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Neuroprotection by the NMDA receptor-associated open-channel blocker memantine in a photothrombotic model of cerebral focal ischemia in neonatal rat

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Abstract

Excessive accumulation of glutamate or other excitatory amino acids and the subsequent overactivity of NMDA receptors is currently thought to lead to neuronal injury in cerebral ischemia. Therefore, antagonists of the NMDA receptor may offer an approach for the treatment of ischemic brain injury. Dizocilpine (MK-801), an NMDA receptor-associated channel blocker, protects neurons in several rodent stroke models. However, this drug has numerous side effects and causes apoptosis of neonatal neurons. Recently, another NMDA receptor-associated channel blocker, memantine, has been shown to ameliorate NMDA-receptor mediated neurotoxicity in neuronal cell cultures and in focal cerebral ischemia models in adult rats without substantial side effects. Memantine has been used clinically in the treatment of Parkinson's disease and spasticity for a number of years. Here we tested the effects of memantine on focal stroke caused by photochemical thrombosis in neonatal rats and demonstrated a neuroprotective effect of memantine in this model. We also found excellent correlation between infarct size determined by magnetic resonance imaging (MRI) and histopathological analysis in the same animals. A single pre-ischemic dose of memantine (20 mg/kg) given 15 min prior to induction of stroke reduced the infarct size by 36.3% when compared to control animals treated with normal saline ($P < 0.0001$). At this dosage, memantine manifests few, if any, neurobehavioral side effects. Thus memantine appears to be both safe and effective in neonatal as well as adult animal models of stroke.

Keywords: Photochemical thrombosis; Focal cerebral ischemia; Memantine; Neonatal, rat

1. Introduction

Several lines of evidence suggest that the excitatory amino acids, glutamate and congeners, may play a critical role in cerebral hypoxic–ischemic injury and neurodegenerative diseases (Rothman and Olney, 1986; Choi, 1988; Lipton, 1992; Lipton and Rosenberg, 1994). In the rat brain under hypoxic–ischemic conditions, excitatory amino acids accumulate to toxic concentrations in the extracellular space (Benveniste et al., 1984). Overactivation of NMDA receptors, a subtype of glutamate receptor, by excitatory amino acids appears to mediate a component of neurotoxicity by opening ion channels and allowing excessive influx of Ca^{2+} (Choi, 1990; Lipton and Rosenberg, 1994; Castillo and Babson, 1998).

Delayed neuronal damage induced by exposure to excessive levels of glutamate is reduced by antagonists of the NMDA receptor (Simon et al., 1984; McDonald et al., 1989). Evidence suggests that uncompetitive more so than competitive NMDA receptor antagonists may be useful in diminishing the injury caused by excessive glutamate following stroke (Levy and Lipton, 1990). Dizocilpine (MK-801), an uncompetitive NMDA receptor antagonist, has been demonstrated to protect neurons from ischemic damage in various animal models (Gill et al., 1987; Rod and Auer, 1989; Swan and Meldrum, 1990). However, this drug impairs normal neuronal function, manifests behavioral side effects, and can cause injury and apoptosis in neonatal neurons at potentially therapeutic concentrations (Olney et al., 1989, 1991; Ikonomidou et al., 1999).

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Accumulating evidence indicates that another drug, memantine (1-amine-3,5-dimethlyadamantane), a well-tolerated drug in clinical use for many years in Europe, is also an uncompetitive NMDA receptor antagonist (Bormann, 1989; Kornhuber et al., 1989). Recently, we demonstrated that memantine produces reversible open-channel block of NMDA receptor-associated channels. The kinetics of memantine action in the channel results in block of excessive NMDA receptor activity but sparing of physiological receptor activity (Chen et al., 1992; Chen and Lipton, 1997; Chen et al., 1998). This selectivity gives memantine an edge over other glutamate receptor antagonists that have recently failed in clinical trials. Additionally, memantine prevents NMDA receptor-mediated neuronal injury in both rat retinal ganglion cells and cortical neurons in vitro and in models of focal cerebral ischemia in vivo (Seif el Nasr et al., 1990; Chen et al., 1992, 1998). These pharmacological and therapeutic properties of memantine suggest that it may protect neurons against hypoxic–ischemic injury.

In human infants, hypoxic–ischemic brain injury is a common medical problem caused by perinatal asphyxia (Volpe, 1993). We therefore studied the effects of memantine on cerebral ischemic injury in the perinatal period using a photo-induced thrombotic stroke model, originally developed for adult rats (Watson et al., 1985; De Ryck et al., 1989; Cai et al., 1998), which we modified for neonatal rats. We also demonstrated a close quantitative correlation between the size of the infarct determined by magnetic resonance imaging (MRI) and by histopathological analysis in the same animals.

2. Materials and methods

2.1. Photoinduction of ischemia and drug treatment

Neonatal Long–Evans rat pups (Charles River, Wilmington, MA, USA), age 7–10 days, weighing 20–27 g were used in these experiments. All animals were housed in the institutional facility and all surgical and handling procedures were approved by the Institutional Animal Care and Use Committee. Rat pups from two litters were randomized in pairs to receive either memantine (20 mg/kg, i.p.; $n = 12$) or buffered normal saline ($n = 12$) 15 min prior to photochemical thrombosis; therefore, for statistical reasons, the rat pups were analyzed as pairs. Memantine was the kind gift of Merz (Frankfurt, Germany). The experiments were performed and analyzed in a masked fashion.

Photo-induced cerebral ischemia was performed based on the method of Watson et al. (1985) with several modifications for neonatal rats. The animals were anesthetized with chloral hydrate (50–100 mg/kg), placed in a stereotaxic holder, and a transverse linear skin incision was made between the left eye and ear. Rose bengal (40 mg/kg, intracardiac administration) was given, and immediately thereafter, a 1-cm long brass collimator (aperture, 2 mm in diameter) was affixed to the skull at a coordinate 3 mm to the left of the bregma. A 560-nm wavelength light from a fibertopic 300 W xenon arc lamp (LC Technology, Sunnyvale, CA, USA) was directed at a focal brain region via the collimator for 3 min. Skull and rectal temperature were monitored during the experiment with a digi-sense thermometer (Cole-farmer, Chicago, IL). Animal rectal temperature was maintained at 35.0 ± 1.0°C and skull temperature was maintained at 34.4 ± 0.2°C during and until 1 h after the experiment by keeping the animals on a heating pad (Seabrook Micro-Temp Pump, Cincinnati, OH). Our previous studies had shown that treatment with memantine in either rat pups or adult rats does not affect temperature or other physiological parameters, including glucose, pH, and arterial blood gases (Chen et al., 1998).

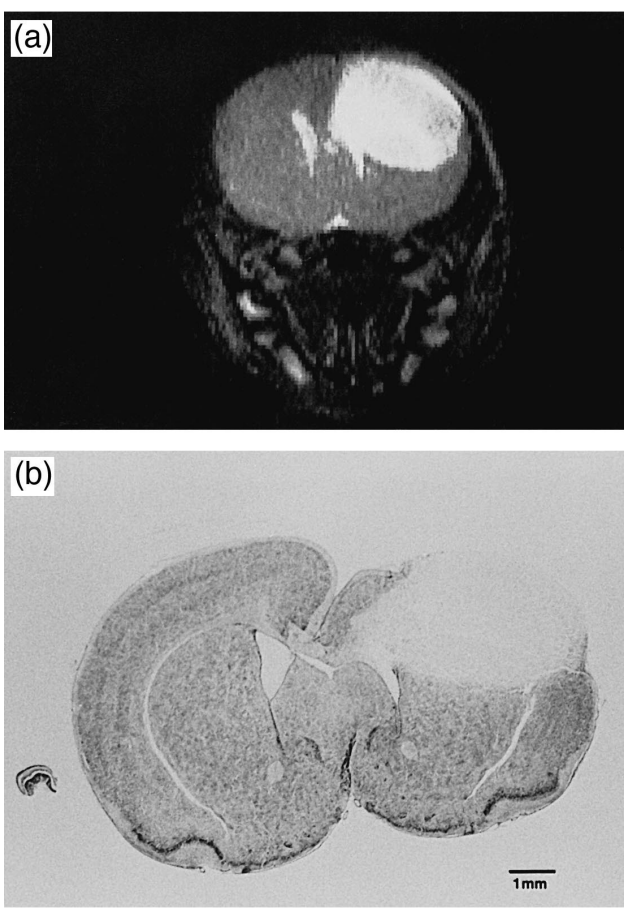

Fig. 1. Comparison of MRI scan and histological appearance of the same section of brain after cerebral infarct. (a) T2-weighted brain MRI scan of a neonatal rat 6 days following an infarct induced by photochemical thrombosis. (b) Coronal section of the same rat brain in the same rostral–caudal region as the MR image reveals a large area of necrosis of both neuronal and glial elements extending from the cortical surface to the lateral ventricle (haemotoxylin and eosin).

After closure of the incision with 6-0 nylon suture, animals were returned to the dam until time of sacrifice.

2.2. MRI quantitation

Six days after injury, the animals ($n = 12$ each group) underwent MRI (Biospec MRI, small bore animal imager, 4.7 T, Brüker, Billerica, MA, USA). We chose to wait 6 days to perform the analysis because preliminary studies had shown that the size of the stroke had stabilized by that time and cerebral edema had resolved. Parameters for the scans of multislice, T2-weighted imaging were as follows: TR = 2500 ms, TE = 100 ms, field-of-view (FOV) = 2.5 × 2.5 cm, acquisition matrix 128 × 128, and slice thickness 1 mm with no interslice gap. A volumetric analysis of the infarct size was performed directly from these images by a masked observer. The percentage of infarct volume was quantified following previously described methods (Manning et al., 1990; Chen et al., 1998).

2.3. Histopathology quantitation

Following MRI scanning, randomly-selected animals ($n = 3$ in each group) were anesthetized with ether and perfused transcardially with 4% paraformaldehyde in phosphate buffered saline, pH 7.4. Brains were embedded in paraffin and serially sectioned at 20 μm. Every 10th section was processed for routine histology and stained with haematoxylin and eosin. The surface area of the infarct tissue was measured from haematoxylin and eosin sections using NIH Image 1.55 analysis software (National Institutes of Health, Bethesda, MA, USA). The infarct area obtained from histopathological sections was then compared with the infarct area determined by MRI for corresponding sections.

2.4. Statistical analysis

A two-tailed *t*-test was performed for comparisons between control and experimental groups.

3. Results

3.1. MRI and histopathological correlation

Six days after photothrombotic insult, T2-weighted MRI scans of all animals revealed a region of high signal intensity in the left fronto-temporal area, representing the area of cerebral infarction. All the lesions had a wedge shaped configuration extending from the left fronto-temporal cortex deep into the white matter and were located at similar vertical and rostral–caudal coordinates (Fig. 1a). Histopathologic analysis on post-injury day 6 confirmed the area of infarct, as visualized on the MRI images. There was an area of uniform necrosis involving both neuronal and glial elements which was wedged shaped, extending

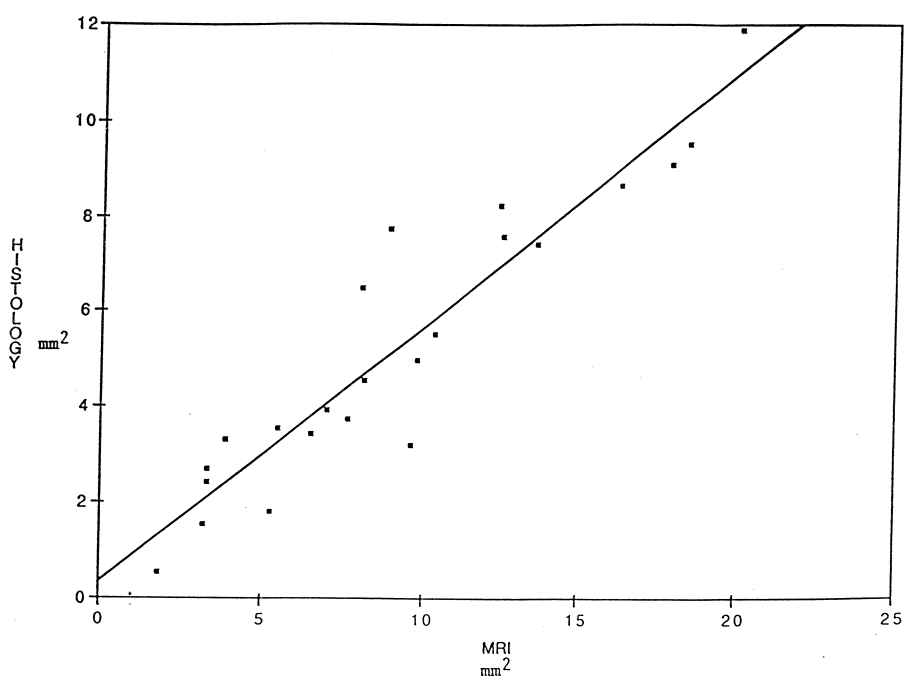

Fig. 2. Correlation of infarct area measured from MRI vs. histologic images in three randomly selected animals. Each point represents area calculated from corresponding sections by MRI and histology. The small difference in absolute areas between MRI and histopathological analysis represents tissue shrinkage during processing for post-mortem histology.

from the cortical surface to the lateral ventricle (Fig. 1b). This well-demarcated infarct was usually surrounded by macrophages that had begun to infiltrate the necrotic zone. Thrombotic material and red blood cell stasis were noted in small vessels, but the larger blood vessels appeared to be patent. The area of the infarct, as determined by MRI, correlated well with the infarct area determined by histological analysis in corresponding brain sections from the same animals ($r = 0.94$; Fig. 2).

3.2. Infarct volumes after memantine

Infarct volumes were obtained from T2-weighted MRI scans. Compared to the saline-treated group, animals receiving memantine sustained significantly smaller infarcts (Fig. 3; Table 1). The mean percentage of the difference in the stroke volumes between the control and memantine-treated groups was 36.3% ($P < 0.0001$).

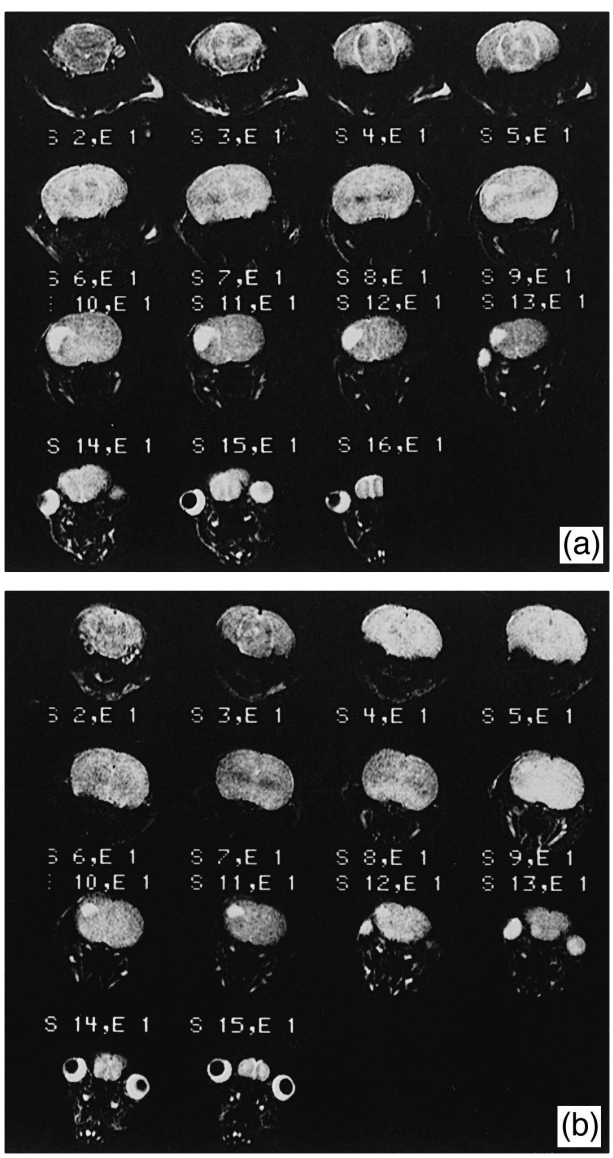

Fig. 3. T2-weighted brain MRI scans obtained 6 days after cerebral infarct in 9-day-old rat pups pretreated with (a) normal saline or (b) memantine (20 mg/kg). Note that the lesions are localized in similar regions in each animal, but the infarcted areas are smaller in the memantine-treated animal.

Table 1
Pre-ischemic treatment with memantine (20 mg/kg) vs. saline

Litter matched animal pair	Percentage of difference in cerebral infarct volume
	Control vs. memantine
1.	22.0
2.	31.8
3.	19.8
4.	51.3
5.	39.9
6.	35.0
7.	30.4
8.	49.0
9.	47.8
10.	36.1
11.	28.8
12.	25.2

Mean ± standard deviation 36.3 ± 11.9, $P < 0.0001$.

4. Discussion

The photothrombotic stroke model in adult rats is well-established and has the distinct advantage of being minimally invasive compared to other procedures that are used to induce focal cerebral ischemia (Watson et al., 1985; Rogers and Hunter, 1997). For the first time, we applied this model to neonatal rats in order to study cerebral hypoxia–ischemia in the perinatal period. In our studies, stereotaxic localization of the light source permitted placement of the photothrombotic lesion in similar areas of the brain in all of the rat pups, allowing valid comparison of pharmacologic manipulations in the control and experimental groups. Additionally, the lesion size and location within animals in the same treatment group was found to be very reproducible.

The rat pups in this model manifest a focal stroke on MRI scan that correlated well with the histopathological analysis of the completed infarction (Fig. 2). These results extend to the neonate previous correlations of MRI and histopathology in adult models (Allegrini and Sauer, 1992). The correlation of MRI data and histopathological data suggests that an MRI-based quantification of stroke volume in living subjects can be used to assess the effectiveness of neuroprotective therapy in human stroke trials, including those designed to study the prevention of perinatal encephalomalacia due to hypoxic–ischemic insults in the newborn (Volpe, 1993).

Excessive accumulation of excitatory amino acids and the subsequent overactivity of NMDA receptors have been associated with neuronal injury in cerebral ischemia. In the

neonatal cortex, where the relative number of NMDA receptors is higher than in adult (McDonald and Johnston, 1990; Monyer et al., 1994; Sheng et al., 1994), NMDA receptor-mediated hypoxic–ischemic injury may play an important role in perinatal asphyxia. To support this hypothesis further, we report here that treatment with memantine, an uncompetitive NMDA receptor antagonist, attenuated infarct size by 36.3% in neonatal rats undergoing photo-induced cerebral ischemia. In our study, memantine was given 15 min prior to induction of the stroke because previous pharmacokinetic studies had indicated that peak brain concentrations were achieved ~30 min after i.p. administration (Wesemann et al., 1982). The memantine dosage was selected to produce a concentration of memantine in brain parenchyma similar to that achieved in humans taking the drug for Parkinson's disease, while minimizing potential side effects (Wesemann et al., 1983; Chen et al., 1998). Higher concentrations of memantine may have diverse effects on multiple neurotransmitter systems including dopaminergic, cholinergic, and serotonergic neurons (Maj, 1982; Warnick et al., 1982). However, in the present study, we chose a lower dose of memantine that had been reported to be effective in reducing infarct size during focal cerebral ischemia in adult rats with minimal side-effects (Chen et al., 1998). Since memantine is already used clinically in Europe for other clinical indications, trials for human neonatal hypoxic–ischemic injury may be expedited.

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