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The effect of the adrenocorticotropin-(4-9) analogue, ORG 2766, and of dizolcipine (MK-801) on infarct volume in rat brain

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Abstract

The purpose of this study was to evaluate whether the synthetic adrenocorticotropin-(4-9) (ACTH-(4-9)) analogue ORG 2766, HMet(O_2)-Glu-His-Phe-D-Lys-Phe-OH, which has been shown to have beneficial effects on both the recovery from experimentally induced lesions of the central nervous system and peripheral nerve degeneration, has a protective effect on focal ischemic neuronal damage. The NMDA receptor antagonist dizolcipine (MK-801), a very potent neuroprotective drug, was used as positive reference compound. Isoflurane-anesthetized rats had the middle cerebral artery occluded using either an intravasal or an extravasal technique, because pilot experiments had shown differences in the severity of ischemia for the two middle cerebral artery occlusion techniques. MK-801, 500 μg kg^{-1} min^{-1}, or saline was administered i.v. 30 min after occlusion of the middle cerebral artery. In the ACTH-(4-9) analogue/saline group, 10 and 150 μg/kg of the analogue, or saline was injected s.c. both directly after and 24 h after occlusion. The ACTH-(4-9) analogue treatment had no effect on the infarction volume in either model of middle cerebral artery occlusion, whereas MK-801 caused a significant reduction in the volume of cortical infarction in both models. We conclude that, although ORG 2766 is known to enhance the recovery from experimentally induced lesions of the central nervous system through a neurotrophic action and has proven to have significant beneficial effects on peripheral nerve regeneration, it did not prevent ischemic neuronal damage after intravasal or extravasal middle cerebral artery occlusion in rats. The results with MK-801, which caused significant reductions in the volume of cortical infarction in both models of middle cerebral artery occlusion, with clearly the largest reduction in the intravasal middle cerebral artery occlusion model, again indicate that there are differences in the severity of the cerebral ischemia which the two models produce in the rat brain.

Keywords: Cerebral ischemia; Middle cerebral artery; ORG 2766; (Rat)

1. Introduction

Peptides derived from adrenocorticotropin (ACTH) and their analogues are promising neurotrophic and neuroprotective peptides, as has been demonstrated in in vivo and in vitro studies (Bär et al., 1990; Gispen et al., 1994; Hol et al., 1994). It has not only been shown that these ACTH-peptides have significant beneficial effects on peripheral nerve regeneration (Verhaagen et al., 1987; Van der Zee et al., 1991; Hamers et al., 1993; Duckers et al., 1993), but also that they enhance the recovery from experimental central nervous system lesions. For example, the synthetic ACTH-(4-9) analogue, ORG 2766 (HMet(O_2)-Glu-His-Phe-D-Lys-Phe-OH), attenuated behavioural disturbances associated with lesioning of various central nervous system structures (Landfield et al., 1986; McDaniel, 1993) and considerably accelerated the functional as well as the morphological recovery after a 6-hydroxydopamine lesion in the nucleus accumbens (Wolterink and Van Ree, 1990; Wolterink et al., 1990). Surprisingly, it has not yet been investigated whether treatment with ORG 2766 also has neuroprotective effects in models of focal cerebral ischemia.

Considerable experimental evidence suggests that during cerebral ischemia, the extracellular concentration of endogenous excitatory amino acids such as aspartate and glutamate increases excessively due to enhanced release of

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excitatory amino acids from neurons, to impaired reuptake by neurons and glia, and also through leakage of excitatory amino acids from the cytosol of dying neurons (Benveniste et al., 1984; Hagberg et al., 1985; Silverstein et al., 1986; Herz et al., 1996a). These extracellular excitatory amino acids reach toxic levels, triggering a cascade of molecular events, which lead to irreversible injury (Rothman and Olney, 1986; Choi, 1988). It has been shown that, in primary cell cultures, the ability of glutamate to kill neurons is primarily due to the activation of the NMDA subtype of glutamate receptor. In models of focal cerebral ischemia, blockade of glutamate receptors, as for example by NMDA receptor antagonists such as MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate), produces a marked neuroprotective effect (Ozyurt et al., 1988; Park et al., 1988; Steinberg et al., 1989; Boxer et al., 1990; Smith and Meldrum, 1995).

We decided to investigate the possible neuroprotective effects of ORG 2766, with MK-801 as a positive reference, in two different models of focal cerebral ischemia: intravasal and extravasal middle cerebral artery occlusion. In previous experiments, in which we compared both techniques of middle cerebral occlusion, Herz et al. (1996b) showed that both the volume of cerebral infarction and the distribution of the severity of cerebral blood flow reduction over the cerebral cortex, were significantly different. For this reason, it was interesting to evaluate both MK-801 and ORG 2766 in these two models of middle cerebral artery occlusion. Possible inter-model differences in neuroprotectivity of either substance would again point to a difference in the severity of focal cerebral ischemia produced by the two models. Awareness of such differences in the severity of cerebral ischemia could be of value in the design of an animal model of focal cerebral ischemia with predictive value for the clinical treatment of stroke.

2. Materials and methods

For logistic reasons male Fisher 344 (inbred: Iffa-Credo Broekman, Someren, The Netherlands) rats were used for the extravasal middle cerebral occlusion experiments, and male Wistar rats (outbred: U: WU (CPB), Utrecht, The Netherlands) were used for intravasal middle cerebral artery occlusion experiments (all 350–400 g). The rats had free access to standard laboratory chow and water both before and after surgical intervention. Anaesthesia was induced with 3% isoflurane in a mixture of 70% N_2O and 30% O_2 while the rat was in a flow chamber. Following intubation, the rats were mechanically ventilated (Harvard Rodent Ventilator, Model 683) with 1.5% isoflurane in 70% N_2O/30% O_2. Polyethylene cannulas were introduced in the left jugular vein (PE-50) to allow intravenous bolus drug administration and continuous substitution of saline (1 ml/h) to counteract loss of fluid through mechanical ventilation, and the left femoral artery (PE-10) for assessment of blood pressure (Viggo-Spectramed DT-XX disposable transducer (Viggo-Spectramed, Bilthoven, The Netherlands) and sampling of arterial blood for measurements of P_aCO_2 and P_aO_2, respectively. The P_aCO_2 and P_aO_2 were kept in the normal range (P_aCO_2: 32–40 mmHg and P_aO_2: 95–150 mmHg; Ciba-Corning 288 Blood Gas System). Body temperature was measured by means of a rectal probe, and kept at 37 ± 0.50°C (Harvard, Homeothermic Blanket Control Unit). The design of the experiments was approved by the Experimental Animal Boards of the Medical Faculty of the Utrecht University.

2.1. Middle cerebral artery occlusion

2.1.1. Extravasal middle cerebral artery occlusion

Extravasal occlusion of the middle cerebral artery was performed by a modification of the technique of Tamura et al. (1981), as described in Herz et al. (1996c). Briefly, with the animal placed in the lateral position in a stereotactic apparatus, a standardized craniotomy was made (exposing a long proximal middle cerebral artery segment) and the dura was opened. A 10-0 ethilon ligature was used to occlude the middle cerebral artery as proximally as possible. Hereafter, a long proximal segment of the middle cerebral artery was occluded by bipolar thermocoagulation starting at the place where the middle cerebral artery originates from the internal carotid artery and ending at the place where the middle cerebral artery crosses the inferior cerebral vein.

2.1.2. Permanent intravasal middle cerebral artery occlusion

Permanent intravasal occlusion of the middle cerebral artery was obtained using the intraluminal thread technique (Koizumi et al., 1986; Longa et al., 1989) as modified by Kawamura et al. (1991). In short, after ligation of the left external carotid artery and the left pterygopalatine artery, the left carotid artery was severed, and a thread was introduced into its distal stump and advanced into the left internal carotid artery towards the cerebral circulation until a faint resistance was felt. At that moment, the tip of the thread occludes the lumen of the left anterior cerebral artery, so that it cannot be introduced any further, and the entrance of the left middle cerebral artery out of the internal carotid artery is also occluded by the thread. The occluding thread was a 3-0 Surgipro monofilament polypropylene thread (Auto Suture Nederland, Zeist, The Netherlands), the tip (10 mm) of which had been dipped in boiling xylol. This procedure not only weakens the tip, thus minimizing the risk of perforation of the intracranial part of the internal carotid artery, but also creates a small increase in the diameter of the tip so that the possibility is diminished that blood can leak along the occluding thread into the middle cerebral artery. During the intravasal middle cerebral artery occlusion experiments, we used laser-Doppler flowmetry to establish whether intracranial ad-

vancement of the occluding thread actually resulted in a sudden decrease in cortical blood flow in the middle cerebral artery territory, indicative of a successful middle cerebral artery occlusion.

2.2. Laser-Doppler flowmetry

We used a PeriFlux PF3 (Perimed, Stockholm, Sweden) flowmeter, equipped with a 2-mW helium–neon laser with a wavelength of 632.8 nm. Blood flow oscillations were recorded using the 12-kHz low-pass filter setting and a 0.2-s time constant. Flow values are expressed in arbitrary units (perfusion units, PU). The needleprobe (tip diameter 0.45 mm; PF 302) was mounted on a micromanipulator. A rectangle was carefully drilled through the left parietal bone, with its corners on the coordinates (3P, 3L), (3P, 6L), (8P, 3L) and (8P, 6L) in relation to the bregma. The dura was left intact. The probe was positioned just above the dural surface, and saline was applied to moisten the dura and fill the space between dura and probe.

2.3. Experimental procedure

In the MK-801 group (intravasal middle cerebral artery occlusion: $n = 7$; extravasal middle cerebral artery occlusion: $n = 6$) a bolus infusion of MK-801 (500 μg/kg in 1 min; 500 l/min) or saline (intravasal occlusion: $n = 7$; extravasal occlusion: $n = 6$) (vehicle controls) was administered into the left jugular vein 30 min after occlusion of the middle cerebral artery (MK-801 dosage and time of administration derived from Park et al. (1988)). In the ORG 2766 group (intravasal occlusion: $n = 8$; extravasal occlusion: $n = 7$), 10 μg/kg ORG 276 or saline (vehicle controls; intravasal occlusion: $n = 7$; extravasal occlusion: $n = 7$; injection volume 0.5 ml) was injected subcutaneously both directly after and 24 h after occlusion. In a separate experiment, we tested a 15-fold higher dose of ORG 2766 ($n = 7$), 150 μg/kg s.c. vs. saline ($n = 7$) both directly after and 24 h after intravasal middle cerebral artery occlusion. Blood pressure, and, in the case of intravasal occlusions, also laser-Doppler-assessed cerebral cortical microcirculatory flow, were monitored using a computerized biosignal processing and analysis system (DiSys System, Mediware, Groningen, The Netherlands). Directly after drug administration after occlusion of the middle cerebral artery, the polyethylene cannulas were removed, and the wounds were closed. The rats were then returned to their cages, and after 48 h, were injected i.p. with 90 mg/kg pentobarbital. Following thoracotomy, a blunt metal cannula was placed in the ascending aorta via the left ventricle, and secured. The right atrium was incised, the abdominal aorta was clamped, and the animal was perfused with 100–150 ml of saline and then with 200 ml of fixative (4% formalin in a 0.1 M phosphate buffer). The animals were decapitated, and their brains were placed in the same fixative for at least 1 week. The brain was frozen, and coronal sections, 25 μm thick, were cut throughout the rostrocaudal extent of the brain with a cryostat. Every 10th coronal section was selected, stained with conventional hematoxylin/eosin staining, and mounted on slides under coverslips. A computerized Image Processing System, IBAS (Kontron, Münich, Germany) or a digital image analysis system (TIM, DIFA, Breda, The Netherlands) was used to measure the area of infarction in all sections, both as absolute numbers and as a percentage of total slice area. The total volume of infarction was determined by integration of the infarction areas of the sections and the distance between them (250 μm).

2.4. Statistics

Values are expressed as means ± S.E.M. Differences between data sets were evaluated statistically (SPSS for Windows) by analysis of variance (ANOVA). A $P < 0.05$ was considered to indicate a significant difference.

2.5. Drugs used

Org 2766 (H-Met-(O_2)0-Glu-His-Phe-D-Lys-Phe-OH) was a gift from Organon Int., Oss, The Netherlands.

Table 1
Physiological variables: ORG2766 and MK-801 experiments just before MCA occlusion

	MCA occlusion technique							
	Intravasal				Extravasal			
	Saline	MK-801	Saline	ORG 2766	Saline	MK-801	Saline	ORG 2766
MAP (mmHg)	104 ± 5	97 ± 8	101 ± 5	98 ± 4	93 ± 4	93 ± 3	103 ± 2	101 ± 5
Arterial pH (unit)	7.47 ± 0.01	7.44 ± 0.01	7.44 ± 0.02	7.45 ± 0.01	7.44 ± 0.02	7.45 ± 0.01	7.48 ± 0.01	7.46 ± 0.02
P_aCO_2 (mmHg)	33.4 ± 0.6	35.0 ± 0.8	36.1 ± 1.4	35.3 ± 1.0	36.0 ± 1.6	36.8 ± 0.8	34.4 ± 0.7	34.7 ± 1.4
P_aO_2 (mmHg)	129 ± 6.9	137.3 ± 10.1	139.9 ± 3.2	125.9 ± 10.1	131.0 ± 3.4	127.6 ± 5.4	125.9 ± 2.5	125.2 ± 6.5

Values are means ± S.E.M. MCA, middle cerebral artery. In all groups, physiological variables were measured just before intravasal or extravasal occlusion of the MCA. No significant differences between values for parameters between experimental groups. MK-801 and saline (intravasal): $n = 7$, $n = 7$, respectively; ORG 2766 (10 μg/kg) and saline (intravasal): $n = 8$, $n = 7$, respectively; MK-801 and saline (extravasal): $n = 6$, $n = 6$, respectively; ORG 2766 (10 μg/kg) and saline (extravasal): $n = 7$, $n = 7$, respectively. All extravasal MCA occlusion experiments were with F344 rats and all intravasal MCA occlusion experiments were with Wistar rats.

MK-801 ([+]-5-methyl-10,11-dihydro-5H-dibenzocyclohepten-5,10-imine maleate) was a gift from Merck.

3. Results

3.1. Physiologic variables

Table 1 lists the mean arterial blood pressure, P_aO_2, P_aCO_2 and arterial pH (±S.E.M.) just before middle cerebral artery occlusion in all experimental groups treated with MK-801 and ORG 2766 after intra- or extravasal occlusion, and their vehicle controls. No significant differences in the values of these physiological parameters were observed between these groups. In Fig. 1, the effects of the intravasal occlusion (only intravasal) on the hemodynamic variables are shown during a monitoring period of 2 h. It is seen that the MCA occlusion hardly influenced blood pressure, but increased heart rate and, as expected, decreased cerebral blood flow. No changes in these effects were seen irrespective of the treatment (saline or ORG 2766). Body temperature was controlled and kept constant at 37 ± 0.5°C. Earlier pilot experiments had already shown that the administration of both MK-801 and ORG 2766, under identical experimental conditions, did not produce changes in laser-Doppler-assessed cerebral blood flow.

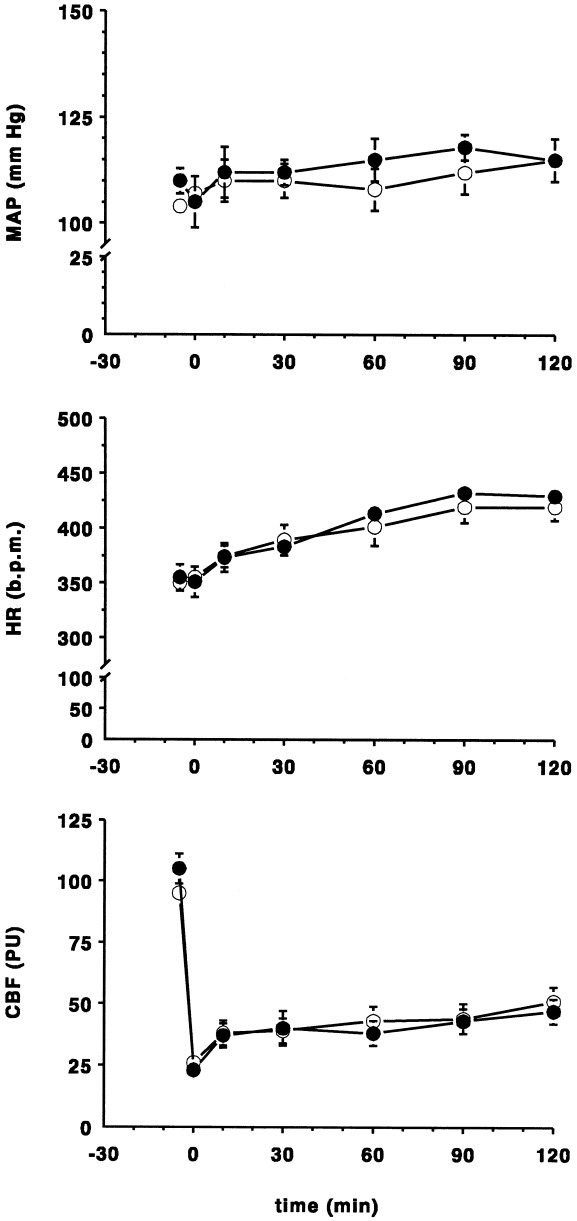

Fig. 1. Effect of intravasal middle cerebral artery occlusion on mean arterial blood pressure (MAP), heart rate (HR) and cerebral blood flow (CBF). Middle cerebral artery occlusion was performed at 0 min. The effects of ORG 2766 (150 μg/kg, s.c.; $n = 7$; ○) on these variables were not different from those in saline-treated rats ($n = 7$; ●).

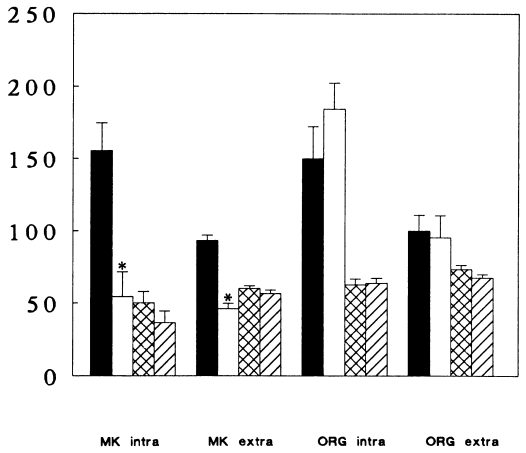

Fig. 2. Effects of MK-801 (MK) and ORG 2766 (ORG) on infarction volume after middle cerebral artery (MCA) occlusion in mm^3 (*Y*-axis). Values: means ± S.E.M. The closed and open bars represent cortical infarction volume in control vehicle or drug-treated animals, respectively. The cross-hatched and hatched bars represent striatal infarction volume in control vehicle or drug-treated animals, respectively. Intra, extra: intravasal or extravasal MCA occlusion respectively. * Denotes significant difference in cortical infarction volume after both techniques of MCA occlusion ($P < 0.05$, ANOVA) between MK-801-treated and vehicle controls.

3.2. Volume of cerebral infarction

Fig. 2 shows the volumes of cerebral infarction in the eight experimental groups. The extravasal occlusion experiments provided no evidence for neuroprotectivity by ORG 2766 (10 μg/kg). MK-801, however, produced a significant ($P = 0.001$) decrease in cortical infarction volume (±50%), but had no significant effect on the volume of striatal infarction. The volumes of cerebral infarction were greater in the intravasal occlusion experiments than in the extravasal occlusion experiments. However, no evidence for neuroprotectivity by ORG 2766 was found in this model either, while MK-801 produced a significant ($P = 0.004$) decrease in cortical infarction volume (±65%), but

had no significant effect on the volume of striatal infarction (Fig. 2).

Treatment with the higher dose of ORG 2766 (150 μg/kg) did not change the infarct volume (215 ± 30 mm^3, $n = 7$) from that measured in saline treated rats (179 ± 25 mm^3, $n = 7$). The infarct volume as a percentage of the left hemisphere was $35 \pm 5\%$ in the ORG 2766-treated rats and $31 \pm 4\%$ in the controls (saline).

4. Discussion

The purpose of this study was to evaluate whether the ACTH-(4-9) analogue ORG 2766, which has been shown to accelerate the recovery from experimentally induced lesions of the central nervous system (Landfield et al., 1986; Wolterink and Van Ree, 1990; Wolterink et al., 1990; McDaniel, 1993), and to have significant beneficial effects on peripheral nerve regeneration (Verhaagen et al., 1987; Van der Zee et al., 1991; Hamers et al., 1993; Duckers et al., 1993), has a protective effect on neuronal damage caused by focal ischemia. In all these studies, the effective dosage for ORG 2766 was 10 μg/kg s.c., once daily, which is consistent with the effective dose range of 1–10 μg/kg s.c. (once daily) established by Wolterink and Van Ree (1990). Furthermore, ACTH/ORG 2766 can bind to NMDA receptors in the rat hippocampus (Trifiletti and Pranzatelli, 1992), while, from the behavioural aspect ORG 2766 in the dose scheme shown, can counteract the overactivation of the NMDA receptor (Spruijt et al., 1994). Since we found no effect in the cerebral ischemia model (vide infra) we also tested a 15-fold higher dose of ORG 2766 (150 μg/kg). ORG 2766 has no direct cardiovascular actions (De Wildt et al., 1993), and is therefore only expected to have direct neurotrophic/neuroprotective properties. As a positive reference compound in the two models of focal cerebral ischemia, we chose the NMDA receptor antagonist, MK-801, which is a potent neuroprotective drug (Ozyurt et al., 1988; Park et al., 1988; Steinberg et al., 1989; Boxer et al., 1990; Dezsi et al., 1992; Gill et al., 1992; Hatfield et al., 1992; Collaço-Moraes et al., 1994). The two models of focal cerebral ischemia we used, intravasal (with an intraluminal thread; Herz et al., 1996b) and extravasal (after craniotomy) middle cerebral artery occlusion, differ in more aspects than the technique used for vessel occlusion. The intravasal occlusion in Wistar rats results in a large but variable volume of cerebral infarction, while the extravasal occlusion technique in Wistar rats results in a relatively small volume of cerebral infarction with only slight variability (Duverger and MacKenzie, 1988; Herz et al., 1996b,c). Clearly, the two models produce a different severity of cerebral ischemia. It was therefore important to use a positive reference substance such as MK-801, which has been shown to have pronounced neuroprotective effects in models that use extravasal occlusion, so that we could compare the effectivity of MK-801, in terms of neuroprotection, in both models of occlusion with the possible neuroprotectivity of ORG 2766.

MK-801 caused a significant reduction in the volume of cortical infarction in both models of occlusion. The largest reduction, in both absolute numbers and percentage reduction, was observed in the intravasal occlusion model. This different neuroprotective potency of MK-801 in the two models is another argument in favour of the possibility that the intravasal and extravasal models of occlusion differ as to the severity of the cerebral ischemia they produce in the rat brain (Herz et al., in press). It is known that the effectivity of NMDA receptor antagonists in cerebral ischemia appears to depend on the severity of the ischemic insult and its impact on the energy state (Wieloch et al., 1989; Siesjö and Bengtsson, 1989). If complete energy failure occurs, such as in global ischemia or in the core of a focal cerebral infarction, NMDA receptor antagonists are not effective (Edvinsson et al., 1993; Yao et al., 1994). In contrast, NMDA receptor antagonists are clearly efficacious in the penumbra, in which the energy state is less markedly disturbed (Edvinsson et al., 1993; Yao et al., 1994). Our results with MK-801 in both occlusion models do suggest that the volume of the penumbra is much greater after intravasal occlusion than after extravasal occlusion, both in absolute numbers and as percentage of the total volume of cerebral infarction. This can indeed have consequences for the predictive value of both models in the clinical treatment of stroke. For this reason, it could be of importance to evaluate possible neuroprotective substances using both models of middle cerebral artery occlusion.

ORG 2766 treatment, also with the rather high dose of 150 μg/kg, s.c., did not provide neuroprotection in either of the two models of occlusion. Neither cortical nor striatal volume of infarction was affected. There are indications that ORG 2766 might act via the NMDA receptor (Gilchrist et al., 1994; Spruijt et al., 1994). It could therefore very well be that, while the NMDA receptor is strongly stimulated by the enormously increased extracellular concentration of glutamate during cerebral ischemia, no possible modulation of excitatory neurotransmission by ORG 2766 could exert a beneficial influence.

We conclude that, despite the fact that the synthetic ACTH-(4-9) analogue, ORG 2766, is known to enhance the recovery from experimentally induced lesions of the central nervous system, and has proven to have significant beneficial effects on peripheral nerve regeneration, it did not prevent ischemic neuronal damage after intravasal or extravasal middle cerebral artery occlusion in rats. Our results with MK-801, which caused a significant reduction in the volume of cortical infarction in both models of occlusion, with the clearly greatest reduction (in both absolute numbers and percentage of the total volume of infarction) in the intravasal occlusion model, again point to

a difference in the severity of cerebral ischemia that the two models produce in the rat brain.

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