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Prevention of Trauma-Induced Neurodegeneration in Infant and Adult Rat Brain: Glutamate Antagonists

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The mechanisms of neuronal degeneration following traumatic head injury are not well understood and no adequate treatment is currently available for the prevention of traumatic brain damage in humans. Seven day old rat pups were subjected to mechanical percussion of the head. Cortical damage in infant rats was reduced by pre-treatment with the N-methyl-D-aspartate (NMDA) antagonists dizocilpine (MK-801) or 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP). The AMPA antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) did not significantly suppress cortical damage in infant rats. In adult rats, traumatic head injury leads to primary (at impact-cortex) and secondary (distant- hippocampus) damage to the brain. Morphometric analysis demonstrated that both cortical and hippocampal damage was mitigated by pre-treatment with either the NMDA antagonist CPP or the non-NMDA antagonist NBQX. Neither treatment prevented primary damage in the cortex when therapy was started after trauma. Delayed treatment of rats with NBQX, but not with CPP, beginning between 1 and 7 h after trauma prevented the hippocampal damage. No protection was seen when therapy with NBQX was started 10 h after trauma. These data indicate that NMDA antagonists may possess better neuroprotective properties against excitotoxic processes triggered by traumatic brain injury in young individuals whereas AMPA antagonists may be more beneficial in adults.

Keywords: Traumatic brain injury; glutamate antagonists; AMPA antagonist; NMDA antagonists

INTRODUCTION

Traumatic brain and spinal cord injury are major causes of morbidity in modern society for which there are no specific treatments. The medical management of traumatic brain and spinal cord injury is mainly symptomatic and consists of control and support of respiratory

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and cardiovascular systems, control of intracranial pressure, hemodynamic stabilization, and prophylactic anticonvulsant treatment (Kaufman and Dacey, 1994). In many instances, the outlook for survivors is poor, ranging from deterioration of the brain and life-long disability, to epilepsy and persistent vegetative state.

Clinical experience and experimental observations in animals suggest that traumatic brain and spinal cord damage are not final at the time of injury. Brain damage resulting from severe head injury can be classified into primary damage, which occurs at impact and appears immediately or shortly after injury, and secondary damage, which occurs distant to the impact and may not appear until several hours post-injury (Adams, 1992). It appears therefore that CNS trauma triggers a cascade of events which cause primarily unaffected neurons to degenerate at a later time-point.

One of the most promising mechanisms involved in the propagation of traumatic injury in the CNS is an excitotoxic process involving excitatory amino acids such as glutamate. Glutamate is a neurotransmitter in the CNS but has the potential to destroy neurons. In hypoxia/ischemia and CNS trauma glutamate is released more rapidly than it is taken up and accumulates in the extracellular space (Benveniste *et al.*, 1984; Bondoli *et al.*, 1981; Drejer *et al.*, 1985; Faden *et al.*, 1989; Katayama *et al.*, 1990; Nilsson *et al.*, 1990). This accumulation can lead to necrosis of neighbouring neurons and through the resulting edema to increases of intracranial pressure, a well known complication of CNS trauma.

Excitotoxicity is mediated by two general classes of excitatory amino acid receptors, N-methyl-D-aspartate (NMDA) and non-NMDA, the latter being differentially sensitive to kainic acid and/or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). NMDA receptors appear to be primarily responsible for ischemia-induced excitotoxic neuronal injury in the developing brain (Ikonomidou *et al.*, 1989; McDonald *et al.*, 1987; Olney *et al.*, 1989) whereas both NMDA and non-NMDA receptors are likely to contribute to ischemic neurodegeneration in the adult CNS (Mosinger *et al.*, 1991; Sheardown *et al.*, 1990). Most of the research implicating excitotoxic mechanisms in trauma-induced neurodegeneration has focused on the adult CNS (Faden *et al.*, 1989; McIntosh *et al.*, 1990; Panter and Faden, 1992; Toulmond *et al.*, 1993), has examined the mechanisms which underlie primary damage after traumatic head or spinal cord injury (Povlishok, 1993), and how to rescue injured neurons at impact using NMDA antagonists as potential neuroprotectants (Faden *et al.*, 1989; McIntosh *et al.*, 1990; Panter and Faden, 1992; Toulmond *et al.*, 1993). A modest improvement in neurological outcome has been reported but it is likely that the studies have not fully revealed the therapeutic potential of neuroprotective drugs, since traumatic neurodegeneration in the adult CNS may be mediated by different classes of glutamate receptors. For this reason we developed a model of contusion injury to the brain of infant rats, studied the histopathological response to mechanical trauma and evaluated the ability of NMDA and a non-NMDA antagonist to protect the immature brain against this type of brain injury.

Similarly, little is known about the mechanisms that cause traumatic damage in the adult CNS. Primary damage occurs immediately after head or spinal cord injury (Povlishock, 1993). Secondary traumatic damage appears several days after the impact and may be of great clinical relevance (Wrathall *et al.*, 1992), since it may allow greater time

window for therapeutic interventions (Gennarelli, 1984). For these reasons we have investigated the evolution of primary and secondary damage after acute traumatic head injury in adult rats using the contusion device described by Feeney *et al.* (1981). Using this experimental approach, we describe quantitatively cortical and hippocampal damage secondary to traumatic head injury in rats and demonstrate involvement of excitatory amino acid-mediated processes in the evolution of such damage.

MATERIALS and METHODS

Head trauma in young rats

The contusing device consisted of a hollow stainless-steel cylinder 40 cm in length, 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 footplate (2 mm diameter) resting upon the surface of the skull. The center of the footplate was positioned 3 mm anterior and 2 mm lateral to lambda and depressed the skull by 1.5 mm. The weight was allowed to fall onto the footplate from a height of 16.5 cm. Seven day old Sprague-Dawley rats were anesthetized with halothane and placed in a mold that fit the contours of the skull. The contusion impact was delivered unilaterally to the right side of the skull. Animals were allowed to survive 0, 1/2, 1, 2, 4 or 6 hrs after the trauma, then were deeply anesthetized with an overdose of pentobarbital and perfused through the heart and the ascending aorta with a solution of paraformaldehyde (1%) and glutaraldehyde (1.5%) in pyrophosphate buffer. After perfusion for 15 min the brains were removed and processed for light and electron microscopy (Olney, 1971).

The NMDA antagonists dizocilpine maleate (MK-801) and 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonate (CPP), and the non-NMDA antagonist 2.3-dihydro-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) were tested for their ability to protect against trauma-induced brain damage. MK-801 was administered at the dose of 1 mg/kg or 5 mg/kg i.p. either 30 min before or 1 hr after the trauma. CPP was administered i.p. in three separate 30 mg/kg injections at 50 min intervals, one 40 min before and the others at 10 and 60 min after the trauma. NBQX was administered i.p. in three separate 30 mg/kg injections, given 30 min before and 15 and 75 min after the trauma. Control pups for these experiments were subjected to contusion injury but received no neuroprotective drug. Both experimental and control pups were sacrificed and perfused with fixative 4 h after the trauma.

Transverse sections 1 μm thick were cut at 10 μm intervals through the forebrain and the damaged areas were evaluated in their entire anteroposterior extent. Counts of necrotic neurons (NN) were performed in seven coronal sections obtained at 10 μm intervals through the area of maximal damage. The section that displayed the maximal damage (highest NN count) and three consecutive sections anterior plus three consecutive sections posterior to that section were selected. The sum of NN counts obtained from all selected sections in each brain were taken as a measure of the primary lesion size. Cellular profiles counted included neurons exhibiting vacuous swollen perikarya and chromatin clumping in the nucleus (bull's eye profiles) and vacuolated dark neurons. Comparisons between

experimental and control groups were performed by means of Student's t-test on logarithmically transformed NN scores: log (x+1.5). Comparison between more than two groups was performed by means of one way analysis of variance of logarithmically transformed NN scores.

Head trauma in adult rats

The contusing device for adult rat brain injury consisted of a stainless-steel tube 40 cm in length, perforated at 1 cm intervals to prevent air compression in the tube (Feeney *et al.*, 1981). The device was kept perpendicular to the surface of the skull and guided a falling weight onto the footplate resting upon the surface of the dura. A 4.5 mm diameter of the footplate and a 2.5 mm depression of the brain surface were selected to avoid mechanical puncture of the dura. A force of 380 g/cm produced by a 20 g weight was selected to produce brain contusion. Male Fisher 344 rats (Charles River), 200-250 g in weight, were anesthetized with tribromoethanol (Aldrich, 260 mg/kg i.p.). A craniotomy was performed over the left hemisphere and the center of the footplate was stereotaxically positioned 1.5 mm posterior and 2.5 mm lateral to the bregma. Sham controls had identical anesthesia and surgery without the impact.

For morphological examination of the brains by light microscopy, rats were anesthetized with an overdose of pentobarbital (Ceva) and perfused with saline followed by fixative containing 10% formaldehyde, 10% glacial acetic acid, and 80% methanol three days after injury. Brains were allowed to fix *in situ* for 24 h. and were then removed and processed for paraffin embedding. Serial coronal sections of whole brain were cut at 10 μm, and every 10th section was mounted on a glass slide and stained with cresyl violet. For morphological analysis of cortical damage, brain sections were visually inspected at 27 different rostro-caudal levels extending from -0.26 to -6.80 mm according to Paxinos and Watson (1986). The damage in the hippocampus was quantified stereologically at 12 different rostro-caudal levels extending from -2.12 to -6.04 mm (Paxinos and Watson, 1986) throughout its mediolateral axis using a Leitz Aristoplan microscope equipped with Leitz counting grid type 513740 at a magnification x120. Normal neurons were identified by the presence of the typical nuclei with clear nucleoplasm and distinct nucleolus, surrounded by cytoplasm containing Nissl substance. For every hippocampal section, maps of neuronal distribution were constructed and the differences in normal neuron counts between non-injured and injured sides ($D_{R/L}$) were taken for statistical analysis. To quantitatively assess neuronal loss in the hippocampus, an unbiased stereological disector technique (Gundersen *et al.*, 1988) was used to estimate the mean numerical density (N_V) of pyramidal neurons at the rostro-caudal level -2.80 mm (Paxinos and Watson, 1986). An unbiased counting frame (0.05 x 0.05 mm; disector height 0.01 mm) and a high-aperture (100x) oil-immersion objective were used for the sampling. The N_V for each hippocampal subfield was determined with approximately 5-9 disectors. The distal border of the subfield CA1 with the subiculum was considered as a point at which a clearly defined layer of at least 2 pyramidal cells was no longer evident. The proximal borderline between subfields CA1 and CA2 was indicated by the sudden increase in cell size and change in morphology in the latter region. The border between CA2 and CA3 was considered at the point where looser arrangement of

large pyramidal cells goes into densely packed pyramidal cells of the subfield CA3. CA4 neurons were considered as those lying between the superior and inferior dentate granule cell layers including portions of the CA3c pyramidal neurons. An arbitrary line connecting the lateral ends of the dentate granule cell layers was considered a junction between subfields CA3 and CA4.

The treatment regimen with the non-NMDA antagonist NBQX (Novo-Nordisk - 3 x 3 mg/kg and 3 x 30 mg/kg i.p. every hour) was chosen to give a relevant concentration in the brain to interact with AMPA/kainate receptors (Sheardown *et al.*, 1990). Such treatment with NBQX was initiated 2 h before and 1, 4, 7, or 10 h after traumatic injury to the cortex. The NMDA antagonist CPP (Tocris - 3 x 3 mg/kg and 3 x 30 mg/kg i.p. every hour) was given following a similar regimen to that of NBQX to ensure that the antagonist was present in relevant concentrations in the brain to interact with NMDA receptors (Lehmann *et al.*, 1987; Turski *et al.*, 1988). Treatment with CPP was started 2 h before and 1, or 4 h after traumatic cortex injury. NBQX, CPP, or vehicle were administered i.p. in a volume of 0.5 ml/100 g of body weight. For intravenous treatment, NBQX or CPP in the dose of 1 or 10 mg/kg/h, or vehicle were continuously infused via a catheter placed in the femoral vein over 4 hours at 0.4 ml/min by means of Harvard pumps. Intravenous treatment with NBQX or CPP was initiated 1 h after traumatic injury to the cortex.

The experimental data were analysed statistically by means of multivariate analysis of variance or Student's t-test.

RESULTS

Brain trauma in infant rats

Histopathological features. The trauma applied to the infant skull resulted in perforation of the parietal bone, tearing of the dura and indentation of the parietal cortex. Immediately after trauma the indentation was the only finding evident by light microscopy. Necrotic neurons became evident at 30 min following the impact. At 2 h a zone of necrotic neurons that progressively expanded over a pediod of 4-6 h was surrounding the area of impact (Figure 1). The neuropil assumed a spongiform appearance, caused by swelling of dendritic processes. Neurons undergoing edematous degeneration characterized by swelling of the cytoplasm and clumping of the nuclear chromatin were seen at the area of damage (bull's eye profiles). Large pyramidal neurons developed condensation of the nucleus and vacuolization of the cytoplasm. This type of degeneration, also called vacuolar condensation, has been associated with glutamate-induced cytopathology in infant rodents and primates (Olney *et al.*, 1972), cerebral ischemia and status epilepticus (Olney *et al.*, 1986).

Evolution of brain trauma in infant rats. To study the expansion of traumatic damage over time, rat pups were sacrificed at intervals from 0 to 6 h after the impact. The number of necrotic neurons (NN) increased steadily over time indicating continuing progression of damage up to 4 h after impact. Analysis of variance on logarithmically transformed NN scores revealed that the distribution of scores varied significantly ($F_{INF}(1,49)=6.307$;

$p<0.005$). Comparison of scores between pairs of groups revealed that there was significantly more damage at 2 h ($p<0.05$), 4 h and 6 h than at 1/2 h ($p<0.005$), 4 h and 6 h than at 1 h ($p<0.01$; $p<0.005$) and 6 h than at 2 h ($p<0.025$).

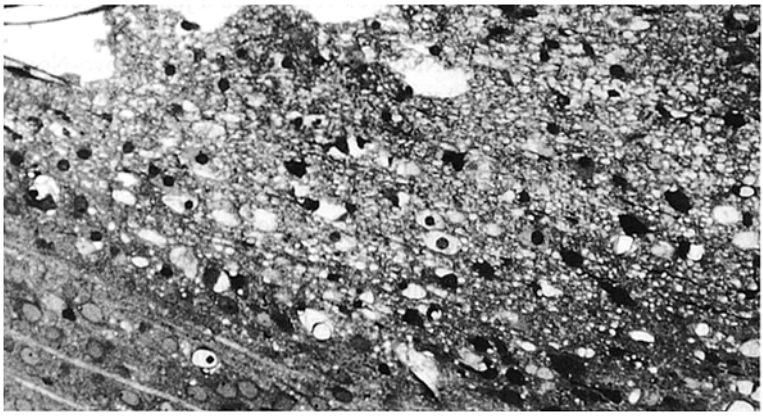

Figure 1. Light micrograph depicting traumatic lesion in the parietal cortex of a 7 day old rat 4 h after the mechanical impact. A zone of acutely degenerating neurons surrounds the area of impact. Mag. x100.

Glutamate antagonists. The NMDA antagonist MK-801, administered i.p. 30 min prior to the traumatic impact at a dose of 1 mg/kg, significantly mitigated traumatic lesions (Table 1).

Table 1. Effects of MK-801, CPP and NBQX on the size of traumatic damage in the frontoparietal cortex of infant rats

	0.5 h pre	1 h after
Vehicle	2.17±0.11	2.41±0.08
MK-801 (ip), 1 mg/kg	1.38±0.2**	1.86±0.15**
Vehicle	2.60±0.08**	2.06±0.15**
MK-801 (ip), 5 mg/kg	-	-
Vehicle	2.77±0.05	-
CPP (ip), 3 x 30 mg/kg	2.1 ±0.13***	-
Vehicle	2.64±0.1	-
NBQX (ip), 3 x 30 mg/kg	2.69±0.09	-

Shown are the means of logarithmically transformed NN-scores ± SEM (numbers of necrotic neurons in the fronto-parietal cortex counted in 7 consecutive sections - log (NN+ 1.5)) in 8-18 animals. Statistical significances were calculated by means of Student's t-test. **$p<0.01$; ***$p<0.001$.

Significant protection with MK-801 was also achieved when the compound was administered 1 h after the impact (Table 1). The higher dose of MK-801 (5 mg/kg) also offered protection but was not more effective than the dose of 1 mg/kg. The competitive NMDA antagonist CPP also ameliorated traumatic damage in pups on preceeding treatment (Table 1). Treatment with the non-NMDA receptor antagonist NBQX, 30 min before and 15 and 75 min after the traumatic impact did not significantly reduce the lesion size compared to controls (Table 1).

Brain trauma in adult rats

Histopathological features:

Cortex. The brains from animals subjected to traumatic injury had no signs of external damage to the cortex, no hemorrhages in the periventricular white matter below the cortex, and no apparent distortion of the hippocampi. Portions of cortex located immediately below the impact site had pronounced cell loss. Treatment with NBQX 3 x 30 mg/kg i.p. initiated 2 h before the impact reduced cortical damage. Similarly, treatment with CPP 3 x 30 mg/kg i.p. initiated 2 h before the impact mitigated the cortical damage as well (Figure 2). When treatment with either NBQX or CPP was delayed, neither drug prevented neuronal degeneration at the site of impact. Intravenous infusions with NBQX 1 and 10 mg/kg/h, and CPP 1 and 10 mg/kg/h 1-5 h after trauma were also ineffective in reducing cortical damage.

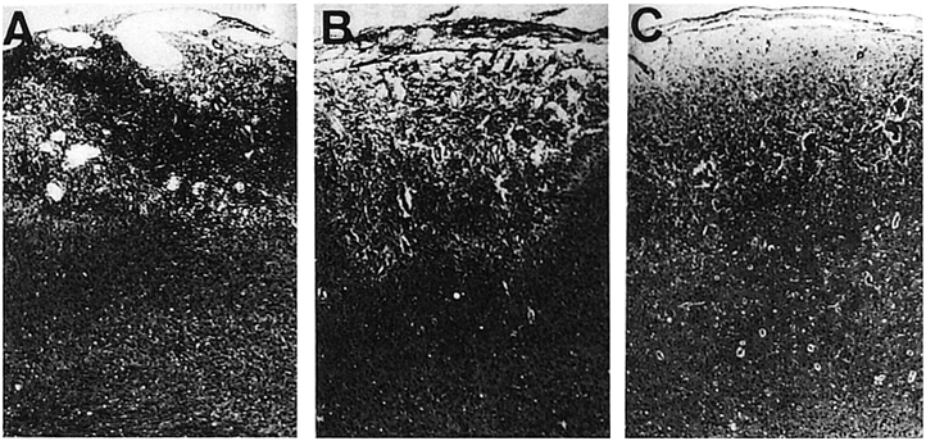

Figure 2. Neuropathological sequelae of mechanical percussion of the rat cortex and protective action of glutamate antagonists NBQX and CPP against neuronal degeneration. A, Parietal cortex with grave breakdown of the cell structure and desintegration of the neuropil 72 h after injury in a rat subjected to i.p. administration of vehicle 2, 1 and 0 h before trauma. B, Destruction of the cortex in a rat subjected to three i.p. injections of NBQX, 30 mg/kg 2, 1 and 0 h before traumatic injury. The extent of the damage was reduced. No or few signs of neuronal degeneration were seen in deep cortical layers. C, Protective action of CPP in a rat subjected to three i.p. injections of NBQX 30 mg/kg 2, 1 and 0 h before trauma. The damage was restricted to external layers of the cortex. Survival time: 72 h. Cresyl violet stain. Mag. x 10.

Hippocampus. Traumatic injury to the cortex led to neuronal damage in the hippocampal CA3 subfield on the side ipsilateral to the impact (Figure 3). Contralateral hippocampus remained unaffected. Neuronal cell loss was restricted to subfield CA3 as revealed by normal neuron counts (Figure 3) and estimation of N_V (Table 2). No changes in the counts and N_V of pyramidal cells, or in morphology were detected in hippocampal subfields CA1, CA2 and CA4.

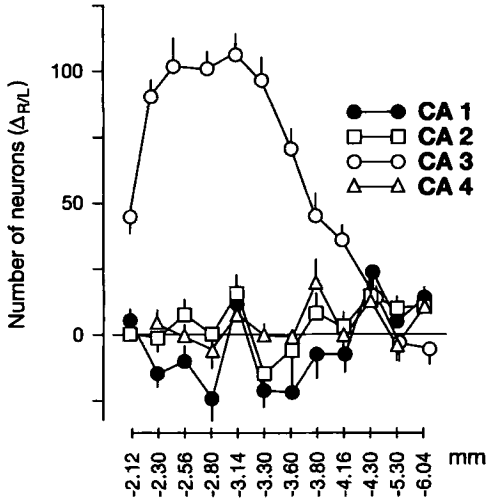

Figure 3. Hippocampal pathology following head trauma in rats. Shown are mean ±SEM difference ($D_{R/L}$) between the counts of normal neurons on non-injured (R) and injured (L) side in hippocampal subfields CA1 - CA4 (n=14) at AP levels -2.12 through -6.04 mm (Paxinos and Watson, 1986). Normal neuron counts varied from 158±10 (AP -2.12) to 1370±28 (AP -6.04) in CA1, from 38±3 to 141±10 in CA2, from 138±6 to 805±32 in CA3, and from 60±2 to 151±17 in CA4. ANOVA on the counts of normal neurons revealed significant main effect ($F_{CA3}(1,11)=5.01$, $p<0.0001$) indicating that traumatic injury to the cortex resulted in secondary cell loss in the subfield CA3, but not in subfields CA1, CA2 and CA4. On the side contralateral to the cortex injury, numerical density (N_V) of pyramidal cells in the hippocampus at AP -2.80 mm reached 0.396 x $10^6/mm^3$ for CA1, 0.293 x $10^6/mm^3$ for CA2, 0.183 x $10^6/mm^3$ for CA3, and 0.159 x $10^6/mm^3$ for CA4, and was significantly different from N_V in CA3 on the impact side only (N_V=0.084 x $10^6/mm^3$; $p<0.001$, Student t-test).

Table 2. Effect of antecedent and delayed treatment with NBQX and CPP on numerical density (N_v) of pyramidal cells in the hippocampal subfield CA3 at AP -2.80 in rats subjected to cortical trauma.

Ipsilateral x $10^6/mm^3$	2/1/0 h pre	1/2/3 h after	1-5 h after
Vehicle	0.087±0.005	0.084±0.003	0.081±0.011
NBQX (ip)			
3 x 3 mg/kg	0.131±0.007***	0.127±0.003***	
3 x 30 mg//kg	0.181±0.009***	0.173±0.003***	
CPP (ip)			
3 x 3 mg/kg	0.145±0.004***	0.079±0.005	
3 x 30 mg/kg	0.177±0.005***	0.088±0.009	
NBQX (iv)			
1 mg/kg/h			0.093±0.005
10 mg/kg/h			0.167±0.007***
CPP (iv)			
1 mg/kg/h			0.087±0.007
10 mg/kg/h			0.085±0.008

Shown are means ± SEM x $10^6/mm^3$ of N_v of pyramidal cells in the hippocampal subfield CA3 on the site ipsilateral to cortex injury in 4-14 rats. On contralateral site N_v values varied from 0.183 to 0.189 x $10^6/mm^3$ and did not significantly differ from each other. Student's t-test, ***$p<0.001$.

Table 3. Time-window for protection offered by NBQX and CPP against neuronal degeneration in the hippocampal subfield CA3 after cortical trauma in rats as revealed by analysis of numerical density (Nv) at AP level-2.80. NBQX was administered i.p. 1, 2 and 3 h, 4, 5, and 6 h, 7, 8 and 9 h, and 10, 11 and 12 h after cortical impact. CPP was administered i.p. 1, 2 and 3 h, and 4, 5 and 6 h after cortical injury.

	NBQX 3 x 30 mg/kg (ip)	CPP 3 x 30 mg/kg (ip)
Vehicle	0.083±0.001	0.085±0.002
1/2/3 h after	0.173±0.003***	0.088±0.009
4/5/6 h after	0.151±0.005***	0.087±0.007
7/8/9 h after	0.127±0.013***	n.t.
10/11/12 h after	0.085±0.005	n.t.

Shown are the means ± SEM x $10^6/mm^3$ of N_v of pyramidal cells in the hippocampal subfield CA3 on the side ipsilateral to cortex injury in 6-27 rats. On contralateral side N_v values varied from 0.183 to 0.185 x $10^6/mm^3$ and did not significantly differ from each other. Student's t-test; ***$p<0.001$.

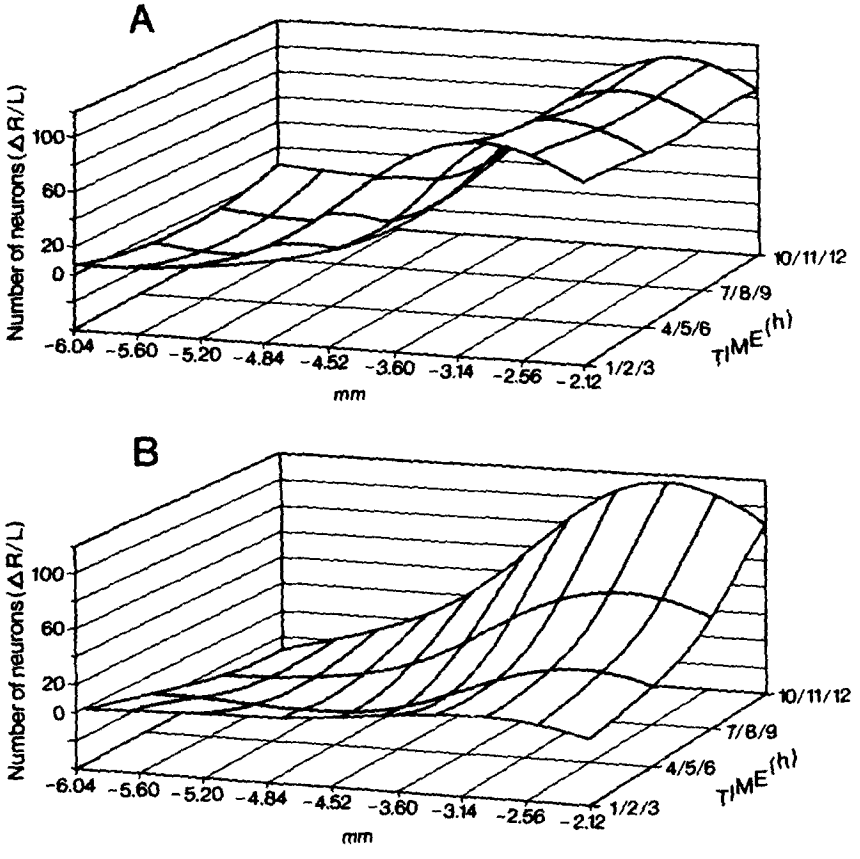

Figure 4. Therapeutic time-window (three-dimensional) against neuronal degeneration in the hippocampal subfield CA3 after cortical trauma in rats offered by NBQX (B) and vehicle (A). NBQX, 30 mg/kg, or vehicle were administered i.p. 1, 2 and 3 h, 4, 5 and 6 h, 7, 8 and 9 h, and 10, 11 and 12 h after cortical impact. Shown are mean ± SEM difference ($D_{R/L}$) between the counts of normal neurons on non-injured (R) and injured (L) side at AP levels -2.12 through -6.04 mm (Paxinos and Watson, 1986). ANOVA on differences between counts of normal neurons in the subfield CA3 for different treatment regimens with NBQX showed that the main effect of treatment regimen was significant ($F_{REG}(36,114)=2.80$, $p<0.0001$) with multiple comparisons, revealing that NBQX prevented cell loss in the subfield CA3 when treatment was initiated 1, 4 or 7 but not 10 h after impact.

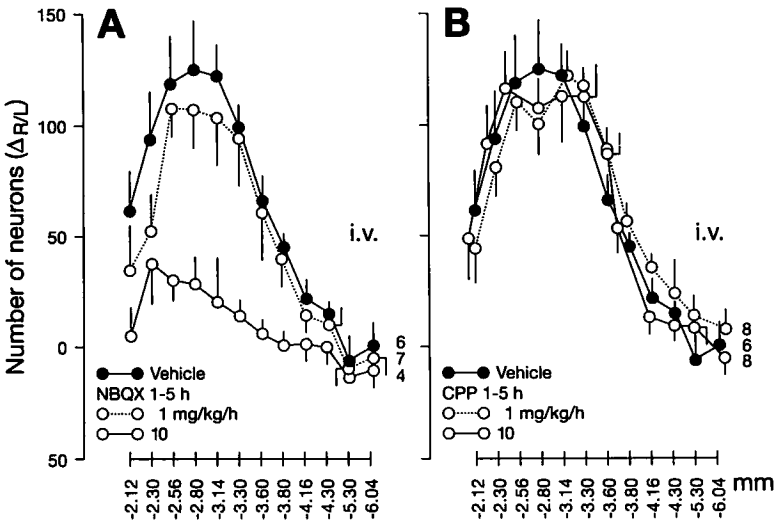

Figure 5. Dose relationship of protective action of NBQX and CPP against neuronal degeneration in the hippocampal subfield CA3 induced by traumatic injury to the cortex in rats. NBQX, CPP or vehicle were administered i.v. by means of continuous infusion between 1 and 5 h after cortical impact. Circles in A and B represent mean ± SEM difference ($D_{R/L}$) between the counts of normal neurons on non-injured (R) and injured (L) site at AP levels -2.12 through -6.04 mm (Paxinoc and Watson, 1986). ANOVA on differences between counts of normal neurons in the subfield CA3 showed that the main effect of treatment was significant ($F_{iv}(18,10)=2.82$, $p<0.001$) with multiple comparisons, revealing that NBQX, 10 mg/kg/h, prevented cell loss in the subfield CA3 while vehicle and NBQX, 1 mg/kg, provided no protection. CPP did not offer protection against cell loss in the hippocampal CA3 subfield.

Glutamate antagonists. NBQX. Table 2 shows that the AMPA antagonist NBQX conferred substantial protection against damage to the hippocampal subfield CA3 induced by traumatic injury to the cortex. Pre-treatment with 3 x 30 mg/kg NBQX administered i.p. 2/1/0 h before impact offered almost total protection against damage to the hippocampus, while 3 x 3 mg/kg was less active (Table 2). Surprisingly, delayed treatment with NBQX was also protective (Table 2). The protective effect of NBQX was dose-dependent. Protection observed after treatment with 3 x 30 mg/kg administered i.p. 1/2/3 h after impact was more pronounced than that found after 3 x 3 mg/kg (Table 2). Similarly, protection was seen after treatment with 3 x 30 mg/kg administered i.p. 4/5/6 h after impact (Table 3). The protection time-window for NBQX extended up to 7 h, since the treatment with 3 x 30 mg/kg administered i.p. 7/8/9 h after impact still protected against damage to the hippocampus (Table 3; Figure 4). No protection against damage to the hippocampal subfield CA3 was detected when treatment with NBQX was started 10 h after the impact (Table 3). Continuous i.v. infusions of NBQX were protective with 10 mg/kg/h but not with 1 mg/kg/h (Table 2; Figure 5).

CPP. Treatment with 3 x 30 mg/kg CPP applied i.p. 2/1/0 h before impact offered pronounced protection, while 3 x 3 mg/kg was less effective against the damage to the hippocampal subfield CA3 (Table 2). Treatment with 3 x 30 mg/kg CPP administered i.p. 1/2/3 h or 4/5/6 h (Table 3) after impact conferred no protection against damage to the hippocampus. No protection against damage to the hippocampus was seen after i.v. administration of CPP in doses of 1 and 10 mg/kg/h infused between 1 and 5 h after traumatic impact (Table 2).

DISCUSSION

Primary traumatic brain damage in infant rats

In the infant rat brain, trauma, like hypoxia/ischemia, causes cytopathological reaction that resembles that caused by subcutaneous administration of glutamate (Olney, 1971; Olney *et al.*, 1972). Neurons subjacent to the impact site began within 30 min to display signs consistent with glutamate-induced degeneration. The degenerative reaction expanded progressively over 4 h. Treatment with NMDA receptor antagonists MK-801 and CPP was neuroprotective against traumatic brain injury in infant rats whereas the AMPA/KA receptor antagonist NBQX was ineffective. These results can be interpreted in the context of evidence suggesting that NMDA receptors in the immature rat brain undergo a period of hypersensitivity between the 6th and 10th postnatal days during which time neurons are hypervulnerable to the excitotoxic action of NMDA and to hypoxia/ischemia (Ikonomidou *et al.*, 1989; McDonald *et al.*, 1988). In contrast, during this developmental period kainic acid is relatively non-toxic when injected directly into the brain. Age-dependent differences in the subunit composition of the NMDA receptors probably account for the different sensitivities to agonists (Monyer *et al.*,1994).

Primary and secondary traumatic brain damage in adult rats

Traumatic percussion of the cortex in adult rats led to primary damage at impact and to consistent secondary damage in the hippocampus. Morphometric analysis has shown that the most rostral and medial aspects of the subfield CA3 in the dorsal hippocampus were severely affected, whereas caudal aspects remained intact. The damage to the hippocampus was seen on the side ipsilateral to the impact. Cortical damage extended through the dorsomedial aspects of the frontoparietal cortex overlying the caudate nucleus.

Blockade of NMDA-mediated excitation by the antagonist CPP before traumatic injury reduced cortical damage and prevented degeneration of pyramidal neurons in the hippocampus. The protection of the hippocampal pyramidal neurons occurred although it is well recognized that the subfield CA3 possesses relatively less NMDA versus AMPA/KA receptors (Monaghan and Beaton, 1991) suggesting sufficiently high sensitivity of the region to NMDA-mediated excitation and high protective potential of the NMDA antagonist CPP against secondary damage. On delayed treatment (1 h after the impact), the NMDA antagonist CPP had no protective action either in the cortex or in the hippocampus. Although the NMDA antagonists were previously reported to protect brain tissue in diverse models of primary tissue damage after head or spinal cord injury in rodents on antecedent

and shortly delayed (up to 15 min) treatment (Faden *et al.*, 1989; McIntosh *et al.*, 1990), no study addressed systematically the question of the duration of neuroprotective action of NMDA antagonists on delayed treatment in trauma models both against primary and secondary damage (Faden and Salzman, 1992; Ikonomidou and Turski, 1995). The present study based on a comprehensive analysis of the primary and secondary damage to the cortex and hippocampus after head trauma suggests that protective action of NMDA antagonists such as CPP may be short, not extending beyond 1 h after the injury.

Blockade of AMPA/KA-mediated excitation by the non-NMDA antagonist NBQX before or between 1 and 7 h after traumatic injury prevented degeneration of pyramidal neurons in the hippocampal subfield CA3. NBQX was also protective in the cortex when administered before but not after trauma. These results suggest that in adult rats, activation of both NMDA- and non-NMDA-dependent mechanisms is involved in the evolution of primary damage to the cortex, whereas non-NMDA mechanisms are essential for the evolution of secondary damage to the hippocampal CA3 subfield after trauma.

Furthermore, this study documents a narrow therapeutic time-window for glutamate antagonists CPP and NBQX in the prevention of primary cortical damage after trauma, and shows a broad therapeutic time-window for the non-NMDA antagonist NBQX but not the NMDA antagonist CPP in the prevention of secondary damage in the hippocampus. An important aspect is that on delayed treatment, no protection was detected in the cortex at the impact side under therapy with NBQX, even though this treatment was sufficient to protect secondary damage in the hippocampus. In contrast, on antecedent treatment, NBQX reduced the damage in the cortex and in the hippocampus.

Excitotoxic mechanisms in traumatic brain injury

The mechanisms by which physical trauma triggers release and extracellular accumulation of endogenous excitotoxins are not clear. One possible explanation is that damaged neurons and glia leak glutamate and aspartate into the extracellular compartment that can depolarize and damage neighbouring neurons. Depolarization would, in addition, impair glutamate uptake systems and contribute to persistence of excitotoxic glutamate concentrations in the extracellular compartment for longer periods of time. Depolarization of glial cells by increased extracellular potassium concentrations could also promote glutamate release from glial cells and further impairment of glial uptake mechanisms for glutamate. Focal swelling at the lesion site may in addition compress blood vessels, produce local ischemia and deficient energy supply as a complicating factor.

Mechanisms underlying secondary damage in hippocampus after traumatic injury remain unknown. No evidence of hypoxia, ischemia or hyperthermia has been registered during the acute period after trauma since blood pressure, oxygenation and body temperature remained normal. No overt seizures were observed during the acute period after trauma and following recovery from anesthesia. There is, however, reason to believe that secondary damage seen in the hippocampus after cortical trauma may be dependent on the release of excitatory amino acids. Long-term monitoring of glutamate and aspartate concentrations in the hippocampal subfield CA3 by means of *in vivo* microdialysis has shown increases of 300-700% within initial 40-60 min after cortical injury on the impact side (Bernert *et al.*, 1994).

No changes in the concentrations of glutamate and aspartate were recorded in the CA3 subfield contralateral to cortex injury (Bernert *et al.*, 1994). If this mechanism underlies secondary damage in the hippocampus after cortex trauma, then it may well be that rapid changes in glutamate and aspartate metabolism lead to activation of reversible mechanisms (mediated by AMPA/KA) that may lead to cell death and remain operant over 7-10 h. Unusual effectiveness of delayed treatment with NBQX but not with CPP suggests that such mechanisms may indeed be involved. Although at the present time very little evidence can be offered in support of such a hypothesis, there is evidence that AMPA receptors may modify their configuration to increase sensitivity to glutamate or to increase channel permeability to Ca^{2+} after ischemia (Pellegrini-Giampietro *et al.*, 1992; see, however, Monyer *et al.*, 1991). Furthermore, Ca^{2+}/calmodulin-dependent protein kinase II may translocate to synaptic junctions in vulnerable hippocampal neurons for up to 24 h after transient ischemia leading to sensitization of co-localized AMPA receptors which may result in delayed neuronal death (McGlade-McCulloh *et al.*, 1993; Hu and Wieloch, 1995). Consequently, NBQX has been shown to prevent neuronal damage in the hippocampal subfield CA1 up to 12 h following global forebrain ischemia (Li and Buchan, 1993). Such considerations suggest that trauma or ischemia may eventually activate a cascade of long-lasting changes in glutamate receptor function in preferentially sensitive regions of the brain which are essential for delayed neuronal injury to occur.

Therapeutic implications

Traumatic head injury in infant rats was mitigated by NMDA antagonists whereas AMPA antagonist were ineffective. Furthermore, NMDA antagonists showed a long time-window in infant rats subjected to head trauma. In adult rats, brain damage induced by head trauma can be much better controlled by AMPA but not by NMDA antagonists. AMPA antagonists show a long therapeutic time-window in adult rats subjected to traumatic head injury.

These results are consistent with the observation that the immature brain is extremely vulnerable to glutamate toxicity and to conditions such as hypoxia/ischemia (Ikonomidou and Turski, 1995; Olney and Ikonomidou, 1991). This phenomenon has been attributed to hypersensitivity of NMDA receptors in young age (Grafe, 1994; Ikonomidou *et al.*, 1989; McDonald and Johnston, 1990). The hypothesis of NMDA receptor hypersensitivity is strengthened by findings showing that in the infant rat brain the age of peak sensitivity to hypoxia/ischemia coincides with the age of peak sensitivity to NMDA, and that neuronal groups most sensitive to NMDA are the ones most likely to undergo hypoxic/ischemic degeneration at a given age (Ikonomidou *et al.*, 1989). Similarly to traumatic brain damage, glutamate-induced (Ikonomidou *et al.*, 1989) and hypoxic/ischemic (Olney *et al.*, 1989) brain damage in the infant rat brain are also very effectively prevented by NMDA receptor antagonists. In contrast, AMPA antagonists, are not very effective against hypoxic/ischemic (Hagberg *et al.*, 1992) or traumatic brain damage in infant rats.

Different expression patterns of NMDA receptor subunits at different ages most likely account for the hypersensitivity of NMDA receptors in young age. Seeburg and his associates described that the NR2D subunit of the NMDA receptor that displays a long

offset decay and weak Mg^{2+} block is highly expressed in rats at the age of seven days which coincides with the age of peak sensitivity to NMDA (Monyer *et al.*, 1994).

These observations raise the hope that NMDA antagonists may be effective against hypoxic/ischemic and traumatic damage in the human pediatric population. It is also quite reassuring that NMDA antagonists are devoid of neurotoxic properties (vacuolization of pyramidal neurons in the cingulate/retrosplenial cortex) in infant rats (Farber *et al.*, 1992; Fix *et al.*, 1994; Olney *et al.*, 1991). Thus, the use of NMDA antagonists as neuroprotective agents in the human pediatric population is a promising perspective for the near future although potential side effects such as respiratory and cardiovascular depression need to be considered.

In contrast to NMDA receptors, AMPA/kainate receptors possess potent neurotoxic properties in the adult mammalian brain (Campochiaro and Coyle, 1978). The epileptogenic properties of kainic acid are much less pronounced in infants compared to adult rats (Albala *et al.*, 1984; Ben-Ari *et al.*, 1984; Cavalheiro *et al.*, 1983). Similar observations were made in humans. It is quite remarkable that children in East-South Asia treated with oral kainate for ascariasis in the 1950's never developed poisoning or memory disturbances (Takemoto, 1978) whereas domoate, a kainate analog found in cultured blue mussels Mytilus edulis, caused an outbreak of food poisoning accompanied by disseminated brain damage in adults and aging humans in Canada in the 1980s (Pearl *et al.*, 1990; Teitelbaum *et al.*, 1990).

In conclusion, it is suggested that NMDA antagonists may possess better neuroprotective properties against excitotoxic processes triggered by traumatic brain injury in infants whereas AMPA antagonists may be more beneficial in adult and ageing individuals.

REFERENCES

Adams, J.H. (1992). Dead injury. In (J.H. Adams, and L.W. Duchen, eds) Greenfield's Neuropathology, (Arnold, London), pp. 106-152.

Albala, B.J., Moshe, S.L., and Okada, R. (1984). Kainic acid-induced seizures: a developmental, study. *Dev. Brain Res.* 13:139-148.

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

Ben-Ari, Y., Tremblay, E., Berger, M., and Nitecka, L. (1984). Kainic acid seizure syndrome and binding sites in developing rats. *Dev. Brain Res.* 14:284-288.

Bernert, H., Mathias, S., Löschmann, P.-A., and Turski, L. (1994). Traumatic brain injury, prevented by the AMPA antagonist NBQX. *Soc. Neurosci. Abstr.* 20:977.

Bondoli, A., Barbi, S., Camaioni, D., Della Morte, F., Magalini, S.I. (1981). Plasma and cerebrospinal fluid free amino acid concentrations in post-traumatic cerebral oedema in patients with shock. *Resuscitation* 9:119-124.

Campociaro, P., and Coyle, J.T. (1978). Ontogenetic development of kainate neurotoxicity: correlates with glutamatergic innervation. *Proc. Natl. Acad. Sci. USA* 75:2025-2029.

Cavalheiro, E.A., de Feo, M.R., and Ricci, G.F. (1983). Intracortical and intrahippocampal injections of kainic acid in developing rats: an electrographic study. *Electroencephalogr. Clin. Neurophysiol.* 56:480-486.

Drejer, J., Benveniste, H., Diemer, N.H., and Schousboe, A. (1985). Cellular origin of ischemia-induced glutamate release from brain tissue an vivo and *in vitro*. *J. Neurochem.* 45: 145-151.

Faden, A.I., Demediuk, P., Panter, S.S., and Vink, R. (1989). The role of excitatory amino acids and NMDA receptors in traumatic brain injury. *Science* 244:798-800.

Faden, A.I., and Salzman, S. (1992). Pharmacological strategies in CNS trauma. *Trends Pharmacol. Sci.* 13:29-35.

Farber, N.B., Price, M.T., Labruyere, J., Fuller, T.A., and Olney, J.W. (1992). Age dependency of NMDA antagonist neurotoxicity. *Soc. Neurosci. Abst.* 18:1148

Feeney, D.M., Boyeson, M.G., Linn, R.T., Murray, H.M., and Dail, W.G. (1981). Responses to cortical injury: I. Methodology and local effects of contusions in the rat. *Brain Res.* 211:67-77.

Fix, A.S., Horn, J.W., Wightman, K.A., Johnson, C.A., Long, G.C., Storts, R.W. *et al.* (1993). Neuronal vacuolization and necrosis induced by the noncompetitive N-methyl-D-aspartate (NMDA) antagonist MK-801 (dizocilipine maleate): A light and electron microscopic evaluation of the rat retrosplenial cortex. *Exp. Neurol.* 123:204-215.

Grafe, M.R. (1994). Developmental changes in the sensitivity of the neonatal rat brain to hypoxic/ischemic injury. *Brain Res.* 653:161-166.

Gennarelli, T.A. (1984). Emergency department management of head injuries. *Emerg. Med. Clin. North Am.* 2:749-760.

Gundersen, H.J.G., Bagger, P., Bendtsen, T.F., Evans, S.M., Korbo, L., Marcussen, N., Moller, A., Nielsen, K., Nyengaard, J.R., Pakkenberg, B., Sorensen, F.B., Vesterby, A. and West, M. J. (1988). The new stereological tools: Disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* 96:857-881.

Hagberg, H., Diemer, N. H., and Andiné, P. (1992). Hypoxic-ischemic brain damage in the newborn rat: effect of NMDA- and AMPA-receptor antagonists. *Biol. Neonat.* 62:299.

Hu, B.-R., and Wieloch, T. (1995). Stress-induced inhibition of protein synthesis initiation: modulation of initiation factor 2 and guanine nucleotide exchange factor activities following transcient cerebral ischemia in the rat. *J. Neurochem.* 64:277-284.

Ikonomidou, C., Labruyere, J., Benz, A., and Olney, J. W. (1989). Antagonistic pharmacology of glutamate neurotoxicity in infant rat brain. *Soc. Neurosci. Abstr.* 15:767.

Ikonomidou, C., Mosinger, J.L., Shahid Salles, K., Labruyere, J., and Olney, J.W. (1989). Sensitivity of the developing rat brain to hypobaric/ischemic damage parallels sensitivity to N-methyl-aspartate neurotoxicity. *J. Neurosci.* 9:2809-2818.

Ikonomidou, C., and Olney, J. W. (1994). Developmental excitotoxicity for explaining neuropsychiatric disturbances. *Neuropsychopharmacology* 10:448S.

Ikonomidou, C., Price, M.T., Mosinger, J.L., Friedrich, G., Labruyere, J., Shahid Salles, K., Olney, J.W. (1989). Hypobaric-ischemic conditions produce glutamate-like cytopathology in infant rat brain. *J. Neurosci.* 9:1693-1700.

Ikonomidou, C., and Turski, L. (1995). Excitotoxicity and neurodegenerative diseases. *Curr. Opinion Neurol.* 8:487-497.

Katayama, Y., Becker, D.P., Tamura, T., and Hovda, D.A. (1990). Massive increase in extracellular potassium and the indiscriminate release of glutamate following concussive brain injury. *J. Neurosurg.* 73:889-900.

Kaufman, B.A., and Dacey, R.G. (1994). Acute care management of closed head injury in childhood. *Ped. Ann.* 23:18-28.

Lehmann, J., Schneider, J., McPherson, S., Murphy, D.E., Bernard, P., Tsai, C. *et al.* (1987). CPP, a selective N-methyl-D-aspartate (NMDA)-type receptor antagonist: characterization *in vitro* and *in vivo*. *J. Pharmacol. Exp. Ther.* 240:737-746.

Li, H., and Buchan, A.M. (1993). Treatment with an AMPA antagonist 12 hours following severe normothermic forebrain ischemia prevents CA1 neuronal injury. *J. Cereb. Blood Flow Metab.* 13:933-939.

McDonald, J.W., Silverstein, F.S., and Johnston, M.V. (1987). MK-801 protects the neonatal brain from hypoxic/ischemic damage. *Eur. J. Pharmacol.* 140:359-361.

McDonald, J.W., Silverstein, F.S., and Johnston, M.V. (1988). Neurotoxicity of N-methyl-D-aspartate is markedly enhanced in developing rat nervous system. *Brain Res.* 459:200-203.

McDonald, J.W., and Johnston, M.V. (1990). Physiological and pathological roles of excitatory amino acids during central nervous system development. *Brain. Res. Rev.* 15:41-70

McGlade-McCulloh, E., Yamamoto, H., Tan, S.-E., Brickey, D.A., and Soderling, T. S. (1993). Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. *Nature* 362: 640-642.

McIntosh, T.K., Vink, R., Soares, H., Hayes, R., and Simon, R. (1990). Effect of noncompetitive blockade of N-methyl-D-aspartate receptors on the neurochemical sequelae of experimental brain injury. *J. Neurochem.* 55:1170-1179.

Monaghan, D.T., and Beaton, J.A. (1991). Diversity and organization of excitatory amino acid receptors in the CNS. In (V.I. Teichberg, and L. Turski, eds.) *Excitatory Amino Acids and Second Messenger Systems*, Springer, Berlin, pp. 1-16.

Monyer, H., Burnashev, N., Laurie, D.J., Sakmann, B., and Seeburg, P.H. (1994). Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. *Neuron* 12:529-540.

Monyer, H., Nellgard, B., Seeburg, P.H., and Wieloch, T. (1991). Differential regulation of AMPA receptor splice variants in the CA1 region following ischemia. *Soc. Neurosci. Abstr.* 17:184.

Mosinger, J.L., Price, M.T., Bai, H.Y., Xiao, H., Wozniak, D.F., and Olney, J.W. (1991). Blockade of both NMDA and non-NMDA receptors is required for optimal protection against ischemic neuronal degeneration in the *in vivo* adult mammalian retina. *Exp. Neurol.* 113:10-17.

Nilsson, P., Hillered, L., Pontén, U., and Ungerstedt, U. (1990). Changes in cortical extracellular levels of energy-related metabolites and amino acids following concussive brain injury in rats. *J. Cereb. Blood Flow Metab.* 10:631-637.

Olney, J.W. (1971). Glutamate-induced neuronal necrosis in the infant mouse hypothalamus: An electron microscopic study. *J. Neuropathol. Exp. Neurol.* 30:75-90.

Olney, J.W., Collins, R.C., Sloviter, R.S. (1986). Excitotoxic mechanisms of epileptic brain damage. *Adv. Neurol.* 44:857-877.

Olney, J.W., and Ikonomidou, C. (1991). Excitotoxic mechanisms operating in youth and old age. In (A. Kozikowski, ed.) *Neurobiology of the NMDA Receptor*, VCH, New York, pp. 187-202.

Olney, J.W., Ikonomidou, C., Mosinger, J.L., and Friedrich, G. (1989). MK-801 prevents hypobaric-ischemic neuronal degeneration in infant rat brain. *J. Neurosci.* 9:1701-1704.

Olney, J.W., Labruyere, J., Price, M.T. (1989). Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science* 244:1360-1362.

Olney, J.W., Labruyere, J., Wang, G., Sesma, M.A., Wozniak, D.F., Price, M.T. (1991). NMDA antagonist neurotoxicity: mechanism and protection. *Science* 254:1515-1518.

Olney, J.W., Sharpe, L.G., and Feigin, R.D. (1972). Glutamate-induced brain damage in infant primates. *J. Neuropathol. Exp. Neurol.* 31:464-488.

Panter, S.S., and Faden, A.I. (1992). Pretreatment with NMDA antagonists limits release of excitatory amino acids following traumatic brain injury. *Neurosci. Lett.* 136:165-168.

Paxinos, G., and Watson, C. (1986). The Rat Brain in Stereotaxic Coordinates (Academic Press, San Diego, CA).

Pearl, T.M., Bedard, L., Kosatsky, T., Hockin, J.C., Todd, E.C.D., and Remis, R.S. (1990). An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. *N. Engl. J. Med.* 322:1775-1780.

Pellegrini-Giampietro, D.E., Zukin, R.S., Bennett, M.V.L., Cho, S., and Pulsinelli, W.A. (1992). Switch in glutamate receptor subunit gene expression in CA1 subfield of hippocampus following global ischemia in rats. *Proc. Natl. Acad. Sci. USA* 89:10499-10503.

Povlishock, J.T. (1993). Traumatic brain injury. The pathobiology of injury and repair. In (A. Gorio) *Neuroregeneration*, Raven, New York, pp. 185-216.

Sheardown, M.J., Nielsen, E.O., Hansen, A.J., Jacobsen, P., and Honore, T. (1990). 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline: a neuroprotectant for cerebral ischemia. *Science* 247:571-574.

Takemoto, T. (1978). Isolation and structural identification of naturally occurring excitatory amino acids. In (E. McGeer, J.W. Olney, and P. McGeer, eds.) *Kainic Acid as a Tool in Neurobiology*. Raven, New York, 1-17.

Teitelbaum, J.S., Zatorre, R.J., Carpenter, S., Gendron, D., Evans, A.C., Gjedde, A., Cashman, N.R. (1990). Neurologic sequelae of domoic acid intoxication due to the ingestion of contaminated mussels. *N. Engl. J. Med.* 322:1781-1787.

Toulmond, S., Serrano, A., Benavides, J., and Scatton, B. (1993). Prevention by eliprodil (SL 82.0715) of traumatic brain damage in the rat. Existence of a large (18h) therapeutic window. *Brain Res.* 620:32-41.

Turski, L., Klockgether, T., Turski, W.A., Ikonomidou, C., Schwarz, M., and Sontag, K.-H. (1988). Muscle relaxant action of excitatory amino acid antagonists: Sites of action. In (E.A. Cavalheiro, J. Lehmann, and L. Turski, eds) *Frontiers in Excitatory Amino Acid Research*. Liss, New York, pp. 343-350.

Wrathall, J.R., Teng, Y.D., Choiniere, D., Mundt, D.J. (1992). Evidence that local non-NMDA receptors contribute to functional deficits in contusive spinal cord injury. *Brain Res.* 586:140-143.