Neurotoxicity Research, 2003, VOL. 5(7). pp. 475-490

F.P. Graham Publishing Co.

Neuropathological and Biochemical Features of Traumatic Injury in the Developing Brain

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(Received 19 August 2003; Revised 26 September 2003; In final form 26 September 2003)

Trauma to the developing brain constitutes a poorly explored field. Some recent studies attempting to model and study pediatric head trauma, the leading cause of death and disability in the pediatric population, revealed interesting aspects and potential targets for future research.

Trauma triggers both excitotoxic and apoptotic neurodegeneration in the developing rat brain. Excitotoxic neurodegeneration develops and subsides rapidly (within hours) whereas apoptotic cell death occurs in a delayed fashion over several days following the initial traumatic insult. Apoptotic neurodegeneration contributes in an age-dependent fashion to neuronal injury following head trauma, with the immature brain being exceedingly sensitive. In the most vulnerable ages the apoptosis contribution to the extent of traumatic brain damage far outweighs that of the excitotoxic component.

Molecular and biochemical studies indicate that both extrinsic and intrinsic mechanisms are involved in pathogenesis of apoptotic cell death following trauma. Interestingly, in infant rats a pancaspase inhibitor ameliorated apoptotic neurodegeneration with a therapeutic time window of up to 8 h after trauma.

These results help explain unfavorable outcomes of very young pediatric head trauma patients and imply that regimen which target slow active forms of cell death may comprise a successful neuroprotective approach.

Key words: Apoptosis; Excitotoxicity; Neuroprotection

INTRODUCTION

Traumatic brain injury (TBI) constitutes a major cause of morbidity and mortality in the industrialized world (Goldstein, 1990; Sosin *et al.*, 1995; Thurman *et al.*, 1999). According to the National Center for Injury Prevention and Control, an estimated 1.5 million Americans sustain TBI each year. As a result, 50,000 people die, 230,000 are hospitalized and survive and an estimated 80,000-90,000 people experience the onset of long-term disability every year (Thurman *et al.*, 1999). A large proportion of TBI patients are never hospitalized but may suffer varying degrees of cognitive impairment, behavioral and personality changes, irritability, post-traumatic vertigo, sleep disturbances, attentional deficits and headaches.

Although children under six years of age sustain TBI more frequently than any other age group (Adelson and Kochanek, 1998; Diamond, 1996), there has been limited research focusing on traumatic injury to the developing brain. The assumption that pathophysiology of TBI is identical in the adult and developing central nervous system is incorrect. Clinical studies suggest that age decidedly influences both morbidity and mortality after head injury in children, with those under 4 years of age showing the worst outcomes (Mahoney *et al.*, 1983; Koskiniemi *et al.*, 1995; Adelson and Kochanek, 1998). A study by Koskiniemi and colleagues (1995) demonstrated that in a cohort of children suffering severe head injury prior to the age of 4 years none was able to work independently outside a structured environment years later. Children older than 4 years at the time of injury had a significantly better outcome. Differences in the mechanisms by which TBI

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ISSN 1029 8428 print/ ISSN 1476-3524 online.

was sustained and the higher incidence of non-accidental closed head traumata in the very young (Kraus *et al.*, 1987; James, 1999) may partly account for these findings. Nevertheless, it is important to consider whether the developing brain may be more vulnerable to suffering irreversible neuronal loss and/or axonal injury and, if so, what mechanisms may be involved.

In this review we will first summarize recent clinical, neuropathological and biochemical data pertaining to TBI in infants, toddlers and young children. Then we will present own experimental work on TBI in the developing rat brain with focus on cellular injury and the involved pathomechanisms.

CLINICAL AND NEUROPATHOLOGICAL FEATURES OF TBI IN INFANTS AND CHILDREN

People under the age of 18 years constitute the majority of victims of TBI. In children there are two peak periods of incidence: early childhood (less than 5 years of age) and mid-to-late adolescence (Luerssen *et al.*, 1988). Ten to 15% of children with head trauma suffer severe head injury with the majority of survivors having permanent deficits.

TBI can be grouped into different types, depending on the mechanism of injury: focal versus diffuse, closed head versus penetrating injuries and primary versus secondary injuries. Diffuse injuries are more common in children than focal injuries and closed head injuries account for the majority of cases in children.

Child abuse tends to occur more often in the very young (less than 4 years) and may even be the major cause of severe brain injury in this group, representing almost two-thirds of severe brain injury cases in the 0- to 4-year old range in some series. Shaken baby syndrome, which can be associated with head impact, is most common between 3 and 6 months of age (Barlow and Minns, 1993) and results in a high mortality rate (10-40%), acute neurological signs and poor neurological outcome, mental retardation, cerebral palsy, blindness, epilepsy and major behavioral problems (Oliver, 1975; Jaspan *et al.*, 1992; Bonnier *et al.*, 1995; 2002; Duhaime *et al.*, 1996; Shaver *et al.*, 1996).

Another major cause of head trauma among infants, toddlers and young children are falls. In older children, falls and assaults result in less than 20% of TBI.

Primary traumatic injury elicits a secondary response from the brain as a reaction to that injury, which is believed to contribute to the diffuse cerebral swelling and tissue damage seen following pediatric TBI. This secondary response includes loss of cerebral autoregulation, breakdown of the blood-brain barrier, intracellular and extracellular edema, and ischemic brain injury. Intracranial hypertension, ischemia and vasospasm are thought to contribute to progression of the injury. A strong association between diffuse brain swelling and hypoxemia or early hypotension has been reported (Aldrich *et al.*, 1992). The very young developing brain can be particularly susceptible to extensive damage and have a higher likelihood for worse outcome. In addition, in inflicted brain injury, the most common form of brain injury in infants and young children, the injuries tend to be multiple and diffuse.

There are few reports in the literature on neuropathology of infant head injury. In shaken baby syndrome, edema, bleeding, infarcts, white matter contusional tears, and axonal injury have been reported (Zimmerman *et al.*, 1979; Vowles *et al.*, 1987; Jaspan *et al.*, 1992; Duhaime *et al.*, 1998). In other studies, contusional tears but no axonal injury in infants were described (Lindenberg and Freytag, 1969). Neuronal eosinophilia, interpreted as hypoxic-ischemic changes, is a very common finding in one study by Shannon *et al.* (1998), in which some axonal injury was also found in infants and toddlers less than 18 months of age.

In a recent study by Geddes *et al.* (1999) the findings in the brains of a series of 37 infants aged 9 months or less, all of whom died from inflicted head injuries, and 14 control infants who died of other causes were summarized. Surprisingly, the most common histological finding was severe and widespread neuronal damage. In this particular study, widespread traumatic axonal injury was only found in association with multiple skull fractures. This is in contrast to earlier reports stating that diffuse axonal injury is one of the inevitable and devastating sequelae of shaken baby syndrome (Brown and Minns, 1993; Munger *et al.*, 1993; David, 1999). The only location where focal axonal damage was consistently found in the series by Geddes *et al.* was the craniocervical junction, the neuropathology being that of stretch injury from cervical hyperextension/flexion. The authors concluded that damage to this area could account for the observed apnea, which could in turn lead to hypoxic damage and brain swelling (Geddes *et al.*, 2001a).

Thus, head injury in infants in the series by Geddes *et al.* was shown to result primarily in neuronal damage, and to a much lesser extent in diffuse axonal injury. According to the authors, this finding could be explained in one of two ways: either the unmyelinated axon of the immature cerebral hemispheres is relatively resistant to traumatic damage, or, in shaking-type injuries, the brain is not exposed to the forces necessary to produce diffuse axonal injury (Geddes *et al.,* 2001b).

Similarly, in a series on inflicted head injury including older victims (aged 20 days to 8 years), severe hypoxic brain damage was present in 77% of the cases. Children over 1 year of age tended to have larger subdural hemorrhages than infants and, in the few cases where traumatic axonal injury was present, patterns of hemispheric white matter damage more akin to those reported in adults were found. Overall and contrary to what one might expect, diffuse axonal injury was an uncommon sequel in both infants and children with inflicted brain injury, whereas widespread neuronal damage (eosinophilia and shrinkage, interpreted as neuronal hypoxia-ischemia) was a very frequent finding (Geddes *et al.*, 2001b).

These two studies raised the assumption that there are significant differences between the pathology of non-accidental head injury in children and adults but also between children of different ages. They also support the notion that pathophysiology of TBI may show age-dependent variations and underline the need for animal models that will allow studying developmental aspects of TBI.

BIOCHEMICAL FEATURES OF TBI IN INFANTS AND CHILDREN

In a study by Bayir *et al.* (2002) antioxidant reserves and oxidative stress in cerebrospinal fluid after severe TBI were assessed in infants and children. Among the eleven studied patients in that group there were five aged 2 months to 4 years. The authors found evidence for marked and progressive compromise of antioxidant defenses and free radical mediated lipid peroxidation, suggesting that these markers could be used to assess the effect of therapies on oxidative stress in patients after TBI. They also suggested that defining the role of oxidative stress in the pathophysiology of TBI in infants and children could help with the development of novel, clinically applicable therapies.

Other biochemical markers that have been studied in children with TBI include serum and cerebrospinal fluid concentrations of S100B and neuron specific enolase (Berger *et al.*, 2002), adenosine levels in the cerebrospinal fluid (Robertson *et al.*, 2001) and concentrations of interleukins 6 and 10 (Bell *et al.*, 1997). All these parameters were found elevated following TBI. Interestingly, neuron specific enolase and S100B concentrations in the CSF of children with TBI showed a unique time course in inflicted brain injury. In this group both an early peak and a late peak of NSE and S100B concentrations were observed, the first occurring at a median of 11 hours after injury and the second one occurring at a median of 63 hours after injury. Interestingly, the group of children assigned to that group were 0.2- 1.8 years old. Such biochemical findings raise the assumption that in inflicted brain injury in infants and toddlers there appears to be a wave of early cell death occurring within hours after injury and a second wave of delayed cell death occurring days after injury, which may represent an important therapeutic target.

In a study by Clark *et al.* (2000a) increases of oligonucleosomes and the antiapoptotic protein bcl-2 in cerebrospinal fluid of infants and children after severe TBI were described. Most profound increases of oligonucleosomes were detected on the second day after trauma. Interestingly, bcl-2 levels showed a significantly higher increase in patients who survived versus those who died. The authors concluded that bcl-2 may participate in the regulation of cell death after TBI in infants and children and that the increase in bcl-2 seen in patients who survived is consistent with a protective role for this anti-apoptotic protein after TBI (Clark *et al.*, 2000a).

HOW TO MODEL PEDIATRIC HEAD TRAUMA

The developing mammalian brain undergoes a period of rapid growth, during which synaptogenesis and physiological cell death, the prototypic example of apoptosis, takes place. This brain growth spurt period begins in the human in the 6th month of pregnancy and extends up to the third year of life. In rodents, this period runs during the first three postnatal weeks, in piglets it also begins prenatally and includes the first 100 days of life. To model phenomena that take place in the developing brain of human infants, toddlers and young children, one needs to study animals during the comparable developmental period. Such experiments have been performed in rats and piglets.

Among the several models that have been developed for studying brain trauma, three have been widely used: the fluid percussion model (McIntosh *et al.*, 1989), the controlled cortical impact model (Dixon *et al.*, 1991) and the weight drop model (Feeney *et al.*, 1981).

In the fluid percussion model, rapid injection of small volumes of saline into the closed cranial cavity against the dura induces brain injury. The pressure pulse is delivered through a craniotomy lateral or central to the midline and induces a short-lasting intracranial pressure increase and tissue deformation. This model has been used in rats, rabbits, cats, pigs and mice. The neuropathology described in this model consists of cortical contusion, selective hippocampal neuronal loss, axonal

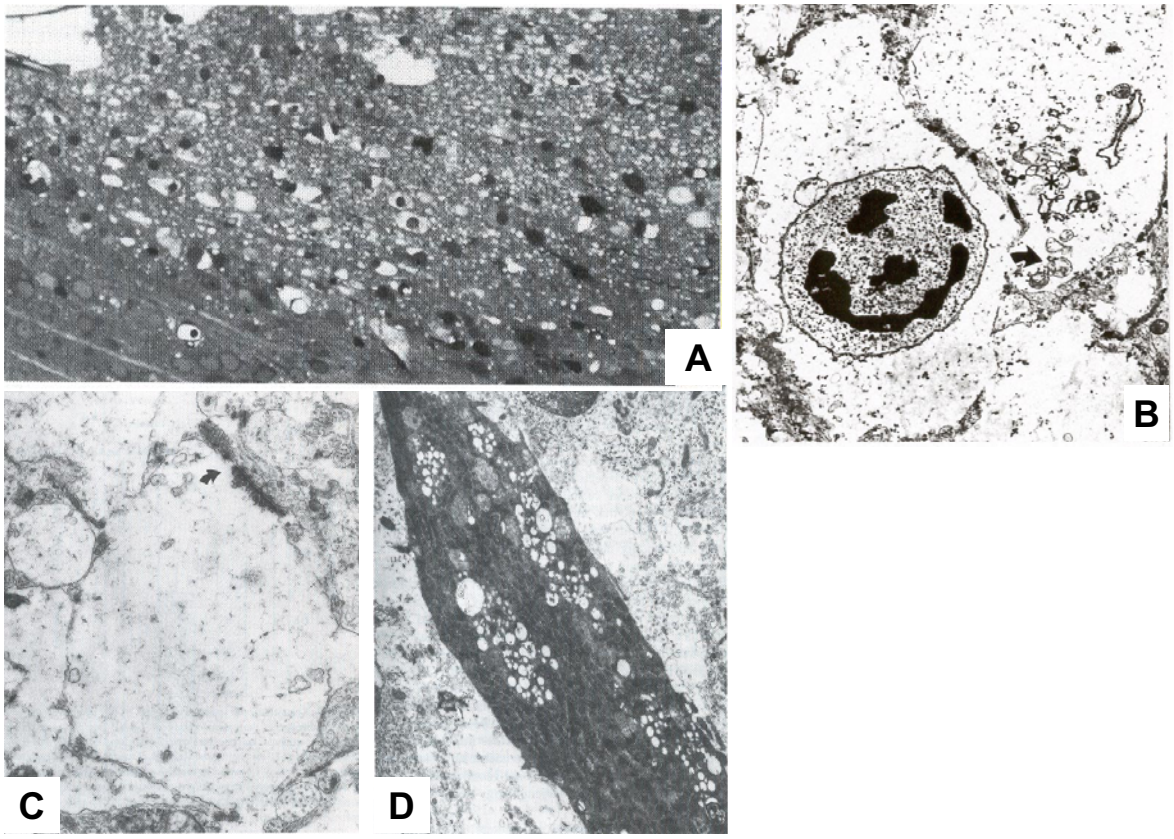

FIGURE 1 Light- and electronmicrographs depicting features of acute excitotoxic neurodegeneration following trauma to the 7 day old rat brain. (**A**) Light micrograph depicting traumatic lesion in the parietal cortex 4 h after the mechanical impact. The areas of necrosis consist of degenerating neurons with swollen cytoplasm and pyknotic nuclei (methylene blue azur II stain, X530). (**B**) Electron micrograph from the traumatized cortex of a rat pup 4 h after trauma showing an acutely degenerating neuron. The cytoplasm is massively swollen as are intracellular organelles, the endoplasmic reticulum (star) and mitochondria (arrow). The nucleus demonstrates clumps of nuclear chromatin. Magnification x6,500. (**C**) Electron micrograph taken at 2 h after trauma depicting a swollen dendrite with several areas of membrane breakdown. A presynaptic axon terminal appears intact, it contains synaptic vesicles, is of normal size and is forming an axodendritic synapse with the degenerating dendrite (arrow). Degeneration of dendritic elements with preservation of presynaptic axons is a typical feature of excitotoxic lesions. Magnification x9,000. (**D**) Pyramidal neuron undergoing dark cell degeneration 2 h after mechanical trauma. The cytoplasm is condensed and contains several vacuoles. Magnification x14,000.

injury and regional cerebral edema.

In the controlled cortical impact model, a pneumatically driven piston directly impacts the animal´s brain through a craniotomy positioned lateral or central to the midline. This model has been described in adult rats and mice. Tissue deformations can be well controlled by adjusting the depth and velocity of impact, which produces a cortical contusion, hippocampal cell loss and cognitive dysfunction.

In the weight drop model, injury is produced by a metal rod which falls through a guide tube onto the animal´s skull or the exposed brain. Weight and height of the rod determine injury severity. In this model, as in the other two, a cortical contusion, hippocampal cell loss and cognitive dysfunction are produced.

Attempts to model pediatric head trauma were initially made by Prins and Hovda (Prins *et al.*, 1996; Prins and Hovda, 1998) and Adelson and colleagues (Adelson *et al.*, 1996; 1997) who adopted the lateral fluid percussion and the closed head injury model, initially described by Marmarou and colleagues (Marmarou *et al.*, 1994), to 17-day-old rats.

Our group studied the response of the rat brain to head trauma in even earlier developmental stages in an attempt to examine mechanisms that contribute to unfavorable outcomes of very young pediatric patients to head trauma. For that purpose, a model using the weight drop device described by Allen for the spinal cord (Allen, 1911) and by Feeney for the brain (Feeney *et al.*, 1981) was developed. In the following we will

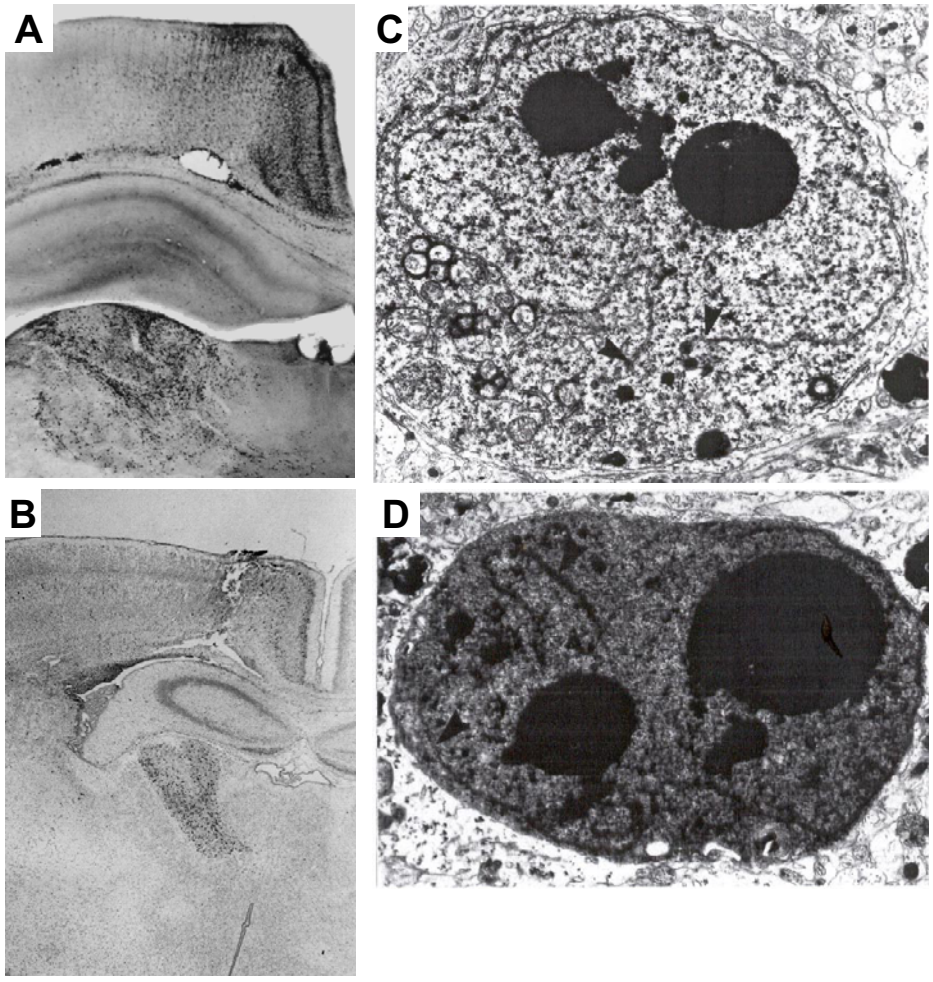

FIGURE 2 Light- and electronmicrographs depicting features of delayed apoptotic neurodegeneration following trauma to the 7 day old rat brain. Silver-positive (**A**, DeOlmos cupric silver staining) and TUNEL-positive (**B**) cells in the brains of 8 day old rats subjected to head trauma on day 7. (**C, D**) Electron microscopic evaluation of the cingulate cortex 16 h after parietal 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. The neuron in **C** 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 (arrow heads). In this early stage cytoplasmic organelles appear essentially normal. As the apoptotic process evolves (**D**), the nuclear membrane decomposes into fragments (arrow heads), the contents of the nucleoplasm and cytoplasm freely intermix and the entire cell becomes uniformly condensed. In later stages, apoptotic bodies are formed and these are extruded into the neuropil (not shown). Finally, both the main cell mass and the apoptotic bodies are transformed into shrunken amorphous masses of debris and are phagocytized. Magnifications: **C**, x10,500; **D**, x9,750.

review neuropathological and biochemical data we obtained using this head trauma model in developing rats.

NEUROPATHOLOGICAL FINDINGS IN TBI IN THE DEVELOPING BRAIN

Using the weight drop device initially in 7 day old rats, we found that mechanical trauma to the immature brain causes an acute excitotoxic lesion within the area of impact which rapidly expands within 4 h after trauma (Ikonomidou et. al., 1996) (FIG. 1). This local excitotoxic response is followed by disseminated cell death affecting many brain regions ipsi- and contralateral to the trauma site, which is detected by means of DeOlmos silver staining (DeOlmos and Ingram, 1971) and TUNEL staining for a period of hours after the excitotoxic degeneration has run its course (FIG. 2). Delayed cell death is detected in frontal, parietal, cingulate and retrosplenial cortices, laterodorsal, mediodorsal and ventral thalamic nuclei, hippocampal dentate gyrus, subiculum and striatum (Bittigau *et al.*, 1999; Pohl *et al.*, 1999).

Examination of histological sections by TUNEL staining revealed that TUNEL positive cells displayed a similar distribution pattern. By morphometric analysis, densities of silver positive- and TUNEL-positive cells obtained from parallel sections within affected brain areas did not significantly differ from each other. Thus, cells which degenerated in a delayed fashion after head

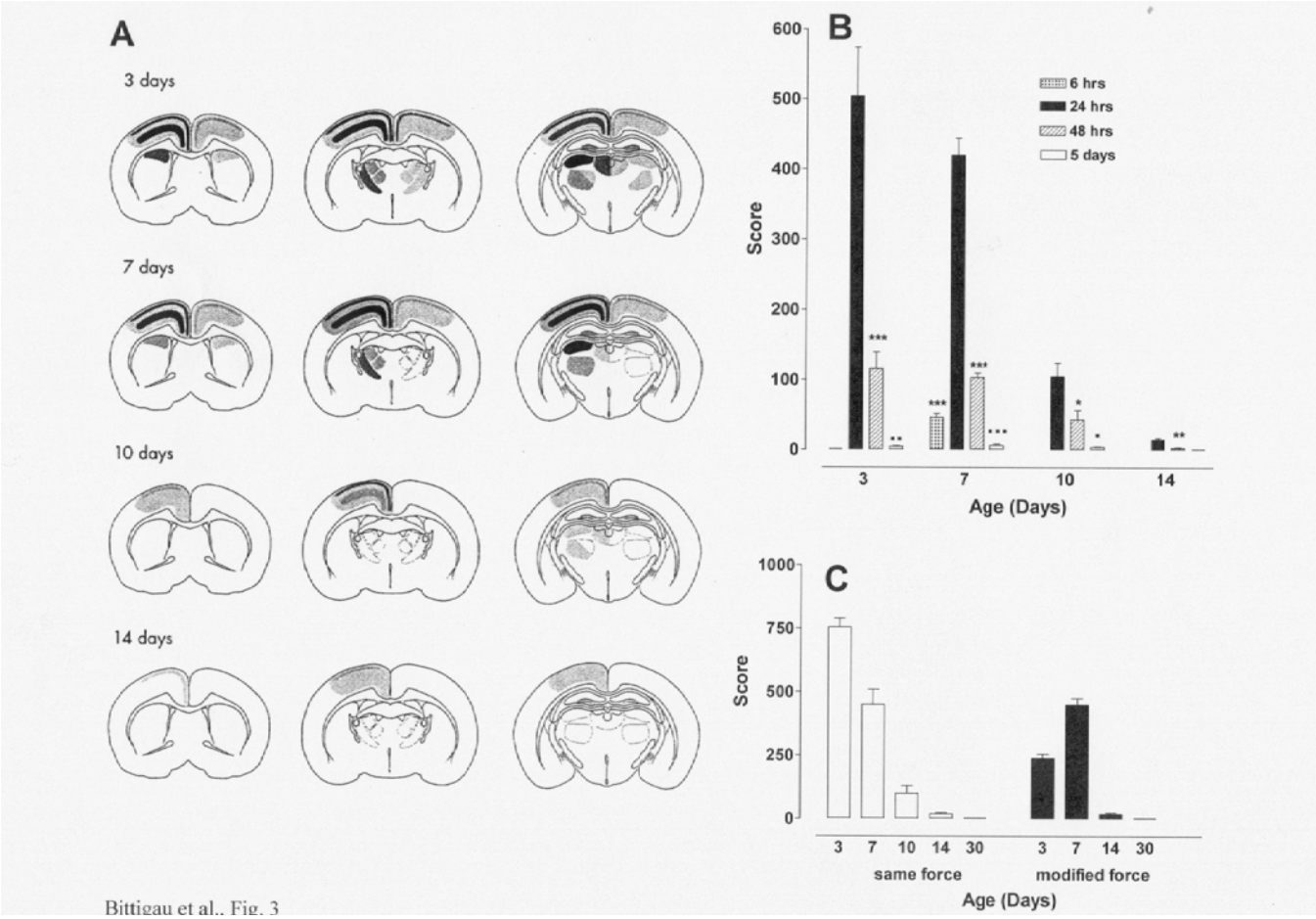

FIGURE 3 (**A**) Schematic illustration depicting the distribution patterns of TUNEL positive cells (shaded areas) in different age groups at 24 h after trauma. Darker tones indicate higher densities of degenerating cells. Rats were traumatized on postnatal days 3, 7, 10, 14 and 30. (**B**) Time course of apoptotic cell death following head trauma in different age groups. Morphological analysis was performed by means of the stereological optical dissector in sections stained with the DeOlmos cupric silver method. Rats were given a cumulative severity score for apoptotic damage. Depicted are mean scores ± SEM from 5-7 rats. Brains were analysed at 24, 48 h or 5 days after trauma, in 7 day old rats also at 6 h after trauma. Apoptotic cell death in all ages studied showed a maximum at 24 h after trauma. (**P* <0.05, ****P* <0.001 compared to 24 h; •*P* <0.05, ••*P* <0.01, •••*P* <0.001 compared to 48 h; Student´s *t* test). (**B**) Severity of posttraumatic apoptotic cell death in relation to age. Quantitation of damage was performed by means of the stereological optical dissector in sections stained with the TUNEL method. Each region was given a score 1 for every 1000 degenerating cells/mm^3 (1000 cells mm^3=1) and the scores from 14 regions ipsilateral and 14 regions contralateral to the trauma were added to give a cumulative severity score for the brain. Depicted are mean scores ± SEM from 5-9 rats. Open bars represent mean scores from rats traumatized with the same mechanical force. Black bars represent mean scores from rats which were traumatized using the same pressure/brain weight ratio in all age groups. In both groups, severity of apoptotic damage at 24 h after trauma was significantly (*P* <0.001; Student´s *t* test) higher in 3 and 7 day old rats as compared to 10, 14 or 30 day old animals.

trauma in the 7 day old rat brain displayed nuclear DNA-fragmentation (Bittigau *et al.*, 1999).

To confirm the apoptotic nature of this delayed degenerative reaction to trauma in the 7 day old rat brain, and taking into account that degenerating neurons dying by a non-apoptotic process can also show TUNEL positivity (Ishimaru *et al.*, 1999), large numbers of degenerating cells were examined by electron microscopy. In all regions examined (frontoparietal and cingulate/retrosplenial cortices, thalamus, caudate nucleus), the cells undergoing delayed degeneration displayed ultrastructural changes characteristic of apoptosis. The first detectable ultrastructural changes consisted of clumping of nuclear chromatin and mild to moderate condensation of the entire cell. Nuclear chromatin became transformed into flocculent densities which formed one or more large electron dense spherical balls (FIG. 2C, 2D). The nuclear envelope separated into fragments, and finally the cell became unpartitioned with nucleoplasmic contents freely intermingling with cytoplasmic contents. Large chromatin masses migrated often towards 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 identical to changes seen in neurons undergoing physiological cell death, a natural apoptotic process by which redundant or unsuccessful neurons are deleted from the developing brain (Ishimaru *et al.*, 1999) and meet established criteria for diagnosing apoptosis (Wyllie *et al.*, 1980). At 24 h after trauma, cells in both early and late stages of apoptosis were detected, indicating that the process of cell suicide was still progressing. We did not identify cells undergoing non-apoptotic degeneration by electron microscopy in affected brain regions at 16-24 h after trauma, which indicates that, at those times, apoptosis is the predominant form of degeneration, whereas up to 6 h after trauma excitotoxic degeneration primarily takes place in the infant rat brain (Ikonomidou *et al.*, 1996; Bittigau *et al.*, 1999).

These results demonstrate that both acute excitotoxic and slower active or apoptotic cell death occur in the context of traumatic brain damage in developing rats.

AGE-DEPENDENT DISTRIBUTION PATTERNS AND SEVERITY OF APOPTOTIC CELL DEATH FOLLOWING TRAUMA

We studied age dependent severity of apoptotic neurodegeneration following TBI by performing two series of experiments, one in which the brains of rats at all ages (3-30 days old) were subjected to the same mechanical force and one in which the mechanical force was modified in order to achieve the same pressure/brain weight ratio (Bittigau *et al.*, 1999). In both series we detected apoptotic cell death at 24 h after head trauma in 3-14 day old rats. Silver and TUNEL stains gave similar distribution patterns in all age groups. The age-dependent distribution patterns are shown in figure 3.

When the same force of 160gcm was used to traumatize pups of all age groups, severity of apoptotic cell death was highest in 3 day old rats and decreased significantly with increasing age (FIG. 3C). Even when the force was adjusted to provide the same pressure/brain weight ratio in all age groups, distant apoptotic damage in older animals remained minimal as compared to 3 and 7 day old rats. Under this experimental condition (fixed pressure/brain weight ratio), 7 day old rats were most vulnerable to distant apoptotic damage triggered by mechanical trauma (FIG. 3C).

Apoptotic cell death reached a peak at 24 h following trauma in the brains of pups at all ages studied and was non-detectable at 5 days after the insult (FIG. 3B).

In our studies in infant animals, we found that, at a given developmental age, highest densities of apoptotic cells following trauma were detected in areas that also displayed highest densities of cells undergoing physiologic cell death (Bittigau *et al.*, 1999). This possibly indicates that neurons and glia may be most vulnerable to die via apoptosis when exposed to an exogenous insult during a certain period of their maturation and differentiation process. It has been proposed that the ratio of pro- versus antiapoptotic factors within a cell primarily determines its vulnerability and likelihood to undergo active cell death (Kroemer, 1997; Hengartner, 2000). Thus, the ontogenetically regulated expression of potential proapoptotic factors early in development, such as c-jun, c-fos, p53 (Ferrer *et al.*, 1996), which, under physiological conditions, promote differentiation of immature neurons and glia may, under pathological circumstances, predispose these same cells to undergo suicide. P53, for example, promotes cell differentiation but initiates apoptotic deletion following irradiation (Borovitskaya *et al.*, 1996; Norimura *et al.*, 1996; Almog and Rotter, 1997). Progressive axonal injury and secondary axotomy after trauma may be an additional mechanism that promotes neuronal apoptosis, due to deafferentiation and loss of trophic support (Maxwell *et al.*, 1997).

Why the same traumatic insult that triggers no detectable apoptotic cell death in the 30-day-old rat brain gives rise to a massive, disseminated apoptotic response in infant rats is unclear. Certainly age-specific differences in the degree of myelination and brain water content will allow traumatic forces to transmit more easily to deeper brain structures the more immature the brain is at the time of injury. Interestingly, the infant rat brain is most sensitive to apoptotic neurodegeneration following trauma during the *N*-methyl-D-aspartate (NMDA) -receptor-hypersensitivity period (McDonald *et al.*, 1988; Ikonomidou *et al.*, 1989), with peak vulnerability on postnatal day 7. This exactly coincides with the age at which the infant rat brain is most sensitive to NMDA-excitotoxicity. In view of reports that NMDA receptor stimulation may trigger both excitotoxic and apoptotic neurodegeneration depending on intensity of stimulation (Bonfoco *et al.*, 1995), the question arises whether stimulation of NMDA receptors by endogenous glutamate, may promote apoptosis following trauma in infant rats. However, while attempting to block this apoptotic response with NMDA antagonists, we observed an unexpected potentiating effect (Pohl *et al.*, 1999), indicating that NMDA receptor blockade promotes apopto-

sis and NMDA receptor stimulation may be neuroprotective against apoptosis. Radical scavengers and antioxidants elicited a protective effect against delayed apoptotic cell death in the infant rat TBI model (Pohl *et al.*, 1999), suggesting contribution of free radicals in pathogenesis of this form of neurodegeneration.

Other factors likely to complicate tissue injury and potentially further entertain apoptotic deletion are progressive axonal injury and secondary axotomy after trauma with resulting deafferentation, decrease in the levels of neurotrophic factors and loss of trophic support, activation of glial cells and inflammatory pathways involving death receptors. Our attempts to explore some of these mechanisms will be illustrated in the following.

PATHWAYS LEADING TO APOPTOTIC NEURODEGENERATION

Apoptosis can be initiated by diverse signals and executed via different biochemical pathways (Hengartner, 2000). Triggers include growth factor deprivation, DNA damage, cytokine production and activation of death receptors, as well as release of cytochrome c from the mitochondria into the cytoplasm. Although biochemical pathways differ considerably, they all converge upon activation of effector caspases (Krammer, 2000; Meier *et al.*, 2000; Nicholson, 2000; Rich *et al.*, 2000; Savill and Fadok, 2000; Yuan and Yankner, 2000).

An intrinsic and extrinsic apoptotic pathway have been defined, the first initiated by release of cytochrome c into the cytoplasm and the second by activation of death receptors. Cytochrome c release leads to activation of effector caspases via recruitment of caspase-9 (Hengartner, 2000). Aggregation of the death receptor Fas (CD95/Apo-1), a member of the TNF-α superfamily, follows Fas ligand binding and leads to formation of a death-inducing signaling complex (DISC): Fas itself, an adapter protein named Fas associated death domain (FADD) and the inactive form of caspase-8 (Martin-Villalba *et al.*, 1999). After formation of the DISC, procaspase-8 is proteolytically cleaved, activated and released from the DISC (Chinnaiyan *et al.*, 1995; Muzio *et al.*, 1996; Medema *et al.*, 1997; Krammer, 2000). Caspase-8 then activates downstream caspases, such as caspase-3, which execute the cell.

Activation of caspases comprises a subsequent critical step within the apoptotic cascade. Caspases contribute to cell cleavage via inactivation of nuclease inhibitors and survival proteins, direct disassembly of cell structures, destruction of proteins involved in cytoskeleton regulation and inactivation of proteins involved in DNA repair and replication (Thornberry and Lazebnik, 1998).

To investigate involvement of the intrinsic apoptotic pathway in the pathogenesis of apoptotic neurodegeneration following trauma to the developing brain, we analyzed changes in the expression of antiapoptotic proteins of the bcl-2 group that decrease mitochondrial membrane permeability, changes in cytochrome c immunoreactivity in the cytosolic fraction and changes in caspase-9 activity in the infant rat brain trauma model. To investigate involvement of the extrinsic pathway, Fas-expression and caspase-8 activity in brain tissue were measured. To investigate the role of neurotrophins, endogenous mRNA levels for neurotrophin-3 (NT-3) and brain derived neurotrophic factor (BDNF) were analyzed. Finally, to test the potential benefit of caspase inhibition in TBI to the developing brain, the pancaspase inhibitor z-VAD.FMK was administered to infant rats and neurodegeneration was quantitated. Our findings indicate that trauma leads to activation of the intrinsic and the extrinsic apoptotic pathways in the developing rat brain and that inhibition of effector caspases confers neuroprotection over a time window of at least 8 h after trauma.

TRAUMA-INDUCED CHANGES IN CASPASE-3-LIKE ENZYMATIC ACTIVITY AND DNA-BREAKDOWN IN THE INFANT RAT BRAIN

Head trauma induced significant elevations of caspase 3/CPP32-like activity and DNA fragmentation (oligonucleosomes) in extracts from ipsilateral cingulate and parietal cortex, thalamus, striatum and hippocampus as measured at 24 h after trauma (Bittigau *et al.*, 1999).

These findings indicate that activation of CPP32-like caspases play a critical role in active cell deletion following trauma to the immature brain. In contrast to the adult brain, CPP32-like proteolytic activity rose not just up to 130%, as described in a study by Yakovlev *et al.* (1997), but up to 2,830% of control values. Together with our histological data, this highlights the disproportionately large magnitude of the apoptosis contribution to posttraumatic brain damage in the immature brain. Using combined optical dissector stereology and volumetry we calculated that in the brains of 7 day old rats subjected to head trauma, millions of cells were dying an apoptotic death in the brain at 24 h after trauma as opposed to a few thousand cells dying an excitotoxic death at 4 h after trauma (Bittigau

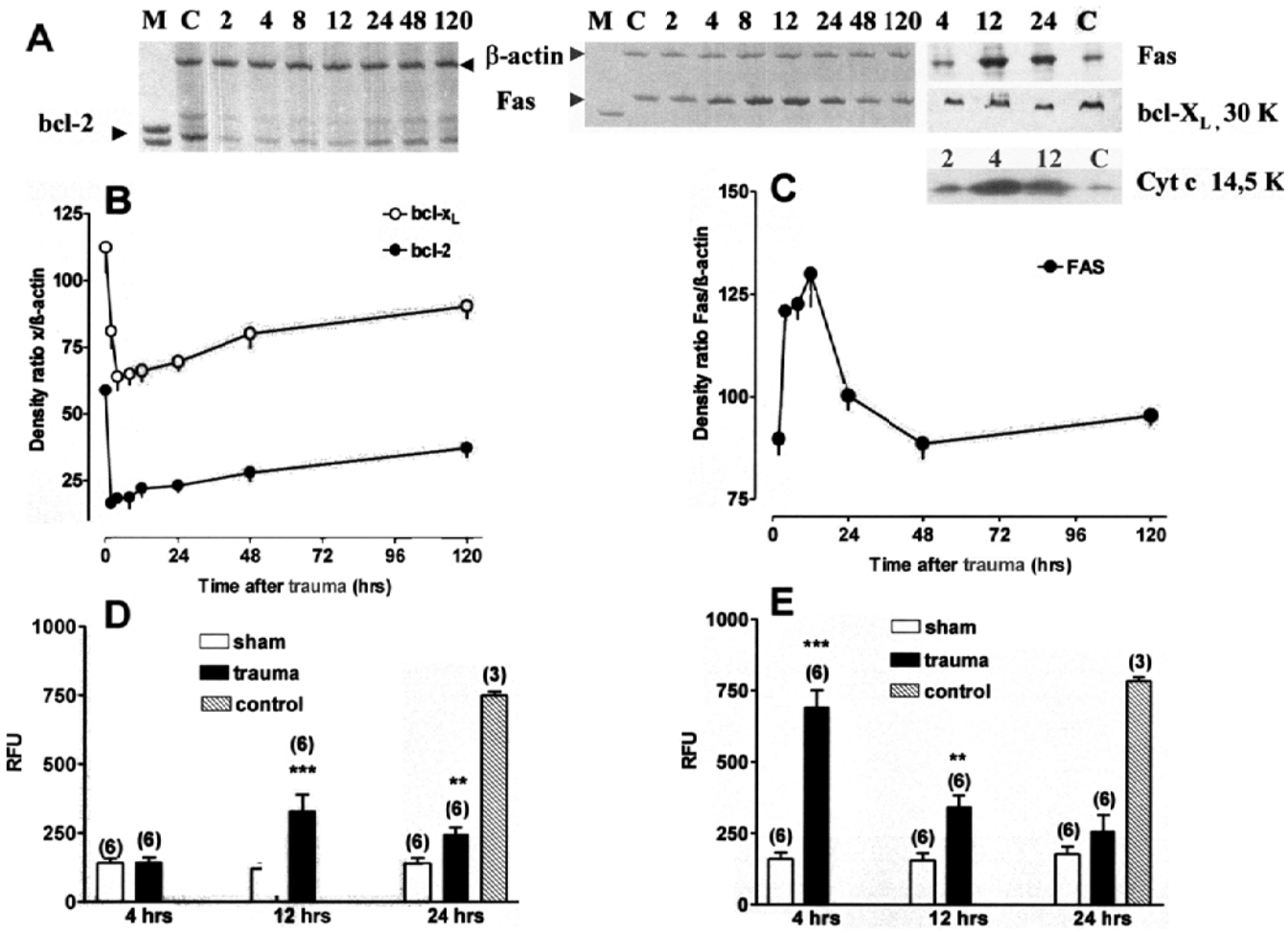

FIGURE 4 (**A**) Bcl-2 (left) and Fas (right) mRNA expression in right thalamus (ipsilateral to trauma site) in a sham operated rat at 0 hrs after trauma and in rats subjected to head trauma at 2-120 h after trauma. mRNA was reverse transcribed to cDNA, amplified by polymerase chain reaction using specific primers for bcl-2 and ß-actin and subjected to polyacrylamide gel electrophoresis and silver staining. There is obvious decrease in mRNA levels for bcl-2. There is an increase in mRNA levels for Fas at 4 h, peaking at 12 h after the insult and lasting up to 24 h. These are representative gels from a series performed to analyze bcl-2 and Fas mRNA expression. On the right, Western blot analysis for Fas and bcl-x_L in brain extracts prepared from the thalamus of sham operated rats (C) and rats traumatized on day 7, at 4-24 h after trauma. Representative blots of a series performed (thalamus, striatum, cortex), demonstrating an increase in Fas and decrease in bcl-x_L protein expression 4 h after trauma in the ipsilateral thalamus. Western blot analysis of cytochrome c immunoreactivity in the cytoplasmic fractions of brain extracts taken from the thalamus of a sham operated rat (C) and rats traumatized on day 7, at 2, 4 and 12 h after trauma is also shown. There is an increase in cytochrome c immunoreactivity in the cytosolic fraction by 2 h after trauma. (**B**) The results of densitometric analysis of bcl-2 and bcl-x_L specific mRNA-bands on the gels from traumatized rats are presented in reference to ß-actin. Data represent the ratio (%) of the density of the bcl-2 or bcl-x_L band to the ß-actin band ± SEM. Two way ANOVA revealed that trauma had a highly significant effect on the mRNA-levels for bcl-2 [$F(7,44)=40.9$, $P<0.001$] and bcl-x_L [$F(7,44)=31.71$, $P<0.001$] in the thalamus. There was a highly significant effect of time (posttraumatic interval) on the mRNA-levels for bcl-2 [$F(1,44)=155.1$, $P<0.001$] and bcl-x_L [$F(1,44)=648.8$] in the thalamus as well, compared to sham operated rats. (**C**) The results of densitometric analysis of the Fas specific mRNA bands from the thalamus of traumatized rats are presented in reference to ß-actin. Data represent the ratio (%) of the density of the Fas band to the ß-actin band ± SEM. Comparison between sham rats and rats subjected to brain trauma by ANOVA revealed that trauma had a highly significant effect on Fas mRNA levels in the thalamus [$F(1,44) = 804.5$, $P<0.001$]. (**D**) Caspase-9 activity in cytosolic protein extracts from thalamus of sham rats and rats subjected to head trauma. Specimen were analyzed at 4, 12 and 24 h after trauma or sham surgery. Caspase-9-like activity was measured fluorometrically, using the specific substrate LEHD and by determining accumulation of free aminotrifluoromethyl coumarin (AFC). Data are expressed in relative fluorescent units (RFU) as means ± SEM after subtraction of the appropriate buffer controls. The numbers in parentheses represent the number of specimen in each group. Two way ANOVA revealed that trauma had a highly significant effect on caspase-9 activity in the thalamus [$F(1,30) = 17.56$, $P<0.001$]. The grey column depicts recombinant caspase-9 activity which served as control. (**E**) Caspase-8 activity in cytosolic protein extracts from right thalamus in rats subjected to head trauma compared to sham rats. Specimens were analyzed at 4, 12 and 24 h after trauma or sham surgery. Caspase-8 like activity was measured fluorometrically using the specific substrate IETD and by determining accumulation of free AFC. Data are expressed in relative fluorescent units (RFU) as the mean ratios ± SEM of signal obtained in specimen from traumatized and sham operated brains after subtraction of the appropriate buffer controls. The numbers in parentheses represent the number of specimen in each group. Two way ANOVA revealed that trauma had a highly significant effect on caspase-8 activity in the thalamus [$F(1,30) = 63.27$, $P<0.001$]. The grey column depicts recombinant caspase-8 activity which served as control.

et al., 1999). Since apoptotic cells can be detected histologically for a few hours (clearance time, 2 h and 20 min) (Thomaidou *et al.*, 1997) and apoptosis is occurring in the infant rat brain for several days after trauma, the numbers of cells eventually deleted by this mechanism are much higher.

ACTIVATION OF THE INTRINSIC APOPTOTIC PATHWAY BY TRAUMA

Downregulation in the Expression of Antiapoptotic Genes

The expression of bcl-2 and bcl-x_L, two proteins with antiapoptotic properties which have been shown to decrease mitochondrial membrane permeability was first investigated at the transcriptional level. Trauma triggered marked and rapid downregulation in the expression of bcl-2- and bcl-x_L-specific mRNA in thalamus and cingulate cortex, which was evident within 2 h following the insult, persisted up to 48 h and demonstrated a slow, incomplete recovery by 120 h after trauma (FIG. 4A, 4B).

Downregulation of bcl-2 family members was confirmed at the protein level in that immunoreactivity of bcl-x_L was analyzed by Western blotting in brain extracts from thalamus, striatum and cortex. Decreased levels of bcl-x_L protein were found in these areas at various time points after trauma (FIG. 4A).

Cytochrome c Release and Caspase-9 Activation

Cytochrome c immunoreactivity was analyzed by Western blotting in the cytosolic fraction of brain extracts from thalamus, striatum and cortex of 7 day old rats subjected to head trauma. Cytochrome c immunoreactivity increased at 2 h after trauma in the cytosolic fraction (FIG. 4A), at a time point when no signs of delayed neurodegeneration were detectable by histological techniques.

The activity of the initiator caspase-9 was measured in thalamic tissue using the specific substrate (LEHD) in sham rats and rats subjected to head trauma. Compared to sham operated rats, there was a significant increase of caspase-9 activity in the thalamus in rats subjected to head trauma at 12 and 24 h after trauma (FIG. 4D).

Activation of the intrinsic apoptotic pathway has previously been reported in *in vivo* trauma models (Morita-Fujimura *et al.*, 1999; Raghupathi *et al.* 2000; Keane *et al.* 2001). How cytochrome c manages to cross the mitochondrial membrane is not understood. In all proposed models (Hengartner, 2000), members of the bcl-2 family play a key role in that they decrease mitochondrial membrane permeability and prevent release of cytochrome c into the cytoplasm (Nicholson, 2000). In the infant rat brain we demonstrate downregulation of the expression of bcl-2 and bcl-x_L following trauma, which is expected to result in increased permeability of the mitochondrial membranes. Changes at the mRNA levels correlated with decreased protein levels (Felderhoff-Mueser *et al.*, 2002). Reasons for decreased expression of antiapoptotic bcl-2 family proteins remain unclear. Transcription of antiapoptotic bcl-2 family members is influenced by CREB, whose activity level is regulated by growth factors (Xing *et al.*, 1996). It has been shown that release of trophic factors and their trophic effects on developing neurons depend upon the level of neuronal activity (Mc Callister *et al.*, 1996; Liou and Fu, 1997). Spreading depression triggered by trauma disrupts physiological synaptic activity. Even in the presence of increased neurotrophin levels, it is possible that disruption of physiological synaptic activity may lead to impairment of intracellular neurotrophin-initiated signaling pathways and result in decrease in the transcription of survival genes.

APOPTOSIS BY DEATH RECEPTOR ACTIVATION (EXTRINSIC PATHWAY) FOLLOWING TRAUMA TO THE DEVELOPING BRAIN

Changes in expression of the death receptor Fas following trauma to the 7 day old rat brain were determined at defined time points post-injury. Trauma triggered increase of Fas-mRNA levels at 4 h after trauma which lasted up to 24 hours and subsequently decreased to pre-trauma levels (FIG. 4A). Increased protein levels for Fas were found in the ipsilateral thalamus, striatum and cortex starting at 4 hours after trauma, with this increase being most pronounced at 12 and 24 h after trauma (FIG. 4A).

In the intact developing brain there is moderate physiological expression of Fas. Fas immunoreactivity increased particularly in the cortex and in the thalamus ipsilateral to the injury after trauma (FIG. 4A, 4C). At 48 h, reduction of Fas immunoreactivity occurred in affected brain regions, possibly reflecting evacuation of cells that overexpressed the receptor (Felderhoff *et al.*, 2002).

To further confirm activation of the extrinsic apoptotic pathway following trauma, caspase-8 activity was measured fluorometrically in the thalamus using the specific caspase-8 substrate IETD. Trauma had a highly significant effect on caspase-8 activity in the thala-

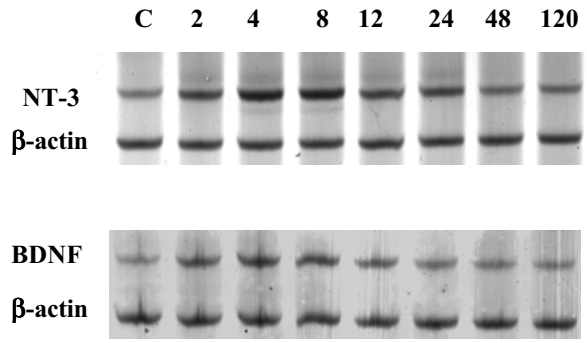

FIGURE 5 NT-3 and BDNF mRNA expression in right thalamus (ipsilateral to trauma site) in a sham operated rat (C) at 0 h after surgery, and rats subjected to head trauma at 2, 4, 8, 12, 24, 48 and 120 h after trauma. There is an increase in mRNA levels for both neurotrophins at 4 h, with peaks between 4-8 h after the insult and decline to control values by 48 h after trauma. The ß-actin band shows equal signal intensity in all columns, verifying cDNA integrity.

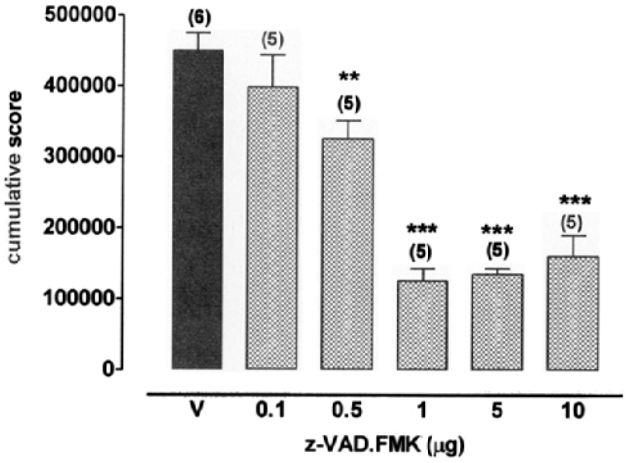

FIGURE 6 Neuroprotective effect of z-VAD.FMK in infant TBI. Seven day old rats received 0.1-10 μg z-VAD.FMK (grey columns) or DMSO vehicle (V, dark column) i.c.v. at 2 h after trauma and were perfused at 24 h after trauma (n=6 per group). In sections stained by the DeOlmos technique, the frontal, parietal, cingulate, retrosplenial cortices, caudate nucleus, thalamus, dentate gyrus and subiculum were subjected to morphometric analysis by stereological dissector method, estimating numerical densities of silver positive cells. Each region was given a score of 1000 for every 1000 degenerating cells/mm^3, and the scores of 14 regions ipsilateral to the trauma side were added to a cumulative score. Columns represent cumulative scores ± SEM (numbers in parentheses represent the number of animals per group). ANOVA revealed that the effect of treatment with z-VAD.FMK was significant [$F(5,30) = 30.06$, $P<0.0001$]. Pairwise comparisons revealed that the dose of 0.5 mg/kg z-VAD.FMK significantly protected from apoptotic neurodegeneration following trauma to the developing brain. $^{**}P<0.01$; $^{***}P<0.001$ compared to DMSO-treated rats, Student´s *t*-test.

mus after trauma (FIG. 4E). Previous studies have demonstrated Fas expression in the context of trauma to the adult brain and in infant hypoxia-ischemia (Nakashima *et al.*, 1999; Beer *et al.*, 2000; Felderhoff-Mueser *et al.*, 2000). Our findings imply Fas receptor involvement in activation of caspase-8 after neonatal brain trauma but do not exclude the possibility that other death receptors and their ligands (TNF, TRAIL) may also contribute to activation of this extrinsic apoptotic pathway.

A cross talk between the extrinsic pathway and the mitochondria has been postulated (Hengartner, 2000). Activation of caspase-8 leads to proteolysis of the proapoptotic protein bid. Truncated bid enters the mitochondria and promotes cytochrome c release. Time course studies suggest that activation of caspase-8 occurs early (within 4 h) after trauma. Therefore, it is possible that caspase-8 mediated bid cleavage may constitute one additional mechanism to facilitate release of cytochrome c into the cytoplasm.

Trauma Triggers Transcription of Neurotrophins

To determine whether downregulation of neurotrophins may contribute to apoptotic neurodegeneration after trauma, mRNA levels for BDNF and NT-3 were analyzed by RT-PCR in brain samples from rats subjected to head trauma at the age of 7 days. Trauma triggered increase of NT-3 and BDNF specific mRNA. This effect was evident at 2 h after trauma, demonstrated a peak at 8 h and returned to basal levels by 48 h after trauma (FIG. 5).

In accordance, BDNF immunoreactivity was quite prominent in the cortex and thalamus ipsilateral to the injury at 24 h after trauma (Felderhoff-Mueser *et al.*, 2002).

Thus, our studies did not demonstrate downregulation in the expression of the neurotrophins BDNF and NT-3. In contrast, neurotrophin mRNA levels and immunoreactivity increased within hours after trauma, suggesting that neurotrophin-upregulation may represent an endogenous compensatory mechanism to counteract neuronal destruction in the developing central nervous system and provide modes for regeneration and repair.

PHARMACOLOGICAL INTERVENTIONS IN DEVELOPMENTAL BRAIN TRAUMA

NMDA Antagonists Block Excitotoxic but Enhance Trauma-induced Apoptotic Neurodegeneration

To assess whether antagonists at the NMDA receptor may have neuroprotective effects in the neonatal head trauma model, we administered NMDA antagonists to

7 day old rats and evaluated the extent of excitotoxic and apoptotic neurodegeneration following such treatment.

Quantitative evaluation 4 h after trauma of the brains of 7-day-old rats treated with the NMDA antagonist dizocilpine 1 h prior to trauma revealed reduction of the extent of excitotoxic damage in the parietal cortex (Ikonomidou *et al.*, 1996; Pohl *et al.*, 1999).

Quantitative evaluation of the brains 24 h after head trauma however revealed that the apoptotic degeneration was more severe in rats treated with dizocilpine than in those subjected to vehicle (Pohl *et al.*, 1999). NMDA antagonists also gave rise to apoptotic degeneration in the brains of 7-day-old sham-operated rats, indicating that they promote physiological apoptosis at this age. 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 would be expected if the combined effect were due to a simple additive mechanism (Pohl *et al.*, 1999).

These results indicate that NMDA antagonists have opposite effects on the two components of TBI in the developing brain; they reduce the relatively small excitotoxic component, while markedly increasing the substantially larger apoptotic component, the net effect being increased neurodegeneration. Thus, NMDA antagonists are singularly unsuitable for neuroprotection in TBI to the developing brain.

Treatment with the Pancaspase Inhibitor z-VAD.FMK Blocks Trauma-induced Apoptotic Cell Death

To assess the neuroprotective potential of caspase-inhibition in TBI in infant rats, the pancaspase inhibitor z-VAD.FMK was administered intracerebroventricularly (i.c.v.) to 7 day old rats in doses of 0.1-10 µg at 2 h after trauma. Treatment with z-VAD.FMK had a significant effect on severity of apoptotic brain damage following trauma. Rats receiving a single dose of z-VAD.FMK displayed a reduction in cumulative scores for degenerating cells (FIG. 6) compared to rats subjected to head trauma and i.c.v. injection of DMSO.

The therapeutic time window for z-VAD.FMK was at least 8 h, since delayed administration of z-VAD.FMK up to 8 h following trauma resulted in lower scores for apoptotic brain damage compared to vehicle treated rats (Felderhoff-Mueser *et al.*, 2002).

In order to exclude the possibility that caspase inhibition might delay but not inhibit apoptosis, we injected z-VAD.FMK (1 µg) 2 h after trauma to 7 day old rats and analyzed their brains at 24, 48 h and 5 days after trauma. A protective effect of z-VAD.FMK was still evident at 48 h after trauma in comparison to vehicle. At 5 days after trauma densities of degenerating cells in both vehicle and z-VAD.FMK treated rats were equally low and did not significantly differ between groups (Felderhoff *et al.*, 2002)

Finally, to provide additional evidence that treatment with z-VAD.FMK offered lasting protection against TBI, we injected 7 day old rats subjected to brain trauma i.c.v. with the protective dose of 1 µg z-VAD.FMK or vehicle at 2 h after trauma. All animals were sacrificed 7 days after trauma or sham surgery without transcardial perfusion, the forebrains were hemisected and their weights monitored. Trauma resulted in significant weight reduction of the right (traumatized) hemisphere in vehicle treated rats compared to the non traumatized left side (Felderhoff-Mueser *et al.*, 2002). Treatment with z-VAD.FMK resulted also in a significant but less pronounced reduction of right hemispheric weights compared to vehicle treated traumatized rats and sham operated rats. These time studies suggest amelioration of the neurodegenerative response to trauma (Felderhoff *et al.*, 2002).

In adult animal models, caspase inhibition may confer neuroprotection in cerebral ischemia (Hara *et al.*, 1997; Endres *et al.*, 1998; Fink *et al.*, 1998; Himi *et al.*, 1998). Furthermore, early treatment of experimental pneumococcal meningitis with z-VAD.FMK was shown to have a beneficial effect, whereas delayed application of this compound did not result in substantial reduction of neuronal loss (Braun *et al.*, 1999). In traumatic injury to the adult brain, z-VAD.FMK and the selective caspase-3 inhibitor z-DEVD.FMK can block neuronal death (Yakovlev *et al.*, 1997; Clark *et al.*, 2000b). In hippocampal and cortical neuronal cultures, the cell permeable pancaspase inhibitor boc-aspartyl(OMe)-fluoromethylketone (BAF) and the more selective caspase-8 inhibitor IETD-FMK (IETD) reduced Fas-induced apoptosis (Felderhoff-Mueser *et al.*, 2000). The only existing *in vivo* study on caspase inhibition in the immature brain demonstrated neuroprotection with the pancaspase inhibitor BAF in an infant model of hypoxic-ischemic injury (Cheng *et al.*, 1998). Our data indicate a beneficial effect of caspase inhibition in brain trauma for neuronal death occurring distant to the impact site. More importantly, the protective effect could be achieved even when the compound was administered in a delayed fashion, indicating relevance in the clinical setting.

TRAUMA ACTIVATES THE ENDOGENOUS CANNABINOID LIGAND-RECEPTOR SYSTEM

Transcriptional mRNA expression and ligand binding capacity of cerebral cannabinoid receptors were assessed at defined time points post-trauma and compared to levels of the endogenous cannabinoid receptor ligands, anandamide and 2-arachidonoyl glycerol (2-AG). While loss of a neuron-specific mRNA marker was observed after induction of head trauma, levels of cannabinoid CB_1 receptor mRNA transcription and ligand binding capacity were upregulated in the ipsilateral cerebral cortex. These alterations were most prominent in the proximity of the impact site of the contusive force (Hansen *et al.*, 2001a). Accumulation of anandamide and its precursor, but not 2-AG, was apparent in the ipsilateral cortex after induction of head trauma, in particular 24 hours post-injury (Hansen *et al.*, 2001a,b), which suggest an enhanced activity of the endogenous cannabinoid receptor-ligand system in the developing brain as a response to contusive head trauma.

Since central presynaptically located cannabinoid receptors suppress the activity of a range of neurotransmitter systems, including glutamatergic and GABAergic neurotransmission (Schlicker and Kathmann, 2001; Wilson and Nicoll, 2002), and endogenous cannabinoids are found in much higher concentrations when neurons are in an injured state (Hansen *et al.*, 2000), it is believed that these substances and their receptors may constitute a putative endogenous response aiming at dampening the destructive impact of brain insults (Hansen *et al.*, 2000; Piomelli *et al.*, 2000). Paradoxically, exogenously induced overactivation of neuronal cannabinoid receptors can also induce apoptotic-like neurodegeneration, presumably depending on the cell type, cell developmental state and degree of activation (Downer *et al.*, 2001; Guzmán *et al.*, 2001). Also, blockade of cannabinoid receptors induces neuroprotection in a developmental model of NMDA-induced excitotoxic brain damage (Hansen *et al.*, 2002). It therefore remains to be established under what circumstances and in which direction intrinsically upregulated cannabinoid ligand-receptor function affects pro-survival signaling pathways in the immature brain and whether the endogenous cannabinoid system may represent a target for pharmacotherapy in pediatric TBI.

CONCLUSIONS

Traumatic injury to the developing central nervous system has two major components, an acute excitotoxic component at the site of the insult and a delayed apoptotic component affecting the impact site as well as deeper brain structures. The number of brain cells affected by the apoptotic component is disproportionally larger than the number of cells degenerating by an excitotoxic mechanism (Bittigau *et al.*, 1999). Given our findings, targeting the downstream effectors of neuronal apoptosis in the acute phase of the insult has therapeutic potential in the treatment of traumatic injury to the immature brain. Antiapoptotic therapies may give cells enough time to establish intrinsic protection systems and restore cellular homeostasis and function (Han and Holtzman, 2000). However, since apoptosis is also a physiological process in the developing brain, studies addressing the long-term functional effects following caspase inhibition appear to be potential targets for future research (Gillardon *et al.*, 1999).

Acknowledgements

This work was supported by BMBF grant 01KO95151TPA3 and a Rahel-Hirsch award from the Humboldt University.

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