Temporal Pattern of Neurodegeneration, Programmed Cell Death, and Neuroplastic Responses in the Thalamus After Lateral Fluid Percussion Brain Injury in the Rat

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Abstract
The effects of traumatic brain injury (TBI) on the thalamus are not well characterized. We analyzed neuronal degeneration and loss, apoptosis, programmed cell death—executing pathways, and neuroplastic responses in the rat thalamus during the first week after lateral fluid percussion injury (LFPI). The most prominent neurodegenerative and neuroplastic changes were observed in the region containing the posterior thalamic nuclear group and ventral posteromedial and posterolateral thalamic nuclei ipsilateral to the LFPI. There was progressive neurodegeneration in these regions, with maximal neuronal loss on Day 7. Increases in numbers of apoptotic cells were detected on Day 1 and were enhanced on Days 3 and 7 after TBI. There was unchanged expression of active caspase-3 at all postinjury time points, but there was increased expression of apoptosis-inducing factor (AIF) on Day 7. The AIF nuclear translocation was detected on Day 1 and was maximal on Day 7. Total thalamic synaptophysin expression was unchanged, but immunostaining intensities were increased at all time points after TBI. Decreased growth-associated protein-43 expression and signal intensity were observed on Day 1. Our results suggest that progressive neuronal damage and loss, AIF signaling pathway–dependent programmed cell death, and limited neuroplastic changes occur in the rat thalamus during the first week after LFPI induction.

Key Words: Apoptosis, Neurodegeneration, Neuroplasticity, Rat, Thalamus, Traumatic brain injury.

INTRODUCTION
Traumatic brain injury (TBI) is the leading cause of death and disability in individuals younger than 45 years worldwide and represents a serious public health problem (1–3). Although our knowledge of the pathobiology of TBI has improved in the last decade, much is not understood and there are no effective therapies at present (4–7). The complex pathobiology of TBI includes the primary injury of the brain tissue and cerebral vasculature caused by the direct mechanical impact and secondary events, including excitotoxicity, ischemia, free radical generation, inflammation, and apoptosis, which lead to cell death and behavioral deficits (3, 8–13).

Traumatic brain injury affects many brain regions (8, 14, 15). Cerebral cortex and hippocampus are the most examined brain structures to date, although some studies have shown that the TBI-induced damage is also present in other brain regions (4, 15).

The thalamus, the brain relay center, is included in the regulation of many cognitive, sensory, motor, and behavioral functions. This brain structure directs incoming information to different cortical, subcortical, and cerebellar regions via numerous efferent projections (16). Therefore, damage of the thalamus and its projection fibers can result in widespread and extensive functional impairment (17). The thalamus is relatively remote from the injured cortex and is, therefore, less disposed to direct contusion after TBI; nevertheless, several studies suggest that disruption of the thalamic integrity may occur in brain trauma and affect prognosis and the development of posttraumatic sequelae (16, 18–20). Although thalamic damage has been reported in both human and experimental TBI, results were shown as descriptive and/or semiquantitative data and were related only to single rather than multiple time points after TBI in most of the published studies (8, 15, 16, 18–22). Moreover, the mechanisms by which the cell death and structural damage occur in this region in TBI are not sufficiently elucidated. In particular, TBI induced both caspase-dependent and caspase-independent programmed cell death pathways in different experimental models, mostly in the cortex and the hippocampus (23–25), but which of these pathways is involved in neuronal death in the thalamus is unknown. Moreover, the temporal patterns of post-TBI synaptic plasticity alterations in the thalamus (on which recovery of the brain functions strongly depends) in the lateral fluid percussion injury (LFPI) model of brain trauma have not previously been investigated. This model closely replicates the closed head brain injury typically found in victims of motor vehicle accidents or falls (26). It is characterized by primarily cortical damage and diffuse injury of subcortical neuronal tissue and is, therefore, a highly clinically relevant and widely accepted TBI model (27).

The purpose of this study was to investigate some of the changes related to the damage and recovery in the thalamus at 1, 3, and 7 days after moderate LFPI. Points of our interest
were the extent of neurodegeneration, neuronal cell loss and apoptosis, roles of caspase-dependent and caspase-independent pathways in programmed cell death, as well as the levels of some indicators of synaptic plasticity.

MATERIALS AND METHODS

Animals and Treatment

Experiments were performed on adult male Wistar Hannover rats weighing from 350 to 450 g. Rats were maintained on a 12-hour light-dark cycle and allowed free access to food and water. All experiments were performed between 10:00 AM and 2:00 PM in a silent room at a temperature of 22°C to 24°C. All animal procedures were approved by the Faculty Ethical Committee and conducted in agreement with the national laws and rules (NN 135/06; NN 37/13; NN 50/13) and with the guidelines set by the European Community Council Directive of November 24, 1986 (86/609/EEC). All efforts were made to minimize animal suffering.

Traumatic brain injury in rats was induced using the LFPI model (28). Animals were anesthetized with isoflurane (4% induction, 2% maintenance) in nitrous oxide/oxygen (2:1) mixture. On fixing the animal in the stereotaxic frame, the skin covering the skull was incised and a 5-mm craniotomy was made over the left parietal cortex, maintaining the dura mater intact. A plastic female Luer Lock adapter was placed at the craniotomy site, fixed with dental cement, and used to connect the animal to the injury device (VCU Biomedical Engineering Facility, Richmond, VA). After the appearance of withdrawal reflex to paw pinch, TBI of moderate severity (1.8 atm) was induced by injection of a transient (duration 21–23 milliseconds) saline pulse into the epidural space. Only the rats with postinjury apnea duration of less than 60 seconds were included in the analyses. After the injury, the Luer Lock fitting was removed, the skin was sutured, and an antibiotic ointment was applied to the wound. Rats were then returned to their home cages and provided free access to food and water. The animals were randomly divided into experimental groups (n = 4–7 per group; total number of animals used = 46) and killed at 1, 3, or 7 days after the LFPI. Sham-operated control group rats had craniotomies but were not injured. Those animals were killed 1 day after the sham procedure.

Tissue Preparation

For histochemical analyses, the animals (n = 20) were deeply anesthetized with sodium thiopental and perfused transcardially with PBS followed by fixative, 4% paraformaldehyde in PBS. Brains were removed and stored in the fixative solution for 20 to 22 hours at 4°C and embedded in paraffin. With the aim to compare the rats’ thalami of different experimental groups at the same anatomic level, the tissue sectioning was done at approximately 3.8 mm posterior to the bregma according to Paxinos and Watson (29). Tissue sections were 3 µm thick. All histologic analyses were done for the region containing posterior thalamic nuclear group (PO) and ventral posteromedial (VPM) and ventral posterolateral (VPL) thalamic nuclei (Fig. 1). Thalamic tissue ipsilateral and contralateral to the brain injury was analyzed by Fluoro-Jade B fluorescence, whereas only the thalami ipsilateral to the brain trauma procedures were examined by the other histologic methods. For Western blotting analyses, animals (n = 26) were decapitated, their brains quickly removed, and whole thalami ipsilateral to the brain trauma were dissected, frozen in liquid nitrogen, and stored at −80°C until use.

Fluo-Jade B Fluorescence

For Fluoro-Jade B staining, the slides were deparaffinized in xylene, dehydrated in ethanol, and then treated for 10 minutes with a 0.06% potassium permanganate solution. Sections were rinsed twice with dH2O for 1 minute and incubated in 0.001% Fluoro-Jade B (Chemicon, Millipore, Billerica, MA)–0.1% acetic acid staining solution for 20 minutes in the dark. After rinsing in dH2O thrice for 1 minute and air-drying, sections were immersed in xylene and coverslipped. Brain sections were examined under ultraviolet light with a fluorescein isothiocyanate fluorescence filter cube (Olympus BX 51 microscope, Olympus DP 70 digital camera; Olympus, Tokyo, Japan). Quantification of the Fluoro-Jade B–stained neurons was done in the thalamus at 200× final magnification. For each animal, 3 photomicrographs of the thalamic sections were done. For each photomicrograph, 3 regions of interest (ROIs) of the approximate size of 0.064 mm² were analyzed (total number of ROIs per animal = 9). Average number of Fluoro-Jade B–positive cells was calculated for every animal and for the experimental groups.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) histochemistry using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Millipore) was performed according to the manufacturer’s instructions. In brief, brain tissue sections were deparaffinized, washed in PBS, and pretreated with proteinase K (20 µg/mL) for 15 minutes at room temperature (RT). After washing in PBS, equilibration buffer was applied on the specimens for 10 seconds, followed by 1-hour incubation with labeling solution of working-strength concentration.
terminal deoxynucleotidyl transferase enzyme in a humidified chamber at 37°C. After 15 seconds of agitation in a Coplin jar, sections were incubated for 10 minutes in the working-strength stop/wash buffer to terminate the labeling reaction and subsequently washed thrice in PBS. For visualization of the DNA fragments, slides were incubated in working-strength antidigoxigenin conjugate for 30 minutes at RT, mounted with antifade mounting medium, and examined by fluorescence microscopy. Quantification of TUNEL-positive cells in the aforementioned region was done at 400× final magnification, with an area of each microscopic field of approximately 0.14 mm². Three photomicrographs from 1 section per animal were analyzed.

Fluorescent Immunohistochemistry and Laser Scanning Confocal Microscopy

Deparaffinized and rehydrated sections were blocked in Tris-buffered saline containing 5% serum, 5% bovine serum albumin, and 0.025% Triton X-100. The sections were then incubated overnight at 4°C with the primary antibody in Tris-buffered saline containing 1% serum, 1% bovine serum albumin, and 0.025% Triton X-100. Primary antibodies were mouse monoclonal anti–neuron-specific nuclear protein (NeuN; Millipore), rabbit polyclonal anti–apoptosis-inducing factor (AIF) (Abcam, Cambridge, MA), mouse monoclonal anti-synaptophysin (SYP; Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti–growth-associated protein-43 (GAP-43; Sigma-Aldrich, St. Louis, MO). On the next day, sections were incubated for 2 hours at RT with the appropriate secondary antibodies: biotinylated rabbit anti-mouse antibody (Dako, Glostrup, Denmark) or DyLight 594–conjugated goat anti-rabbit antibody (Pierce, Rockford, IL). To the sections incubated with biotinylated secondary antibody DyLight 594-conjugated streptavidin was added for 30 minutes at RT. The samples immunolabeled for AIF were counterstained with DAPI. On some sections immunolabeled with anti-SYP, after the secondary antibody incubation, streptavidin–horseradish peroxidase complex was applied, followed by 3,3′-diaminobenzidine chromogen (DAB; Dako) with hematoxylin counterstain. Negative controls were treated identically but with omission of the primary antibodies.

Sections marked with fluorophores were examined by epifluorescence microscopy using the appropriate light filter cube. Quantification of the NeuN-stained neurons and of SYP and GAP-43 signal fluorescence intensity determinations in the thalami were conducted on images acquired at 200× magnification; AIF signals and subcellular localizations were observed on photomicrographs taken at 400× magnification. Photomicrographs of DAB-stained thalamic sections were recorded by light microscopy at 1,000× magnification, with oil immersion objective using the aforementioned microscope.

For quantification of NeuN-immunostained neurons, 3 thalamic sections from each animal were photographed and ROIs of approximately 0.1 mm² were analyzed (total number of ROIs per animal = 9). The average number of NeuN-positive neurons was calculated for every animal and for the experimental groups.

For quantification of cells with AIF-positive cytoplasm or nuclei, counterstaining with DAPI was used. Three photographs per section (~0.14 mm²) per animal were analyzed. The AIF-positive cells with either cytoplasmatic or nuclear AIF localisation were counted in each photograph.

For quantification of the immunofluorescent signals of SYP and GAP-43, 3 ROIs (~0.036 mm² per section, 3 sections per animal) were selected and analyzed. Conditions of microscopy and photography were maintained constant throughout the experiment. Immunoreactivity was quantified by measuring the integrated optical density ([IOD] intensity of fluorescence per unit of surface area of ROI), and the results were corrected for background by subtracting the average intensity of an area on each slide that showed no fluorescence and appeared black on images. The corrected total fluorescence (CTF) was calculated according to this formula:

$$CTF = IOD - \left(\text{area of selected ROI} \times \text{mean fluorescence of the background}\right).$$

Thalamic sections double stained for the AIF and DAPI were further visualized by confocal microscopy. Images were taken with Zeiss LSM700 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) by 40× Plan Apochromat oil immersion objective lenses and using the 0.5-μm z sectioning.

For analyses of confocal images from the recorded z sections, the central section of a single cell that contained the most pronounced DAPI nuclear staining was selected. On that image, a line through the cell was drawn to perform intensity profile plotting for the AIF (red fluorescence) and DAPI (blue fluorescence) staining and thus determine the distribution of AIF in the subcellular compartments.

Western Blotting

Protein expression in the samples of the thalami was analyzed by Western blotting. Briefly, protein samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes. After 1 hour blocking with an appropriate blocking buffer at RT, membranes were incubated with primary antibody solutions overnight at 4°C. Primary antibodies used were rabbit polyclonal anti–cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), rabbit polyclonal anti-AIF (Abcam), mouse monoclonal anti-SYP (Santa Cruz Biotechnology), mouse monoclonal anti–GAP-43 (Sigma-Aldrich), and mouse monoclonal anti–β-actin (Santa Cruz Biotechnology). The following day, membranes were incubated for 1 hour with appropriate biotinylated secondary antibodies (Dako Cytomation), followed by streptavidin–horseradish peroxidase conjugate application for 30 minutes, all at RT. Blots were developed with Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Signals were detected using the Kodak Image Station 440CF, and the bands intensities were quantified by the Kodak 1D Image Analysis Software (Eastman Kodak, Rochester, NY).

Laboratory Data and Statistical Analyses

Numbers of Fluoro-Jade B–, NeuN−, and TUNEL-positive cells and cells with positive cytoplasmic or nuclear
AIF immunostaining are expressed per square millimeter. For densitometric analyses of protein expression, the values are expressed as relative optical densities of the related bands, corrected for the corresponding β-actin contents. Synaptophysin and GAP-43 immunofluorescence intensity levels are expressed as percentages (%) of the control group (100%).

Statistical analyses were performed using the Statistica software version 12.0 (StatSoft, Inc, Tulsa, OK). Data were analyzed using the nonparametric statistical Kruskal-Wallis analysis followed by the Mann-Whitney U test. Results are expressed as mean ± SEM. In all comparisons, \( p < 0.05 \) was considered to indicate statistical significance.

**RESULTS**

**Neurodegeneration and Neuron Loss**

To determine neurodegenerative changes after LFPI, Fluoro-Jade B staining was used. Fluoro-Jade B–stained neurons were not observed in the thalami contralateral to the brain trauma (Fig. 2A–D) or in the thalami ipsilateral to the sham brain injury (Fig. 2E). In contrast, degenerating neuronal cell bodies and their processes were demonstrated in the thalami ipsilateral to the LFPI; the most numerous were in the region containing the PO, VPM, and VPL at all time points after the trauma (Fig. 2F–H). Kruskal-Wallis test revealed significant overall changes in the number of Fluoro-Jade B–stained cells in this ipsilateral thalamic area (Fig. 2I; \( p < 0.001 \)). The number of Fluoro-Jade B–stained neurons in the ipsilateral thalami of the injured animals was significantly increased in relation to the control group at all days after LFPI; the values were also significantly higher on Days 3 (170.89 ± 7.42 cells/mm²) and 7 (195.56 ± 17.68 cells/mm²) versus Day 1 (53.50 ± 7.41 cells/mm²) after the injury. Because Fluoro-Jade B staining revealed no degenerative neurons in the thalami contralateral to LFPI, for all other examinations (i.e. histologic and Western blotting), samples from the ipsilateral thalami were used.

In the injured animals, the fewest NeuN-positive neurons were observed in the region containing the PO, VPM, and VPL. The NeuN immunostaining in this thalamic area was less pronounced in the traumatized rats at all time points after LFPI versus the staining in the control group (Fig. 3A–D).Statistical analysis of these groups’ results revealed that the average number of thalamic NeuN-positive cells on Days 1 (449.94 ± 20.83 cells/mm²), 3 (381.56 ± 23.82 cells/mm²), and 7 (290.67 ± 31.49 cells/mm²) after LFPI were significantly lowered in relation to the control group on Days 3, 5, and 7 (Days 1-7) after LFPI. Because Fluoro-Jade B staining revealed no degenerative neurons in the thalami contralateral to LFPI, for all other examinations (i.e. histologic and Western blotting), samples from the ipsilateral thalami were used.

**Programmed Cell Death**

To assess apoptosis levels in the thalami ipsilateral to the LFPI, TUNEL histochemistry combined with DAPI staining was performed. In control rats, the thalamus exhibited few, if any, TUNEL-positive cells (Fig. 4A) in contrast to the injured animals, in which apoptotic cells were observed in the mentioned

**FIGURE 2.** Neurodegeneration in the thalamus after lateral fluid percussion injury (LFPI). (A–H) Representative photomicrographs of the Fluoro-Jade B–stained region containing posterior thalamic nuclear group and ventral posteromedial and ventral posterolateral thalamic nuclei contralateral (A–D) or ipsilateral (E–H) to the brain trauma in rats of the control group (Control) and in the animals killed at 1 day (LFPI 1d), 3 days (LFPI 3d), and 7 days (LFPI 7d) after LFPI. Scale bar = 500 μm. Insets show higher-magnification images from the related sections. (I) Histogram shows the number of Fluoro-Jade B–positive cells per square millimeter in the abovementioned thalamic region ipsilateral to the brain trauma for each group expressed as mean ± SEM (n = 5 rats per group). * \( p < 0.05 \), significantly different from the Control; # \( p < 0.05 \), significantly different from the LFPI 1d group.
brain structure, especially in the PO, VPM, and VPL thalamic regions, at each time point after LFPI (Fig. 4B–D). It is evident that the number of TUNEL-positive cells was higher in the rats killed 3 and 7 days after TBI in relation to their number detected on Day 1 after the brain trauma (Fig. 4B–D). The number of TUNEL-positive cells in the PO, VPM, and VPL of the injured rats on Days 1 (54.02 ± 5.92 cells/mm²), 3 (122.27 ± 5.66 cells/mm²), and 7 (161.53 ± 17.47 cells/mm²) after TBI was significantly increased in relation to the result obtained in the animals of the control group (11.27 ± 4.18 cells/mm²) (Fig. 4E; p < 0.001). The numbers of TUNEL-labeled cells in the thalamic region on 3 and 7 days after TBI were also significantly higher versus the number 1 day after TBI (Fig. 4E).

To determine whether LFPI-induced apoptosis was mediated by the caspase-3 programmed cell death pathway, we measured caspase-3 expression levels in samples of whole ipsilateral thalami (Fig. 5A). There was no significant difference in the levels of cleaved caspase-3 expressions in the thalami between the rats of the control group and the groups of the injured animals killed at all investigated time points after LFPI (p = 0.349). Because this result indicated that the thalamic post-LFPI programmed cell death is not likely to be caspase-3 dependent, we assessed levels of AIF expression in the thalamic tissue samples. Densitometric measurements of immunoblots showed a statistically significant elevation of the AIF protein level in the thalamic tissue ipsilateral to the LFPI only on Day 7 after injury versus its value in the control conditions (p = 0.036) (Fig. 5B).

Furthermore, because Fluoro-Jade B and NeuN immunostaining and TUNEL histochemistry revealed the most significant changes after LFPI in the PO, VPM, and VPL, we investigated translocation of the AIF from the mitochondria/cyttoplasm to the cell nuclei in the previously mentioned thalamic area. Apoptosis-inducing factor immunoreactivity was not detected in the nuclei but was observed in the cytoplasm of a few thalamic cells in the control rat group (Fig. 6A). In contrast, AIF immunoreactivity within nuclei of thalamic cells as well as in their cytoplasm was pronounced in the traumatized rats killed at all time points investigated; the highest nuclear AIF immunoreactivity was detected at 7 days after TBI (Fig. 6B–D). Significant differences in the number of the thalamic cells with AIF-positive cytoplasm (p = 0.006) and nuclei (p = 0.001) were demonstrated. The number of thalamic cells with AIF cytoplasmic localization was significantly increased at 1 (20.07 ± 5.14 cells/mm²), 3 (14.00 ± 2.09 cells/mm²), and 7 days (29.87 ± 4.26 cells/mm²) after TBI versus the numbers detected in control group animals (6.07 ± 1.90 cells/mm²) (Fig. 6E).

The percentages of cells with nuclear AIF staining were also significantly increased at 1 day (30.33 ± 6.56 cells/mm²), 3 days (30.80 ± 3.25 cells/mm²), and 7 days (95.20 ± 6.32 cells/mm²) after TBI compared with the number in the control conditions (0.00 cells/mm²). Moreover, counts of AIF-stained cell nuclei at 7 days after TBI were higher versus their numbers at earlier time points after injury.

To confirm the changes in AIF subcellular localization, laser scanning confocal microscopy of the sections with AIF immunofluorescent staining (which were counterstained with DAPI for the detection of the cell nuclei) was conducted. Only weak cytoplasmic AIF immunopositivity was detected in the thalamic section of the control animal group (Fig. 7A). In the animal killed 7 days after LFPI, both cytoplasmic and nuclear clear positive AIF immunofluorescent signals were observed (Fig. 7B–D). Representative images show cells with cytoplasmic AIF immunofluorescence (Fig. 7B), as well as cells with apparent chromatin condensation (Fig. 7C, D), with strong perinuclear (Fig. 7C) or nuclear (Fig. 7D) AIF signal, corresponding to different stages of apoptosis. In the lower panels of Figure 7E and F, plot profiles of the lines drawn across the body of a cell with strong cytoplasmic but weak nuclear AIF immunofluorescent signal (Fig. 7E) as well of a cell with nuclear AIF translocation (Fig. 7F), both taken in an animal 7 days after the brain injury, are presented. The histograms show that, in a cell with apparent nuclear condensation (Fig. 7F), colocalization of the AIF and DAPI signal was detected, whereas...
the cell with normal DAPI nuclear distribution and predominant cytoplasmic AIF immunopositivity does not show colocalization (Fig. 7E).

**Neuroplasticity**

Representative immunoblots and their densitometric analyses for the marker SYP in the whole thalamic tissue for each experimental group are shown in Figure 8A; there were no significant differences among the samples (p = 0.122). However, there was a remarkable increase in the intensity of SYP immunoreactivity in ipsilateral thalami of the injured rats at all time points investigated after LFPI that was most pronounced in the PO, VPM, VPL regions (Fig. 8B). The immunostaining was more pronounced in the injured rats at 1, 3, and 7 days after LFPI versus that in the control group. Synaptophysin signal intensities in the investigated thalamic region were significantly great at 1 (137.87% ± 7.53%), 3 (160.81% ± 7.86%), and 7 days (123.25% ± 2.15%) after TBI versus the control (100% ± 2.05%) (Fig. 8C; p = 0.014). Moreover, the maximal SYP intensity was on Day 3 after LFPI and decreased thereafter, although it was still increased relative to the control on Day 7. In sections immunostained for SYP and visualized by DAB staining, SYP immunoreactivity was predominantly diffuse and only some visible SYP granular staining around neuronal perikarya was evident in the controls. By contrast, in the injured rats, increased SYP immunoreactivity and granular SYP accumulation surrounding apparently normal as well as degenerated neuronal cells were demonstrated on Day 3 after TBI (Fig. 8D).

By Western blot, the level of GAP-43 expression in the whole ipsilateral thalami of the injured rats on Day 1 after TBI was significantly lowered in comparison with its level in the control animals (p = 0.042; Fig. 9A). The GAP-43 immunofluorescent signal intensity was less prominent in the investigated thalamic region of the rat killed at Day 1 in relation to the control and all other rats with LFPI (Fig. 9B). Quantification of the GAP-43 signal intensities showed a significant effect of the injury (p = 0.033), that is, signal intensity of the mentioned marker was significantly lowered in comparison with its level in the control animals in the PO, VPM, and VPL on Day 1 after LFPI (Fig. 9C).

**DISCUSSION**

Here, we report that the thalamus, particularly the PO, VPM, and VPL regions, is severely damaged with restricted neuroplastic responses in the week after moderate LFPI in rats. Alterations in the thalamus contralateral to the LFPI were not detected.

**Neurodegeneration and Neuron Loss**

Lateral fluid percussion injury induced significant neuronal damage and loss in the ipsilateral thalamus during the first week after the induction of the brain trauma, as demonstrated using Fluoro-Jade B, a marker of irreversibly injured neurons undergoing degeneration (8, 30). This damage was evident already on Day 1 and was even more pronounced 3 and 7 days after LFPI. In some previous studies, Fluoro-Jade B–positive staining was also observed in the thalami after LFPI in the rat at time points ranging from 1 to 15 days after the insult (15, 30–33). In some of these studies, the staining was semiquantified (30, 33) or examined only as a strain-related difference (32), whereas Sato et al (15) also showed a maximal number of thalamic Fluoro-Jade B–positive neurons at 7 days after injury. Unlike our results, this number was significantly higher versus values obtained on Day 1, but also on Day 3 after injury. The differences in the 2 studies may be caused by methodological differences, that is, Sato et al (15) used a more severe LFPI that was induced at a much more lateral position in comparison with our study.

To corroborate the results of the Fluoro-Jade B staining analysis, we applied NeuN immunohistochemistry to assess neuron preservation. We demonstrated a time-dependent significant decrease in neuron numbers in the investigated thalamic region versus control sham-operated animals. Approximately 25% of neurons were lost already at 1 day after the TBI induction; at 3 days, nearly 37% of neurons had died, and that number was further increased to more than 50% at 7 days after injury. Although previous studies assessed neuronal loss in the thalamus using the same TBI model, other methods such as acid fuchsin, cresyl violet, thionin, and silver staining were used; the results were descriptive and/or semiquantitative and at other time points after TBI (9, 15). To our knowledge, ours is the first report regarding neuronal loss in the thalamus obtained by immunostaining for NeuN in this TBI model.

These 2 sets of results indicate that, after the early initial wave of neuronal death detected already within the first 24 hours after injury, there was a later onset of cell death mechanism that contributed to additional cell loss at 3 and 7 days after TBI. Therefore, our further focus was directed to determination of cell death mechanisms that could contribute to the prolonged thalamic cellular damage.

**Programmed Cell Death**

We used TUNEL staining in combination with DAPI nuclear counterstaining to quantify the level of apoptotic changes in the thalamus after TBI. The TUNEL–positive cells were observed in the injured thalami, particularly in the PO, VPM, and VPL, at all time points investigated after the TBI. Apoptotic cells were demonstrated as early as 24 hours after the TBI, and there were more apoptotic cells at later time points. Conti et al (8) previously reported that the number of thalamic TUNEL positive cells in the injured rats was similar to that found in the sham-operated control rats during the first week after LFPI. The differences between this and our study may be caused by the rat strains used. Namely, our experiments were done on Wistar Hannover rat strain, whereas Conti et al (8) used Sprague-Dawley rats. A major effect on injury severity and recovery in the LFPI brain trauma was previously described in Sprague-Dawley versus Long Evans rats (34) and between Fisher 344 and Sprague-Dawley rats (32).

To identify the types of programmed cell death–executing pathways underlying apoptotic cell death, we determined cleaved caspase-3 and AIF protein expression. Levels of expression of these proteins, as well as of SYP and GAP-43, were determined in whole ipsilateral thalami samples. After the initial surge of necrotic cell death in the injury core located in the parietal cortex and adjacent subcortical tissue after TBI (35, 36), a significant portion of neurons and other types of
brain cells die because of initiation of caspase-dependent and caspase-independent programmed cell death mechanisms (8). Activation of caspase-dependent programmed cell death culminates with the activation of the main executioner protease, caspase-3, which seems to be the major effector caspase in neuronal apoptosis (37). This protein is involved in post-traumatic cell death in different brain regions both in humans (38, 39) and in experimental TBI (23, 24, 40, 41). We found that activated cleaved caspase-3 protein expression was not different at 1, 3, and 7 days after LFPI versus the result obtained in the control group. This result contrasts with the observations of Keane et al (42) who detected cleaved caspase-3-positive cells with apparent apoptotic morphology in the thalamus at 3 and 7 days after the LFPI in the rat. The difference in this and our study may be caused by distinctions in the experimental procedures, that is, Keane et al (42) used immunohistochemistry and did not quantify the overall level of caspase-3 expression.

We observed significant changes both in the levels and cellular localizations of AIF in the thalamus after TBI. Apoptosis-inducing factor is a ubiquitously expressed flavoprotein, the mature form of which is found in the mitochondrial intermembrane space (43, 44). Under normal physiologic conditions, AIF activity is required for the proper functioning of the respiratory chain. After the activation of the death-promoting stimulus, AIF translocates from the mitochondria into the cytosol and then to the nucleus where it mediates chromatin condensation and large-scale fragmentation of DNA (45–50). Several studies have suggested that AIF translocation to the nuclei promotes caspase-independent cell death induced by different stimuli such as glutamate toxicity, oxidative stress, hypoxia, or ischemia (50–53).

To our knowledge, our study is the first report on AIF expression levels and intracellular localization changes in the LFPI model of brain trauma and the first to show AIF alterations in the thalamus in a TBI model. We demonstrated increased

FIGURE 4. Apoptosis in the thalamus ipsilateral to lateral fluid percussion injury (LFPI). (A–D) Extent of apoptosis in the region containing posterior thalamic nuclear group and ventral posteromedial and ventral posterolateral thalamic nuclei in rats of the control group (Control) and in the animals killed at 1 day (LFPI 1d), 3 days (LFPI 3d), and 7 days (LFPI 7d) after LFPI. Representative photomicrographs of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)– and DAPI-stained sections for each group. Scale bar = 100 μm. (E) Histogram shows the number of TUNEL-positive cells per square millimeter in the abovementioned thalamic region for each group expressed as mean ± SEM (n = 5 rats per group). *p < 0.05, significantly different from the Control; #p < 0.05, significantly different from the LFPI 1d group.
AIF  |  DAPI  |  Merge

Control

LFPI 1d

LFPI 3d

LFPI 7d

E

E

AIF positive cells/mm²

Control  |  LFPI 1d  |  LFPI 3d  |  LFPI 7d

Cytoplasm  |  Nucleus

*  |  #  |  *  |  #

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thalamic AIF expression at 7 days after TBI. Moreover, in the area of the PO, VPM, and VML of the control brains, AIF immunoreactivity was found in the cytoplasm of only few thalamic cells but not in their nuclei. The number of thalamic cells with AIF-positive cytoplasm and nuclei in this thalamic region was significantly increased versus controls at all days after TBI. The highest number of thalamic cells with AIF-positive nuclear immunostaining in the most damaged thalamic region was observed on Day 7 after TBI. This value was also significantly higher in comparison with the level of thalamic cells with AIF-positive nuclei detected in animals with TBI and killed at earlier time points after TBI.

Changes in subcellular localization of AIF were additionally confirmed using confocal microscopy. Analyses of

FIGURE 7. Confocal microscopy analysis of subcellular distribution of the apoptosis-inducing factor (AIF) in the thalamus ipsilateral to lateral fluid percussion injury (LFPI). (A–D) Microphotographs of thalamic sections containing posterior thalamic nuclear group and ventral posteromedial and ventral posterolateral thalamic nuclei from a control rat (A) and of those from a rat killed 7 days after LFPI (LFPI 7d) (B–D). Arrows point to the cells with different types of AIF staining. Scale bar = 10 μm. (E, F) In the lower panel, fluorescence intensity profiles, measured along the lines drawn across individual cells with different subcellular distributions of AIF in an injured rat killed at Day 7 after LFPI. Photomicrographs and line scan plot profiles of red (AIF) and blue (DAPI) fluorescent signal in the cell with cytoplasmic (E) and a cell with nuclear (F) AIF staining are displayed. AU, arbitrary units.

FIGURE 6. Apoptosis-inducing factor (AIF) activation in the thalamus ipsilateral to lateral fluid percussion injury (LFPI). Extent of AIF immunofluorescence in the region containing posterior thalamic nuclear group and ventral posteromedial and ventral posterolateral thalamic nuclei in rats of the control group (Control) and in the animals killed at 1 day (LFPI 1d), 3 days (LFPI 3d), and 7 days (LFPI 7d) after LFPI. (A–D) Representative photomicrographs of anti-AIF– and DAPI-stained sections for each group. Insets show higher-magnification images from the related group sections. Arrows point to cells with cytoplasmic staining; arrowheads show cells with nuclear AIF staining. Scale bar = 100 μm. (E) Histogram shows the number of the cells with AIF-positive nuclei and the cytoplasm per square millimeter in the abovementioned thalamic region for each group expressed as mean ± SEM (n = 4–5 rats per group). * p < 0.05, significantly different from Control comparing separately cells with AIF-stained cytoplasm or nuclei; # p < 0.05, significantly different from the LFPI 1d and LFPI 3d groups comparing cells with AIF-stained nuclei.
the sections from the rat killed 7 days after TBI revealed different types of AIF staining patterns, some of which are related to changes in nuclear integrity, that is, in the cell with pronounced cytoplasmic AIF staining, the DAPI staining pattern in the nucleus was diffuse. By contrast, in the cells with perinuclear and/or nuclear AIF-positive staining, chromatin condensation was observed. This finding is in accordance with previous studies, which demonstrated that, after nuclear translocation, AIF binds to the DNA directly and, together with other proteins (e.g. histone H2AX and cyclophilin A) initiates chromatin condensation and large-scale DNA fragmentation (25, 54). Our results of plot profile analyses of AIF versus DAPI staining within a single cell confirmed this notion because, in the cell with the pyknotic nucleus, AIF translocation was detected. In contrast, in the cell with normal nuclear morphology, predominantly cytoplasmic accumulation of this protein was observed.

Taken together, our results of the Western blotting analyses and the immunofluorescence data demonstrated a significant increase in AIF expression and maximal AIF nuclear transfer in the thalamus from the injured rats at 7 days after LFPI. As previously mentioned, our study also showed increased number of AIF-positive cells with nuclear signals at 1 and 3 days after the injury but no change in total AIF protein levels at these time points. This discrepancy may be because of the fact that the level of AIF expression was detected in the extracts of the whole thalamic tissue including cell cytoplasm and nuclei. Unlike Western blotting, AIF immunofluorescent staining enabled the detection of different AIF subcellular localizations and that is why it was possible that the total thalamic AIF expression levels were unchanged on Days 1 and 3 after TBI when nuclear AIF-positive immunostained fractions were increased. This suggestion is in accordance with the previously published finding of better availability of AIF protein to the applied antibodies after its release into the cytosol than during its stay in the intermembrane space of the mitochondria (47).

Neuroplasticity

Traumatic axonal injury is commonly found in TBI cases regardless of injury severity, and it is a good predictor of both poor survival and long-term outcome (5). Because axonal damage, along with the neuronal and glial cell body damage, affects and interrupts signal transmission, cytoskeletal reorganization and axonal extension are important mechanisms that may enable functional improvement after CNS injuries (55, 56). Neuroplastic regenerative processes, although crucial for post-injury recovery after brain trauma, are insufficiently known.

Synaptophysin is a major integral protein of synaptic vesicles and is closely related to axonal reorganization and synaptogenesis (57). To our knowledge, the present study is the first report on thalamic SYP expression in the LFPI model. We showed that the brain trauma did not influence the level of the SYP expression in the tissue of the whole thalami but that it induced significant increase in the thalamic SYP immunoreactivity intensity in the PO, VPM, and VPL areas during the whole first week after the TBI induction. Therefore, we suggest that TBI provoked SYP redistribution to the most damaged thalamic region to compensate for significant neuronal loss. Maximal SYP immunoreactivity was detected on Day 3, after which it decreased on Day 7 to a level that was still significantly higher in comparison with its value in the control conditions. Given that the SYP is often used to quantify the number of terminals during neuroanatomic remodeling, we suggest that the increased SYP immunostaining visible after TBI could be related to posttraumatic accumulation of this protein in presynaptic vesicles as well as to increased number of synapses in the affected thalamic nuclei. The decrease of the SYP signal intensity in the injured thalami on Day 7 compared with its maximal level detected on Day 3 after TBI could be the result of the detachment of synaptic terminals after neuronal cell death. Thus, the decrease in SYP immunoreactivity coincided with maximal neuronal loss obtained also on Day 7 after the TBI. Furthermore, not only quantitative but also qualitative changes in the SYP staining pattern were revealed in the injured thalamic sections in which SYP granular accumulation was more pronounced as opposed to predominantly diffuse SYP immunoreactivity observed in the control conditions.

Growth-associated protein-43 is a phosphoprotein of the nerve terminal membrane, one of the growth-related structures involved in CNS development and also in regeneration after various injuries of the adult CNS, including seizures (58, 59), neuronal axotomy (60), spinal cord injury (61, 62), cerebral ischemia (63, 64), and TBI (65–70). Postinjury upregulation in GAP-43 mRNA and protein expression seems to correlate with the neuronal capacity for processes extension and may, therefore, be crucial for determining which neurons are capable of axonal regeneration or sprouting (55). Because this protein has a key role in restructuring of axonal connections and synapse formation, it has been used as a marker of synaptic plasticity (71).

Our study is the first one in which temporal pattern of GAP-43 expressions in the thalamus during the first week after LFPI was investigated. We found that GAP-43 expression level

FIGURE 8. Synaptophysin (SYP) expression in the thalamus ipsilateral to lateral fluid percussion injury (LFPI) in rats of the control group (Control) and in animals killed at 1 day (LFPI 1d), 3 days (LFPI 3d), and 7 days (LFPI 7d) after LFPI. (A) Representative immunoblots of each group and the SYP expression levels (SYP/β-actin ratios). Data represent mean ± SEM (n = 5 rats per group). (B) Representative photomicrographs of the anti-SYP-stained region containing posterior thalamic nuclear group and ventral posteromedial and ventral posterolateral thalamic nuclei for each group. Scale bar = 200 μm. (C) Histogram shows the SYP signal intensity levels (% of the Control) in the abovementioned thalamic region for each group expressed as mean ± SEM (n = 5 rats per group). * p < 0.05 versus Control. (D) Representative photomicrographs of SYP qualitative changes in the thalamic area investigated. Arrows point to neurons surrounded by diffuse weak SYP immunoreactivity in the control animal (Control). Arrowheads show marked granular SYP accumulation enclosed the thalamic neurons in the injured rat killed at Day 3 after LFPI (LFPI 3d). Scale bar = 25 μm.
FIGURE 9. Growth-associated protein-43 (GAP-43) expression in the thalamus ipsilateral to lateral fluid percussion injury (LFPI) in rats of the control group (Control) and in animals killed at 1 day (LFPI 1d), 3 days (LFPI 3d), and 7 days (LFPI 7d) after LFPI. (A) Representative immunoblots of each group and GAP-43 expression levels (GAP-43/β-actin ratios). Data represent mean ± SEM (n = 4–5 rats per group). * p < 0.05 significantly different versus Control. (B) Representative photomicrographs of the anti-GAP-43-stained region containing posterior thalamic nuclear group and ventral posteromedial and ventral posterolateral thalamic nuclei for each group. Scale bar = 200 μm. (C) Histogram shows the GAP-43 signal intensity levels (% of the Control) in the abovementioned thalamic region for each group expressed as mean ± SEM (n = 4–5 rats per group). * p < 0.05, significantly different versus Control.
in the whole thalamus and its immunoreactivity in the PO, VPM, and VPL area were initially decreased and then returned to close to the control values on Day 7 after TBI. Previously, Emery et al (72) demonstrated unchanged thalamic GAP-43 immunoreactivity on Day 2 after LFPI.

Taking into consideration our results regarding the thalamic levels of SYP and GAP-43 immunoreactivity, it seems that neuroplastic possibly regenerative responses in this brain structure were limited in our experimental conditions. Also, these results suggest heterogeneity of different neuroplastic responses to the brain trauma.

In summary, we demonstrated temporal changes in the extent of neurodegeneration and neuronal loss, programmed cell death, and synaptic reorganization processes in the rat thalamus during the first 7 days after LFPI. In particular, the area containing PO, VPM, and VPL showed remarkable neurodegeneration, neuronal loss, apoptotic changes, and neuroplastic responses. Thalamic neuronal degeneration and cell loss progressed up to 7 days after the brain trauma. Interestingly, in the thalamus, unlike in some other brain regions such as the cortex, caspase-3-dependent apoptotic pathway did not seem to have an important role in post-TBI cell death. In the investigated brain region, the predominantly activated programmed cell death pathway seems to be related to the mitochondrial release and nuclear translocation of AIF leading to caspase-independent apoptosis. In addition, only some neuroplastic and potentially regenerative changes in the rat thalamus during the first week after LFPI were detected. The latter finding points out the insufficient potential of the injured thalamic tissue to promote significant regeneration in the mentioned period after the brain injury induction. Further studies are needed to elucidate the complete pathophysiological mechanisms involved in thalamic post-TBI damage, neurodegeneration, and regeneration that are essential for the development of new therapeutic strategies for the treatment of this condition.

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