it seemed most pertinent to address the issue of whether memantine can interfere with ischaemic tolerance in the 3-NP model of chemical ischaemic tolerance.

In previous studies, administration of the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV) *ex vivo* was claimed to reverse chemical ischaemic tolerance induced by administration of 3-NP 24 h before the *in vitro* experiment (Kasischke *et al.*, 1996). It seems to us that 24 h after administration of the mitochondrial toxin, any tolerance to ischaemia should already have been established (Riepe *et al.*, 1997) and it is therefore difficult to explain how subsequent administration of APV should have been effective (Weih *et al.*, 2000).

Specifically, two separate issues have been addressed: (1) Does chronic infusion of memantine at a dose (20 mg/kg/day s.c.) leading to therapeutically-relevant plasma concentrations (1 μ M) hamper chemically evoked (3-NP, 20mg/kg i.p.) ischaemic tolerance tested in hippocampal slices *ex vivo*? (2) Does short exposition (5 min) of hippocampal slices to the competitive NMDA receptor antagonist D-APV (100 μ M) block chemically-induced ischaemic tolerance in the same preparation as reported previously by Kasischke *et al.* (1996)?

METHODS

Male Sprague-Dawley rats (100-120 g) were kept on a

12:12 h light:dark cycle with food and water *ad libitum*. Experiments were performed according to the animal rights commission allowance (Hessen).

In the chronic memantine-treated group, animals received a s.c. implant of Alzet 2ML2 osmotic pumps delivering memantine at 20 mg/kg/day s.c. - a dose previously shown to mimic the therapeutic pharmacokinetic plasma profile in man (Misztal *et al.*, 1996). Prior to implantation, filled pumps were placed in saline for 24 h in an incubator at 37°C. After 3 days of memantine infusion the animals were sacrificed for the *in vitro* part of the experiment where memantine treatment was continued by adding 1 μ M to the bath solution - i.e., at the upper level of plasma concentrations achieved under therapeutic conditions (Parsons *et al.*, 1998).

The (+)MK-801 group was treated similarly except that (+)MK-801 was applied via the pumps at a dose of 2 mg/kg/day and slices were then incubated further with 0.1 μ M. Although a therapeutically-relevant dose of (+)MK-801 is not known, we chose this regime for the following reasons. Firstly, (+)MK-801 is around 10-fold more potent than memantine against acute damage in this model (Frankiewicz *et al.*, 2000) although higher absolute concentrations of both were required due to the severe nature of such acute models. Secondly, previous studies on ischaemic tolerance have used acute doses of 1-3 mg/kg. (+)MK-801 administered at 2 mg/kg/day via osmotic minipumps almost certainly produces lower plasma concentrations because the half life can be estimated from behavioural effects to be similar to or even shorter than memantine, i.e., ≤ 3 hours (W. Danysz, personal communication).

Control rats and those receiving 3-NP alone were implanted with the osmotic minipumps delivering saline. In all four treatment groups (saline, 3-NP, memantine + 3-NP and (+)MK-801 + 3-NP), chemical ischaemic tolerance was induced by injection of 3-NP (20 mg/kg i.p.) - 48 h after implantation of the osmotic minipumps and 24 h prior to the *in vitro* experiment.

Hippocampal slices (400 μ m) were prepared as reported previously (Frankiewicz *et al.*, 2000). Slices without the CA3 region were placed in an interface chamber and perfused at a rate of 0.8 ml x min⁻¹ with artificial cerebrospinal fluid (aCSF) at 33°C in an oxygen-enriched (95% O₂/5% CO₂) humidified atmosphere. After initial incubation for 30 min in the recording chamber, a glass-recording electrode (1-2 M Ω) was positioned in the dendritic layer of area CA1 to record extracellular field excitatory postsynaptic potentials (fEPSPs), or in the cellular area of CA1 to record population spikes respectively. A concentric, stimulating

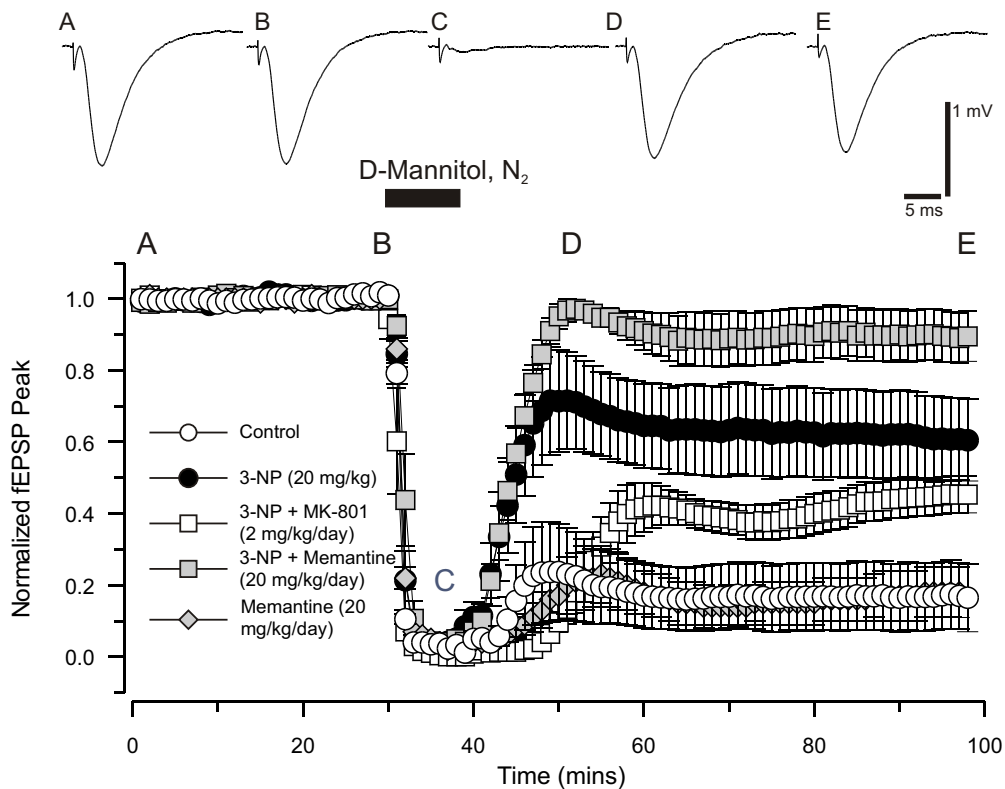


Figure 1 3-NP (20 mg/kg i.p., $n=9$) injected 24 h before experiments in hippocampal slices significantly facilitates recovery of fEPSPs after *in vitro* hypoxia/hypoglycaemia (dark bar) in comparison to control group ($n=11$). Chronic infusion of memantine (20 mg/kg/day) starting 2 days prior to 3-NP and thereafter *ex vivo* in the bath solution (1 μ M) tended to enhance this effect ($n=8$). Similar pre-treatment with (+)MK-801 (2 mg/kg/day, $n=9$) tended to reduce ischaemic tolerance. The effects of (+)MK-801 and memantine were significantly different ($p < 0.005$). ANOVA on mean of the last 10 min of recording (DF=3,33 $F=12.15$, $p < 0.001$) - all *posthoc* tests, Tukey pairwise comparisons. Traces were averaged in groups of 4 consecutive responses (4 x 15s = 1 min) and then normalized with respect to the grouped average of responses during the 30 min prior to hypoxic insult and have been plotted as means (\pm SEM) against time. The representative traces illustrate the recovery of fEPSPs after hypoxia/hypoglycaemia (3-NP, 20mg/kg, + memantine group). The time course of the recording session is shown (A through E).

electrode was placed 500-700 μ m away from the recording electrode to activate the Schaffer collateral commissural fibres. Extracellular recordings were made in response to constant voltage (20-25 V, i.e. ≈ 200 -250 μ A, square pulse for 20 μ sec) single shock stimulation once every 15 sec. Stimulus intensities were adjusted to evoke fEPSPs or population spikes of half maximal amplitude. To evoke hypoxia/hypoglycaemia, slices were perfused with glucose-free aCSF (glucose was replaced with an equimolar concentration of D-mannitol), deoxygenated with 95% N_2 /5% CO_2 for 7 min. Additionally, the base unit of the chamber was bubbled with the same gas mixture, to keep slices in a humid but oxygen-free atmosphere. Averaged peak fEPSPs (mV) or population spike amplitudes (mV) were normalized with respect to the 30 min control period prior to hypoxia/hypoglycaemia. Recovery from ischaemic injury was assessed 51-60 min following re-introduction of oxygen and glucose.

RESULTS

In agreement with previous data (Frankiewicz *et al.*, 2000) infusion of memantine at 20 mg/kg/day s.c. followed by 1 μ M *ex vivo* had no effect on hypoxia/hypoglycaemia-induced suppression of fEPSPs in hippocampal slices, an *in vitro* model of severe acute ischaemic insult such as that likely to occur in stroke (FIG. 1). It should be noted that similar treatment is neuroprotective in numerous animal models of chronic excitotoxicity which are more relevant for its use in Alzheimer's disease (see Parsons *et al.*, 1998).

Injection of 3-NP (20 mg/kg i.p.) - 24 h prior to the *in vitro* experiment - significantly protected against hypoxia/hypoglycaemia-induced suppression of fEPSPs in hippocampal slices ($62.2 \pm 12.2\%$ vs. control of $16.8 \pm 9.4\%$, $p < 0.005$), whereas 3 days pre-treatment with memantine (20 mg/kg/day - Alzet minipumps) actually tended to enhance recovery further ($89.7 \pm 7.2\%$, $p < 0.001$ vs. control, but this effect

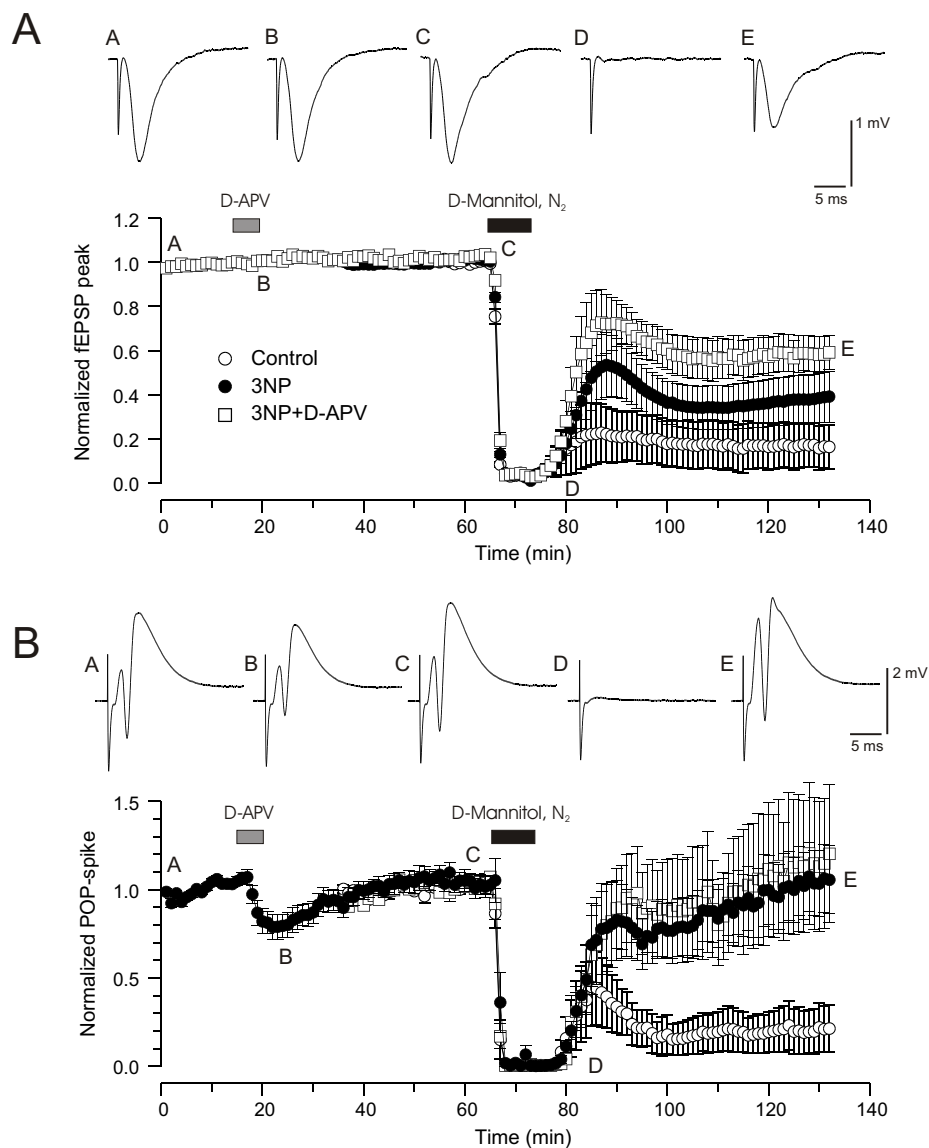


Figure 2 Acute exposure to D-APV. (A) 3-NP injected 24 h before *in vitro* experiments antagonizes hypoxia/hypoglycaemia-induced (dark bar) suppression of fEPSP in hippocampal slices ($n=8$) in comparison to control group ($n=7$). Under the same conditions short incubation (5 min) with D-APV (100 μ M) - grey bar - 45 min before the hypoxic/hypoglycaemic insult did not influence the recovery of neuronal function ($n=8$). (B) 3-NP injected 24 h before *in vitro* experiments antagonizes hypoxia/hypoglycaemia-induced (dark bar) suppression of population spike amplitude in hippocampal slices (black circles, $n=11$), when compared to the control group (open circle, $n=8$). Under the same conditions short incubation (5 min) with D-APV (100 μ M) - grey bar - 45 min before the hypoxic/hypoglycaemic insult had no further effect on neuronal recovery ($n=10$). The raw data illustrate a representative example of the recovery of fEPSPs (A) and population spike (B) respectively after hypoxic insult (3-NP + D-APV group) (for details see FIG. 1).

was not significantly different vs. 3-NP $p=0.184$ FIG. 1). As expected, 3 days pre-treatment with (+)MK-801 (2 mg/kg/day -Alzet minipumps) tended to reduce ischaemic tolerance ($45.3 \pm 4.3\%$, $p=0.23$ vs. control, FIG. 1). It should be stressed that 3-NP + MK801 was not significantly different from control or 3-NP alone. However, the difference in the level of recovery seen with memantine and (+)MK-801 was highly significant ($p < 0.005$).

In a separate set of experiments 3-NP injection also

protected against the hypoxia/hypoglycaemia-induced suppression when fEPSP ($48.6 \pm 2.8\%$ vs. $16.9 \pm 1.1\%$ of control, $p < 0.05$) or pop-spike amplitudes ($102.9 \pm 32.4\%$ vs. $19.5 \pm 11.3\%$ of control, $p < 0.05$) were analyzed. Short (5 min) exposure of the slices to D-APV (100 μ M) 45 min prior to hypoxia/hypoglycaemia did not influence this ischaemic tolerance regardless of the parameter analyzed ($57.5 \pm 8.6\%$ for fEPSP and $94.1 \pm 18.5\%$ for pop-spike respectively) (FIGs. 2A, 2B).

DISCUSSION

The present study shows that memantine - a moderate affinity NMDA receptor antagonist - does not inhibit 3-NP induced ischaemic tolerance to hypoxia/hypoglycaemia. In contrast, (+)MK-801 did partially block this chemically-induced tolerance, in agreement with the finding that (+)MK-801 inhibits ischaemic tolerance when injected 1 h before preconditioning ischaemia in gerbils (Kato *et al.*, 1992). It is likely that a more pronounced effect of MK-801 on ischaemic tolerance would have been observed if we had also used a similar high acute dose of 3 mg/kg (see later). Although this supports the notion that NMDA receptor activation is involved in the chain of events leading to the establishment of ischaemic tolerance, it highlights the need for cautious interpretation of such results with regards to the use of NMDA receptor antagonists *per se* in indications where ischaemic tolerance may be important.

In our previous *in vitro* studies we showed that (+)MK-801 was not able to differentiate between physiological and pathological activation of NMDA receptors (Frankiewicz and Parsons, 1999; Frankiewicz *et al.*, 2000). In fact, physiological NMDA receptor-mediated synaptic plasticity was far more affected than pathological effects (*ibid.*). Oxidative metabolic stress evoked by injection of 3-NP triggers conditions under which NMDA receptor activation leads to the development of ischaemic tolerance. It is very likely that injection of (+)MK-801 preceding 3-NP administration almost completely blocks this process, especially at the very high acute dose (3 mg/kg) used in the study by Kato *et al.* (1992) which would have been high enough to completely block any ongoing NMDA receptor-related activity, both pathological and physiological. Strong deficits in learning and memory in different animal paradigms have already been observed after doses as low as 0.05 mg/kg (Venable and Kelly, 1991).

In the present study we observed clear chemically-induced ischaemic tolerance despite semi-chronic treatment with memantine using ALZET osmotic minipumps (20 mg/kg/day). This route of drug application achieves stable brain concentrations of memantine of around 1 μ M (Misztal *et al.*, 1996). Such concentrations have been found to be neuroprotective in several animal models of chronic neurodegeneration (Wenk *et al.*, 1995; Misztal *et al.*, 1996; Zajackowski *et al.*, 1996) and this effect was not accompanied by any apparent deficits in learning and memory (Zajackowski *et al.*, 1996). We also previously showed that memantine can preferentially block tonic low level pathological activation of NMDA receptors

by e.g. 3-NP *in vitro* at concentrations leaving their physiological function undisturbed (Frankiewicz and Parsons, 1999; Frankiewicz *et al.*, 2000; Brown *et al.*, 2003). Indeed, memantine actually reverses deficits in LTP and learning in animal models by restoring a pathologically disturbed signal to noise ratio (Zajackowski *et al.*, 1997; Frankiewicz and Parsons, 1999; Danysz *et al.*, 2000) and produces a symptomatic improvement in cognition in demented patients (Winblad and Poritis, 1999; Reisberg, 2000).

The present experimental design reflects the therapeutic situation in which memantine is continuously present. It seems unlikely that the lack of negative effects of memantine on ischaemic tolerance were due to compensatory neuroprotective effects of memantine *ex vivo* because similar treatment without 3-NP was not neuroprotective *per se* in this severe model of acute excitotoxic insult. In fact, acute administration of memantine in this same model is only neuroprotective at concentrations some 10 times higher ($IC_{50} = 14.1 \mu$ M) than those shown to be effective in models of more mild but, chronic excitotoxicity (Frankiewicz *et al.*, 2000).

Addressing the other issue of this study, we could not reproduce the results obtained by (Kasischke *et al.*, 1996). In our hands, short (5 min) exposure of hippocampal slices to 100 μ M D-APV did not have any influence on ischaemic tolerance evoked by injection of 3 NP, 24 h prior to the *in vitro* experiment, independent on the parameter analysed (FIG. 2). We decided to repeat this experiment because we could not see a rationale for the effect observed by Kasischke *et al.* (1996). It seems that 24 h after administration of mitochondrial toxin, tolerance to ischaemia should already have been established (Riepe *et al.*, 1997; Weih *et al.*, 2000). The postulated role of NMDA receptors in this phenomenon would be triggering of a certain Ca^{2+} -related biochemical cascade, rather than tonic engagement in this process. This is in line with the accepted role of the NMDA receptor as a coincidence detector being activated under specific conditions of synchronised neuronal activity. There is evidence that the induction of ischaemic tolerance is also associated specifically with secondary biochemical changes like an alteration of binding activity of AP-1 protein preceded by synthesis of the Fos/Jun protein or activation of c-Jun NH₂-terminal kinases (JNK) (Morgan and Curran, 1991; Kapinya *et al.*, 2000; Sugino *et al.*, 2000). Thus, administration of NMDA receptor antagonists at this late stage should not affect ischaemic tolerance.

CONCLUSIONS

We conclude that both tonic low level blockade of the NMDA receptor by the moderate affinity channel blocker memantine and brief application of D-APV shortly before hypoxia/hypoglycaemia do not abolish the ischaemic tolerance acquired following administration of 3-NP. In contrast, the high affinity uncompetitive NMDA receptor antagonist, (+)MK-801, does reduce ischaemic tolerance in the same model when present at relatively high concentrations during the induction period.

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