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In Vivo Protection against NMDA-induced Neurodegeneration by MK-801 and Nimodipine: Combined Therapy and Temporal Course of Protection

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Neuroprotection against excitotoxicity by a combined therapy with the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 and the L-type Ca^{2+} channel blocker nimodipine was examined using an in vivo rat model of NMDA-induced neurodegeneration. Attention was focused on the neuroprotective potential of this combined drug treatment *before* and *after* NMDA-exposure. NMDA was unilaterally injected in the magnocellular nucleus basalis (MBN). Neuronal damage was assessed 12 days after the NMDA-injection by measuring the reduction of cholinergic cortical fibres that originate from the MBN neurons. In controls that received no drug treatment, NMDA-exposure damaged MBN neurons such that 66% of the cholinergic terminals were lost in the ipsilateral parietal cortex. Pretreatment with a nimodipine diet (860 ppm) combined with application of MK-801 (5 mg/kg i.p.) *before* NMDA-exposure reduced fibre loss by 89% thereby providing a near complete neuroprotection. Combined therapy of MK-801 (5 mg/kg i.p.) and nimodipine (15 mg/kg i.p.) 8 min *after* NMDA-infusion reduced neuronal injury by 82%, while the same combination given 2 h *after* the excitotoxic treatment still yielded a 66% protection against neurotoxic damage invoked by NMDA. In conclusion, the present data show that a dual blockade of NMDA-channels and voltage-dependent calcium channels (VDCC's) up to 2 h after NMDA-exposure is able to provide a significant protection against NMDA-neurotoxicity.

Key words: calcium antagonists, combined therapy, excitotoxic, neuroprotection, temporal course

EXCITATORY amino acids have been implicated as important mediators in the process of neuronal cell death in hypoxia, ischaemia and chronic neurodegenerative diseases (Choi *et al.*, 1995; Coyle & Puttfarken, 1993; Dodd *et al.*, 1994; Maragos *et al.*, 1987; Rothman & Olney, 1986). During cerebral ischaemia excessive release of the excitatory neurotransmitter glutamate is observed (Benveniste *et al.*, 1984), which leads to an uncontrolled rise of intracellular free Ca^{2+} (Dubinsky, 1993) and ultimately to neuronal necrosis (Randall & Thayer, 1992). Massive entry through various channels of Ca^{2+} into neurons is considered to be the final common pathway leading to irreversible neuronal damage, since persistent high levels of cytosolic free Ca^{2+} may evoke dysregulations of Ca^{2+} activated processes which are detrimental to neurons (Clapham, 1995). Both N-methyl-D-aspartate (NMDA) receptor-operated ion channels and voltage-dependent Ca^{2+} channels (VDCCs) appear to be largely responsible for the massive Ca^{2+} influx during excitotoxic insults (Garcia *et al.*, 1994; Hartley *et al.*, 1993; Lobner & Lipton, 1993; Rothman & Olney, 1995). During the initial phase of ischaemia Ca^{2+} influx is shown to be mediated primarily by stimulated NMDA-channels followed by a secondary influx involving other Ca^{2+} channels (Lobner & Lipton, 1993) activated by voltage changes or intracellular messenger systems (Chetkovich *et al.*, 1991; Garcia *et al.*, 1994; Skeen *et al.*, 1994). Agents that

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block the Ca^{2+} entry through activated NMDA-channels have been demonstrated in several ischaemia model studies to decrease neuronal damage (Gill *et al.*, 1987; Meldrum & Garthwaite, 1990; Siesjö *et al.*, 1992). Other reports indicate that also blockade of the VDCCs and notably the L-type channels by dihydropyridines provide a remarkable neuroprotection in experimental ischaemia (Bellemann *et al.*, 1983; Langley & Sorkin, 1989; Nuglisch *et al.*, 1990; Rami & Krieglstein 1994; Scriabine *et al.*, 1989).

In the present study we evaluated combined treatment with a noncompetitive NMDA-receptor antagonist (MK-801) and a voltage dependent L-type Ca^{2+} channel blocker (nimodipine) for its neuroprotective effects against NMDA-induced neuronal injury. Both MK-801 (dizocilipine) and nimodipine (a 1,4-dihydropyridine derivate, BAY E 9736) have been demonstrated to attenuate brain injury following experimental ischaemia and excitotoxic insults (Gill *et al.*, 1987; Luiten *et al.*, 1995; McDonald *et al.*, 1990; Nakamura *et al.*, 1993; Nuglisch *et al.*, 1990).

In the currently used model NMDA was unilaterally injected in the cholinergic cell groups of the magnocellular nucleus basalis (MBN), which have axonal projections to the ipsilateral cortex (Fig. 1). Neuronal injury was measured on the reductions of cholinergic axonal fibres in the injected hemisphere, whereby the cholinergic innervation of the intact contralateral side served as internal control. In our previous study this in vivo model was proven to be very suitable and reliable for quantitative assessment of neuroprotective properties of potentially beneficial agents (Luiten *et al.*, 1995).

First we examined dose dependent neuroprotective effects of pretreatment with MK-801 to select an appropriate dose for testing a combined therapy with nimodipine. Secondly the neuroprotective impact of the combination therapy was compared to the effects of single drug treatments with MK-801 and nimodipine. Finally we investigated the temporal course of neuroprotection, by measuring the degree of neuroprotection of both drugs applied at several time points *before* and *after* NMDA-exposure.

Materials and Methods

Subjects and treatments

The experiments of the present study were carried out on a total of 51 young adult (3 month old) male Wistar rats (average body weight 320 g) bred in our own facilities. The animals were housed at a standard temperature of 23 ± 1°C in a light controlled room (lights on from 07.00 h to 19.00 h). All rats had free access to either standard food pellets (Sniff, Soest, FRG) or (in case of nimodipine treatment) to identical pellets containing nimodipine at a concentration of 860 ppm.

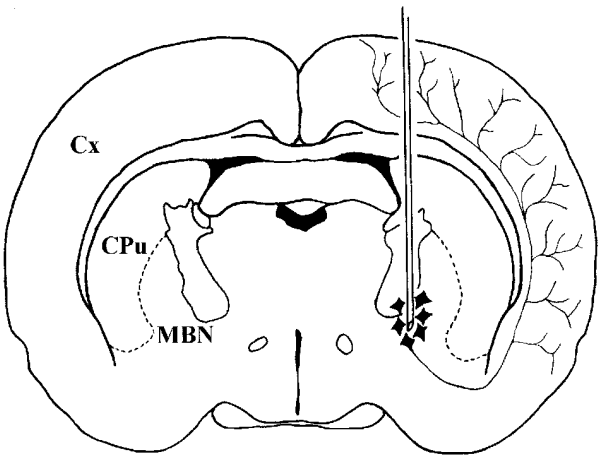

Figure 1. The position of the needle in the MBN, also illustrating cholinergic cell bodies and an axonal fibre projection to the cortex. Abbreviations: Cx, cortex; CPu, caudate putamen; MBN, magnocellular nucleus basalis.

Animals were divided and allocated to two experiments. In the first experiment the neuroprotective effects of three different doses MK-801, applied 2 h *before* NMDA-infusion, were determined to select an appropriate dose for testing a combined treatment with nimodipine. In the second experiment the neuroprotective effects of a combined therapy with MK-801 and nimodipine were compared with the effects of single treatments with either of these drugs against NMDA-neurotoxicity. To study the temporal profile of potential neuroprotection, in the latter experiment combined and single drug therapy was also applied 8 min and 2 h *after* NMDA-injection. These time points were chosen based on previous reports, studying the temporal course of excitoxicity processes due to NMDA exposure, showing an initial calcium influx within 30 min after NMDA-application (Van Lookeren Campagne *et al.*, 1994; Tymianski *et al.*, 1993). The 8 min interval was chosen for practical reasons. At this moment the animals could be removed from the stereotaxic frame, so that a proper i.p. injection could be performed.

Experiment I

To study the effects of three different doses MK-801 on NMDA-induced neuronal injury, 22 animals were divided into four groups: (1) controls with NMDA-injections in the MBN, but receiving no drug treatment at all ($n = 6$); (2) rats receiving 1 mg/kg MK-801 ($n = 5$); (3) rats receiving 3 mg/kg MK-801 ($n = 6$); and (4) a group of animals receiving 5 mg/kg MK-801 ($n = 6$). MK-801 was dissolved in phosphate buffered saline, pH 7.4 (PBS) and i.p. injected 2 h *before* NMDA-infusion. The controls of the first experiment also served as controls for the second experiment.

Table 1. Summary of the different drug treatments in experiment II against NMDA-toxicity

	Pretreatment	Posttreatment	
		8 min	2 h
Nimodipine	$n = 6$	$n = 7$	$n = 6$
MK-801	$n = 6$	$n = 6$	$n = 6$
Nimodipine + MK-801	$n = 6$	$n = 8$	$n = 6$

Experiment II

From the results of the first experiment the dose of 5 mg / kg MK-801 was selected for further study of the neuroprotection by a combined therapy of MK-801 and nimodipine. For investigating the temporal course of protection, besides pretreatment, drugs were applied 8 min and 2 h *after* NMDA-exposure in different groups. For pretreatment with nimodipine, rats received food pellets containing 860 ppm nimodipine starting 2 weeks *prior* to NMDA-injection. The concentration 860 ppm was chosen because it was shown in a number of studies to be an optimal dose yielding neuroprotective effects (Schuurman *et al.*, 1987; Scriabine *et al.*, 1989; De Jong *et al.*, 1992), without affecting brain metabolism (Harper *et al.*, 1981), blood pressure or circulation parameters (Kazda *et al.*,1985). During the period of nimodipine application in food, the daily food intake was measured. Posttreatment with nimodipine consisted of an 15 mg / kg i.p. injection with nimodipine dissolved in a mixture of ethanol and polyethylene glycol (2:1). The various groups in experiment II are summarized in Table 1.

Surgical procedure

Excitotoxic damage to the MBN in all cases was carried out unilaterally in the right hemisphere, such that the intact left hemisphere served as a control for each individual case. The animals were anaesthetized with a combination of sodium pentobarbital (30 mg / kg i.p.) and hypnorm (Duphar, Weesp, Netherlands, 0.4 mg / kg i.m.). The anticholinergicum atropine-sulphate (0.5 mg / kg i.p.) was used to limit salivary bronchial secretion. Rats were positioned in a stereotaxic frame, the head skin was retracted and a small hole was drilled in the skull. For the toxin injections a 5 µl Hamilton syringe was positioned in the MBN at the coordinates 1.5 mm posterior to bregma and 3.2 mm lateral to the midline (defined by the atlas of Paxinos & Watson, 1986). At two dorso-ventral positions, 6.2 mm and 7.0 mm ventral to the dura, 0.5 µl PBS containing 30 nmol of a racemic mixture of N-methyl-D,L-aspartate (Sigma, St. Louis) was slowly injected in steps of 0.1 µl, so that a total of 60 nmol was injected in the MBN. After each injection the needle was left in situ for 5 min to allow for diffusion and to limit spread of the toxin solution during withdrawal of the needle. The dose of 60 nmol was shown in a previous study (Luiten *et al.*, 1995) to result in a 66% reduction of cholinergic fibres projecting to the ipsilateral cortex. Therefore this dose was considered as suitable for studying neuroprotective potentials of the used compounds. After surgery, all animals survived for 12 days until sacrifice and histological processing.

Tissue processing and histochemistry

Fixation of the brain was carried out by transcardial perfusion with 300 ml fixative composed of 2.5% paraformaldehyde, 0.05% glutardialdehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB, pH 7.4) at a perfusion speed of 20 ml / min, which was preceded by a short prerinse of heparinized saline. The brains were removed from the skull and cryoprotected by overnight storage at 4°C in 30% sucrose in 0.1 M PB. Subsequently, the brains were coronally sectioned on a cryostat microtome at a thickness of 20 µm. Before sectioning, the left hemisphere (control side) was marked by punching a small needle hole in the corpus callosum. The free floating brain sections were postfixed by immersion in a 2.5% glutardialdehyde solution in PBS overnight at 4°C. Hereafter, the cholinergic fibres were visualized by staining for the presence of acetylcholinesterase (AChE) according to Hedreen *et al.* (1985) using a silver-nitrate intensification procedure. Finally, the sections were mounted, dehydrated, cover-slipped and examined by light microscopy.

To determine the effect of the neuronal injury in the MBN, the cholinergic fibre density was measured in layer V of the posterior somatosensory cortex in both the left (control side) and right (injection side) hemispheres. Fibre densities were assessed by means of counting fibre crossings with the lines of an in-built ocular counting grid, described in detail by Luiten *et al.* (1995). Crossing fibres were counted in three sections, representing a cortical region receiving the densest cholinergic innervation from the damaged MBN division (Luiten *et al.*, 1987). Comparison between the counted fibre crossings at the injected side and counted values at the intact control side revealed the percentage fibre reduction, which was considered to be proportional to the degree of neurotoxic cell damage. This way a relative value was obtained that was insensitive to individual variations between cases that might occur for technical or other reasons. Percentage neuroprotection for each experimental drug treatment was calculated by the ratio of fibre reduction after NMDA-injection with [X] and without drug application [C], according to the following formula:

$$\% \text{ protection} = 100(1-(\% \text{ fibre reduction [X]} / \% \text{ fibre reduction [C]}))$$

Statistical analysis

To examine a dose-dependent influence of MK-801 on fibre reduction after NMDA-induced injury one-way analysis of variance (ANOVA) was performed. ANOVA was carried out also to define the temporal profile of neuroprotection by combined therapy with nimodipine and MK-801. The non-parametric Mann-Whitney-U test (STATS program) was used for comparison between the effects of the different treatments on fibre reduction after neurotoxic injury of the MBN. A P value less than 0.05 was considered to indicate statistical significance. Data are presented as mean ± standard error of the mean (SEM).

Results

Experiment I

Unilateral injections of NMDA in the MBN complex of non-drug treated animals (controls) resulted in a profound decrease of cholinergic fibres in a large part of the ipsilateral cortex, corresponding with the unilaterally organized anatomical pattern of MBN projections to the cortex in the rat. In the majority of the animals NMDA induced seizures which appeared after surgery when consciousness was regained. The seizures disappeared again within 2 h. A 66.2 ± 3.3% reduction of fibre density was measured 12 days after the NMDA-infusion. All doses of MK-801 applied 2 h *before* MBN injection significantly attenuated NMDA-induced cortical fibre reduction compared to controls ($P < 0.01$, Fig. 2). Fibre loss was reduced in a dose-dependent way (one-way ANOVA $P < 0.001$). The dose of 1 mg / kg MK-801 revealed a neuroprotection of 37.7%, while for 3 mg / kg and 5 mg / kg MK-801 neuroprotection was calculated as 46.0% and 72.3%, respectively.

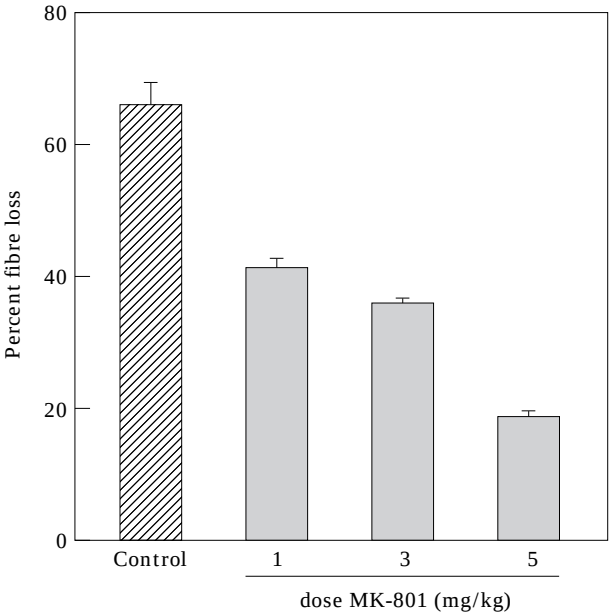

Figure 2. The effect of three different dosages MK-801 on percentage fibre loss 12 days after injection of NMDA in the MBN. MK-801 was injected 2 h *prior* to the injection of NMDA. Controls received no drug-treatment *before* NMDA infusion. Values are means (± SEM) of 5–6 animals in each group and analysed using the non parametric Mann-Whitney-U-test; $P < 0.01$ for all MK-801 doses vs Control.

Experiment II

The dose of 5 mg / kg MK-801 from the first experiment was selected for further investigating a combined treatment of MK-801 and nimodipine. In case of nimodipine application in food pellets (860 ppm), food intake ranged from 20 to 26 g / day, which signified a daily nimodipine intake of 17 to 22 mg. Treatment with a nimodipine diet starting two weeks *before* NMDA-infusion attenuated the NMDA-induced cortical fibre reduction from 66.2 ± 3.3% (controls) to 47.2 ± 5.6% ($P < 0.01$), providing a neuroprotection of 28.8% (Fig. 3, Table 2).

Combination of pretreatments of MK-801 together with nimodipine potently antagonized NMDA-induced neuronal injury and subsequent cholinergic fibre decline. Denervation values were strikingly reduced to only 6.7 ± 1.5% ($P < 0.01$ vs control) and approached our previously found fibre reductions (4.7%) in sham operated animals infused with vehicle solution (Luiten *et al.*, 1995). The neuroprotective effects of the combined treatment were significantly higher ($P < 0.01$) than the effects of single drug treatments. Application of nimodipine together with MK-801 yielded a protection of 89.4% against the excitotoxic effects of NMDA. Concerning single drug

Table 2. Percentage fibre loss (±SEM) after NMDA-exposure and calculated neuroprotection (between brackets) by single and combined pre-/posttreatment with nimodipine and MK-801

	Pretreatment	Posttreatment	
		8 min	2 h
Nimodipine	47.2 ± 5.6% (29%)	38.4 ± 3.5% (42%)	59.8 ± 4.1% (10%)[ns]
MK-801	18.3 ± 0.9% (72%)	17.7 ± 2.3% (73%)	30.0 ± 2.6% (55%)
Nimodipine + MK-801	6.7 ± 1.5% (89%)	12.0 ± 0.7% (82%)	22.9 ± 1.2% (66%
Control	66.2 ± 3.3% (0%)		

[ns] not significant; for all other values $P < 0.01$ vs control.

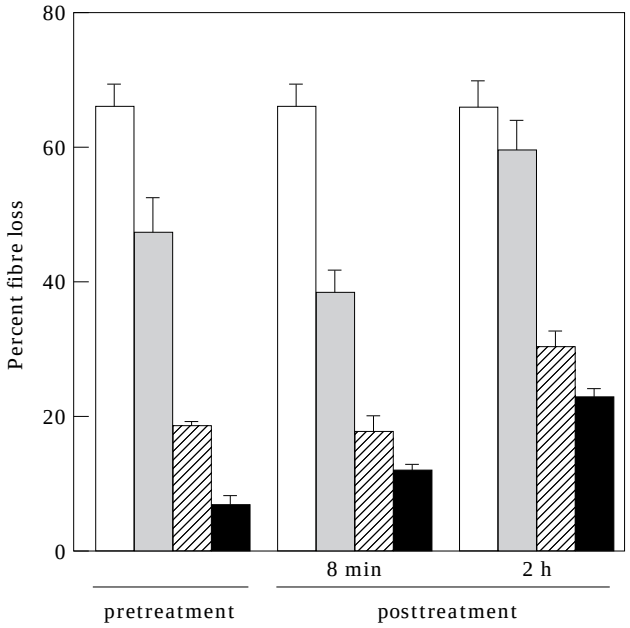

Figure 3. The effect of single and combined pre-/post-treatment with nimodipine and MK-801 on percentage fibre loss 12 days after injection of NMDA in the MBN. Controls received no drug-treatment *before* and *after* NMDA-exposure. Nimodipine pretreatment consisted of a nimodipine diet (860 ppm), while posttreatment existed of an i.p. injection with nimodipine (15 mg/kg). MK-801 (5 mg/kg) was i.p. injected. (□) control; (▒) nimodipine; (▨) Mk-801; (■) nimodipine + Mk-801. Values are means (±SEM) of 6–9 animals in each group and analyzed using the non parametric Mann-Whitney-U-test; $P < 0.05$ for each therapy vs Control, except for Nimo vs Control 2 h posttreatment ($P = 0.07$).

therapy, protective effects of posttreatment 8 min after NMDA-exposure were not significantly different from the effects of pretreatment. However, combined treatment with nimodipine and MK-801 pretreatment was significantly ($P < 0.01$) more effective than treatment 8 min *after* NMDA-injection. With regard to the start of the combined therapy or single drug application of nimodipine (15 mg/kg i.p.) and MK-801 (5 mg/kg i.p.) *after* NMDA-injection, the degree of protection showed a time-dependent effect (ANOVA, $P < 0.01$). Combined nimodipine/MK-801 treatment 8 min *after* NMDA-injections revealed 81.9% protection. Application 2 h later resulted in significantly more fibre loss but protection values still measured up to 65.5%. Single drug treatments showed significantly lower protective results at both time points ($P < 0.01$). It was noteworthy that nimodipine alone applied 2 h after NMDA-exposure revealed no significant protection, whereas combined with MK-801 it resulted in significantly ($P < 0.01$) higher protection values compared to single MK-801 application.

Discussion

In the present study we demonstrated that the combined therapy with a non-competitive NMDA-antagonist (MK-801) and an L-type VDCC blocker (nimodipine) exerted an additional protective influence against NMDA-induced toxicity to cholinergic neurons in the nucleus basalis. The additional protective effect of MK-801 and nimodipine has also been reported in models of ischaemia and in tissue trauma in culture (Greenberg *et al.*, 1990; Rod & Auer, 1992; Uematsu *et al.*, 1991; Regan & Choi, 1994). Furthermore, the degree of protection showed a time-dependent effect with regard to the start of the drug treatment after the NMDA-injection.

A 73% protection achieved by the highest MK-801 dose (5 mg/kg) applied *before* NMDA-injection was further increased to 89% by additional nimodipine treatment. In a previous study we already showed that nimodipine treatment *prior* to NMDA-exposure provided almost 30% neuroprotection indicating the involvement of activated VDCCs in the NMDA-induced neurodegenerative process (Luiten *et al.*, 1995). The present data suggest that also in case of a strong neuronal protection against NMDA-induced neuronal damage by NMDA-antagonists, simultaneous blockade of VDCC-mediated Ca^{2+} currents can additionally and significantly inhibit the process of neuronal damage.

In the initial phase of NMDA-overstimulation as in ischaemic conditions Ca^{2+} influx depends primarily on NMDA-channels (Bading *et al.*, 1995; Garcia *et al.*, 1994). There are good arguments that secondary Ca^{2+} currents through activated VDCC are involved in the later stages of NMDA-induced cell damage (Garcia *et al.*, 1994; Lobner & Lipton, 1993). Several mechanisms may underlie increase of Ca^{2+} currents through VDCCs after overstimulation by NMDA. Firstly, membrane depolarization caused by NMDA-channel activation can trigger additional Ca^{2+} influx through neighbouring VDCCs (Skeen *et al.*,1993). Intracellular messenger systems, including cAMP associated pathways, are thought to be involved in the delayed long-term enhancement of voltage-gated Ca^{2+} currents after NMDA-receptor stimulation (Chetkovich *et al.*, 1991; Garcia *et al.*, 1994; Mironov & Lux, 1992).

Pretreatment with MK-801 and nimodipine yielded a neuroprotection of 89% while application of this

drug combination 8 min and 2 h *after* injection generated a protection of 82% and 66%, respectively. Based on these observations it may be concluded that up to 2 h *after* NMDA-injection a blockade of NMDA-receptor- and VDCC-mediated Ca^{2+} currents is able to protect the majority of neurons against NMDA-induced neurotoxicity. However, these results also indicate that a part of the neurons which benefit from protection by nimodipine and MK-801 when these drugs are given *before* and shortly *after* (8 min) the neurotoxic event, cannot be prevented from neuronal death by the drug application 2 h *after* NMDA-injection. Early neurotoxicity during the first 2 h after NMDA-exposure, exerted by the initial massive Ca^{2+} influx may explain this early irreversible cell damage. Studies in which single neurons in culture were lethally challenged with excitatory amino acids showed an initial Ca^{2+} influx through NMDA-channels followed by a delayed secondary, sustained and irreversible Ca^{2+} rise indicating imminent cell death (Randall & Thayer, 1992; Tymianski *et al.*, 1993). Early blockade of Ca^{2+} influx by NMDA-antagonists was able to prevent this early cell death (Hartley *et al.*, 1993; Tymianski *et al.*, 1993). An initial massive Ca^{2+} transient is considered also to be a critical step in triggering delayed neuronal death. Blockade of NMDA-channels may thus provide the highest degree of neuroprotection in the early phase of excitotoxicity. However, it appears in our currently used in vivo excitotoxicity model that still more than 60% protection can be achieved by blockade of NMDA- and voltage-dependent Ca^{2+} currents at 2 h *after* NMDA-infusion.

Other in vivo studies have also reported a striking neuroprotection by MK-801 alone when applied several hours *after* NMDA-exposure (Foster *et al.*, 1987; Van Lookeren Campagne *et al.*, 1994). It seems likely that in particular neurons in the centre of the NMDA-injection site, where the NMDA-concentration is highest, will be irreversibly affected by an initial NMDA-mediated massive Ca^{2+} influx. By diffusion NMDA will reach other neurons in a later stage and a lower concentration. Therefore remaining majority of neurons may be affected by slow and reversible neurotoxic processes involving Ca^{2+} influxes through activated VDCCs and NMDA-channels. Currently, time dependent regional calcium accumulation is investigated in our NMDA-toxicity model by ^{45}Ca-autoradiograpy, to elucidate the time-dependent process of excitotoxic damage in the NBM.

Based on the observations of delayed enhancement of voltage-dependent Ca^{2+} currents after NMDA-overstimulation (Randall & Thayer, 1992; Tymianski *et al.*, 1993), we would expect a longer lasting neuroprotective potential of nimodipine. Surprisingly, nimodipine alone applied 2 h after NMDA-exposure revealed no significant neuroprotection, compared to a still strongly protective effect established by MK-801. This finding demonstrates that at this stage of excitotoxic insult blockade of activated NMDA-channels is more effective in inhibition of NMDA-neurotoxicity than antagonizing L-type Ca^{2+}-channels. These observations suggest that mainly activated NMDA-channels are involved in the later stages of NMDA-induced neurotoxic processes. However, in this period of delayed cell death nimodipine combined with MK-801 was able to reduce neuronal damage to a significantly higher level compared to the protective effects of MK-801 alone. Presumably, during single NMDA-receptor blockade $[Ca^{2+}]_i$ will still reach lethal levels in some neurons, which can be prevented by an additional VDCC blockade. More detailed analysis of the cytology of cellular damage in the injected region, as currently investigated by us, is necessary to materialize these assumptions.

In conclusion, the present data show that dual blockade of NMDA-channels and VDCCs reveals an additional neuroprotective effect against NMDA-neurotoxicity. The degree of neuroprotection by this therapy is slowly decreased concomitant with the delay of application after NMDA-injection. Early treatment with this drug combination will yield optimal neuroprotection by blocking an initially triggered influx of Ca^{2+}. However, by dual blockade of both classes of Ca^{2+}-channels after this initial phase of Ca^{2+}-overload, there is still a significant potential for reversing NMDA-induced neurotoxic processes.

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References

Bading H, Segal MM, Sucher NJ, Dudek H, Lipton SA, Greenberg ME (1995) N-Methyl-D-aspartate receptors are critical for mediating the effects of glutamate on intracellular calcium concentration and immediate early gene expression in cultured hippocampal neurons. Neuroscience 64:653–664

Bellemann P, Schade A, Towart R (1983) Dihydropyridine receptor in rat brain labeled with [3H] nimodipine. Med Sciences 80:2356–2360

Benveniste H, Drejer J, Schousboe A, Diemer NH (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem 43:1369–1374

Chetkovich DM, Gray R, Johnston D, Sweatt JD (1991) N-Methyl-D-aspartate receptor activation increases cAMP levels and voltage-gated Ca^{2+} channel activity in area CA1 of hippocampus. Neurobiology 88:6467–6471

Choi DW (1995) Calcium still center-stage in hypoxic-ischemic neuronal death. TINS 18:58–60

Clapham DE (1995) Calcium signaling. Cell 80:259–268

Coyle JT, Puttfarken P (1993) Oxidative stress, glutamate, and neurodegenerative disorders. Science 262:689–695

De Jong GI, Traber J, Luiten PGM (1992) Formation of cerebrovascular anomalies in the ageing rat is delayed by chronic nimodipine application. Mech Ageing Dev 64:255–272

Dodd PR, Scott HL, Westphalen RI (1994) Excitotoxic mechanisms in the pathogenesis of dementia. Neurochem Int 25:203–219

Dubinsky JM (1993) Intracellular calcium levels during the period of delayed excitotoxicity. J Neurosci 13:623–631

Foster AC, Gill R, Kemp JA, Woodruff GN (1987) Systemic administration of MK-801 prevents N-methyl-D-aspartate induced neuronal degeneration in rat brain. Neurosci Lett 76:307–311

Garcia DE, Cavalié A, Lux HD (1994) Enhancement of voltage-gated Ca^{2+} currents induced by daily stimulation of hippocampal neurons with glutamate. J Neurosci 14:545–553

Gill R, Foster AC, Woodruff GN (1987) Systemic administration of MK-801 protects against ischemia-induced hippocampal neurodegeneration in the gerbil. J Neurosci 7:3343–3349

Greenberg JH, Uematsu D, Araki N, Hickey WF, Reivich M (1990) Cytosolic free calcium during focal cerebral ischemia and the effects of nimodipine on calcium and histologic damage. Stroke 21:IV72–IV77

Harper AM, Craigen L, Kazda S (1986) Effect of the calcium antagonist nimodipine on cerebral blood flow and metabolism in the primate. J Cereb Blood Flow Metab 1:349–356

Hartley DM, Kurth MC, Bjerkness L, Weiss JH, Choi DW (1993) Glutamate receptor-induced $^{45}Ca^{2+}$ accumulation in cortical cell culture correlates with subsequent neuronal degeneration. J Neurosci 13:1993–2000

Hedreen JC, Bacon SJ, Price DL (1985) A modified histochemical technique to visualize acetylcholinesterase containing axons. J Histochem Cytochem 33:134–140

Kazda S, Garthoff B, Krause HP, Schlossmann K (1985) Cerebrovascular effects of the calcium antagonistic dihydropyridine derivate nimodipine in animal experiments. Arzneim-Forsch 32:331–338

Langley MS, Sorkin EM (1989) Nimodipine, a review of its pharmacological and pharmacokinetic properties and therapeutic potential in cerebrovascular disease. Drugs 37:669–699

Lobner D, Lipton P (1993) Intracellular calcium levels and calcium fluxes in the CA 1 region of the rat hippocampal slice during in vitro ischemia: relationship to electrophysiological cell damage. J Neurosci 13:4861–4871

Luiten PGM, Gaykema RPA, Traber J, Spencer Jr DG (1987) Cortical projections of magnocellular basal nucleus subdivisions as revealed by anterogradely transported *Phaseolus vulgaris* leucoagglutinin. Brain Res 413:229–250

Luiten PGM, Douma BRK, Van der Zee EA, Nyakas C (1995) Neuroprotection against NMDA-induced cell death in rat nucleus basalis by Ca^{2+} antagonist nimodipine. Influence of aging and developmental drug treatment. Neurodegeneration 4:307–314

Maragos WF, Greenamyre T, Penney JB, Young AB (1987) Glutamate dysfunction in Alzheimer's disease: an hypothesis. TINS 10:65–68

McDonald JW, Silverstein FS, Cardona D, Hudson C, Chen R, Johnston MV (1990) Systemic administration of MK-801 protects against N-methyl-D-aspartate- and quisqalate mediated neurotoxicity in perinatal rats. Neuroscience 36:589–599

Meldrum B, Garthwaite J (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. TIPS 11:379–387

Mironov SL, Lux HD (1992) Glutamate selectively increases the high-threshold Ca^{2+} channel current in sensory and hippocampal neurons. Brain Res 580:341–344

Nakamura K, Hatakeyama T, Furuta S, Sakai S (1993) The role of early Ca^{2+} influx in the pathogenesis of delayed neuronal death after brief forebrain ischemia in gerbils. Brain Res 613:181–192

Nuglisch J, Karkoutly C, Mennel HD, Rosberg C, Krieglstein J (1990) Protective effect of nimodipine against neuronal damage in rat hippocampus without changing postischemic cerebral blood flow. J Cereb Blood Flow Metab 10:654–659

Paxinos G, Watson C (1986) The Rat Brain in Stereotaxic Coordinates. New York, Academic Press

Rami A, Krieglstein J (1994) Neuronal protective effects of calcium antagonists in cerebral ischemia. Life Sci 55:2105–2113

Randall RD, Thayer SA (1992) Glutamate-induced calcium triggers delayed calcium overload and neurotoxicity in rat hippocampal neurons. J Neurosci 12:1882–1895

Regan RF, Choi DW (1994) The effect of NMDA, AMPA / kainate, and calcium channel antagonists in traumatic cortical neuronal injury in culture. Brain Res 633:236–242

Rod MR, Auer RN (1992) Combination therapy with nimodipine and dizocilipine in a rat model of transient forebrain ischemia. Stroke 23:725–732

Rothman SM, Olney JW (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. Ann Neurol 19:105–111

Rothman SM, Olney JW (1995) Excitotoxicity and the NMDA receptor—still lethal after eight years. TINS 18:57–58

Schuurman T, Klein H, Beneke M, Traber J (1987) Nimodipine and motor deficits in the aged rat. Neurosci Res Comm 1:9–15

Scriabine A, Schuurman T, Traber J (1989) Pharmacological basis for the use of nimodipine in central nervous system disorders. FASEB J 3:1799–1806

Skeen GA, Twyman RE, White HS (1994) The dihydropyridine nitrendipine modulates N-Methyl-D-aspartate receptor channel function in mammalian neurons. Mol Pharmacol 44:443

Siesjö BK (1992) Pathophysiology and treatment of focal cerebral ischemia. II: Mechanism of damage and treatment. J Neurosurg 77:337–357

Tymianski M, Charlton MP, Carlen PL, Tator CH (1993) Source specificity of early calcium neurotoxicity in cultured embryonic spinal neurons. J Neurosci 13:2085–2104

Uematsu D, Araki N, Greenberg JH, Sladky J, Reivich M (1991) Combined therapy with MK-801 and nimodipine for protection of ischemic brain damage. Neurology 41:88–94

Van Lookeren Campagne M, Verheul JB, Nicolay K, Balázs R (1994) Early evolution and recovery from excitotoxic injury in the neonatal rat brain: A study combining magnetic resonance imaging, electrical impedance, and histology. J Cereb Blood Flow Metab 14:1011–1023