First publ. in: European Journal of Neuroscience 23 (2006), 9, pp. 2611-2622

Neuroprotective properties of memantine in different *in vitro* and *in vivo* models of excitotoxicity

Christiane Volbracht,[1] Johan van Beek,[1] Changlian Zhu,[2,3] Klas Blomgren[2,4] and Marcel Leist[1]
[1]Department of Disease Biology, H. Lundbeck A/S, Ottiliavej 9, 2500 Valby, Denmark
[2]Arvid Carlsson Institute for Neuroscience, Institute of Neuroscience and Physiology, Göteborg University, Sweden
[3]Department of Pediatrics, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China
[4]Department of Pediatrics, The Queen Silvia Children's Hospital, Göteborg, Sweden

Keywords: Alzheimer's disease, apoptosis, excitotoxicity, hypoxia-ischemia, memantine

Abstract

The pathogenesis of stroke, trauma and chronic degenerative diseases, such as Alzheimer's disease (AD), has been linked to excitotoxic processes due to inappropriate stimulation of the *N*-methyl-D-asparate receptor (NMDA-R). Attempts to use potent competitive NMDA-R antagonists as neuroprotectants have shown serious side-effects in patients. As an alternative approach, we were interested in the anti-excitotoxic properties of memantine, a well-tolerated low affinity uncompetitive NMDA-R antagonist presently used as an anti-dementia agent. We explored in a series of models of increasing complexity, whether this voltage-dependent channel blocker had neuroprotective properties at clinically relevant concentrations. As expected, memantine protected neurons in organotypic hippocampal slices or dissociated cultures from direct NMDA-induced excitotoxicity. However, low concentrations of memantine were also effective in neuronal (cortical neurons and cerebellar granule cells) stress models dependent on endogenous glutamate stimulation and mitochondrial stress, i.e. exposure to hypoxia, the mitochondrial toxin 1-methyl-4-phenylpyridinium (MPP^+) or a nitric oxide (NO) donor. Furthermore, memantine reduced lethality and brain damage *in vivo* in a model of neonatal hypoxia-ischemia (HI). Finally, we investigated functional rescue (neuronal capacity to migrate along radial glia) by memantine in cerebellar microexplant cultures exposed to the indirect excitotoxin 3-nitropropionic acid (3-NP). Potent NMDA-R antagonists, such as (+)MK-801, are known to block neuronal migration in microexplant cultures. Interestingly, memantine significantly restored the number of neurons able to migrate out of the stressed microexplants. These findings suggest that inhibition of the NMDA-R by memantine is sufficient to block excitotoxicity, while still allowing some degree of signalling.

Introduction

Agents that specifically block the pathological stimulation of glutamate receptors, mainly the *N*-methyl-D-aspartate receptor (NMDA-R), might be anticipated to restore physiological function of neurons and thereby have disease-modifying effects in Alzheimer's disease (AD). Previous attempts to use NMDA-R antagonists to retard the progression of AD have been hindered by the psychotomimetic and cardiovascular effects of these drugs. However, the uncompetitive NMDA-R antagonist memantine (1-amino-3,5-dimethyladamantane) has not been associated with such adverse effects (reviewed in Parsons *et al*., 1999a; Rogawski, 2000). Memantine has a K_I-value of 0.5 μM at the phencyclidine binding site of the NMDA-R in human frontal cortex (Kornhuber *et al*., 1989). Under therapeutic conditions in patients (daily maintenance doses of 20 mg) the serum levels of memantine range in similar concentrations from 0.5 to 1.0 μM, whereas levels in free cerebrospinal fluid are 20–50% lower due to albumin binding in serum (Kornhuber & Quack, 1995). The tolerability of the clinically used moderate-affinity blocker memantine is believed to be dependent on its faster blocking/unblocking kinetics, and the relatively greater voltage sensitivity (Parsons *et al*., 1999a). Memantine binds at the same depth as Mg^{2+} in the NMDA-R channel. However, it has a somewhat less pronounced voltage-dependency than Mg^{2+} and is therefore more effective in blocking tonic pathological activation of the NMDA-R at moderately depolarized membrane potentials. On the other hand, following strong synaptic activation, memantine, like Mg^{2+}, leaves the NMDA-R channel due to its voltage-dependency and fast unblocking kinetics (reviewed in Kornhuber & Weller, 1997; Lipton, 2006). These properties of memantine may allow selective inhibition of pathological NMDA-R activity by chronic mild membrane depolarization, while permitting normal physiologic function following synaptic release of glutamate after pronounced membrane depolarization. Two distinct mechanisms, release of endogenous glutamate and sensitization of neurons towards glutamate stimulation, contribute to excitotoxicity (Henneberry *et al*., 1989; Choi & Rothman, 1990). Self-enhancing processes have been suggested to account for excitotoxic neuronal death. Conditions leading to an impairment of mitochondrial function and energy failure will be followed by impairment of ion pumps and partial hypopolarization and opening of voltage-dependent ion channels. This in turn sensitizes neurons towards glutamate stimulation by releasing the Mg^{2+} blockade of the NMDA-R (Henneberry *et al*., 1989). Increased NMDA-R-mediated Ca^{2+} influx can elevate energy demand further, enhance depolarization, trigger further Ca^{2+} increase and favour release of endogenous glutamate (Szatkowski & Attwell, 1994). This putatively self-propagating process results in loss of intracellular Ca^{2+} homeostasis and excitotoxicity (Henneberry *et al*.,

Correspondence: Dr Christiane Volbracht, as above.
E-mail: cvo@lundbeck.com

1989; Zeevalk & Nicklas, 1991). These events are thought to underlie neuronal loss in neurodegenerative diseases such as AD. The aim of this study was to investigate the neuroprotective properties of memantine in excitotoxic stress models of varying complexity and the contribution of neuronal depolarization. We wanted to know whether memantine has the ability to rescue neurons at all from excitotoxic damage in a broad range of models and whether the concentrations required are coherent and relevant to the clinical situation.

Experimental procedures

Materials

All reagents were purchased from Sigma–Aldrich, unless otherwise stated.

Animals

Seven-day-old specific pathogen-free BALB/c mice were obtained from Harlan (Horst, Netherland). Wistar rat pups (three-, six-, or nine-day-old) were obtained from Charles River (Sulzfeld, Germany). Time-pregnant C57BL/6J mice were purchased from M & B (Lille Skensved, Denmark). All animal experiments were performed in accordance with the guidelines of the Danish National Committee on Animal Research Ethics, the Ethical Committee of Göteborg (#94–2003), and the European Communities Council Directive #86/609 for the Care of Laboratory Animals.

Hippocampal slice cultures

Hippocampal slice cultures were prepared and grown using the interface method (Stoppini *et al*., 1991) as previously described (Rekling, 2003). Briefly, six-day-old rat pups were deeply anaesthetized by injection of 100–200 μL 5% pentobarbital-sodium (i.p.) and decapitated. Brains were removed and immersed into precooled dissecting medium consisting of Gey's Balanced Salt Solution supplemented with glucose (6.5 g/L). The middorsal segments of the hippocampi were dissected out and cut transversely into 400-μm slices on a McIlwain tissue chopper. The slices were transferred to sterile porous membrane units (Millicell 12 mm CM PTFE 0.4 μm, Millipore), which were transferred to 24 well plates (Nunc) on top of 200 μL culture medium [50% Minimum Essential Medium with Earle's salts, 25% heat inactivated horse serum, and 25% Hanks Balanced Salt Solution, L-glutamine (1 mM), HEPES acid (10 mM), HEPES sodium salt (10 mM), glucose (6.5 g/L), penicillin (5000 units/L), streptomycin (5 mg/L) and amphotericin B (0.31 μg/L), pH 7.25]. Slices were cultured for 2 weeks at 36 °C in 98% relative humidity with 5% CO_2 before used for experiments. The culture medium was changed three times a week. All solutions were purchased from Life Technologies (Gibco-BRL Invitrogen, San Diego, CA, USA).

Cerebellar granule cell culture

Murine cerebellar granule cell (CGC) cultures were isolated from seven-day-old BALB/c pups and cultured as described previously (Volbracht *et al*., 1999). Briefly, mice pups were decapitated and cerebelli isolated. Dissociated neurons were plated on 100 μg/mL poly L-lysine coated dishes at of approximately 0.6×10^6 cells/cm^2 (1 800 000 cells/mL, 500 μL/well, 24-well plate) and cultured in Eagle's basal medium (BME; Biowest, Nuaillé, France), supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 20 mM KCl and 100 U/mL penicillin and 0.1 mg/mL streptomycin (Gibco-BRL Invitrogen). Cytosine arabinoside (10 μM) was added 48 h after plating. The proportion of glia cells in the cultures was less than 5%, as assessed by an antibody against glia-fibrillary-acidic protein (GFAP). Neurons were used without further medium changes at 7 days *in vitro* (DIV). Cultures were exposed to NMDA, or the nitric oxide (NO) donor *S*-nitroso-L-glutathione (GSNO), or 1-methyl-4-phenylpyridinium (MPP^+) in their own medium. The culture medium was exchanged for a controlled salt solution (120 mM NaCl, 25 mM HEPES, 25 mM KCl, 1.8 mM $CaCl_2$, 4 mM $MgCl_2$, 15 mM glucose) 4 h after the start of the treatment, and the cells were left in this medium (usually for 20 h) without re-addition of any inhibitors before toxicity assays were performed. All inhibitors were added 30 min before exposure to the excitotoxins.

Cortical neurons

Murine cortical neurons (CTX) were isolated from day 14–16 fetal C57BL/6 mice as described previously (Hertz, 1989). Briefly, time-pregnant mice were killed by cervical dislocation, foetuses removed and decapitated. Cerebral hemispheres were isolated and dissociated neurons were plated on 100 μg/mL poly D-lysine coated dishes at a density of approximately 0.25×10^6 cells/cm^2 (800 000 cells/mL; 100 μL/well, 96-well plate) and cultured in Neurobasal medium supplemented with 2% B-27 supplement without antioxidants, 0.5 mM L-glutamine and 100 U/mL penicillin and 0.1 mg/mL streptomycin (all solutions from Gibco-BRL Invitrogen). Neurons were fed every third day by replacing half of the medium. CTX were used at 7 days *in vitro* (DIV). The proportion of glia cells in the cultures was less than 10%, as assessed by an antibody against glia-fibrillary-acidic protein (GFAP).

Cerebellar microexplant cultures

Cerebellar microexplant cultures were obtained from postnatal day three rats and cultured in serum-free conditions. Four to five pups were rapidly decapitated, and cerebelli were isolated and immersed into ice-cold phosphate buffered saline (pH 7.4) supplemented with 0.65% glucose. Each cerebellum was cut into smaller sections and triturated two times with a 1 mL insulin syringe via a 23G (0.6 mm) needle, before spinning down at $60 \times \boldsymbol{g}$, 4 °C for 2 min in a 2 mL tube. Each cell pellet was resuspended in ice-cold START V medium (Biochrom, Berlin, Germany) supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin (Gibco-BRL Invitrogen) and centrifuged ($60 \times \boldsymbol{g}$, 4 °C, 2 min) again. Cell pellets were reconstituted into 500 μL START V medium and tubes were kept on ice before single cell suspension samples were combined in 50 mL tubes for plating. Cell suspension (50 μL) was seeded onto poly D-lysine (0.1 mg/mL, MW 30 000–70 000) coated cover glasses (square 18/18 mm) placed into six-well plates (Nunc) by spreading almost to the edges of the cover slips. Seeded cells were allowed to attach for 1–2.5 h (seeding period) at 34 °C before 1.5 mL of START V medium was added to each well. Micoexplants were cultured at 34 °C in 5% CO_2 with 98% relative humidity for 72 h. As excitotoxic injury, microexplant cultures were treated directly after the seeding period with 3-nitropropionic acid (3-NP, 0.5 mM) for 72 h, inhibitors were added simultaneously with the excitotoxin.

Oxygen-glucose deprivation (OGD)

Cultures were transferred to an ischemia chamber (Coy Laboratory Products Inc, Grass Lake, Michigan, USA) containing a gas mixture of

1% O_2, 5% CO_2 and 94% N_2. CTX were changed to glucose free N2-MEM culture medium (MEM medium with addition of 2 mM L-glutamine, 1 mM pyruvic acid and 0.22% $NaHCO_3$ and supplemented with 1% egg albumin, 1% B-27 without antioxidants and 1% N2) 12 h before placement into the ischemia chamber at DIV 7. All reagents were obtained from Gibco-BRL Invitrogen. CGC cultures were exposed to OGD in their own medium without any medium changes at DIV 7. Cultures were placed into in a humidified 37 °C incubator within the ischemia chamber and exposed to ODG for 30 min (CGC) and 14 h (CTX). After the OGD period, cultures were checked for toxicity by phase contrast microscopy and glucose (20 mM) was added to the culture medium. Cultures were kept under normoxic conditions for an additional 6 h (CGC) or 24 h (CTX) before cytotoxicity was assessed either by counting dead and live neurons or by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrasodium bromide (MTT) reduction assay. All inhibitors were added 30 min before exposure to OGD.

Cytotoxicity assays

Cell death in organotypic hippocampal cultures was assessed by cellular uptake of propidium iodide (PI) using an automated fluorescent scanning method (Fluoroskan Ascent FL, ThermoLabsystems) as previously described (Rekling, 2003). Briefly, each well on a 24-well plate is scanned at 43 overlapping points with a 530 nm excitation beam with a diameter of 3 mm. The emitted fluorescence was passed through a 645 nm filter, integrated over 20 ms, and the results were expressed in relative fluorescent units (RFU). The maximal value of 43 readings from an individual well was determined, and this single value was used in the further calculations. For toxicity experiments two readings from the cultures were made: (i) baseline reading prior to treatment (i.e. 24 h after application of culture medium supplemented with 1.5 μM PI), and (ii) insult reading 24 h after the insult with 1.5 μM PI present throughout the 24-h period. The relative cell death was calculated as percentage of the control insult with vehicle to the test drug present, using the formula:

$$\text{Relative cell death}(\%\ \text{of insult with vehicle}) = \left(\frac{\frac{\sum_{i=1}^{n} (\text{IFD}_i - \text{BFD}_i)}{n}}{\frac{\sum_{j=1}^{n} (\text{IFV}_j - \text{BFV}_j)}{n}} \right) \times 100 \quad (1)$$

IFD is insult fluorescence in well i with test drug, BFD is baseline fluorescence in well i with test drug, IFV_j is insult fluorescence in well j with vehicle present, BFV_j is baseline fluorescence in well j with vehicle present, and n is the number of wells.

To assess plasma membrane integrity and nuclear morphology of CGC and CTX, cultures were double stained with the chromatin dyes SYTOX (0.5 μM, noncell permeable, green-fluorescent) and H-33342 (1 μg/mL, cell permeable, blue-fluorescent). Alternatively, cells were loaded with 0.5 μM calcein acetoxymethyl ester (calcein-AM) for 5 min (cells with intact membranes display green fluorescence) in the presence of 1 μM propidium iodide (cells with broken membranes exhibit nuclear red fluorescence) and 1 μg/mL H-33342. Apoptosis was characterized by scoring highly fluorescent condensed nuclei. Approximately 300 cells were counted in three different fields in three different culture wells, and experiments were repeated in at least three different preparations. All dyes were obtained from Molecular Probes (Eugene, OR). In addition, the percentage of viable cells was quantified by their capacity to reduce MTT after incubation with 0.5 mg/mL MTT for 60 min.

To assess viable cells migrating out of cerebellar microexplants, cultures were loaded with calcein-AM (0.5 μM) for 5 min in the presence of 1 μM propidium iodide and viable cells (green fluorescence) were counted. Alternatively, cultures were fixed and migrating cells were counted under phase-contrast optics at 200×. Approximately 200 cells were counted in four different fields in three different culture wells, and experiments were repeated in at least three different preparations. Additionally, fixed cultures were stained for neurofilament or MAP-2 (to identify neurons) and glia fibrillary acidic protein (GFAP, to identify glia cells) and nuclei were stained with H-33342.

Immunofluorescence

After 72 h, cerebellar microexplants were rinsed in PBS and fixed for 2–3 min in increasing concentrations of paraformaldehyde (0.4% PFA; 1.2% PFA; 3% PFA; 4% PFA), followed by digestion step with RNase (1 μg/mL) at 37 °C for 10 min. Fixed cells were permeabilized in 0.1% Triton X-100 for 10 min. Immunostaining was performed using the following antibodies: monoclonal mouse anti-MAP-2 (clone HM-2, 1 : 500; Dako, Glostrup, Denmark), monoclonal mouse anti-neurofilament (clone 2F11, 1 : 500; Dako) and polyclonal rabbit anti-GFAP (1 : 1000; Dako) followed by appropriate secondary antibodies conjugated with either Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes). Nuclei were counterstained with 250 ng/mL H-33342. Fluorescent images were collected and analysed using a MRC-1024 MP laser-scanning confocal microscope (Bio-Rad, Hercules, CA).

Neonatal hypoxia-ischemia

Unilateral hypoxia-ischemia (HI) was induced in Wistar rats on postnatal day 9 (P9), using the Rice-Vannucci model (Rice *et al*., 1981; Hagberg *et al*., 1994). Rats were anaesthetized with halothane (3.0% for induction and 1.0–1.5% for maintenance) in a mixture of nitrous oxide and oxygen (1 : 1). The left common carotid artery was cut between double ligatures of prolene sutures (6–0). After surgery, the wounds were infiltrated with a local anaesthetic, and the pups were allowed to recover for 60–90 min. The duration of anaesthesia and surgery was < 5 min for each pup. Pups were randomly assigned to two groups and treated immediately after surgery (60–90 min before hypoxia-ischemia), whilst recovering from anaesthesia. Thirty-seven pups received memantine intraperitoneally at a dose of 20 mg/kg (10 μL/g, 2.0 mg/mL in saline) while the other 37 pups received saline (10 μL/g) intraperitoneally. Memantine- and vehicle-treated pups were kept together under a heating lamp to maintain their body temperature (rectal temperature of around 35 °C). The litters were placed in a chamber perfused with a humidified gas mixture (7.8% oxygen in nitrogen) for 35 min. After hypoxic exposure, the pups were returned to their biological dams and were allowed to recover for 72 h.

Gross morphology score

Three days after HI, pups were deeply anaesthetized with 50 mg/mL phenobarbital and perfusion fixed with 5% formaldehyde in 0.1 M phosphate buffer through the ascending aorta for 5 min. After dissecting out the brain, the degree of injury was evaluated by inspection of the brain surface according to a method modified from Yager *et al*. (1992). Grade 0, normal, equal size of the two hemispheres and no visible white lesion. Grade 1, a small white lesion plaque. Grade 2, hypotrophy and large cysts in the ipsilateral

hemisphere. Grade 3, only parasaggital visible tissue left in the whole midline. Grade 4, total loss of the ipsilateral hemisphere. Except for very mild injuries, this score shows a linear correlation with the neuropathological score and the infarct volume (Wang *et al*., 2004).

Statistics

An unpaired Student's *t*-test was used when comparing the gross morphology score between memantine- and vehicle-treated animals. A chi-square test was used to compare the mortality rate. A P-value < 0.05 was considered statistically significant. Toxicity tests were run at least in triplicate and repeated in three to eight cell preparations. Statistical significance was calculated on the original data sets using One-way ANOVA followed by the Tukey's test. A P-value < 0.05 was considered statistically significant.

Results

Protection by memantine from NMDA-induced toxicity in organotypic hippocampal slices and cerebellar granule cells

Memantine has a medium inhibitory concentration of approximately 2 μM on the NMDA-R in most electrophysiological studies (Rogawski & Wenk, 2003). The situation is less clear, when a much more distal endpoint, i.e. excitotoxic neuronal death, is chosen. A high percentage of NMDA-R may need to be inhibited in order to affect excitotoxicity at all, and memantine's concentration-effect curve with respect to cell death may be dramatically right-shifted. To explore this, we started off with an excitotoxicity assay in organotypic rat hippocampal slice cultures. To assess cell death, the cultures were bathed in propidium iodide (PI) and assessed for intactness and baseline fluorescence readings before the start of the experiment. Then they were treated simultaneously with 10 μM NMDA and NMDA-R antagonists [(+)MK-801 or memantine], and 24 h later excitotoxicity was quantified by measurement of the PI fluorescence increase over baseline. NMDA alone increased fluorescence readings approximately ten-fold above baseline (Fig. 1A and B). As expected, cotreatment with (+)MK-801 resulted in complete protection from NMDA-induced toxicity, with PI fluorescence remaining at background levels. In this experimental system, memantine blocked cell death with an EC_{50} of approximately 1 μM (Fig. 1A). This indicates, that in this organotypic tissue culture system already a partial block of the NMDA-R system by memantine can have profound beneficial effects.

Next, we used the well-established model system of murine cerebellar granule cell (CGC) cultures. These neurons are glutamatergic and undergo postnatal differentiation with predominant expression of NMDA type glutamate receptors (Cox *et al*., 1990). A direct excitotoxicity assay was performed here also. However, these neurons grow under high potassium concentrations, which lead to a partial depolarization. Cellular depolarization may sensitize to excitotoxicity and reduce the protective effects of memantine in stressed neurons. We treated CGC with 100 μM NMDA to trigger approximately 80% cell death, and viability was assessed either by the neuronal capacity to reduce the dye MTT (Fig. 2A) or cell death was determined by counting nuclei with condensed chromatin (Fig. 2B). As positive control, NMDA-R were blocked by addition of 20 mM $MgCl_2$, which completely protected from NMDA-induced toxicity in CGC. Treatment with memantine (1 μM) resulted in partial protection from NMDA-induced cell death (Fig. 2B), while concentrations of 10 μM and above resulted in an almost complete block of the NMDA effect (Fig. 2A and B). Thus, it appears that memantine can have a significant protective effect in the 1–10 μM range, even when a direct NMDA-R agonist is applied under slightly depolarizing conditions.

Protection by memantine from indirect excitotoxicity triggered by the mitochondrial toxins MPP^+ and NO

Next, we intensified the challenge using stress models, which are dependent on energy-depletion and excitation of neurons by release of endogenous glutamate (Leist & Nicotera, 1998; Nicotera *et al*., 1999). It has been established previously that the mitochondrial toxin MPP^+ or the NO donor *S*-nitrosoglutathione (GSNO) induce excitotoxic apoptosis in CGC (Leist *et al*., 1997a; Leist *et al*., 1997b; Leist *et al*., 1998; Volbracht *et al*., 2001). Neuronal death is triggered by energy failure, and strictly dependent on endogenous glutamate release and NMDA-R activation (Fig. 3A). Exposure of CGC to 100 μM GSNO (Fig. 3B) or 50 μM MPP^+ (Fig. 3C) led to excitotoxic apoptosis of $>50\%$ of all neurons in culture. Cell death was quantified on the population level by the use of the dye MTT. In addition, apoptotic cells characterized by nuclear morphology and integrity were counted individually and the results of both assays were in good agreement (data not shown). As described earlier, NMDA-R blockage by (+)MK-801 resulted in complete protection from indirect excitotoxicity in these models. Treatment with memantine resulted in a concentration-dependent protection from excitotoxic cell loss with an EC_{50} of approximately 2.5 μM for GSNO (Fig. 3B) and approximately 5 μM for MPP^+ treatment (Fig. 3C). Concentrations of 5 μM and higher always led to significant protection (Fig. 3B and C). In some experiments, the protective effect of memantine was followed in greater detail by staining of cultures with calcein-AM. This allowed assessment of the neurite integrity and other aspects of cellular morphology (Fig. 3D). Treatment with memantine (10 μM) completely prevented MPP^+-induced (not shown) or GSNO-induced damage on the level of neurites and somata (Fig. 3D). Thus, in this model of combined excitotoxicity and energy depletion, low micro molar memantine concentrations were protective.

Protection by memantine from OGD in CGC and cortical neurons

OGD is another model where a close relationship between energy deficiency and excitotoxicity has been established (Choi & Rothman, 1990). Here, we exposed two different neuronal cultures, CGC (Fig. 4A) and CTX (Fig. 4B) to OGD and investigated the neuroprotective properties of memantine. CGC were exposed to OGD for 30 min followed by a recovery phase with addition of glucose under normoxic conditions for 6 h. OGD led to 50% cell death, which was completely prevented by (+)MK-801 and 6,7-dinitroquinoxaline-2,3-dione (DNQX) pretreatment. Here we used both NMDA-R and AMPA-R antagonists as positive control to completely shut down signalling via all ionotropic glutamate receptors. Memantine treatment resulted in 60% (10 μM) or complete (50 μM) protection from toxicity (Fig. 4A). A low concentration of 1 μM had no effect in this experimental system. Essentially similar results were obtained in CTX. These were exposed to OGD for 14 h followed by a recovery phase with addition of glucose under normoxic conditions for 24 h. This led to approximately 60% cell death, and total block of all ionotropic glutamate receptors with (+)MK-801 and DNQX rescued all neurons (Fig. 4B). Memantine protected concentration-dependently with an EC_{50} of approximately 2 μM from cell death (Fig. 4B). Here, a concentration as low as 2.5 μM reduced OGD-induced neuronal loss significantly.

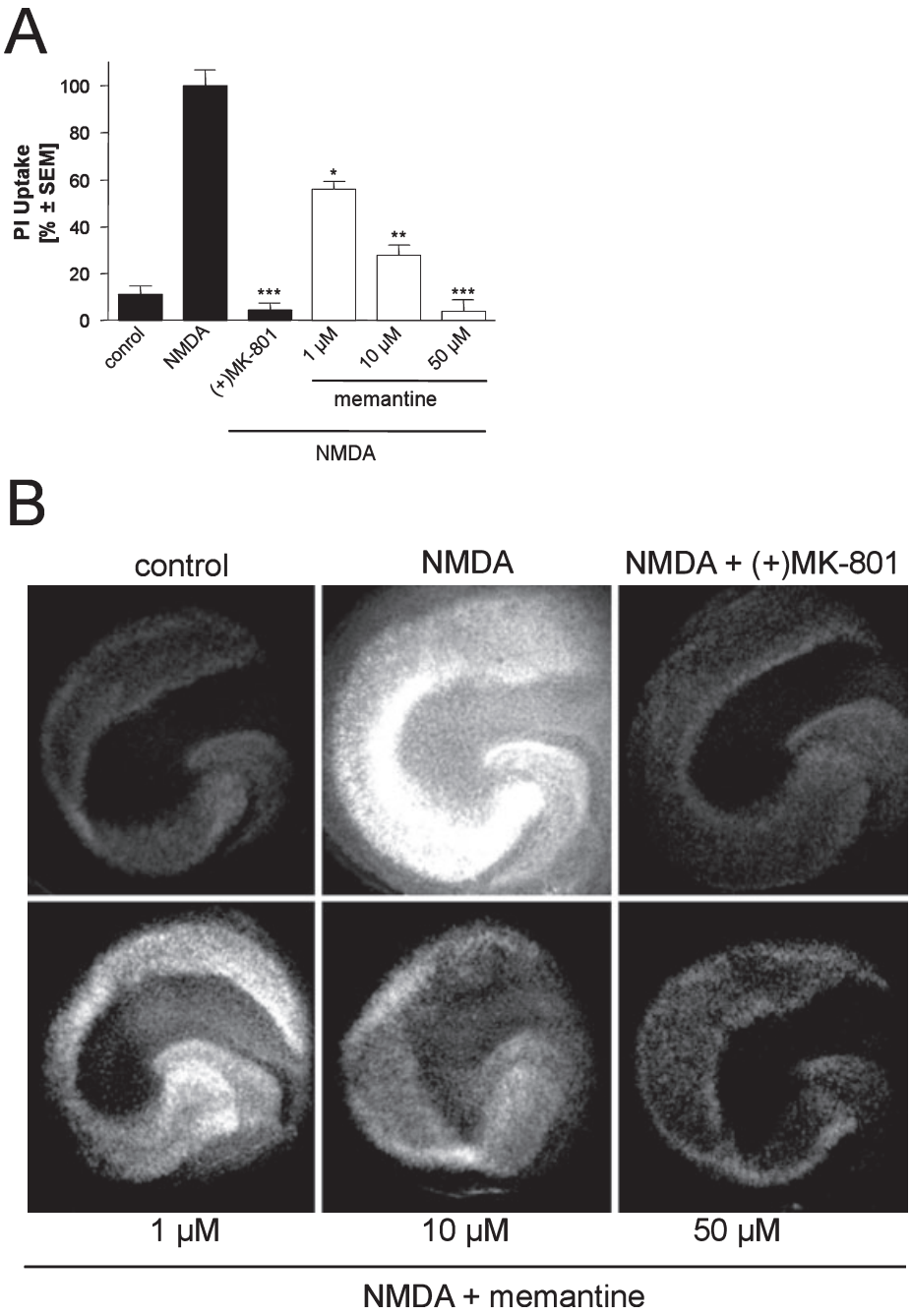

FIG. 1. Protection by memantine from NMDA-induced toxicity in organotypic hippocampal slices. (A) Organotypic rat hippocampal slice cultures were treated with 10 µM NMDA in the absence or presence of (+)MK-801 (10 µM) or memantine (1, 10, 50 µM) for 24 h. Cell death was assessed by measuring propidium iodide uptake using a fluorescent plate reader ($n = 4$). Data are means ± SEM; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, One-way ANOVA followed by the Tukey's test. NMDA treatment plus glutamate receptor blocker was tested vs. NMDA treatment alone. (B) Representative example images with width of image frame corresponding to 2.5 mm.

Protection from neonatal HI by memantine

We investigated whether memantine also shows some protection in a hypoxic-ischemic insult *in vivo*. For this purpose we used a well-established neonatal HI model. We subjected neonatal rat pups to unilateral HI. Immediately after ligation of the left carotid artery pups were randomly assigned to memantine or vehicle treatment ($n = 37$ pups/group), treated intraperitoneally with memantine (20 mg/kg) or with saline (10 µL/g) and exposed to 7.8% oxygen for 35 min. The mortality rates after HI was four times higher in the vehicle group ($P < 0.05$) than in the memantine-treated group (Table 1). The brain damage was evaluated by gross morphological scoring 3 days after HI (Table 1). This gives an indication of infarct sizes, because the scores show a virtually linear correlation with both measurements of infarct volumes and with histopathological scoring (Wang *et al*., 2004). The gross morphological injury was 24% smaller in the memantine-treated animals compared with the vehicle-treated animals (score of 2.1 ± 1.16 vs. 2.76 ± 0.92, respectively; $P = 0.014$; Table 1). Thus, memantine showed a relatively small,

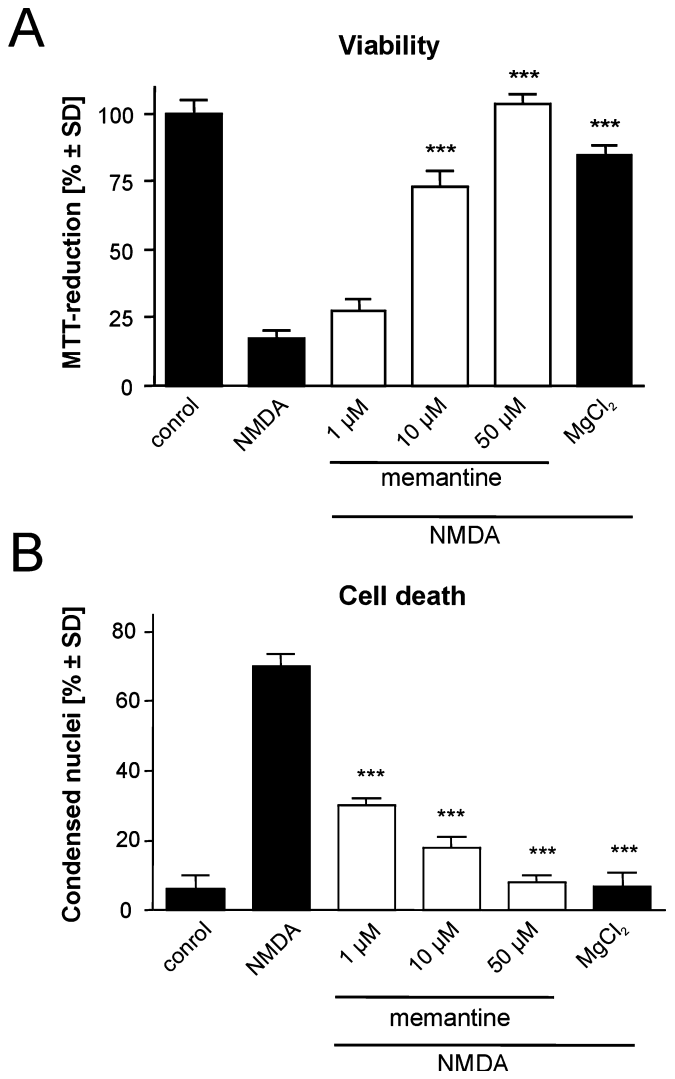

FIG. 2. Protection by memantine from NMDA-induced toxicity in cerebellar granule cells. Murine CGC were incubated with 100 µM NMDA in the absence or presence of $MgCl_2$ (20 mM) or memantine (1, 10, 50 µM) for 4 h. (A) The percentage of viable cells was quantified by their capacity to reduce MTT ($n = 3$). Data are means ± SD; ***$P < 0.001$, One-way ANOVA followed by the Tukey's test. NMDA treatment plus glutamate receptor blocker was tested vs. NMDA treatment alone. (B) Cell death was assessed in neurons stained with H-33342 and is expressed as percentage of nuclei with condensed chromatin ($n = 3$). Data are means ± SD; ***$P < 0.001$, One-way ANOVA followed by the Tukey's test. NMDA treatment plus glutamate receptor blocker was tested vs. NMDA treatment alone.

but significant protection in this *in vivo* model where excitotoxicity plays a prominent part (Hagberg *et al*., 1994; Puka-Sundvall *et al*., 2000).

Protection by memantine from excitotoxic insult in cerebellar microexplant cultures

Finally, we investigated protective effects of memantine in a model allowing the assessment of functional rescue, i.e. restoration of active cellular function. For this, we established cerebellar microexplant cultures. After an excitotoxic insult triggered by the mitochondrial toxin 3-NP, functional survival was measured on the basis of the neuronal capacity to migrate along elongated processes of Bergmann radial glial cells. Cerebellar granule cells survive and differentiate when cultured as microexplants in conditioned serum-free medium. One day after seeding, small clusters of cells showed small processes, which increased in number and length as the culture period proceeded (data not shown). After 3 days, cultures exhibit several long, radially orientated glial fibrillary acidic protein (GFAP)-positive processes extending from the microexplant (Fig. 5A). Radial alignment and GFAP staining suggested that these processes were most likely Bergman glial fibers. Along these fibers, we found increasing numbers of small bipolar cells migrating from the edges of the microexplant to the periphery. These cells stained positive for the neuronal markers MAP-2 (Fig. 5A) and neurofilament (data not shown). We identified these cells as migrating granule cells guided along the Bergmann glial processes (Fig. 5A). Notably, this directed movement of granule cells is dependent on NMDA-R activity, as shutdown of NMDA-R by (+)MK-801 or 5-aminophosphovalerate (APV) has been shown to negatively interfere with the neuronal migration in microexplant cultures (Komuro & Rakic, 1993; Fig. 5B).

We determined the number of migrating cells 3 days after seeding. Control cultures contained approximately 250 cells migrating from the edge of the microexplant to the periphery (Figs 5B and 6A). After treatment with the excitotoxin 3-NP (0.5 mM), we observed approximately 50% fewer neurons migrating out of the explants compared to the solvent-treated control cultures. Growth factors such as insulin-like growth factor 1 (IGF-1, 20 ng/mL) protected from excitotoxic cell loss in this model, resulting in 40% recovery from 3-NP injury (Figs 5B and 6A). A similar effect (45% recovery) was revealed with the AMPA-R blocker DNQX (10 µM). As expected, treatment of microexplants with the NMDA-R antagonists (+)MK-801 (10 µM) or APV (100 µM) did not result in functional survival from excitotoxic cell loss, because cells were inhibited in their migratory function (Figs 5B and 6A). Lower (+)MK-801 concentrations of 0.1 and 1 µM were not able to restore 3-NP-impaired neuronal outgrowth either (data not shown). Interestingly, memantine led to a significant, concentration-dependent functional recovery from excitotoxic cell loss. Treatment with 1 µM, 10 µM, or 50 µM memantine resulted in 29%, 38%, or 48% increase of migrating cells, respectively, compared to cultures treated with 3-NP alone (Fig. 6A and B). These findings support the notion of use-dependent NMDA-R inhibition by memantine, which can block under pathological conditions but still allows physiological activation.

Discussion

NMDA-R mediated neurotoxicity is thought to contribute to a broad variety of neurological diseases, including AD. We show here that the clinically used AD drug memantine, at therapeutically relevant doses leading to plasma levels of approximately 1 µM (Kornhuber & Quack, 1995), provided *in vitro* and *in vivo* protection in a variety of model systems relevant to NMDA-R mediated toxicity. The potential of memantine as a neuroprotective agent in preclinical models is well established. For instance, it has been shown, that memantine protected cultured cortical neurons, cerebellar granule cells, retinal ganglion cells and hippocampal neurons against NMDA-R-mediated toxicity (Erdo & Schafer, 1991; Chen *et al*., 1992; Weller *et al*., 1993; Krieglstein *et al*., 1996; Parsons *et al*., 1999b). Half maximal and higher neuroprotection with memantine was usually seen at concentrations of 10–50 µM. Here, we confirm these results, and determined the minimal effective concentrations of memantine. In organotypic hippocampal slice cultures memantine was neuroprotective against NMDA toxicity already at a concentration of approximately 1 µM

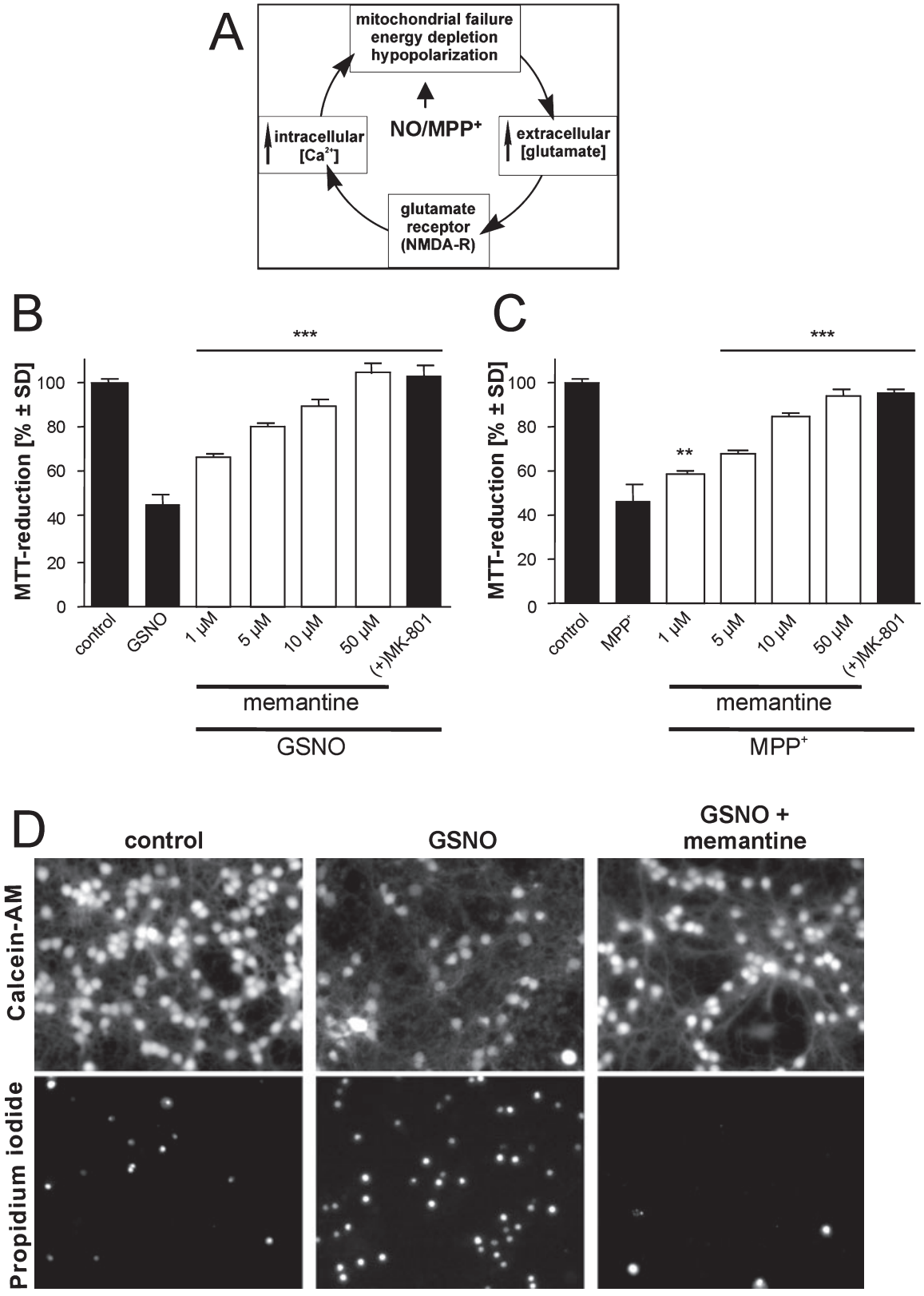

FIG. 3. Protection by memantine from GSNO and MPP^+-induced apoptosis in cerebellar granule cell cultures. (A) Autocrine excitotoxicity in CGC triggered by the mitochondrial toxins nitric oxide (NO) and MPP^+. (B and C) CGC were challenged with 100 µM GSNO (B) or 50 µM MPP^+ (C) in the absence or presence of 2 µM (+)MK-801 or memantine (1, 5, 10, 50 µM) for 4 h. The percentage of viable cells was quantified by their capacity to reduce MTT ($n = 3$). Data are means ± SD; $^{**}P < 0.01$, $^{***}P < 0.001$, One-way ANOVA followed by the Tukey's test. MPP^+ or GSNO treatment plus glutamate receptor blocker was tested vs. MPP^+ or GSNO treatment alone. (D) CGC were challenged with 100 µM GSNO in the absence or presence of 10 µM memantine for 4 h. Neurons were stained with calcein-AM and propidium iodide and fluorescence images corresponding to the same field of observation are displayed. Width of image frame corresponds to 260 µm.

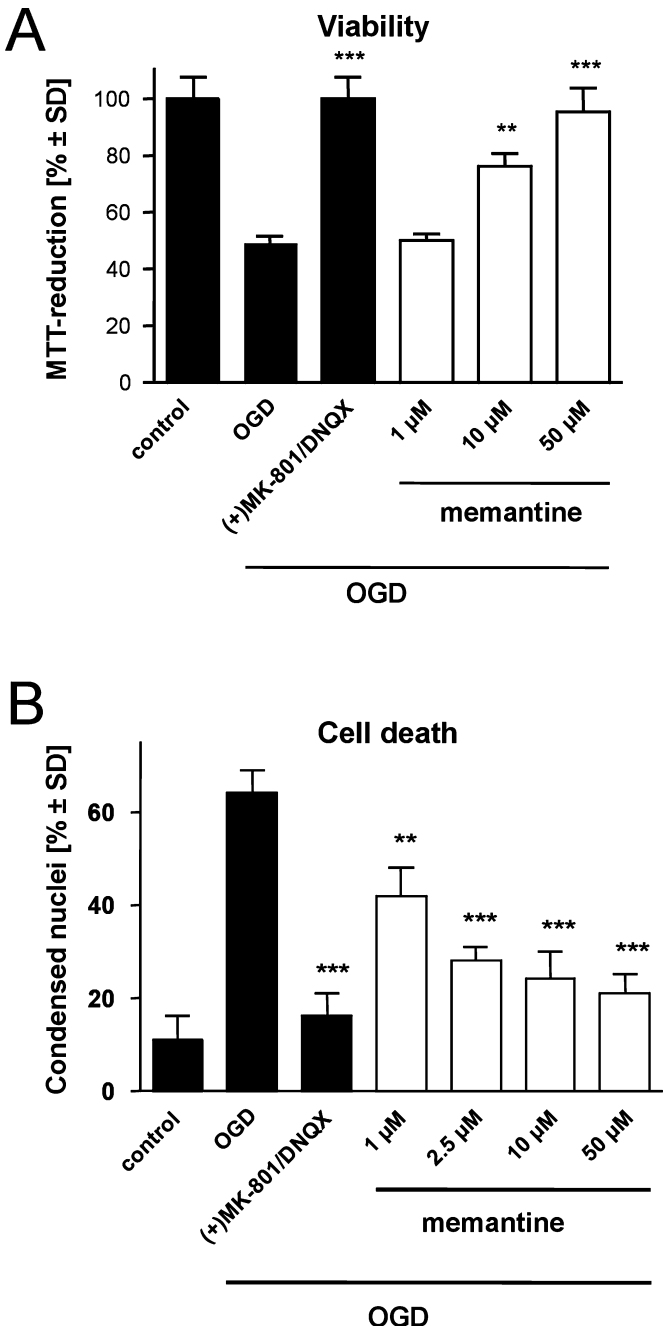

FIG. 4. Protection by memantine from OGD in cerebellar granule cells and cortical neurons. (A) Murine CGC were exposed for 30 min in an ischemia chamber to OGD in the absence or the presence of (+)MK-801 (1 μM) and DNQX (10 μM) or memantine (0, 1, 10, 50 μM). After the OGD period, glucose (20 mM) was added and cultures were kept under normoxic conditions for an additional 6 h. The percentage of viable cells was quantified by their capacity to reduce MTT ($n = 3$). Data are means ± SD; $^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA followed by the Tukey's test. OGD plus glutamate receptor blocker was tested vs. OGD alone. (B) Murine CTX were exposed for 14 h to OGD in the absence or the presence of (+)MK-801 (1 μM) and DNQX (10 μM) or memantine (0, 1, 2.5, 10, 50 μM). After the OGD period, glucose (20 mM) was added and cultures were kept under normoxic conditions for an additional 24 h. Cell death was assessed in neurons stained with H-33342 and expressed as percentage of nuclei with condensed chromatin ($n = 3$). Data are means ± SD; $^{**}P < 0.01$, $^{***}P < 0.001$, One-way ANOVA followed by the Tukey's test. OGD plus glutamate receptor blocker was tested vs. OGD alone.

TABLE 1. Protection by memantine from hypoxia-ischemia *in vivo*

	Vehicle	Memantine
Total number of pups	37	37
Mortality	8	2*
Gross morphology score	2.8 ± 0.92	2.2 ± 1.16*

Unilateral HI was induced in postnatal day 9 rats. After ligation of the left carotid artery, pups were randomly assigned to two groups and treated with memantine (20 mg/kg i.p.) or with saline (10 μL/g). After HI, animals recovered for 72 h and brain damage was evaluated by gross morphology scoring. Mortality data were analysed with the chi-square test, $^{*}P < 0.05$. Gross morphology score data are means ± SD; $^{*}P < 0.05$, unpaired Student's *t*-test.

(Fig. 1). In CGC treated with NMDA the minimal effective concentration of memantine was also 1 μM (Fig. 2). In contrast to the hippocampal slice cultures, CGC were cultured under conditions that facilitate depolarization, which can sensitize to excitotoxicity and reduce the neuroprotection of memantine in stressed neurons. Still, memantine showed protection from direct excitotoxicity in the 1–10 μM range also in neurons under partial depolarization. The lowest concentration resulting in significant protection (1 μM) is in fact within the clinically relevant concentration range (Kornhuber & Quack, 1995) for memantine in Alzheimer's disease patients (Reisberg *et al*., 2003).

In addition to its action as un-competitive NMDA-R antagonist, memantine has been found to antagonize 5-hydroxytryptamine type 3 (Rammes *et al*., 2001) and alpha7 nicotinic acetylcholine receptors (Aracava *et al*., 2005). These effects were seen at concentrations similar to those required for un-competitive antagonistic effects at the NMDA-R (Rammes *et al*., 2001; Aracava *et al*., 2005). However, as the above direct excitotoxic models are strictly dependent on over-activation of NMDA–R, we assume that the neuroprotective effect of memantine in excitotoxicity models is due to its action on the NMDA-R.

The above-mentioned introductory experiments used direct excitotoxic insults. However, excitotoxic pathophysiological mechanisms in neurodegenerative diseases as AD are believed not necessarily to depend only on increased glutamate levels *per se*, but also on increased receptor sensitivity and over-activation by resting levels of glutamate (Albin & Greenamyre, 1992), followed by release of endogenous glutamate (Henneberry *et al*., 1989; Choi & Rothman, 1990). It is likely that glutamatergic neurons are both executors and victims of excitotoxic processes. Energy depletion is among the frequent initiating conditions leading to excitotoxicity, and mitochondrial dysfunction is believed to be one of the most generalized causes favouring the development of neurodegenerative diseases (Beal, 1996). Therefore, we investigated memantine's neuroprotective effects in models that depend on energy depletion and endogenous glutamate release (Leist & Nicotera, 1998; Nicotera *et al*., 1999). The neurotoxin MPP^+ is an inhibitor of mitochondrial respiratory chain complex I (Nicklas *et al*., 1985) and the pathophysiological mediator NO released from GSNO acts as inhibitor of the mitochondrial respiratory chain (Clementi *et al*., 1998), among other cellular targets. Both compounds cause impairment of mitochondrial function and energy failure, triggering a vicious loop strictly dependent on endogenous glutamate release and NMDA-R activation, leading to loss of intracellular Ca^{2+} homeostasis and excitotoxicity in CGC (Leist *et al*., 1997a; Leist *et al*., 1997b; Leist *et al*., 1998; Volbracht *et al*., 2001). Distal mechanisms of the NMDA-R mediated Ca^{2+} increase involve

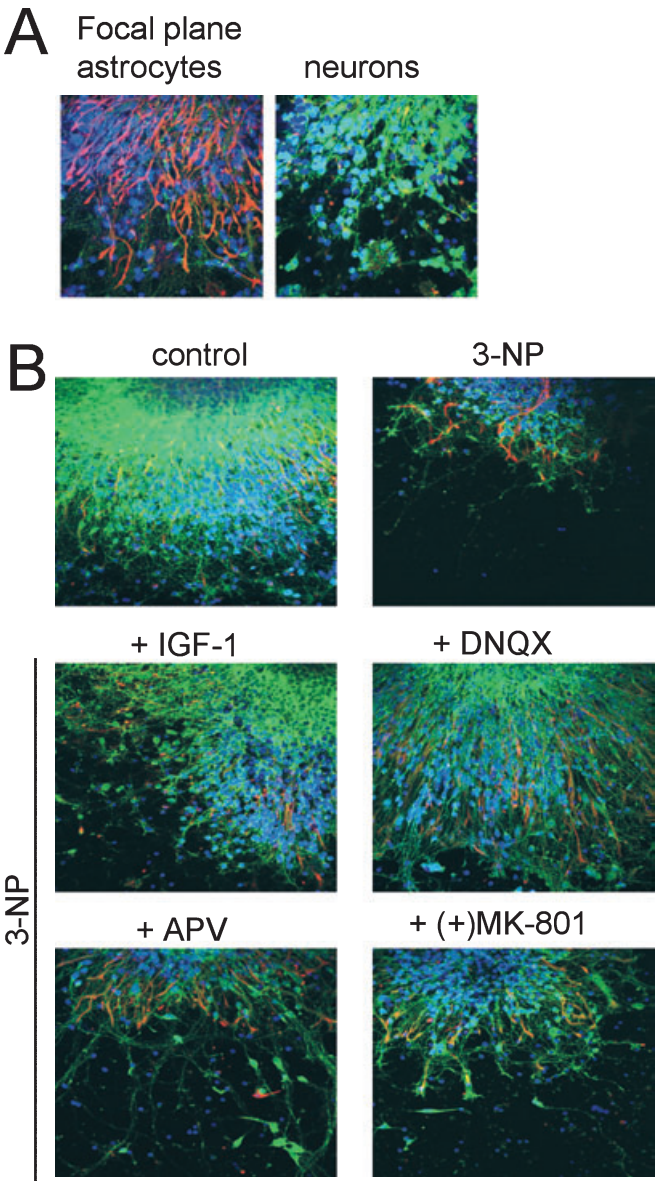

FIG. 5. NMDA–R-dependent neuronal migration in cerebellar microexplant cultures. (A) Rat cerebellar microexplants were cultured for 72 h. Specimens were fixed and subjected to immunofluorescent staining for GFAP (glia fibrillary acidic protein, an astrocyte marker, red channel), and MAP-2 (a neuronal marker, green channel). Nuclei were stained with H-33342 (blue channel). Images were recorded either on the focal plane of astrocytes (bottom of the dish) or on neurons (on top of astrocytes). The width of the image frame corresponds to 160 μm. (B) Microexplant cultures were exposed to 3-NP (0.5 mM) simultaneously with (+)MK-801 (10 μM), APV (100 μM), DNQX (10 μM) or IGF-1 (20 ng/mL) and fixed after 72 h. Specimens were subjected to immunofluorescent staining for GFAP (red channel), and MAP-2 (green channel). Nuclei were stained with H-33342 (blue channel). Images were recorded on the focal plane of neurons and image width corresponds to 320 μm.

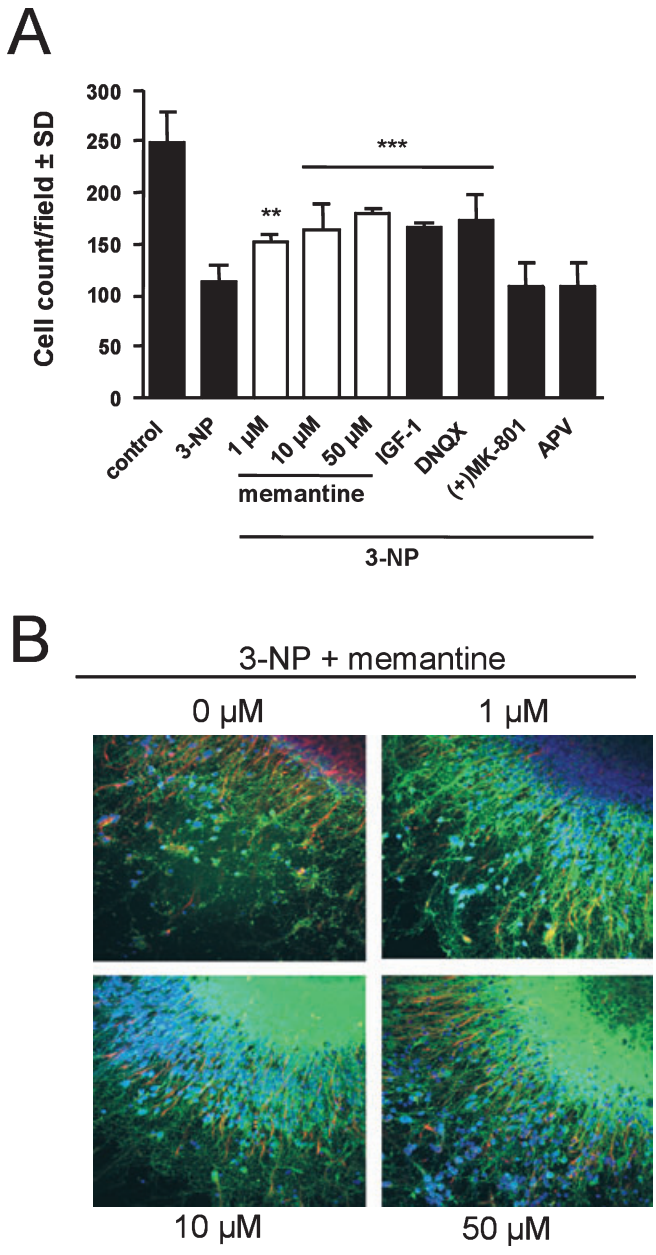

FIG. 6. Memantine protects from excitotoxic insult by 3-NP in cerebellar microexplant cultures. (A) Cerebellar microexplant cultures were exposed to 3-NP (0.5 mM) simultaneously with (+)MK-801 (10 μM), APV (100 μM), memantine (1, 10, 50 μM), DNQX (10 μM) or IGF-1 (20 ng/mL) and fixed after 72 h. Cells migrating out of the microexplants were counted manually by phase contrast microscopy ($n = 12$). Data are means ± SD; $^{**}P < 0.01$, $^{***}P < 0.001$; one-way ANOVA followed by the Tukey's test. 3-NP treatment plus glutamate receptor blocker/IGF-1 was tested vs. 3-NP treatment alone. (B) Specimens exposed to 3-NP (0.5 mM) simultaneously with memantine (0, 1, 10, 50 μM) were subjected to immunofluorescent staining for GFAP (red channel), and MAP-2 (green channel). Nuclei were stained with H-33342 (blue channel). Images were recorded on the focal plane of neurons and image width corresponds to 320 μm.

among others calpain activation (Volbracht *et al*., 2005). In line with this, inhibition of NMDA-R, exocytosis, or calpain activity has been shown to be protective in these models. Only NMDA-R blockage ameliorated all cytotoxic effects (Leist *et al*., 1997a; Leist *et al*., 1997b; Leist *et al*., 1998; Volbracht *et al*., 2001). Here, we show now, that memantine protected concentration-dependently from MPP^+-induced or GSNO-induced excitotoxicity. We determined the minimal effective concentrations of memantine to be in the range of approximately 2 μM (Fig. 3). Compared to direct excitotoxic insults, memantine displayed slightly lower potency in the indirect models, which may be explained by the more severe challenge in the latter. These *in vitro* observations are in good agreement with *in vivo* data. Memantine has been tested in animals against primary insults dependent on mitochondrial impairment and

energy depletion and provided protection from inhibition of mitochondrial function (Schulz *et al*., 1996; Wenk *et al*., 1996).

Another condition where a close relationship between energy deficiency and excitotoxicity has been established is for instance in stroke/ischemia where the lack of oxygen and glucose leads to cellular ATP depletion, causing glutamate release and NMDA-R over-stimulation (Choi & Rothman, 1990). This can be modelled *in vitro* by depriving neuronal cultures of oxygen and glucose for a limited time span. We found neuroprotection from OGD insult by memantine using either cerebellar granule cells as the model system with an EC_{50} of approximately 10 μM (Fig. 4A), or murine cortical neurons, with an EC_{50} of approximately 2 μM (Fig. 4B). Our results are in agreement with others using hypoxia/hypoglycaemia insults *in vitro*. Memantine protected chick cortical neurons against chemically induced ischemia with a minimal effective concentration of 1 μM (Seif el Nasr *et al*., 1990), led to partial neuroprotection in the 4–10 μM range in hippocampal slices exposed to OGD (Pringle *et al*., 2000; Sobrado *et al*., 2004) and blocked hypoxia/hypoglycaemia-induced suppression of excitatory postsynaptic potentials with an EC_{50} of 14 μM (Frankiewicz *et al*., 2000). Taken together with the results obtained in the GSNO and MPP^+ model, we demonstrate here, that memantine is neuroprotective with a minimal effective concentration of approximately 2 μM in indirect excitotoxicity models. These *in vitro* observations also hold true *in vivo*. Memantine was earlier shown to be protective in animal models of global (Seif el Nasr *et al*., 1990; Block & Schwarz, 1996) and focal ischemia (Dogan *et al*., 1999; Stieg *et al*., 1999; Gorgulu *et al*., 2000; Culmsee *et al*., 2004). Although our present work is not focused on *in vivo* effects, we confirmed memantine's neuroprotective action in a model of neonatal HI. Memantine reduced mortality after HI by a factor of four and attenuated brain injury by 24% (Table 1), which is an effect similar in size to earlier studies. To our knowledge, this is the first study demonstrating a protective effect of memantine in this model of perinatal asphyxia.

Despite the potentially damaging effects of high concentrations of glutamate, physiological glutamate activity is absolutely required for normal brain function. Complete block of the NMDA-R leads to a number of serious adverse effects, such as coma, drowsiness, and hallucinations (Koroshetz & Moskowitz, 1996). Glutamate receptors are actively required for long-term potentiation, an experimental paradigm thought to model some molecular aspects of learning and memory (Bliss & Collingridge, 1993). It is hypothesized that reduced glutamatergic neurotransmission in critical brain areas may even contribute to cognitive decline in AD (Greenamyre *et al*., 1985; Greenamyre *et al*., 1988). For these reasons a positive modulation of glutamatergic function might be a promising therapeutic strategy in AD. These conflicting considerations indicate that the prevention of excitotoxicity by glutamate receptor antagonists must be achieved without interfering with the physiological action of glutamate that is required for memory and learning. Therefore, it is of equal significance not only to show protection from excitotoxicity, but also to predict the capacity of neuroprotective NMDA-R antagonists to allow physiological action. Physiologically, NMDA-R are activated transiently by mM concentrations of glutamate (Clements *et al*., 1992), whereas during pathological activation, NMDA-R are believed to be activated by lower concentrations of glutamate but more or less continuously (Szatkowski & Attwell, 1994). Thus, Mg^{2+}, which normally acts as a physiological blocker, is not effective enough to serve this role upon prolonged depolarization during excitotoxic insults. This places low affinity un-competitive NMDA-R antagonist memantine intermediate between Mg^{2+} and high affinity drugs (Parsons *et al*., 1993; Parsons *et al*., 1995). High affinity antagonists such as (+)MK-801 are undesirable as they entail nonselective inhibition of tonic glutamate activity as well as phasic physiological NMDA-R function. Memantine likewise to Mg^{2+} is removed from the NMDA-R channel upon strong synaptic depolarization due to its voltage dependency and rapid unblocking kinetics. However, it does not leave the channel as easily as Mg^{2+} upon prolonged depolarization during chronic excitotoxic insults (Kornhuber & Weller, 1997; Danysz & Parsons, 2003; Lipton, 2006).

We demonstrate here that memantine can block sustained NMDA-R activation under pathological conditions induced by the indirect excitotoxin NP-3 in cerebellar microexplant cultures without inhibiting physiological function (Fig. 6). As the functional parameter in this system, we used neuronal migration, which is dependent on NMDA-R activity. It has been established, that blocking of the NMDA-R by competitive antagonists such as APV and un-competitive antagonists such as (+)MK-801 negatively interferes with neuronal migration (Komuro & Rakic, 1993). The directed neuronal movement is highly dependent on intracellular Ca^{2+} dynamics *via* NMDA-R activation. Other ionotropic glutamate receptors have not been implicated in this process, and AMPA-R blockers did not influence neuronal migration (Komuro & Rakic, 1993). As expected, the NMDA-R antagonists (+)MK-801 and APV did not result in functional rescue from excitotoxic cell loss (Fig. 5). These antagonists might have protected neurons against excitotoxicity induced by NP-3, but then inhibited neuronal migration. On the other hand, the AMPA-R blocker DNQX did not influence neuronal migration and protected from excitotoxicity (Fig. 5). Interestingly, memantine did not interfere with neuronal migration either. Determined by the amount of migrating neurons, memantine led concentration-dependently to the functional survival from excitotoxic cell loss with an EC_{30} of 1 μM (Fig. 6). To our knowledge, this is the first report demonstrating protective effects of memantine against excitotoxicity with preserved NMDA-R-dependent migrational ability. The capacity to protect from excitotoxicity but also to allow neuronal migration distinguishes memantine from the other NMDA-R antagonists used here. When comparing different antagonists, it is important that their different affinities for NMDA-R are considered. Memantine was used at a concentration (50 μM) that was approximately 50 times higher than its dissociation constant on the NMDA-R. On the other hand (+)MK-801 was used at concentrations (0.1, 1 and 10 μM) approximately 30-, 300-, and 3000-fold higher than its affinity for NMDA-R. As the highest memantine and lowest MK-801concentration used here were in a similar range in relation to their individual affinity for the NMDA-R, effects of different occupancies seem unlikely, but cannot be completely excluded. Altogether, we assume from these results that memantine blocks under pathological conditions, but leaves the NMDA-R channel upon for transient physiological activation.

Acknowledgements

The excellent technical assistance of A. Christiansen is gratefully acknowledged. We thank Dr F. Sieg from NeuronZ Limited (Auckland, New Zealand) for providing valuable help establishing the cerebellar microexplant cultures.

Abbreviations

AD, Alzheimer's disease; APV, 5-aminophosphovalerate; CGC, cerebellar granule cells; CTX, cortical neurons; DIV, days *in vitro*; DNQX, 6,7-dinitroquinoxaline-2,3-dione; GSNO, *S*-nitroso-L-glutathione; HI, hypoxia-ischemia; IGF-1, insulin-like growth factor 1; (+)MK-801, [5R,10S]-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine; MPP^+, 1-methyl-4-phenylpyridinium; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrasodium bromide; NMDA, *N*-methyl-D-aspartate; NMDA-R, NMDA receptor; NO, nitric oxide; 3-NP, 3-nitropropionic acid; OGD, oxygen-glucose deprivation.

References

Albin, R.L. & Greenamyre, J.T. (1992) Alternative excitotoxic hypotheses. *Neurology*, **42**, 733–738.

Aracava, Y., Pereira, E.F., Maelicke, A. & Albuquerque, E.X. (2005) Memantine blocks alpha7* nicotinic acetylcholine receptors more potently than *N*-methyl-D-aspartate receptors in rat hippocampal neurons. *J. Pharmacol. Exp. Ther.*, **312**, 1195–1205.

Beal, M.F. (1996) Mitochondria, free radicals, and neurodegeneration. *Curr. Opin. Neurobiol.*, **6**, 661–666.

Bliss, T.V. & Collingridge, G.L. (1993) A synaptic model of memory: long-term potentiation in the hippocampus. *Nature*, **361**, 31–39.

Block, F. & Schwarz, M. (1996) Memantine reduces functional and morphological consequences induced by global ischemia in rats. *Neurosci. Lett.*, **208**, 41–44.

Chen, H.S., Pellegrini, J.W., Aggarwal, S.K., Lei, S.Z., Warach, S., Jensen, F.E. & Lipton, S.A. (1992) Open-channel block of *N*-methyl-D-aspartate (NMDA) responses by memantine: therapeutic advantage against NMDA receptor-mediated neurotoxicity. *J. Neurosci.*, **12**, 4427–4436.

Choi, D.W. & Rothman, S.M. (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu. Rev. Neurosci.*, **13**, 171–182.

Clementi, E., Brown, G.C., Feelisch, M. & Moncada, S. (1998) Persistent inhibition of cell respiration by nitric oxide: crucial role of *S*-nitrosylation of mitochondrial complex I and protective action of glutathione. *Proc. Natl Acad. Sci. USA*, **95**, 7631–7636.

Clements, J.D., Lester, R.A., Tong, G., Jahr, C.E. & Westbrook, G.L. (1992) The time course of glutamate in the synaptic cleft. *Science*, **258**, 1498–1501.

Cox, J.A., Felder, C.C. & Henneberry, R.C. (1990) Differential expression of excitatory amino acid receptor subtypes in cultured cerebellar neurons. *Neuron*, **4**, 941–947.

Culmsee, C., Junker, V., Kremers, W., Thal, S., Plesnila, N. & Krieglstein, J. (2004) Combination therapy in ischemic stroke: synergistic neuroprotective effects of memantine and clenbuterol. *Stroke*, **35**, 1197–1202.

Danysz, W. & Parsons, C.G. (2003) The NMDA receptor antagonist memantine as a symptomatological and neuroprotective treatment for Alzheimer's disease: preclinical evidence. *Int. J. Geriatr. Psychiatry*, **18**, S23–S32.

Dogan, A., Eras, M.A., Rao, V.L. & Dempsey, R.J. (1999) Protective effects of memantine against ischemia-reperfusion injury in spontaneously hypertensive rats. *Acta. Neurochir. (Wien)*, **141**, 1107–1113.

Erdo, S.L. & Schafer, M. (1991) Memantine is highly potent in protecting cortical cultures against excitotoxic cell death evoked by glutamate and *N*-methyl-D-aspartate. *Eur. J. Pharmacol.*, **198**, 215–217.

Frankiewicz, T., Pilc, A. & Parsons, C.G. (2000) Differential effects of NMDA-receptor antagonists on long-term potentiation and hypoxic/hypoglycaemic excitotoxicity in hippocampal slices. *Neuropharmacology*, **39**, 631–642.

Gorgulu, A., Kins, T., Cobanoglu, S., Unal, F., Izgi, N.I., Yanik, B. & Kucuk, M. (2000) Reduction of edema and infarction by memantine and MK-801 after focal cerebral ischaemia and reperfusion in rat. *Acta. Neurochir. (Wien)*, **142**, 1287–1292.

Greenamyre, J.T., Maragos, W.F., Albin, R.L., Penney, J.B. & Young, A.B. (1988) Glutamate transmission and toxicity in Alzheimer's disease. *Prog. Neuropsychopharmacol. Biol. Psychiatry*, **12**, 421–430.

Greenamyre, J.T., Penney, J.B., Young, A.B., D'Amato, C.J., Hicks, S.P. & Shoulson, I. (1985) Alterations in L-glutamate binding in Alzheimer's and Huntington's diseases. *Science*, **227**, 1496–1499.

Hagberg, H., Gilland, E., Diemer, N.H. & Andine, P. (1994) Hypoxia-ischemia in the neonatal rat brain: histopathology after post-treatment with NMDA and non-NMDA receptor antagonists. *Biol. Neonate*, **66**, 205–213.

Henneberry, R.C., Novelli, A., Cox, J.A. & Lysko, P.G. (1989) Neurotoxicity at the *N*-methyl-D-aspartate receptor in energy-compromised neurons. An hypothesis for cell death in aging and disease. *Ann. N.Y. Acad. Sci.*, **568**, 225–233.

Hertz, E., YuA.C.H., Hertz, L., Juurlink, B.H.J. & Schousboe, A. (1989) Preparation of primary cultures of mouse cortical neurons. In Shahar, A., De Vellis, J., Vernadakis, A. & Haber, B., (Eds), *A Dissection and Tissue Culture Manual of the Nervous System*. Alan R. Liss, Inc., New York, pp. 105–108.

Komuro, H. & Rakic, P. (1993) Modulation of neuronal migration by NMDA receptors. *Science*, **260**, 95–97.

Kornhuber, J., Mack-Burkhardt, F., Kornhuber, M.E. & Riederer, P. (1989) [3H]MK-801 binding sites in post-mortem human frontal cortex. *Eur. J. Pharmacol.*, **162**, 483–490.

Kornhuber, J. & Quack, G. (1995) Cerebrospinal fluid and serum concentrations of the *N*-methyl-D-aspartate (NMDA) receptor antagonist memantine in man. *Neurosci. Lett.*, **195**, 137–139.

Kornhuber, J. & Weller, M. (1997) Psychotogenicity and *N*-methyl-D-aspartate receptor antagonism: implications for neuroprotective pharmacotherapy. *Biol. Psychiatry*, **41**, 135–144.

Koroshetz, W.J. & Moskowitz, M.A. (1996) Emerging treatments for stroke in humans. *Trends Pharmacol. Sci.*, **17**, 227–233.

Krieglstein, J., Lippert, K. & Poch, G. (1996) Apparent independent action of nimodipine and glutamate antagonists to protect cultured neurons against glutamate-induced damage. *Neuropharmacology*, **35**, 1737–1742.

Leist, M., Fava, E., Montecucco, C. & Nicotera, P. (1997a) Peroxynitrite and nitric oxide donors induce neuronal apoptosis by eliciting autocrine excitotoxicity. *Eur. J. Neurosci.*, **9**, 1488–1498.

Leist, M. & Nicotera, P. (1998) Apoptosis, excitotoxicity, and neuropathology. *Exp. Cell Res.*, **239**, 183–201.

Leist, M., Volbracht, C., Fava, E. & Nicotera, P. (1998) 1-Methyl-4-phenylpyridinium induces autocrine excitotoxicity, protease activation, and neuronal apoptosis. *Mol. Pharmacol.*, **54**, 789–801.

Leist, M., Volbracht, C., Kühnle, S., Fava, E., Ferrando-May, E. & Nicotera, P. (1997b) Caspase-mediated apoptosis in neuronal excitotoxicity triggered by nitric oxide. *Mol. Med.*, **3**, 750–764.

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

Nicklas, W.J., Vyas, I. & Heikkila, R.E. (1985) Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *Life Sci.*, **36**, 2503–2508.

Nicotera, P., Leist, M. & Manzo, L. (1999) Neuronal cell death: a demise with different shapes. *Trends Pharmacol. Sci.*, **20**, 46–51.

Parsons, C.G., Danysz, W., Bartmann, A., Spielmanns, P., Frankiewicz, T., Hesselink, M., Eilbacher, B. & Quack, G. (1999b) Amino-alkyl-cyclohexanes are novel uncompetitive NMDA receptor antagonists with strong voltage-dependency and fast blocking kinetics: *in vitro* and *in vivo* characterization. *Neuropharmacology*, **38**, 85–108.

Parsons, C.G., Danysz, W. & Quack, G. (1999a) Memantine is a clinically well tolerated *N*-methyl-D-aspartate (NMDA) receptor antagonist – a review of preclinical data. *Neuropharmacology*, **38**, 735–767.

Parsons, C.G., Gruner, R., Rozental, J., Millar, J. & Lodge, D. (1993) Patch clamp studies on the kinetics and selectivity of *N*-methyl-D-aspartate receptor antagonism by memantine (1-amino-3,5-dimethyladamantan). *Neuropharmacology*, **32**, 1337–1350.

Parsons, C.G., Quack, G., Bresink, I., Baran, L., Przegalinski, E., Kostowski, W., Krzascik, P., Hartmann, S. & Danysz, W. (1995) Comparison of the potency, kinetics and voltage-dependency of a series of uncompetitive NMDA receptor antagonists *in vitro* with anticonvulsive and motor impairment activity *in vivo*. *Neuropharmacology*, **34**, 1239–1258.

Pringle, A.K., Self, J., Eshak, M. & Iannotti, F. (2000) Reducing conditions significantly attenuate the neuroprotective efficacy of competitive, but not other NMDA receptor antagonists *in vitro*. *Eur. J. Neurosci.*, **12**, 3833–3842.

Puka-Sundvall, M., Wallin, C., Gilland, E., Hallin, U., Wang, X., Sandberg, M., Karlsson, J., Blomgren, K. & Hagberg, H. (2000) Impairment of mitochondrial respiration after cerebral hypoxia-ischemia in immature rats: relationship to activation of caspase-3 and neuronal injury. *Brain Res. Dev. Brain Res.*, **125**, 43–50.

Rammes, G., Rupprecht, R., Ferrari, U., Zieglgansberger, W. & Parsons, C.G. (2001) The *N*-methyl-D-aspartate receptor channel blockers memantine, MRZ 2/579 and other amino-alkyl-cyclohexanes antagonise 5-HT(3) receptor currents in cultured HEK-293 and N1E-115 cell systems in a non-competitive manner. *Neurosci. Lett.*, **306**, 81–84.

Reisberg, B., Doody, R., Stoffler, A., Schmitt, F., Ferris, S. & Mobius, H.J. (2003) Memantine in moderate-to-severe Alzheimer's disease. *N. Engl. J. Med.*, **348**, 1333–1341.

Rekling, J.C. (2003) Neuroprotective effects of anticonvulsants in rat hippocampal slice cultures exposed to oxygen/glucose deprivation. *Neurosci. Lett.*, **335**, 167–170.

Rice, J.E., 3rd, Vannucci, R.C. & Brierley, J.B. (1981) The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Ann. Neurol.*, **9**, 131–141.

Rogawski, M.A. (2000) Low affinity channel blocking (uncompetitive) NMDA receptor antagonists as therapeutic agents – toward an understanding of their favorable tolerability. *Amino Acids*, **19**, 133–149.

Rogawski, M.A. & Wenk, G.L. (2003) The neuropharmacological basis for the use of memantine in the treatment of Alzheimer's disease. *CNS Drug Rev.*, **9**, 275–308.

Schulz, J.B., Matthews, R.T., Henshaw, D.R. & Beal, M.F. (1996) Neuroprotective strategies for treatment of lesions produced by mitochondrial toxins: implications for neurodegenerative diseases. *Neuroscience*, **71**, 1043–1048.

Seif el Nasr, M., Peruche, B., Rossberg, C., Mennel, H.D. & Krieglstein, J. (1990) Neuroprotective effect of memantine demonstrated *in vivo* and *in vitro*. *Eur. J. Pharmacol.*, **185**, 19–24.

Sobrado, M., Roda, J.M., Lopez, M.G., Egea, J. & Garcia, A.G. (2004) Galantamine and memantine produce different degrees of neuroprotection in rat hippocampal slices subjected to oxygen-glucose deprivation. *Neurosci. Lett.*, **365**, 132–136.

Stieg, P.E., Sathi, S., Warach, S., Le, D.A. & Lipton, S.A. (1999) Neuroprotection by the NMDA receptor-associated open-channel blocker memantine in a photothrombotic model of cerebral focal ischemia in neonatal rat. *Eur. J. Pharmacol.*, **375**, 115–120.

Stoppini, L., Buchs, P.A. & Muller, D. (1991) A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Meth.*, **37**, 173–182.

Szatkowski, M. & Attwell, D. (1994) Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. *TINS*, **17**, 359–365.

Volbracht, C., Chua, B.T., Ng, C.P., Bahr, B.A., Hong, W. & Li, P. (2005) The critical role of calpain versus caspase activation in excitotoxic injury induced by nitric oxide. *J. Neurochem.*, **93**, 1280–1292.

Volbracht, C., Fava, E., Leist, M. & Nicotera, P. (2001) Calpain inhibitors prevent nitric oxide-triggered excitotoxic apoptosis. *Neuroreport*, **12**, 3645–3648.

Volbracht, C., Leist, M. & Nicotera, P. (1999) ATP controls neuronal apoptosis triggered by microtubule breakdown or potassium deprivation. *Mol. Med.*, **5**, 477–489.

Wang, X., Zhu, C., Gerwien, J.G., Schrattenholz, A., Sandberg, M., Leist, M. & Blomgren, K. (2004) The nonerythropoietic asialoerythropoietin protects against neonatal hypoxia-ischemia as potently as erythropoietin. *J. Neurochem.*, **91**, 900–910.

Weller, M., Finiels-Marlier, F. & Paul, S.M. (1993) NMDA receptor-mediated glutamate toxicity of cultured cerebellar, cortical and mesencephalic neurons: neuroprotective properties of amantadine and memantine. *Brain Res.*, **613**, 143–148.

Wenk, G.L., Danysz, W. & Roice, D.D. (1996) The effects of mitochondrial failure upon cholinergic toxicity in the nucleus basalis. *Neuroreport*, **7**, 1453–1456.

Yager, J.Y., Heitjan, D.F., Towfighi, J. & Vannucci, R.C. (1992) Effect of insulin-induced and fasting hypoglycemia on perinatal hypoxic-ischemic brain damage. *Pediatr. Res.*, **31**, 138–142.

Zeevalk, G.D. & Nicklas, W.J. (1991) Mechanisms underlying initiation of excitotoxicity associated with metabolic inhibition. *J. Pharmacol. Exp. Ther.*, **257**, 870–878.