Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 2508–2513, March 1999
Pharmacology

N-Methyl-D-aspartate antagonists and apoptotic cell death triggered by head trauma in developing rat brain

(traumatic head injury/apoptosis/necrosis/excitotoxicity/neurodegeneration)

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Communicated by Martin Lindauer, University of Würzburg, Würzburg, Germany, December 21, 1998 (received for review February 11, 1998)

ABSTRACT **Morbidity and mortality from head trauma is highest among children. No animal model mimicking traumatic brain injury in children has yet been established, and the mechanisms of neuronal degeneration after traumatic injury to the developing brain are not understood. In infant rats subjected to percussion head trauma, two types of brain damage could be characterized. The first type or primary damage evolved within 4 hr and occurred by an excitotoxic mechanism. The second type or secondary damage evolved within 6–24 hr and occurred by an apoptotic mechanism. Primary damage remained localized to the parietal cortex at the site of impact. Secondary damage affected distant sites such as the cingulate/retrosplenial cortex, subiculum, frontal cortex, thalamus and striatum. Secondary apoptotic damage was more severe than primary excitotoxic damage. Morphometric analysis demonstrated that the *N*-methyl-D-aspartate receptor antagonists 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonate and dizocilpine protected against primary excitotoxic damage but increased severity of secondary apoptotic damage. 2-Sulfo-α-phenyl-*N*-*tert*-butyl-nitrone, a free radical scavenger, did not affect primary excitotoxic damage but mitigated apoptotic damage. These observations demonstrate that apoptosis and not excitotoxicity determine neuropathologic outcome after traumatic injury to the developing brain. Whereas free radical scavengers may prove useful in therapy of head trauma in children, *N*-methyl-D-aspartate antagonists should be avoided because of their propensity to increase severity of apoptotic damage.**

Although children under 6 years of age sustain traumatic brain injury more frequently than any other age group (1), there has been a dearth of research focusing on traumatic brain injury to the developing brain. Consequently, very little progress has been made toward understanding the mechanisms and developing neuroprotective measures for traumatic brain damage in children. Current therapy is symptomatic and consists of control and support of cardiovascular and respiratory systems, hemodynamic stabilization, control of intracranial pressure, and prophylactic anticonvulsant treatment (2).

Clinical experience and experimental observations in animals suggest that brain damage resulting from severe head injury can be classified into primary, which occurs at impact and appears immediately or shortly after injury, and secondary, which occurs distant to the impact and may not appear until several hours after injury (3, 4). It is assumed that injured neurons have a potential for recovery and that neurodegeneration triggered by traumatic impact is a dynamic and time-related process (5). According to this viewpoint, early diagnosis and medical support are crucial for the prevention of additional brain damage after head injury.

Studies focusing on the adult brain have shown that the damage associated with head trauma is triggered by an excitotoxic mechanism involving glutamate (6, 7). Accordingly, drugs that block glutamate receptors ameliorate both primary and secondary damage in adult head injury models (7). Neuronal death may also occur by an apoptotic programmed and genomically controlled mechanism (8, 9). No study has addressed involvement of apoptotic cell death in traumatic damage in the developing brain. Therefore, we have developed morphometric methods for detecting primary and secondary damage after traumatic head injury in infant rats by using a modified contusion device initially described by Allen (10) for the spinal cord and by Feeney *et al.* (11) for the brain. Using this experimental approach, we report that mechanical trauma to the developing brain causes primary excitotoxic (nonapoptotic) damage to the cortex and secondary delayed damage that is apoptotic to the cingulate/retrosplenial cortex, frontal cortex, parietal cortex, subiculum, thalamus, and striatum. We describe the type of neurodegenerative response to head trauma in the infant rat brain in terms of its topography, time course, age dependency, and response to neuroprotective treatment and critically evaluate potential implications for the therapy of head trauma in children.

MATERIALS AND METHODS

Traumatic Brain Injury and Contusive Device. Wistar rat pups (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, Germany) were anesthetized with halothane and placed in a mold fashioned to fit the contours of the skull and holding it in the desired attitude. The anesthesia was induced in 4% halothane and maintained in 1.5% halothane in balanced room air (12, 13) until the end of the procedure. A skin incision was made to expose the skull surface. The contusing device consisted of a hollow stainless steel tube 40 cm long, perforated at 1-cm intervals to prevent air compression. The device was kept perpendicular to the surface of the skull and guided a falling weight onto a circular footplate (2.0 mm in diameter) resting upon the surface of the parietal bone. A force of 160 g×cm produced by a 10-g weight was selected to produce brain contusion. The following coordinates in relation to lambda were used for stereotaxic positioning of the footplate onto the exposed parietal bone: 2 mm anterior and 2 mm lateral at the age of 3 days; 3 mm anterior and 2 mm lateral at the age of 7 days; 3.5 mm anterior and 2.5 mm lateral at the ages of 10 and 14 days and 4 mm anterior and 3 mm lateral at the age of 30 days. The contusion force was delivered unilaterally to the right side of the skull. The experiments were performed in accordance with the

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Abbreviations: CPP, 3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonate; MK-801, dizocilpine; NMDA, *N*-methyl-D-aspartate; SPBN, 2-sulfo-α-phenyl-*N*-*tert*-butyl-nitrone; N_v, numerical density; T_n, total number of cells; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling.

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German and United States Animal Welfare Acts and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Morphometry. For morphological analysis rats were anesthetized with an overdose of chloral hydrate and perfused through the heart and ascending aorta for 15 min with a solution of paraformaldehyde (1%) and glutaraldehyde (1.5%) in pyrophosphate buffer (for combined light and electron microscopy) or paraformaldehyde (4%) in phosphate buffer [for terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) or DeOlmos cupric silver staining].

Light Microscopy on Plastic Sections and Electron Microscopy. Brains were sliced in 1-mm-thick slabs, fixed in osmium tetroxide, dehydrated in alcohols, and embedded in araldite. For light microscopy, transverse serial sections, 1–10 μm, were cut and stained with methylene blue/azure II. Subsequently, ultrathin sections were cut and stained with uranyl acetate/lead citrate and examined by electron microscopy.

TUNEL Staining. To visualize nuclei with DNA cleavage, serial coronal sections (70 μm) of the entire brain were cut on a vibratome and residues of peroxidase-labeled digoxigenin nucleotide were catalytically added to DNA fragments by terminal deoxynucleotidyltransferase (ApopTag, Oncor Appligene, Heidelberg, Germany). Subsequently, the sections were counterstained with methyl green. Nuclei displaying DNA cleavage had a dark brown appearance and were surrounded by green-colored cytoplasm.

DeOlmos Cupric Silver Staining. To visualize degenerating cells, coronal sections of the whole brain were stained with silver nitrate and cupric nitrate by the method of DeOlmos and Ingram (14). Degenerating cells had a distinct dark appearance due to the silver impregnation.

Methylene Blue/Azure II Staining. To visualize normal cells, serial coronal sections of the entire brain were stained with methylene blue/azure II. Normal cells were identified by the presence of the typical nuclei with clear nucleoplasm and distinct nucleolus surrounded by homogenous cytoplasm.

Quantitation of the Excitotoxic Damage in the Parietal Cortex. Thirty minutes and 2, 4, 6, and 24 hr after traumatic injury the number of degenerating cells undergoing excitotoxic death within the parietal cortex was determined in methylene blue/azure II-stained sections. For this purpose the volume of the damage in the parietal cortex was determined by means of a video enhanced image analysis system (IMAGE 1.54; National Institutes of Health). Subsequently, mean numerical density (N_v) of degenerating cells in parietal cortex was determined by using an unbiased stereological disector technique (15) under blinded conditions. The total number (T_n) of degenerating cells was then determined by multiplying N_v with volume. The degenerating cells undergoing excitotoxic death were identified by the presence of pyknotic nuclei surrounded by massively swollen cytoplasm (edematous degeneration) or by darkening of the cytoplasm and presence of intracytoplasmic vacuoles (vacuolar condensation) (16).

Quantitation of the Distant Damage in the Brain. The distant damage was quantified in TUNEL or DeOlmos cupric silver-stained sections in the frontal, parietal, cingulate, retrosplenial cortex, caudate (mediodorsal), thalamus (laterodorsal, mediodorsal and ventral nucleus), and subiculum by the method of stereological disector, estimating mean N_v of degenerating cells. An unbiased counting frame (0.05 mm $\times$ 0.05 mm; disector height, 0.07 mm) and a high aperture objective were used for the sampling. The N_v for each brain region was determined with 8–10 disectors under blinded conditions. Furthermore, to assess the severity of the distant damage for every 1,000 degenerating cells per mm^3, a score 1 was given (1,000 cells per mm^3 = 1), and the scores from 13 regions ipsilateral and 13 regions contralateral to the trauma were added to give a cumulative severity score for the brain. The 13 regions within which quantitative analysis of degenerating cell densities was performed were layers II and IV of the frontal, parietal, cingulate, and retrosplenial cortices; caudate nucleus; laterodorsal, mediodorsal and ventral thalamic nuclei; and subiculum.

To determine the total number of cells undergoing apoptotic death within the brain 24 hr after trauma, the numbers of degenerating cells within a total of 26 regions per brain (13 regions ipsilateral and 13 regions contralateral to trauma) were individually calculated by means of disector techniques and stereological volumetry and summed to give a total number of cells undergoing apoptotic death 24 hr after trauma.

Drugs and Treatment Regimen. To determine whether *N*-methyl-D-aspartate (NMDA) antagonists mitigate primary damage in the brain induced by head trauma in developing rats, 3-(carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP; Tocris), a competitive NMDA antagonist, and dizocilpine (MK-801; RBI), a noncompetitive NMDA antagonist, were used. CPP was administered i.p. in a dose of 30 mg/kg 30 min before and 20 and 70 min after trauma, and dizocilpine was given i.p. in a dose of 1 mg/kg 30 min before traumatic injury. High-dose and antecedent treatment regimen were selected to ensure that CPP and dizocilpine were reaching the brain in relevant concentrations (17, 18) before the excitotoxic injury was maximally expressed at the survival time of 4 hr. The effect of 2-sulfo-α-phenyl-*N*-*tert*-butylnitrone (SPBN; Aldrich), a free-radical scavenger, on primary damage was studied as well. SPBN was administered i.p. in a dose of 60 mg/kg 1 hr before traumatic injury.

To determine whether NMDA antagonists protect against the distant damage in the brain induced by head trauma in developing rats CPP was administered i.p. to 7-day-old rats in a dose of 15 mg/kg 60, 110, and 160 min and 9 and 17 hr after trauma. Dizocilpine was given i.p. in a dose of 0.5 mg/kg every 4 hr for 24 hr beginning 1 hr after trauma. This treatment regimen was chosen to give relevant concentrations of antagonists in the brain to interact with NMDA receptors (17, 18). The ability of SPBN to prevent the delayed damage when administered i.p. in a dose of 60 mg/kg 1 and 13 hr after trauma was also tested in 7-day-old rats.

To determine whether NMDA antagonists alone might cause neuropathologic changes in the brains of nontraumatized 7-day-old rats, sham-controls received i.p. injections of CPP in a dose of 15 mg/kg 60, 110, and 160 min and 9 and 17 hr or dizocilpine in a dose of 0.5 mg/kg every 4 hr for 24 hr, and the brains were removed at 24 hr after sham-surgery. In addition we tested whether SPBN alone might interfere with physiological apoptosis by using i.p. administration of 60 mg/kg 1 and 13 hr after sham-surgery in 7-day-old rats. The treatment regimen was chosen to ensure that SPBN was present in relevant concentrations in the brain (19).

Statistics. Statistical analysis of the data was performed by means of analysis of variance followed by Student's *t* test.

RESULTS

Excitotoxic Degeneration at the Site of Traumatic Injury. Shortly after head trauma neurons in parietal cortex subjacent to the site of impact began showing changes that by electron microscopic analysis were identical to those induced in developing brain by glutamate (16). Edematous swelling of neuronal dendrites and cell bodies and disruption of intracytoplasmic organelles were the earliest changes. These changes were followed by clumping of nuclear chromatin and nuclear pyknosis. Analysis of the time course of excitotoxic damage in the parietal cortex revealed a gradual progression of its size between 30 min and 4 hr after trauma with a peak at 4 hr (Table 1). Little or no signs of excitotoxic damage were seen in the brain 24 hr after head trauma (Table 1).

Delayed Apoptotic Degeneration at Distant Sites. TUNEL-positive nuclei started to appear in retrosplenial/cingulate cortex and dorsolateral thalamus 6 hr after trauma, and their numbers were increased at 16 and 24 hr. At 24 hr, the density of TUNEL-positive cells at the distant sites was similar to the density

Table 1. Time course of excitotoxic damage in the parietal cortex in rats subjected to traumatic head injury at the age of 7 days and the effect of NMDA antagonists dizocilpine and CPP and the radical scavenger SPBN on the extent of the damage 4 hr after head trauma

Survival time, hr	T_n	% of maximum	Dizocipline, T_n	CPP, T_n	SPBN, T_n
0.5	1,568 ± 215	9.56			
2	8,118 ± 1,023	49.50			
4	16,400 ± 2,435	100	2,329 ± 506* (14.2%)	6,224 ± 942* (37.95%)	15,100 ± 3,971 (92.07%)
6	14,599 ± 2,842	89.02			
24	210 ± 24	1.28			

Total number of degenerating cells (T_n) in parietal cortex was assessed in methylene blue/azure II-stained sections by multiplying mean numerical density (N_V) with the volume of cortex damage. Cells undergoing excitotoxic death were identified by the presence of pyknotic nuclei surrounded by swollen cytoplasm (edematous degeneration) or by darkening of the cytoplasm and presence of intracytoplasmic vacuoles (vacuolar condensation). Shown are means ± SEMs of T_n of degenerating cells in the parietal cortex on the site ipsilateral to head injury in 6–15 rats. Dizocilpine was administered in the dose of 1 mg/kg i.p. 30 min before head trauma, CPP was given i.p. in the dose of 30 mg/kg i.p. 30 min before and 20 and 70 min after trauma, and SPBN was given in the dose of 60 mg/kg i.p. 30 min before trauma. *, $P < 0.001$; Student's t test.

of degenerating cells detected by silver staining (Fig. 1). The degeneration did occur bilaterally and reached its maximal severity 24 hr after trauma (see Fig. 3). At all survival times studied, 6, 16, 24, and 48 hr and 5 days, the distant damage was more severe in the hemisphere ipsilateral to the site of traumatic injury (Table 2 and Fig. 2). In most severely affected areas such as retrosplenial cortex, subiculum, parietal cortex, cingulate cortex, and laterodorsal thalamus the density of degenerating cells 24 hr after head trauma was up to 23%–33% of the total cell density (Table 2). The extent of the damage in the caudate and ventral thalamus reached only 2% of the entire cell population in these regions (Table 2). The density of spontaneously degenerating cells in sham rats was up to 0.74%–1.67% of the total cell density in the frontal, cingulate, parietal, and retrosplenial cortex, suggesting that physiological programmed cell death was ongoing and that the brain regions with highest inherent apoptosis were matching those with the highest sensitivity to apoptotic degeneration after traumatic injury (Table 2). Because degenerating neurons dying by a nonapoptotic process can be TUNEL-positive (20), we examined the degenerating cells in the cingulate cortex and dorsolateral thalamus by electron microscopy to determine whether the mechanism of cell death was apoptotic. The first detectable ultrastructural changes in cells undergoing delayed degeneration consisted of clumping of nuclear chromatin and mild to moderate condensation of the entire cell. Nuclear chromatin became transformed into flocculent densities that joined together to form one or more large electron-dense spherical balls. In early stages the nuclear envelope separated into fragments that floated randomly about the cytoplasm. In the absence of an intact continuous nuclear membrane, the cell became unpartitioned with nucleoplasmic contents freely intermingling with cytoplasmic contents. The large chromatin masses migrated often toward

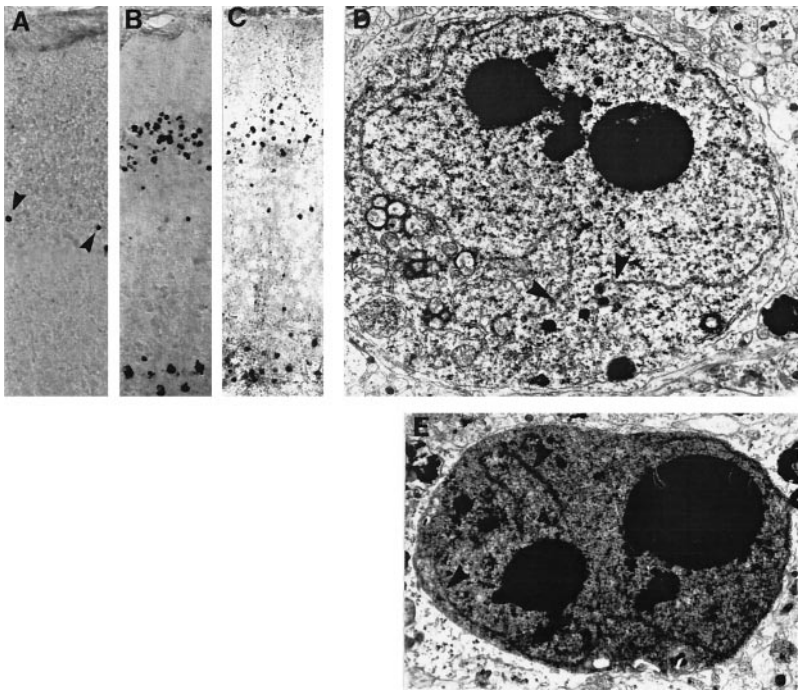

FIG. 1. Apoptotic cell death in the cingulate cortex of an 8-day-old nontraumatized rat (*A*) and a rat subjected to head trauma on postnatal day 7 and sacrificed 24 hr (*B* and *C*) or 16 hr (*D* and *E*) later. In *A* and *B*, the apoptotic cells are detected by TUNEL staining and in *C* apoptotic cells are detected by DeOlmos cupric silver staining. In *D* and *E,* the cell death process is shown by electron microscopy to have the hallmark morphological characteristics of apoptosis. (*A*) In the nontraumatized control brain, an occasional cell is TUNEL-positive (arrowheads), indicating that it is undergoing physiological (programmed) cell death that occurs normally in scattered distribution in the developing brain. (*B* and *C*) A much more robust display of degeneration is detected in the cingulate cortex of traumatized brains, and the pattern of degeneration revealed by TUNEL staining (*B*) is the same as revealed by DeOlmos silver staining (*C*). (*D* and *E*) Electron microscopic evaluation of the cingulate cortex 16 hr after head trauma reveals that the type and sequence of morphological changes meet the classical criteria for apoptosis and are identical to the changes in neurons undergoing physiological cell death in the developing brain (20, 21). The neuron in *D* is showing very early signs of apoptotic cell death, which consist of the formation of electron-dense spherical chromatin masses in the nucleus and a discontinuity in the nuclear membrane (arrowheads), signifying breakdown of the membrane boundary that normally partitions the nucleus from the cytoplasm. In this very early stage, the cell shows only mild condensation, and cytoplasmic organelles appear essentially normal except for some peculiar coated vesicles of undetermined origin. As the apoptotic process evolves (*E*), the nuclear membrane decomposes into fragments (arrowheads) that float randomly about, the contents of the nucleoplasm and cytoplasm freely intermix, and the entire cell becomes uniformly condensed. In later stages, apoptotic bodies containing both cytoplasmic and nucleoplasmic materials are formed and these are extruded into the neuropil (data not shown). Finally, both the main cell mass and the apoptotic bodies are transformed into shrunken amorphous masses of debris and are phagocytized. (Magnification: *A–C*, ×100; *D*, ×10,500; E, ×9,750.)

Table 2. Numerical density (N_V) of cells in frontal, parietal, cingulate, and retrosplenial cortex; mediodorsal, laterodorsal, and ventral thalamus; caudate; and subiculum in rats subjected to traumatic head injury at the age of 7 days

	Sham			Trauma			
Position	Normal cells, N_V normal	Degenerating cells, N_V spontaneous	%	Degenerating cells, N_V ipsilateral	%	Degenerating cells, N_V contralateral	%
Frontal cortex II	0.1897 ± 0.0045	0.0032 ± 0.0003	1.67	0.0198 ± 0.0038*	10.45	0.0101 ± 0.0021*	5.35
Frontal cortex IV	0.1212 ± 0.0103	0.0002 ± 0.00003	0.16	0.0051 ± 0.0010†	4.20	0.0045 ± 0.0010†	3.74
Parietal cortex II	0.2239 ± 0.0134	0.0022 ± 0.0006	0.96	0.0689 ± 0.0081‡	30.77	0.0137 ± 0.0019*	6.12
Parietal cortex IV	0.1360 ± 0.0063	0.0003 ± 0.00008	0.26	0.0852 ± 0.0017†	6.26	0.0009 ± 0.0002*	0.69
Cingulate II	0.1958 ± 0.0113	0.0030 ± 0.0004	1.54	0.0515 ± 0.0088†	26.32	0.0091 ± 0.0017*	4.67
Cingulate IV	0.1281 ± 0.0061	0.0002 ± 0.00003	0.13	0.0098 ± 0.0011‡	7.62	0.0012 ± 0.0003*	0.91
Retrosplenial II	0.2159 ± 0.0139	0.0016 ± 0.0003	0.74	0.0538 ± 0.0080‡	24.93	0.0026 ± 0.0003*	1.21
Retrosplenial IV	0.1230 ± 0.0109	0.0004 ± 0.00007	0.33	0.0415 ± 0.0057‡	33.71	0.0008 ± 0.0001*	0.67
Caudate	0.2225 ± 0.0111	0.0006 ± 0.0001	0.29	0.0048 ± 0.00009†	2.16	0.0029 ± 0.0061*	1.31
Mediodorsal thalamus	0.1683 ± 0.0064	0.0002 ± 0.00004	0.14	0.0015 ± 0.00003‡	0.88	0.0010 ± 0.0002*	0.59
Laterodorsal thalamus	0.1339 ± 0.0131	0.0004 ± 0.00006	0.30	0.0307 ± 0.0072‡	22.95	0.0020 ± 0.0005*	1.53
Ventral thalamus	0.1129 ± 0.0026	0.0001 ± 0.00003	0.11	0.0023 ± 0.0005†	2.04	0.0014 ± 0.0004*	1.22
Subiculum	0.1681 ± 0.0082	0.0004 ± 0.00006	0.22	0.0520 ± 0.0063‡	30.93	0.0033 ± 0.0006†	1.99

Assessment of cell densities were performed 24 hr after head trauma or sham surgery in DeOlmos cupric silver or methylene blue/azure II-stained sections of the whole brain. N_V values of normal and silver-accumulating cells were determined by using the unbiased disector technique and are shown as means ± SEMs × 10^6 cells per mm^3 in 6–16 rats. II and IV stand for cortical layers II and IV.
*, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; Student's t test.

the periphery of the cell and in some cases the cell divided into separate independent bodies consisting of a contingent of cytoplasm and one or more nuclear chromatin balls. This type and sequence of changes are unequivocally apoptotic in that they are identical to those seen in neurons undergoing apoptosis in physiologically developing brain (20) and meet all the criteria set for diagnosing apoptosis (22). It is of interest that 16 hr after trauma, cells in both early and late stages of apoptosis were detected (Fig. 1), which indicates that 16 hr after trauma the process of cell suicide was still progressing.

Extent of Excitotoxic and Apoptotic Cell Death in the Rat Brain After Head Trauma. The evolution of brain damage after head trauma in developing rats is a highly dynamic process. To determine which type of damage, excitotoxic or apoptotic, was more devastating, we chose to compare the numbers of cells dying an excitotoxic or apoptotic cell death at the respective times of their maximal expression. From the time course studies, it was known that excitotoxic degeneration is most prominent at 4 hr after trauma (Table 1). Apoptotic cell death reached its maximum at 24 hr after trauma (Fig. 3.). Estimation of numbers of degenerating cells in the brains of 7-day-old rats subjected to head trauma revealed that at 24 hr after trauma (the time of maximal expression of apoptotic cell death) 2,241,986 ± 336,297 cells (n = 9) were dying an apoptotic death as opposed to 16,400 ± 2,435 cells (n = 12) dying an excitotoxic death at 4 hr after trauma (the point of maximal expression of excitotoxic cell death). This indicates that apoptotic, rather than nonapoptotic, cell death determines neuropathologic outcome after head trauma in the brain of rats at the age of 7 days.

Age Dependency of the Delayed Apoptotic Degeneration. To determine whether susceptibility to apoptotic cell death is dependent upon age, we subjected 3-, 7-, 10-, 14-, and 30-day-old rats to head trauma and evaluated the damage quantitatively 24

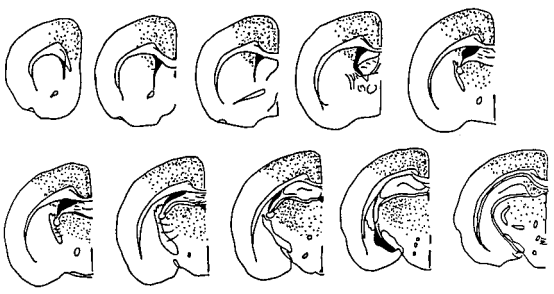

FIG. 2. Distribution pattern of degenerating cells (dots) as shown by DeOlmos cupric silver staining in the brains of developing rats traumatized on postnatal day 7 and sacrificed 24 hr later.

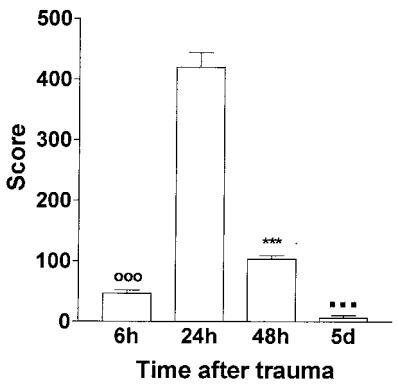

FIG. 3. Time course and severity of delayed disseminated apoptotic death observed in the brains of developing rats traumatized on postnatal day 7 and sacrificed 6, 24, or 48 hr or 5 days later. For quantification purposes, each brain was given a score that was based on determined densities of degenerating cells within 13 regions ipsilateral and 13 regions contralateral to the trauma. Depicted are means ± SEMs in 6–16 animals. Delayed damage was more severe at 24 hr compared with 6 hr (○○○, $P < 0.001$) and 48 hr (***, $P < 0.001$) after trauma. Damage severity continued to decline and was significantly less at 5 days compared with 48 hr after trauma (■■■, $P < 0.001$; Student's t test.).

hr after injury by using the DeOlmos cupric-silver staining. Three- and 7-day-old rats demonstrated the highest vulnerability (Fig. 4). In 10-day- and 14-day-old rats, the severity of distant damage had markedly decreased. At 30 days of age damage was limited to the site of impact and no distant lesions were found (Fig. 4).

Effects of Neuroprotective Drugs on Excitotoxic Degeneration in the Parietal Cortex. Quantitative evaluation 4 hr after trauma of the brains of 7-day-old rats treated with dizocilpine revealed reduction of the extent of excitotoxic damage in the parietal cortex by 86%. CPP reduced excitotoxic damage in the parietal cortex by 62%, whereas SPBN elicited no significant neuroprotective effect (Table 1).

Effects of Neuroprotective Drugs on Apoptotic Degeneration in the Brain. Quantitative evaluation of the brains 24 hr after head trauma revealed that the apoptotic degeneration was more severe in rats treated with dizocilpine than in those subjected to vehicle (Fig. 5). Severity of the apoptotic degeneration in the brains of 7-day-old rats subjected to head trauma reached a mean cumulative score of 382 ± 26 ($n = 26$), whereas that in rats treated with dizocilpine was 780 ± 28 ($n = 13$), demonstrating increase of the damage by 104%. Degeneration in the brains of rats subjected to head trauma and to the treatment with CPP reached a mean cumulative score of 826 ± 37 ($n = 8$) revealing increase in the extent of the damage by 116% (Fig. 5). Highest increases in the extent of apoptotic degeneration was noted in the cingulate cortex ipsilateral (71%) and contralateral (15%), laterodorsal thalamus ipsilateral (56%) and contralateral (6%), frontal cortex ipsilateral (32%) and contralateral (20%), and retrosplenial cortex contralateral (18%) to trauma after treatment with dizocilpine. In the brains of rats subjected to head trauma and treatment with CPP, highest increases were noted in the cingulate cortex contralateral (81%), frontal cortex ipsilateral (69%) and contralateral (44%), subiculum ipsilateral (63%) and contralateral (27%), retrosplenial cortex contralateral (20%), and laterodorsal thalamus contralateral (7%) to the traumatic injury.

NMDA antagonists gave rise to apoptotic degeneration in the brains of 7-day-old sham-operated rats, indicating that they interfered with physiological apoptosis at this age. An apoptotic response to NMDA antagonists was noted in cortical layers II and IV, thalamic nuclei, the caudate, and in the subiculum. Mean cumulative scores for apoptotic damage caused by CPP and dizocilpine in sham-operated rats were 184 ± 6 ($n = 6$) and 236 ± 5 ($n = 6$), respectively. In 7-day-old rats subjected to head trauma and subsequent treatment with NMDA antagonists the severity of apoptotic cell death was higher (by 27% after treatment with dizocilpine and 37% after treatment with CPP) than the expected additive apoptotic response triggered by trauma or the NMDA antagonists alone (Fig. 5).

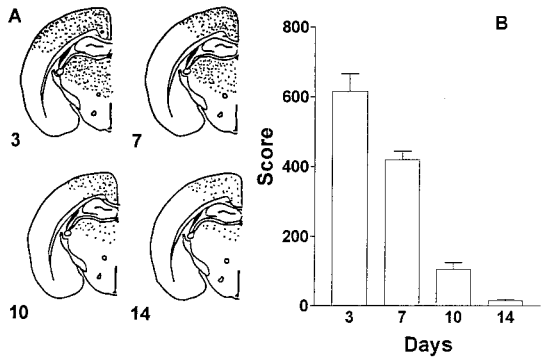

FIG. 4. (*A*) Distribution of degenerating cells (dots) as shown by the DeOlmos cupric silver staining in the brains of developing rats subjected to head trauma on postnatal days 3, 7, 10, or 14. Morphological analysis was performed 24 hr after traumatic injury. (*B*) Severity of delayed posttraumatic cell death in relation to age. Rats were given a score 1 for every 1,000 degenerating cells per mm^3 (1,000 cells per mm^3 = 1) and the scores from 13 regions ipsilateral and 13 regions contralateral to the trauma were added to give a cumulative severity score for the brain. Depicted are the means ± SEMs in 6–16 rats. Three-day-old rats ($n = 11$) were more severely affected than 7-day-old rats ($P < 0.0008$; $n = 16$), 7-day-old rats more severely than 10-day-old rats ($P < 0.0001$; $n = 6$), and 10-day-old more severely than 14-day-old rats ($P < 0.0014$; $n = 6$; Student's t test).

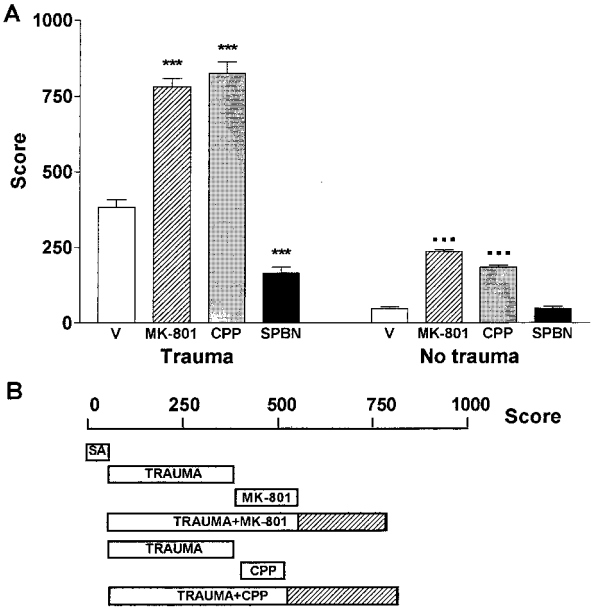

FIG. 5. Potentiation by NMDA antagonists of apoptotic cell death in the brains of rats subjected to head trauma or sham surgery on postnatal day 7 and sacrificed 24 hr later (*A*). Dizocilpine was administered at the dose of 0.5 mg/kg i.p. 60 min after trauma or sham surgery and subsequently every 4 hr. CPP was injected i.p. at the dose of 15 mg/kg at 60 min, 110 min, and 160 min and then at 9 hr and 17 hr after trauma or sham surgery. SPBN was administered i.p. at the dose of 60 mg/kg at 1 and 13 hr after trauma or sham surgery. Nontraumatized rats received the drugs according to the same schedule. Densities of degenerating cells were calculated in 13 brain regions ipsilateral and 13 regions contralateral to trauma and the brains were given a cumulative score that reflects severity of the damage. Dizocilpine ($P < 0.001$) and CPP ($P < 0.001$) potentiated delayed apoptotic damage, whereas SPBN significantly ameliorated apoptotic cell death ($P < 0.001$) compared with vehicle-treated (V) traumatized rats. Surprisingly, both dizocilpine ($P < 0.001$) and CPP ($P < 0.001$) caused a significant amount of apoptosis in the brains of sham-operated 7-day-old rats compared with vehicle-treated (V) sham-operated rats. The severity of apoptotic degeneration caused by head trauma and subsequent treatment with dizocilpine or CPP was higher than the expected additive effect of the apoptotic responses to head trauma or treatment with NMDA antagonists alone. To illustrate this, in *B* the mean scores for apoptotic damage caused by each treatment condition are presented as horizontal bars. SA represents the mean score for spontaneous apoptosis in the brains of vehicle-treated sham rats. Shaded areas represent the amount of damage that reflects potentiation by dizocilpine and CPP of the trauma-triggered component of apoptotic degeneration in the brains of 7-day-old rats. ***, $P < 0.001$ vs. vehicle-treated rats; ■■■, $P < 0.001$ vs. vehicle- treated rats; Student's t test.

In contrast, analysis of cumulative severity scores revealed that SPBN reduced the severity of apoptotic damage in the brain after head trauma in 7-day-old rats by 57% (Fig. 5). Rats subjected to the treatment with SPBN had mean cumulative severity score of 165 ± 20 ($n = 13$) only (Fig. 5). SPBN did not affect physiological apoptosis in the brain of 7-day-old sham-operated rats (Fig. 5).

DISCUSSION

The damage triggered by head trauma in the brain of developing rats has two major components, an acute component at the impact site that by ultrastructural criteria is excitotoxic and nonapoptotic and a delayed component at sites distant to the impact that fulfills ultrastructural criteria for apoptosis. The apoptotic damage that occurs in multiple locations in the brain

affects a larger number of neurons than excitotoxic damage and is likely to make a greater contribution to posttraumatic deficits.

Excitotoxic damage, known to occur in the brain after deleterious insults such as ischemia, trauma, or seizures (status epilepticus), has remained a main focus of research over the past two decades (23, 24). Although apoptosis has been identified as an additional to excitotoxicity mechanism leading to neurodegeneration, our findings show that in the developing rat brain after traumatic injury apoptotic cell death carries higher impact than excitotoxic cell death in determining neuropathologic outcome.

The NMDA antagonists CPP and dizocilpine protect against the excitotoxic but increase the apoptotic component of traumatic brain injury. Because the extent of excitotoxic damage induced by head trauma is minor in comparison to the extent of apoptotic damage, the net effect of NMDA antagonist treatment is a clear-cut worsening of neuropathologic outcome after trauma to the developing brain.

Apoptotic damage triggered by head trauma in the developing brain presumably does not occur as a secondary manifestation causally linked to the primary excitotoxic damage at the impact site because if this were the case, blocking the excitotoxic reaction at the impact site by NMDA antagonists should diminish instead of enhance distant damage. Head trauma may trigger apoptosis either by activating a mechanism that normally promotes programmed cell death or by compromising a mechanism that normally suppresses it. Distant apoptotic damage after head trauma is most severe in areas that show highest densities of spontaneous apoptotic cells in sham rats, such as the cingulate, frontal, parietal, and retrosplenial cortex. This fact suggests that the widespread apoptotic cell death triggered by head trauma in the infant rat brain may be due in part to interference with the programmed cell death process that occurs during a critical developmental period. Regardless which mechanism might explain how trauma elicits apoptotic damage, the fact that this damage is augmented by treatment with NMDA antagonists raises the possibility that glutamate acting via NMDA receptors suppresses programmed cell death in the developing brain. NMDA antagonists may therefore compromise this mechanism, thereby making neurons overly prone to show an apoptotic response to trauma.

Our studies demonstrate that vulnerability to apoptotic cell death in the rat brain is highest in the first two postnatal weeks and decreases thereafter. Although it is uncertain how the rodent and human compare regarding the time window of vulnerability to posttraumatic brain damage, it is possible that humans may be at risk for this type of damage during early childhood, and this signifies that it may be detrimental to use NMDA antagonists in the therapeutic management of pediatric head trauma. Furthermore, such conclusions call into question the practice of administering NMDA antagonists such as ketamine during the stabilization period immediately after head trauma in young children (25, 26). On the other hand, the fact that the delayed pattern of brain damage continued to evolve over 24 hr after head trauma in developing rats suggests that there may be a wide time-window for therapeutic intervention.

The present findings suggest that a free-radical scavenger, SPBN, may have therapeutic value in mitigating delayed apoptotic degeneration triggered by head trauma. These observations are in line with reports on protective action and wide therapeutic time window of SPBN in adult head trauma and ischemia models (27). Treatment with SPBN is beneficial only because apoptosis represents the predominant form of neurodegeneration after head trauma in developing rats. SPBN did not protect against excitotoxic damage, suggesting that it would be useless in a neurodegeneration with a predominant excitotoxic component. Our results call for the necessity to better understand and define the relative contributions of excitotoxicity and apoptosis to the pathogenesis of neurodegenerative syndromes before making a decision on the mode of treatment. Nonetheless, determining relative contributions of these two mechanisms of cell death is of crucial importance also because certain antiexcitotoxic regimen, when used in syndromes with predominant apoptotic component, may elicit unexpected deleterious side effects.

If such conclusions apply to human brain, then they call for careful selection of neuroprotective measures depending on the cell death type predominating in a specific neurodegenerative disorder.

This work was supported by Grant 01KO95151TPA3 from the Bundesministerium für Bildung und Forschung (BMBF).

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