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European Journal of Pharmacology 554 (2007) 18–29
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Neuroprotective activity of selective mGlu1 and mGlu5 antagonists *in vitro* and *in vivo*

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Received 30 April 2006; received in revised form 21 September 2006; accepted 26 September 2006
Available online 10 October 2006

Abstract

The neuroprotective potential of allosteric mGlu5 and mGlu1 antagonists such as 6-methyl-2-(phenylethynyl)-pyridin (MPEP)/[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) and (3-ethyl-2-methyl-quinolin-6-yl)-(4-methoxy-cyclohexyl)-methanone methanesulfonate (EMQMCM), was tested *in vitro* in organotypic hippocampal cultures and in the middle cerebral artery occlusion model of stroke *in vivo*. Both classes of agent have high selectivity toward mGlu sub-types and are active in animal models of various diseases indicating satisfactory CNS penetration. In organotypic hippocampal cultures MPEP showed high neuroprotective potency against sub-chronic (12 days) insult produced by 3-NP with an IC_{50} of c.a. 70 nM. In contrast, although the mGlu1 antagonist EMQMCM was also protective, it seems to be weaker yielding an IC_{50} of c.a. 1 μM. Similarly, in the transient (90 min) middle cerebral artery occlusion model of ischaemia in rats, MTEP seems to be more effective than EMQMCM. MTEP, at 2.5 mg/kg and at 5 mg/kg provided 50 and 70% neuroprotection if injected 2 h after the onset of ischaemia. At a dose of 5 mg/kg, significant (50%) neuroprotection was also seen if the treatment was delayed by 4 h. EMQMCM was not protective at 5 mg/kg (given 2 h after occlusion) but at 10 mg/kg 50% of neuroprotection was observed. The present data support stronger neuroprotective potential of mGlu5 than mGlu1 antagonists.

Keywords: (3-ethyl-2-methyl-quinolin-6-yl)-(4-methoxy-cyclohexyl)-methanone methanesulfonate (EMQMCM); 6-methyl-2-(phenylethynyl)-pyridine (MPEP); [(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP); mGlu1 antagonist; mGlu5 antagonist; Middle cerebral artery occlusion; Stroke; Neuroprotection; *In vivo*; *In vitro*; Organotypic cultures

1. Introduction

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors (GPCRs); the functional unit is a homodimer. They, couple through G proteins either directly to ion channels or to second messenger cascades, via phospholipase C (PLC) or adenylate cyclase (AC) (Schoepp, 2001; Pin and Duvoisin, 1995). Group I mGlu receptors which includes mGlu1 and mGlu5, act through Gq coupled to PLC resulting, upon activation, in stimulation of phosphoinositide hydrolysis and release of Ca^{2+} from intracellular stores (Conn and Pin, 1997). It has been suggested that targeting these receptor sub-types may offer therapeutic potential in many CNS disorders (Spooren et al., 2003). However, the role of group I mGlu in neurodegeneration still remains controversial. Whereas antagonists of group I mGlu have most often been associated with neuroprotection, agonists have been found to either amplify or attenuate neuronal cell death *in vitro* (Copani et al., 1995; Nicoletti et al., 1999; Allen et al., 2000).

Activation of mGlu1 and mGlu5 receptors might promote multiple processes contributing to pathological cascades leading to post-ischaemic neuronal death. These processes include: an increase in neuronal excitability due to the activation of inward cationic currents or the reduction of outward K^+ conductance; direct Ca^{2+} influx from the extracellular space through NMDA receptors and L-type channels (Fagni et al., 2000); an enhancement of the release of glutamate that has been connected with the neurotoxic effects of group I mGlu receptor agonists

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doi:10.1016/j.ejphar.2006.09.061

(Strasser et al., 1998); a potentiation of NMDA and AMPA receptor responses and activation of the MAP kinase pathway via PKC (Calabresi et al., 2001).

The discovery of selective mGlu agonists/antagonists, provided novel tools for addressing the role of individual receptor sub-types and their importance as potential therapeutics (Spooren et al., 2003). The selective mGlu1 antagonists (*S*)-4-carboxypheylglycine (AIDA), 7-(hydroxyimino)-cyclopropa[*b*]chromen-1*a*-carboxylate ethyl ester (CPCCOEt), and (*S*)-(+)-alpha-amino-4-carboxy-2-methylbezeneacetic acid (LY367385) reduced traumatic neuronal injury *in vivo* and *in vitro* (Faden et al., 2001). Moreover, the mGlu1 antagonists AIDA and (*S*)-(+)-2-(3′-carboxybicyclo [1.1.1]pentyl)-glycine (CBPG) attenuated degeneration of vulnerable CA1 pyramidal neurons in gerbils subjected to transient global ischaemia (PellegriniGiampietro et al., 1999a,b), whereas the mGlu5 selective antagonist MPEP (6-methyl-2-(phenylethynyl)-pyridine) failed to provide neuroprotection after i.c.v. administration (Meli et al., 2002). Based on these data and *in vitro* studies, the authors suggested that the activation of mGlu1 but not mGlu5 receptor contributes to post-ischaemic neuronal injury in cortical/neocortical areas (Meli et al., 2002). These findings provided the general basis for a recent review implying a role of mGlu1 rather than mGlu5 receptors in neurodegeneration in ischaemic conditions (Pellegrini-Giampietro, 2003).

However, others, using the middle cerebral artery occlusion ischaemia model of stroke reported that, the mGlu5 antagonist MPEP and a group I agonist (RS)-2-Cholro-5-Hydroxyphenylglycine (CHPG) exerted similar protection when applied i.c.v. 15 min after occlusion (Bao et al., 2001). Protective effect of MPEP was lost if delayed administration of 135 min was used.

Considering the above controversial findings, the present study was aimed to compare the neuroprotective actions of the novel highly selective mGlu1 receptor antagonist (3-ethyl-2-methyl-quinolin-6-yl)-(4-methoxy-cyclohexyl)-methanone methanesulfonate (EMQMCM) with the mGlu5 receptor antagonist [(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP) after systemic administration in middle cerebral artery occlusion model of ischaemia in rats. Additionally, neuroprotective effect of EMQMCM and MPEP were tested in a sub-chronic model of excitotoxicity *in vitro* in organotypic hippocampal slices subjected to 12 days exposure to the mitochondrial toxin 3-nitropropionic acid (3-NP).

The results have been presented, in part, in an abstract form (Baude et al., 2004).

2. Materials and methods

2.1. Subjects

12-day-old Sprague Dawley rats (Janvier, France) were used for the preparation of organotypic slice cultures. In the middle cerebral artery occlusion study, 3-months old male Wistar rats weighing 250–320 g were used (Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland). Before experiments animals were housed in groups of 5 under standard laboratory conditions. After middle cerebral artery occlusion, animals were housed individually until sacrificed. All animals had free access to food and water with the exception of the day before the *in vivo* experiment when they were fasted overnight. The experimental protocol was approved by the Local Animal Care and Use Committee and conforms to the national guidelines for the care and use of animals in research. The study was approved by the Ethical Committee, Regierung-spraesidium Darmstadt, Hessen and performed in accordance with the recommendations and policies of the U.S. National Institutes of Health Guidelines for the Use of Animals.

2.2. 3-NP-induced toxicity in organotypic slice cultures

2.2.1. Organotypic slice cultures preparation

Hippocampal slice cultures were prepared by the interface culture method, previously described by Stoppini et al. (1991) and modified by Bahr et al. (1994, 1995). Briefly, 12-day-old Sprague Dawley rats were killed by decapitation and the hippocampi were dissected out and sliced transversely into 400-μm sections. 10 slices were plated onto 0.4-μm porous membrane inserts (Millicell-CM, Millipore) in 6 well plates (Corning Costar). Each well contained 1 ml of culture medium composed of 50% Basal Eagle medium, 25% Earle's balanced salt solution, 25% horse serum, supplemented with 3 mM glutamine, 20 mM HEPES, 5 mM $NaHCO_3$, 40 mM D-glucose, 0.5 mM ascorbic acid, 2 mM $CaCl_2$, 2.5 mM $MgSO_4$, 1 mg/l insulin, 5 units/ml of penicillin and 5 mg/l streptomycin (all by Sigma). The slices were allowed to mature and stabilize for 2 weeks at 37 °C, humidified air and 5% CO_2. Medium was changed every 2–3 days.

2.2.2. Semi-chronic neuroprotection assay

During the 12 days of intoxication with 3-NP (35 μM) an increasing slice toxicity was induced. To study the neuroprotective potency of the mGlu receptor antagonists, these were co-incubated at various concentrations during the whole exposure period. The neuroprotective effect was quantified biochemically by measuring lactate dehydrogenase (LDH) release into the culture medium after 2, 5, 7, 9, and 12 days of drug exposure. Morphological examinations by phase contrast microscopy, as well as propidium iodide (PI) and Cresyl violet staining at the end of the experiment were used to confirm the LDH results.

2.2.3. LDH efflux assay

As a marker of general cell death *in vitro*, the release of LDH into the culture medium was determined colourimetrically using the CytoTox96R non-radioactive cytotoxicity assay kit (Promega). Resulting absorbances were recorded at a wavelength of 490 nm and corrected for background LDH activity present in the culture medium, as well as for spontaneous LDH release of non-treated control slices. To record the development of neuroprotection, the absorbance was added for each time point and calculated as percentage slice death (3-NP mediated cytotoxicity taken as 100%).

2.2.4. Slice staining

Organotypic cultures were stained with propidium iodide (PI, 10 μg/ml) for 1 h. PI uptake is indicative of significant membrane injury (Macklis and Madison, 1990) and has been correlated with

Table 1
Effect of EMQMCM and MPEP on sub-chronic neurotoxicity of 3-NP in organotypic hippocampal slices

Treatment	7 days	9 days	12 days
EMQMCM	0.06±0.24	1.12±0.39	1.15±0.51
MPEP	0.061±0.013	0.077±0.013	0.060±0.012

Results are expressed as IC_{50}±S.E.M. (μM) for each agent tested for exposure time 7, 9 and 12 days (n=15 slices from several animals).

LDH release and the degree of histological change in glutamate neurotoxicity in slice cultures (Vornov et al., 1994). PI fluorescence was elicited at 546 nm and recorded at 610 nm on an Axiovert 10 inverse fluorescence microscope (Zeiss). Images were taken using a Contax 167 MT camera (Kyocera) and visualised via a laboratory imaging software (Lucia, Nikon).

For Cresyl violet staining slices were fixed for 2 h in 4% paraformaldehyde and cryoprotected in 20% sucrose for at least 1 h, both at +4 °C. After dehydration, slices were stained with Cresyl violet, which recognizes all structures with acidic groups. Nucleus and nucleic acids appear violet, neurons faintly blue.

2.3. Procedure for MCA occlusion study

2.3.1. Surgical procedure and drug administration

Middle cerebral artery occlusion surgeries were performed under general halothane anaesthesia. Transient middle cerebral artery occlusion was induced with the intraluminal filament method (3–0 nylon monofilament suture) as described before (Longa et al., 1989; Zawadzka and Kaminska, 2005). Surgery was performed under halothane anaesthesia (induction with 5% halothane, maintenance with 1.5%–2%, in oxygen), in spontaneously ventilated rats. Rectal temperature was maintained at 36.5 °C–37.5 °C with a heating pad. The right common carotid artery was exposed through a midline incision and carefully dissected from surrounding nerves and fascia, from its bifurcation to the base of the skull. The superior thyroid artery and the occipital artery branches of the external carotid artery were then isolated and coagulated. A 5–0 silk suture was tied loosely around the external carotid artery and a microvascular clip was placed across the external carotid artery adjacent to its origin. A puncture was made and a 3-cm long 3–0 nylon filament with its end rounded by heating (0.25–0.30 mm in diameter) was gently inserted into the external carotid artery lumen. The nylon filament was advanced from the external carotid artery to internal carotid artery lumen and further down to the circle of Willis up to the beginning of anterior cerebral artery approx. 18–20 mm, according to the animal's body weight. In this way, the monofilament passed over the origin of the middle cerebral artery and thereby occluded it and all remaining sources of blood flow from the internal carotid artery, anterior cerebral artery and posterior cerebral artery. A silk suture was tied around the internal carotid artery to immobilize the monofilament. The surgical wound was closed and anaesthesia discontinued. During middle cerebral artery occlusion the animals were allowed to wake up and walk around freely between the onset and offset of occlusion. They were re-anaesthetized 15 min before the removal of the filament. After 90 min of middle cerebral artery occlusion, the filament was withdrawn to allow reperfusion, the incision was closed and anaesthesia discontinued. Apart from intrasurgery measurements, rectal temperature was recorded daily. Sham-operated animals were subjected to similar surgery with exception of middle cerebral artery occlusion.

MTEP and EMQMCM were dissolved in physiological saline. MTEP was administered intraperitoneally (i.p.) at a single dose of 2.5 or 5 mg per kg body weight, 2 or 4 h after the start of middle cerebral artery occlusion. EMQMCM was injected at the dose of 5 or 10 mg per kg body weight (i.p.) 2 h after the start of ischaemia.

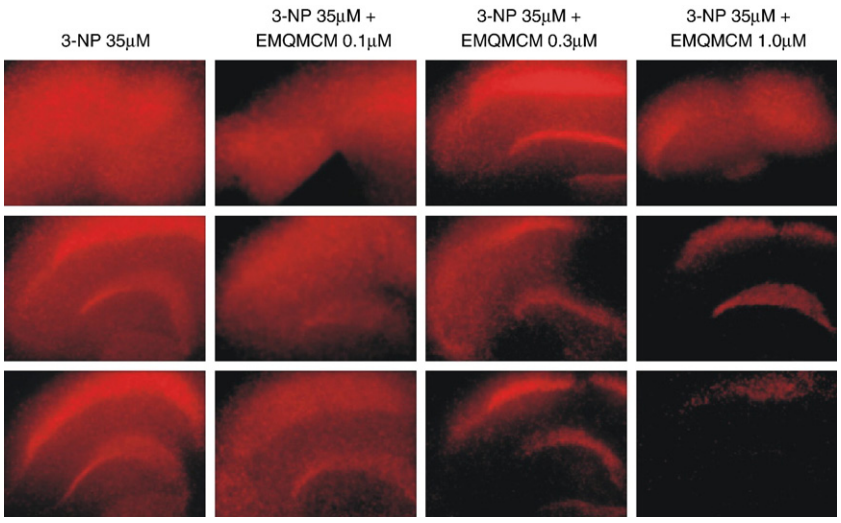

Fig. 1. Protection by EMQMCM vs. 3-nitropropionic acid (3-NP)-induced toxicity shown as PI fluorescence images of hippocampal sub-fields. Slices were exposed to 35 μM 3-NP for 7, 9 or 12 days in the absence or presence of EMQMCM (30–1000 nM). Cultured slices were exposed to 35 μM 3-NP for 12 days in the absence or presence of EMQMCM 0.1–1 μM. Representative images from three different cultures per treatment group are shown in view-field of approximately 3 mm.

2.3.2. Neurological evaluation

The development of infarction in the regions normally supplied by the MCA resulted in neurological deficits which were verified by somatosensory tests performed daily. To examine the postural reflex (Bederson et al., 1986a,b) rats were suspended by the tail and scored according to the following criteria: grade 0 – no observable deficit, grade 1 – forelimb flexion (hemiparesis), grade 2 – decreased resistance to lateral push towards the paretic side (in addition to behaviour in grade 1), grade 3 – contralateral circling towards the paretic side (in addition to behaviour in grade 2), grade 4 – no movement. Next, rats were held close to a table and the ability to extend their forelimbs towards the edge was measured to examine sensorimotor integration in forelimb placing responses to visual, tactile, and proprioceptive stimuli (visual/tactile placing with frontal and lateral surface of paws, each tested separately): grade 0 – no observable deficit, grade 1 – partial or delayed placing (approx. 2 s), grade 2 – absence of placing. Maximal neurological score to obtain in the above tests was 12. The animals were allowed 10 trials before scoring.

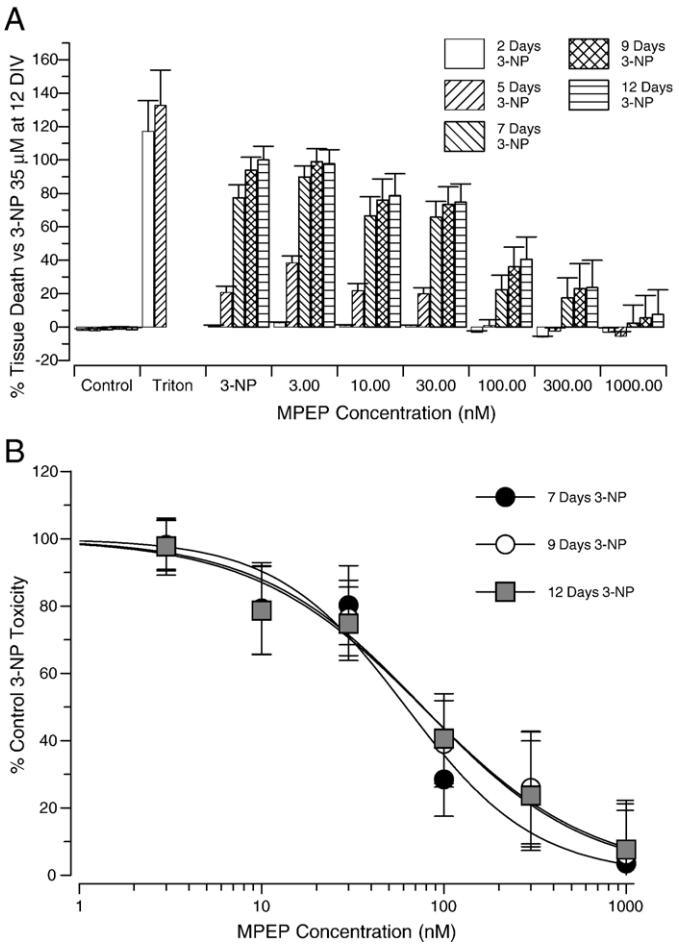

Fig. 2. Hippocampal slices were exposed to 35 µM 3-NP for 2, 5, 7, 9, and 12 days in the absence or presence of MPEP (3–1000 nM) and assessed for lactate dehydrogenase (LDH) release. (A) Data were normalised with respect to maximal 3-NP toxicity seen at day 12 (100%) and background LDH release (0%). (B) Data were normalised with respect to maximal 3-NP toxicity seen at each treatment day (100%) and background LDH release (0%). This representation illustrates the similar neuroprotective effects of MPEP over time (only time points 7, 9 and 12 are shown as at earlier time points toxicity was not sufficient to allow reliable dose response). This representation clearly shows the development of 3-NP toxicity over time. Data are means ± S.E.M. (n = 15, number of slices obtained from several animals).

2.3.3. Staining and infarct volume analysis

24 h after ischaemia rats were deeply anesthetized with an overdose of sodium pentobarbital, decapitated and their brains were removed rapidly. The tissue was sliced into 2-mm-thick coronal sections beginning at the olfactory bulbs using ice-cold rat brain slicing matrix. The slices were stained in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, Germany) for 20 min at 37 °C in the dark. The slices were recorded via digital photography; images were changed into the greyscale mode for more precise quantification of infarct area and analyzed by NIH software ImageJ ver. 1.29. Core injury was defined as tissue completely lacking TTC staining. Infarct volume was obtained by multiplying the values for each area by the slice thickness according to the indirect method (Swanson et al., 1990). Results were expressed in mm^3 (infarction volume).

2.3.4. TUNEL (dUTP nick end labelling) staining and laser scanning cytometry (LSC)

Frozen brain slices were treated with 99% ethanol and 80% acetic acid at a ratio of 2:1 at +4 °C for 30 min. Slices were washed 3 times in PBS and incubated with TUNEL reaction mixture (Roche, In situ cell death detection kit, fluorescein labelled) for 1 h in 37 °C in dark and humid environment. Incubation was followed by rinsing slices 3 times in PBS, dehydration in alcohol and mounting with DPX Mountant for histology (Fluka). Cover slipped brain sections were examined under a fluorescent Olympus IX70 microscope and inverted confocal microscope (DM IRE2, Leica).

Quantification of TUNEL staining results was performed using iCys™ Research Imaging Cytometer with an Olympus IX-71 microscope unit with a bright-field lamp (as described by Szydlowska et al., in press). Fluorescence was measured using Argon laser (488 nm), objective 20× and integral pixel correction. We analysed 5 slices from each brain (at the same levels), 24 h after middle cerebral artery occlusion.

2.3.5. Nissl staining

For Nissl staining slices were fixed for 1 h in 4% paraformaldehyde. After dehydration, slices were stained with Cresyl violet, which recognizes all structures with acidic groups. Nucleus and nucleic acids appear violet, neurons faintly blue.

2.4. Statistical analysis

Statistical analysis for neurological scores and infarct volume evaluation were performed using analysis of variance (ANOVA, Statistica) followed by post-hoc Tukey's test. LSC data analysis, was performed using ANOVA (Statistica) followed by post-hoc Newman–Keuls test.

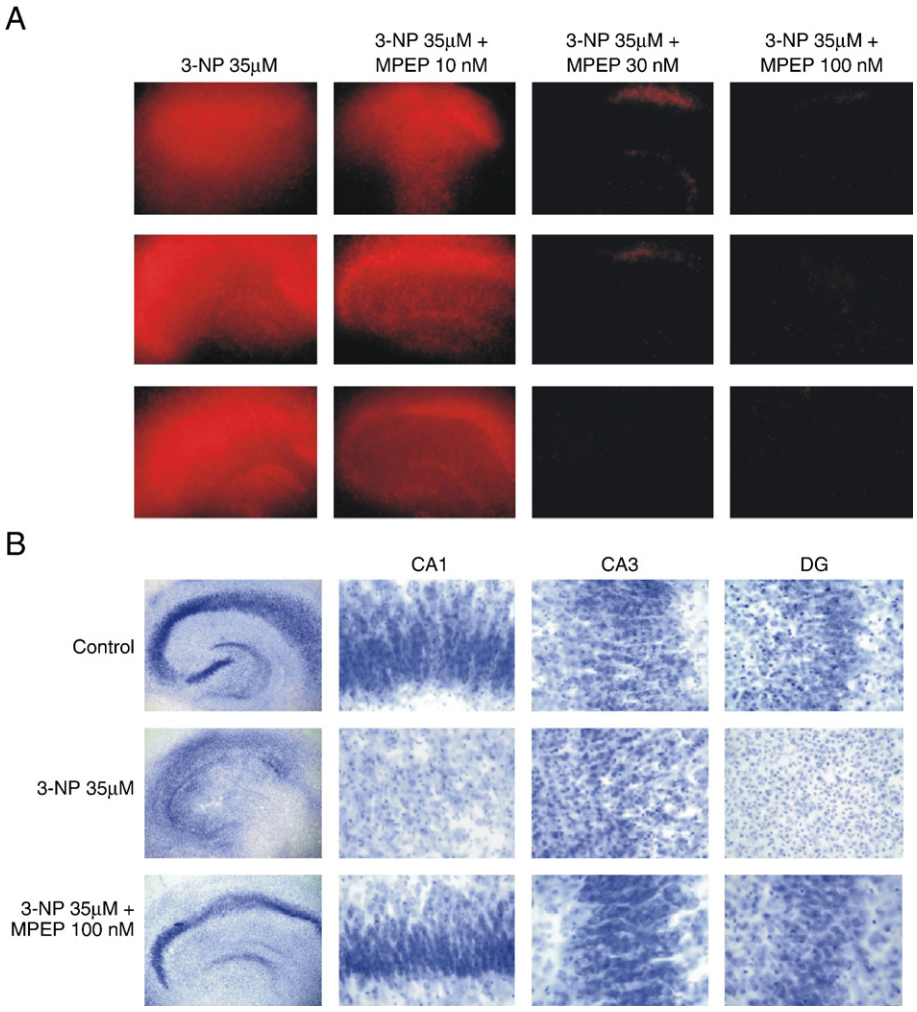

Fig. 3. Protection by MPEP vs. 3-NP-induced slice toxicity in organotypic hippocampal slices after 12 days of exposure. (A) Toxicity shown as PI fluorescence images of hippocampal sub-fields of cultured slices exposed to 35 μM 3-NP for 12 days in the absence or presence of MPEP (10–100 nM). (B) Cresyl violet staining of slice nuclei showing hippocampal sub-fields. Shown are representative images from three different cultures per treatment group are shown in a view-field of approximately 3 mm. Control slices had no PI uptake (not shown).

2.5. Drugs

MTEP hydrochloride, MPEP hydrochloride and EMQMCM mesylate were synthesized by Merz Pharmaceuticals (Frankfurt, Germany).

3. Results

3.1. Neurotoxicity in organotypic cultures

3-NP treatment (35 μM) induced a time-dependent toxicity in organotypic hippocampal cultures. Pronounced toxicity was first seen the following 7th day of exposure (Fig. 2A). The maximum possible slice death compared to Triton X-100 treatment, which results in complete tissue lysis, was between 40 and 80%. The massive LDH release was accompanied by morphological changes, originating in the CA3 region, followed by the CA1 region, involving stratum radiatum and finally spreading over the whole slice. Slices first became swollen before finally shrinking. PI images performed at the end of the experiment revealed extensive hippocampal damage. All pyramidal sub-fields, as far as they could be visualised, showed massive to complete neuronal degeneration.

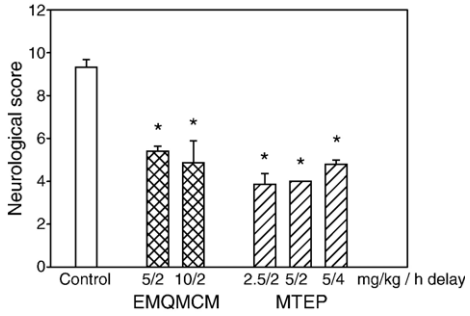

Fig. 4. Effect of EMQMCM and MTEP on neurological score following middle cerebral occlusion (MCAo) in rats. MGlu antagonists were administered i.p. at given doses and time after occlusion. Results are expressed as mean±S.E.M. *$p<0.05$ vs. vehicle ($n=12$ middle cerebral artery occlusion, saline-treated rats and $n=7$ for middle cerebral artery occlusion, drug-treated group; post-hoc Tukey test).

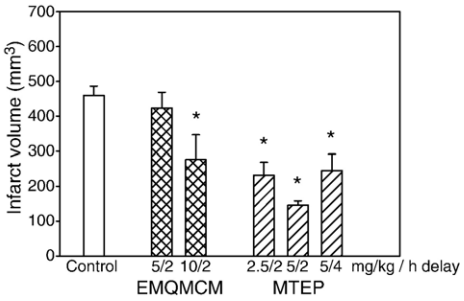

Fig. 5. Effect of EMQMCM and MTEP on total infarct volume produced by middle cerebral occlusion (MCA) in rats. MGlu antagonists were administered i.p. at given doses and time after occlusion. Results are expressed as mean ± S.E.M. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ vs. vehicle ($n=12$ middle cerebral artery occlusion, saline-treated rats and $n=6$ for middle cerebral artery occlusion, drug-treated group; (post-hoc Tukey test).

In the slices with pyramidal sub-fields still visible, Cresyl violet-stain demonstrated a markedly decreased number of neuronal layers and morphologically altered nuclei.

3.1.1. EMQMCM

The mGlu1 antagonist EMQMCM (0.03–1 μM) showed concentration-dependent neuroprotective effects against 3-NP-induced LDH release in organotypic hippocampal slices with IC_{50} c.a. 1 μM (Table 1). These neuroprotective effects were also evident in PI-stained slices (Fig. 1) from which it is apparent that effects were strongest in the CA3 sub-field. These effects were first seen at concentrations that are several hundreds fold higher than those active at mGlu1 receptors (Noeske et al., 2004).

3.1.2. MPEP

MPEP (3–1000 nM) also showed concentration-dependent neuroprotective effects against 3-NP-induced LDH release in organotypic hippocampal slices (Fig. 2A, B) with an approximate IC_{50} of 70 nM (Table 1). These neuroprotective effects were also evident in PI- (Fig. 3A) and Cresyl violet-stained slices (Fig. 3B) and phase contrast microscopy (slice swelling and atrophy, not shown) with a decrease in 3-NP-induced pathology in the CA1 (strongest), CA3 and DG sub-fields. In contrast to EMQMCM, these effects were first seen at concentrations similar to those producing functional antagonism of mGluR5 receptors *in vitro* (Jatzke et al., 2005). At the highest concentration of MPEP, slices showed almost no evident cytotoxicity.

3.2. Middle cerebral artery occlusion in rats

Treatment of rats with either MTEP or EMQMCM under all tested conditions, improved the neurological scores evaluated 24 h after middle cerebral artery occlusion. There was an improvement of postural reflex, visual and tactile placing reactions in MTEP or EMQMCM-treated groups after the recovery periods. The neurological scores recorded at 24 h after onset of ischaemia were significantly better in both the MTEP and EMQMCM-treated groups compared to saline-treated animals (Fig. 4).

Transient occlusion of the middle cerebral artery resulted in ischaemic brain damage in the front parietal somatosensory cortex, the striatum and other areas such as the hypothalamus, corresponding to the territory perfused by the middle cerebral artery. Infarcts were assessed by a vital TTC staining 24 h after middle cerebral artery occlusion with or without MTEP or

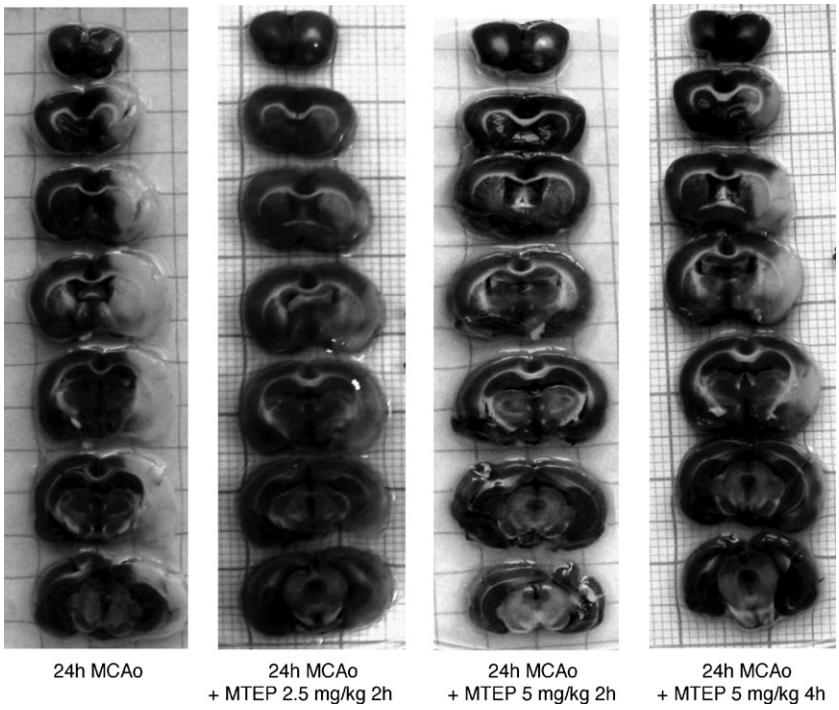

Fig. 6. Representative examples of TTC staining of rat brains following MTEP treatment in animals subjected to middle cerebral occlusion (MCAo). MGlu antagonist was administered i.p. at given doses and time after occlusion. The distribution of infarct areas in the saline and MTEP-treated ischaemic rats at different coronal levels is shown.

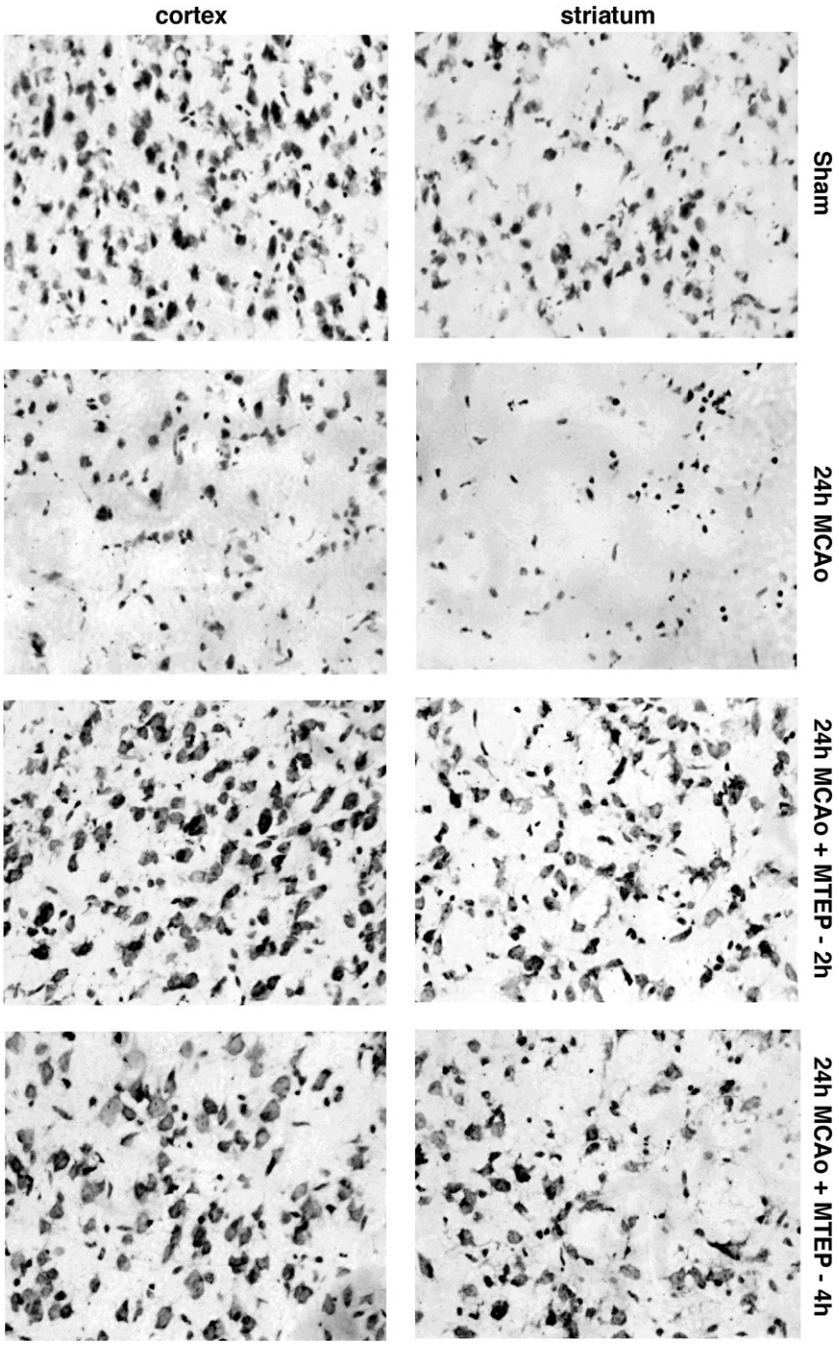

Fig. 7. Neuroprotective effect of MTEP administrated 2 or 4 h after middle cerebral artery occlusion (MCAo). Representative example of rat brain slices after Nissl staining following saline or MTEP treatment in animals subjected to middle cerebral occlusion. Nissl staining revealed almost complete protection when MTEP was administered 2 h after ischaemia comparing to brain slices of animals which did not receive drug. A considerable protective action of MTEP is still visible when the drug was injected 4 h after ischaemia.

EMQMCM (Fig. 5). The volume of cortical damage after 90-min ischaemia and reperfusion for 24 h was significantly reduced by the administration of MTEP: 2.5 mg/kg (25 μmol/kg, 2 h after middle cerebral artery occlusion) or 5 mg/kg (50 μmol/kg, 2 or 4 h after middle cerebral artery occlusion) compared to saline-treated animals (Fig. 5). Mean brain infarct volume in MTEP-treated rats was significantly smaller in drug-treated animals compared to saline-treated rats.

At 5 mg/kg (12.5 μmol/kg) EMQMCM did not significantly change the infarction size when administered 2 h after ischaemia compared to saline-treated animals (Fig. 5). EMQMCM however, did show a significant effect compared to vehicle-treated ischaemic animals, when given to animals at a higher dose of 10 mg/kg (25 μmol/kg). Fig. 6 illustrates the distribution of infarct areas in the saline and MTEP/EMQMCM-treated ischaemic rats at different coronal levels.

Rectal temperature measured over 3 h after the onset of middle cerebral artery occlusion did not change significantly in EMQMCM- or MTEP-treated vs. saline-treated animals subjected to middle cerebral artery occlusion (data not shown).

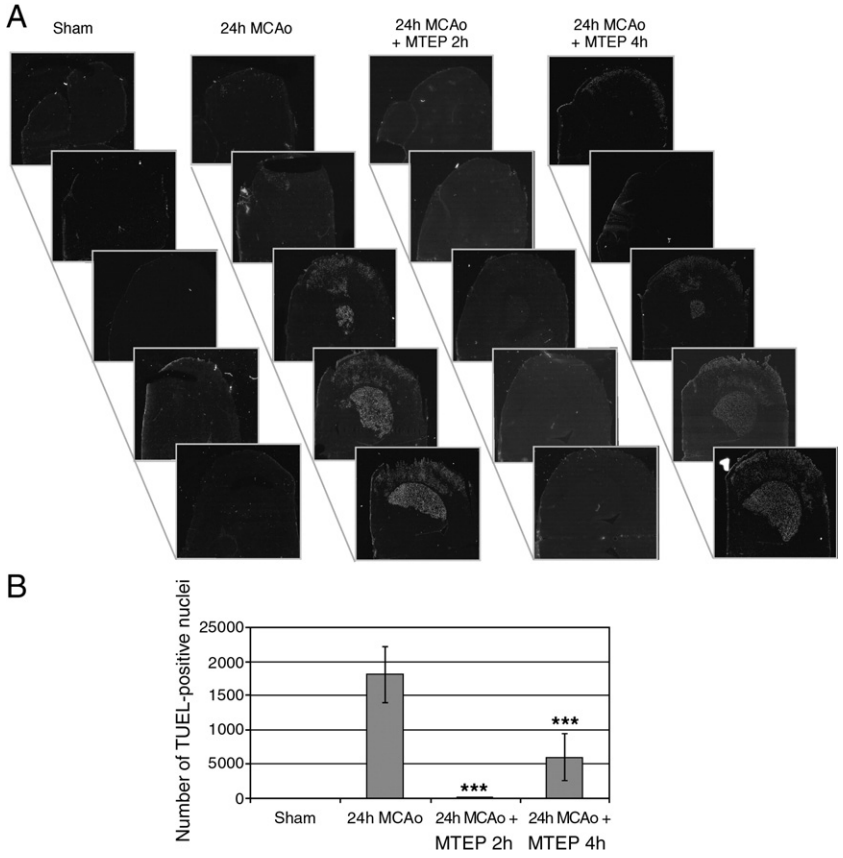

Fig. 8. Administration of MTEP to rats subjected to middle cerebral occlusion (MCAo) reduces DNA fragmentation. TUNEL staining was performed on brain slices from control rats and rats subjected to middle cerebral artery occlusion with or without drug treatment followed by laser scanning cytometer analysis. Panel A. Representative photographs of ischaemic brain hemisphere of rats subjected to various treatments. For each rat, 5 brain slices at different coronal levels were analysed. Panel B. Quantification of TUNEL staining results was performed using iCysTM Research Imaging Cytometer with microscope unit Olympus IX-71 Graph shows a total number of cells with fragmented DNA counted on 5 brain slices from each brain ($n=3$ per group). Results are expressed as mean ± S.E.M. ***$p<0.001$ (post-hoc Newman–Keuls test).

To visualise tissue damage and cell death more precisely than using TTC staining we performed Nissl staining (Fig. 7). The damage of the brain after 24 h of ischaemia was clearly visible in the striatum and in the cortex. In the striatum (ischaemic core) the tissue structure was completely destroyed. 5 mg/kg MTEP administration after 2 h revealed its very strong protective action. The number and the morphology of cells did not change compared to the brains of control rats. When the drug was administered 4 h after ischaemia, it still revealed a neuroprotective action, although not as complete as when administered 2 h after ischaemia.

For better evaluation of the neuroprotective effect of MPEP action, we performed TUNEL staining. TUNEL staining allows visualising cells with fragmented DNA. Every single nuclei was counted using Laser Scanning Cytometer (LSC). TUNEL-positive nuclei were counted on 5 brain slices from each brain, on different stroke levels.

Quantitative TUNEL staining analysis clearly showed a neuroprotective effect of 5 mg/kg MPEP (administered 2 and 4 h after ischaemia, Fig. 8). Inhibition of DNA fragmentation in MPEP-treated animals was superior to the neuroprotective effect evaluated with TTC staining. In sham-operated animals, no TUNEL-positive nuclei could be detected, however 24 h after ischaemia we observed numerous dying cells. In a group of animals which received MPEP 2 h after ischaemia we have found only single dying cells. Protection by MPEP injected 4 h after ischaemia was not as pronounced, but still the number of TUNEL-positive cells was 3 times lower than in vehicle treated animals.

4. Discussion

The present study shows neuroprotective effects of the selective mGlu1 and mGlu5 antagonists EMQMCM and MTEP/MPEP both *in vitro* (organotypic cultures subjected to 3NP insult) and *in vivo* (middle cerebral artery occlusion model of ischaemia). To our knowledge, the present data are the first presentation of mGlu1 and mGlu5 antagonists in an *in vitro* model of chronic excitotoxicity. Moreover, the efficacy of these highly specific antagonists has not been evaluated in the middle cerebral artery occlusion model of ischaemia previously. In the present study, both *in vitro* and *in vivo*, the mGlu5 antagonist seemed to be more potent at least in case of infarct size evaluation. It is also noteworthy that MTEP was active when given even 4 h after occlusion. The neuroprotective effects of MTEP were not connected with changes in body temperature.

For better evaluation of MTEP neuroprotective action administered 2 and 4 h after ischaemia, we performed

histological analysis (Nissl staining), which demonstrates a complete neuroprotection in the cortex and the striatum when drug was applied 2 h after middle cerebral artery occlusion and a considerable protection when MTEP application is delayed. Neuroprotective effects in the striatum following drug treatment commencing 2 h after middle cerebral artery occlusion is a rare feature because this area is not spared by application of other neuroprotective drugs. We also used a novel, automated approach – laser scanning cytometry – allowing us to quantify TUNEL-positive nuclei in brain slices. TUNEL staining identifies dying cells with fragmented DNA and is a more precise method to visualise cell death. TTC staining visualises metabolically active cells and lack of staining indicates not only dead or dying cells but also cells with impaired metabolic function. However, some cells which are not labelled may be transiently inactive but will not ultimately die. TUNEL staining–laser scanning cytometry analysis allowed us to detect a stronger neuroprotective action of MTEP than using measurements of brain damage visualised by TTC staining.

EMQMCM is an uncompetitive mGlu1 antagonist with an IC_{50} value of about 3 nM (Lesage et al., 2002). It is of course possible that the apparent lower efficacy in the middle cerebral artery occlusion model in the present study is due to pharmacokinetic reasons such as high plasma protein binding, fast metabolism or elimination. However, at doses similar to those tested in the present experiments (5 mg/kg or lower) EMQMCM induces clear behavioural effects e.g. anxiolysis or memory impairment and receptor occupancy studies *ex vivo* with closely related agents indicate that at 5 mg/kg nearly 100% of mGlu receptors should be blocked 1 h after administration (Steckler et al., 2005; Pietraszek et al., 2005; Gravius et al., 2005). More importantly, the *in vivo* potency of MTEP and EMQMCM seems to be very similar (starting effective doses are usually 2.5–5.0 mg/kg). Compared to MPEP, MTEP has been claimed to be more potent and selective *in vitro* and more potent in *in vivo* tests for anxiety (Cosford et al., 2003; Anderson et al., 2003; Busse et al., 2004).

The 3-NP sub-chronic toxicity in organotypic hippocampal cultures used in the present study models some aspects of moderate excitotoxicity possibly relevant for chronic neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's chorea and ALS. In this model, MPEP was very efficacious with an *in vitro* potency similar to that known to interact with rat mGlu5 receptors e.g. IC_{50} of around 30 nM against both [^{3}H]MPEP binding in rat cortical membranes and quisqualate-induced Ca^{2+} influx in cultured rat astrocytes (Pagano et al., 2000; Jatzke et al., 2005; Muhlemann et al., 2006). On the other hand, although EMQMCM had some positive effects, these were first seen at concentrations 300 fold higher than those known to interact with mGlu1 receptors. As such, it seems likely that mGlu5 receptors are involved in this type of excitotoxicity whereas the effects of EMQMCM might have been mediated via interactions with a different target than mGlu1 receptors.

In contrast, previous *in vitro* studies in similar culture systems suggest neuroprotective effects for mGlu1 antagonists but not for mGlu5 antagonists in different, more severe forms of excitotoxicity such as oxygen glucose deprivation which are more relevant for acute global ischaemic insults (Pellegrini-Giampietro, 2003). It is very difficult to reconcile such a juxtaposition of activities in these different severities of insults in similar tissue culture preparations — one might rather expect greater activity in the more mild insults used in the present study. This could indeed be the explanation why MPEP was active in the present *in vitro* experiments but an alternative explanation must be found for the lack of activity of the selective mGlu1 antagonist used here e.g. different mechanisms of oxygen glucose deprivation — and 3-NP-induced excitotoxicity.

It should be also taken into account that most mGlu1 antagonists tested previously were competitive antagonists, often with a mixed spectrum of activity at different mGluRs, including Gi coupled receptors: (*S*)-4C3HPG (RS-4-carboxy-3-hydroxyphenylglycine) (Buisson and Choi, 1995), AIDA, (*S*)-CBPG ((*S*)-(+)-2-(3′-carboxybicyclo[1.1.1]pentyl)-glycine) (PellegriniGiampietro et al., 1999a,b; Allen et al., 2000, 2001; Meli et al., 2002), (*S*)-4CPG ((*S*)-4-carboxyphenylglycine) (Allen et al., 2000, 2001). It has been claimed that LY367385 (Moroni et al., 2002; Meli et al., 2002) and 3-MATIDA (3-methyl-aminothiophene dicarboxylic acid) (Cozzi et al., 2002; Moroni et al., 2002) have greater selectivity for mGlu1 over mGlu5 receptors and both have also been shown to be neuroprotective *in vitro*. However, they are also weak compounds (low μM affinity) and selectivity for mGlu1 vs. e.g. group II/III receptors cannot be guaranteed at the high concentrations required for neuroprotection in these studies. On a similar line, the only non-competitive antagonist used thus far was CPCCOEt and this is also weak (Hermans et al., 1998; Litschig et al., 1999) and was tested at high concentrations (10–30 μM) (Meli et al., 2002) that may conceivably affect other receptors, especially considering possible differences in potencies for allosteric antagonists at rat vs. human receptors (Lavreysen et al., 2003; Mabire et al., 2005) (Parsons unpublished data). In contrast, unpublished data from our laboratories confirmed that EMQMCM is a potent non-competitive antagonists at both human (IC_{50}=9 nM) and rat mGlu1 receptors (IC_{50}=1 nM) and is selective in this regard e.g. IC_{50} rat mGlu5>100 μM, rat mGlu2, 4 6, and 8<30% maximal agonist activation at 10 μM (Wroblewski, personal communication). To our knowledge, the present study is the first to use a highly potent and selective mGlu1 antagonists for such neuroprotection experiments.

The role of mGlu1 and mGlu5 receptors in ischaemic brain damage which is highly controversial issue, was the topic of a recent review (Pellegrini-Giampietro, 2003). Targeted disruption of mGlu1*a* gene failed to affect neuronal injury after middle cerebral artery occlusion in mice (Ferraguti et al., 1997). Moreover, the mGlu1 antagonist (3aS,6aS)-6*a*-naphtalen-2-ylmethyl-5-methyliden-hexahydro-cyclopenta[*c*]furan-1-on (BAY 36-7620) did not have neuroprotective effects in the middle cerebral artery occlusion model in rats and only a tendency was observed after triple i.v. bolus application mg/kg, given immediately, 2 and 4 h after occlusion (De Vry et al., 2001). In contrast, other mGlu1 antagonists 3-MATIDA and LY367385 ((*S*)-2-methyl-4-carboxyphenlglycine) attenuated

neuronal injury following oxygen glucose deprivation *in vitro* and reduced brain damage after middle cerebral artery occlusion following systemic injection in rats (Moroni et al., 2002). Under similar ischaemic conditions (middle cerebral artery occlusion for 2 h) a neuroprotective effect was also found following treatment with the structurally very similar mGlu1 antagonist R128494 given 10 min after reperfusion at 2 mg/kg and followed by 5 × 5 mg/kg injections over 4 h (Lesage et al., 2002).

Following global ischaemia in gerbils AIDA (i.p.) failed to protect CA1 neurons from neurodegeneration (Rao et al., 2000), but in another study LY367385 was active providing c.a. 50% neuroprotection (Bruno et al., 1999).

Similarly controversial are the data on mGlu5 antagonists. In one study MPEP, in contrast to AIDA or LY367385, has been shown to exert no protective effect following global ischaemia in gerbils (Meli et al., 2002). However, because of the i.c.v. mode of administration used, these negative results should be interpreted with caution since the penetration gradient to the site of major insult (hippocampus) is not known and may differ greatly depending on the lipophylicity of the substances used and the relevance of such approach for prediction of therapeutic utility is limited. Similar concerns regard the report by Bao et al. (2001) demonstrating that MPEP (i.c.v.) diminishes neuronal injury and improves neurological recovery following middle cerebral artery occlusion. Interestingly in this study the mGlu5 agonist, CHPG also induced neuroprotection which may reflect interaction with necrotic vs. processes. Following global ischaemia in gerbils, systemic administration of either MPEP or MTEP was protective against ischaemic neuronal damage (Rao et al., 2000; Makarewicz et al., 2006). Although some controversy exists concerning the use of TTC as measure of evaluation and 24-h evaluation interval, we have previously compared an infarct volume using TTC and Nissl staining at 24, 48, 72 h after middle cerebral artery occlusion and found a very good correspondence of the infarct areas (Kaminska, not published). This in line with e.g. report by Bederson et al. (1986a,b).

It has been suggested, that at least for traumatic brain injury, the neuroprotective effects of MPEP involves antagonistic effects at NMDA receptors (Movsesyan et al., 2001). It should however, be stressed that in the present and previous studies (Makarewicz et al., 2006) with the more selective antagonist MTEP (Cosford et al., 2003) neuroprotective effects were also seen. Moreover, the claim by Movsesyan et al. that MPEP has relevant activity at NMDA receptors has been challenged recently (Popoli et al., 2004). Our own data support the notion that direct interactions with NMDA receptors are probably not relevant for these effects of either MTEP or MPEP. (MPEP and MTEP IC_{50}s in NMDA patch clamp from hippocampal neurones were 160 and 267 μM respectively (unpublished data) i.e. nearly 10,000 fold higher than functional antagonism at mGlu5 receptors — e.g. IC_{50}s in cultured rat astrocytes were 19 and 16 nM respectively (Jatzke et al., 2005).

Recently Makarewicz et al. (2006) studied the neuroprotective effect of EMQMCM and MTEP, using the 3-min forebrain ischaemia model in Mongolian gerbils and the hypoxia/ischaemia model in 7-day old rats. In this study the drugs were administered i.p. three times every 2 h, beginning 30 min after hypoxia, at doses of 1.25, 2.5 and 5.0 mg/kg. In the hypoxia/ischaemia model EMQMCM (starting at 2.5 mg/kg) but not MTEP showed neuroprotective activity while following global ischaemia in gerbils both substances showed protection (MTEP at 2.5 mg/kg and EMQMCM at 5 mg/kg). However, it has been suggested that post-ischaemic hypothermia may be partially involved in the mechanism of neuroprotection following EMQMCM in gerbils (ibid.). In general, it has been demonstrated in several studies that under normoxia small variations in the brain temperature after middle cerebral artery occlusion parallel the rectal temperature and in turn measurements of body temperature is a reliable and adequate method to evaluate possible drug effect on body and brain temperature. In the present study tested drugs do not significantly affect body temperature, and no extensive body worming was required and in such conditions rectal temperature reflects well temperature in the brain (Laptook et al., 2001).

The functional *in vivo* part of the present study is rather consistent with previous reports showing neuroprotective effects of mGlu1 antagonists in ischaemia models, but does not support their superiority over mGlu5 antagonists. In contrast, *in vitro* and *in vivo* infarct size data rather tend to suggest higher potency of mGlu5 antagonists.

Although, both mGlu1 and mGlu5 can be physically and functionally coupled to NMDA receptors, their effect on NMDA receptor function might vary between brain structures (Awad et al., 2000; Attucci et al., 2001; Mannaioni et al., 2001; Heidinger et al., 2002). For example, in the CA1 regions of the hippocampus stimulation of mGlu5, but not mGlu1, potentiates NMDA-evoked currents (Doherty et al., 1997; Mannaioni et al., 2001). However, in the CA3 region of the hippocampus stimulation of both mGlu1 and mGlu5 enhances NMDA receptor function (Benquet et al., 2002) but mGlu5 potentiates NMDA-evoked current *via* a G protein activation whereas mGlu1 enhance NMDA function *via* G protein-independent mechanism involving Src tyrosine kinase activation (Benquet et al., 2002). These findings suggest, that an indirect inhibitory influence at NMDA receptors may contribute to the neuroprotective effects of mGlu1/5 antagonists, however no hypothermia was observed in ischaemic conditions after mGlu antagonists in contrast to NMDA receptor antagonists (Buchan and Pulsinelli, 1990; Corbett et al., 1990).

In fact, it has been suggested that interaction with NMDA receptors may be one of several mechanisms whereby inhibition of mGlu1 and mGlu5 receptors attenuate neurodegeneration in ischaemia conditions. Multiple effects seem to be involved such as a decrease in neuronal excitability due to change in K^+ channels; decrease in Ca^{2+} efflux from intracellular, inhibition of glutamate release and increase in GABA release (Pellegrini-Giampietro, 2003).

In conclusion, the present data support neuroprotective potential of both mGlu1 and mGlu5 antagonists in ischaemia but in contrast to a recent review (Pellegrini-Giampietro, 2003) do not point to stronger activity of mGlu1 antagonists. Moreover, data from 3-NP hippocampal slice model indicate stronger involvement of mGlu5 receptors in milder forms of excitotoxicity.

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