Differential Effects of FK506 on Structural and Functional Axonal Deficits After Diffuse Brain Injury in the Immature Rat

Ann Mae DiLeonardi, PhD, Jimmy W. Huh, MD, and Ramesh Raghupathi, PhD

Abstract
Diffuse axonal injury is a major component of traumatic brain injury in children and correlates with long-term cognitive impairment. Traumatic brain injury in adult rodents has been linked to a decrease in compound action potential (CAP) in the corpus callosum, but information on trauma-associated diffuse axonal injury in immature rodents is limited. We investigated the effects of closed head injury on CAP in the corpus callosum of 17-day-old rats. The injury resulted in CAP deficits of both myelinated and unmyelinated fibers in the corpus callosum between 1 and 14 days postinjury (dpi). These deficits were accompanied by intra-axonal dephosphorylation of the 200-kDa neurofilament subunit (NF200) at 1 and 3 dpi, a decrease in total NF200 at 3 dpi and axonal degeneration at 3 and 7 dpi. Although total phosphatase activity decreased at 1 dpi, calcineurin activity was unchanged. The calcineurin inhibitor, FK506, significantly attenuated the injury-induced NF200 dephosphorylation of NF200 at 3 dpi and axonal degeneration at 3 and 7 dpi but did not affect the decrease in NF200 protein levels or impaired axonal transport. FK506 had no effect on CAP deficits at 3 dpi but exacerbated the deficit in only the myelinated fibers at 7 dpi. Thus, in contrast to adult animals, FK506 treatment did not improve axonal function in brain-injured immature animals, suggesting that calcineurin may not contribute to impaired axonal function.

Key Words: Axonal injury, Calcineurin, Compound action potential, FK506, Immature rat, Neurofilament, Traumatic brain injury.

INTRODUCTION
Traumatic brain injury (TBI) is a leading cause of death in infants and children and results in long-lasting cognitive deficits in survivors (1, 2). The primary pathologic process in brain-injured children is diffuse axonal injury, which has been correlated with long-term negative cognitive outcome (3–5). Diffuse axonal injury in animals is manifested as traumatic axonal injury (TAI), which results from a combination of mechanoporation of the axolemma, focal loss of microtubules, ionic dysregulation, impaired axonal transport, and neurofilament compaction (6–12). Axonal swellings containing accumulations of amyloid precursor protein (APP), synaptophysin or compacted neurofilaments, and retrograde tracer studies provide evidence of TAI in multiple white matter tracts of adult brain-injured animals (12–15). We and others have shown that traumatic injury to the immature brain results in TAI that is manifested as axonal swellings containing APP or neurofilaments (16–20). Over time, the injured axons undergo secondary axotomy and degeneration (21, 22), which occur in both immature (19) and adult animals (13).

Traumatic axonal injury in adult rodents has been linked to functional deficits observed as a decrease in compound action potential (CAP) within the corpus callosum and cerebellum (13, 23–25). Compound action potentials across the corpus callosum are characterized by a 2-peak waveform that represents the field potential of myelinated axons and unmyelinated axons (26). Myelinated and unmyelinated fibers can be identified based on recruitment (threshold of activation), refractoriness (minimum time between 2 action potentials), and susceptibility to 4-aminopyridine, which increases the duration and amplitude of unmyelinated axons but has no effect on myelinated axons (26). Diffuse brain injuries in adult rats result in deficits in CAP of both myelinated and unmyelinated axons at 1 day. Although the myelinated axons recover by 7 days, deficits are sustained in unmyelinated fibers (23, 24, 27). In contrast to these observations, concussive brain injury in adult mice led to a lasting decrease (up to 14 days) in CAP of myelinated fibers, suggesting that there are species differences (13). Because there have been no studies evaluating axonal function after diffuse brain trauma in immature rodents, our first goal was to determine the effect of closed head injury on CAP in the corpus callosum of the 17-day-old rat.

Increased calcium entry into axons has been suggested as a potential mechanism underlying both CAP deficits and TAI (8, 28–31). Inhibition of either calpain (a calcium-activated protease) or calcineurin (a calcium-dependent phosphatase) has been successful in attenuating CAP deficits and axonal injury after diffuse brain injury in the adult rat (27, 32, 33). Traumatic brain injury in the immature rat leads to neurofilament compaction (16), which may result from proteolysis (via calpain) or dephosphorylation (via calcineurin) of the side-arm domains of neurofilament subunits (34–36). Diffuse brain injury or optic nerve stretch in adult rats resulted in dephosphorylation of the 200-kDa neurofilament subunit (37–39), implicating calcineurin, which can dephosphorylate neurofilaments (40, 41), and is increased after TBI.
Moreover, the calcineurin inhibitors FK506 and cyclosporin A have been reported to reduce the burden of axonal injury after diffuse brain trauma in adult animals (32, 33, 46–49) and to decrease neurofilament disruption after axon stretch in vitro (50). Our previous observations of neurofilament compaction in axonal swellings in the injured immature rat brain led us to determine whether axonal neurofilaments would undergo dephosphorylation and to hypothesize that FK506 would limit the extent of neurofilament dephosphorylation and reverse CAP deficits after diffuse brain trauma in immature rats.

**MATERIALS AND METHODS**

**Brain Injury**

Seventeen-day-old male and female Sprague-Dawley rat pups (33 ± 4 g [mean ± SD]; Charles River Laboratories, Wilmington, MA) were anesthetized with isoflurane (5%) and subjected to diffuse brain injury, as previously described (19). Sham-injured animals were anesthetized and surgically prepared but did not receive an injury. The animals used to characterize the alterations in axonal conductance and neurofilament expression are listed in Table 1; those used for the characterization of the temporal alterations in neurofilament dephosphorylation were part of a larger study evaluating histopathological alterations and behavioral deficits after diffuse head injury in immature rats (19). In the experiments evaluating the effect of FK506, brain-injured animals received intraperitoneal injections of vehicle (20% methanol in 1 × PBS) or 10 or 25 mg/kg FK506 (AG Scientific, San Diego, CA) immediately and 6 hours after injury (Table 2). In preliminary studies, 1- and 5-mg/kg doses were tested based on efficacy reported in previous studies (33, 46, 47, 51); however, because of a lack of effect of these doses on neurofilament dephosphorylation and intra-axonal accumulation of APP, the doses listed were tested. All surgical procedures were done in accordance with the rules and regulations of the Institutional Animal Care and Use Committee of Drexel University College of Medicine and were in compliance with the Guide for the Care and Use of Laboratory Animals. Animals were placed on a heating pad to maintain body temperature at 37°C throughout the procedures and recovery.

**CAP Measurements**

Animals were anesthetized, brains were rapidly removed, and 450-μm-thick coronal slices containing the corpus callosum were prepared as described by Reeves et al (23) (Fig. 1A). Evoked CAPs were recorded across the corpus callosum (Fig. 1B) using an Axocapm 2B amplifier, digitized at 100 kHz, and stored on disk for analysis. Constant current pulses (200-microsecond duration) were used to evoke CAPs in the corpus callosum, and the current where the CAP reached its maximum was defined as the maximum current for each individual slice; intensity of the current was decreased by five 100-μA steps to construct input-output curves. The amplitude of the N1 component of the CAP (representing myelinated fibers) was quantified as the difference from the first positive peak to the first negative peak (Fig. 1C). To determine the amplitude of the N2 component (representing unmyelinated fibers), an arbitrary line was drawn relative to baseline and a tangent dropped to the second negative peak (Fig. 1C). Duration was determined as the time from the first to the second positive peak for the N1

### TABLE 1. Animals for Experiments on Temporal Pattern Changes After Diffuse Brain Injury

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Experiment</th>
<th>Group</th>
<th>Time Point, days</th>
<th>n</th>
<th>Weight (mean ± SD), g</th>
<th>Apnea (mean ± SD), seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axonal conduction</td>
<td>Time course</td>
<td>Sham</td>
<td>1</td>
<td>6</td>
<td>34 ± 2</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sham</td>
<td>7</td>
<td>8</td>
<td>33 ± 3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sham</td>
<td>14</td>
<td>7</td>
<td>31 ± 3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>1</td>
<td>8</td>
<td>35 ± 3</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>7</td>
<td>8</td>
<td>35 ± 3</td>
<td>7 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>14</td>
<td>7</td>
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<tr>
<td>Neurofilament dephosphorylation (immunohistochemistry)</td>
<td>Time course from Huh et al (19)</td>
<td>Sham</td>
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<td>4</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>4</td>
<td>36 ± 3</td>
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<tr>
<td></td>
<td></td>
<td>Sham</td>
<td>7</td>
<td>4</td>
<td>38 ± 2</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>1</td>
<td>4</td>
<td>35 ± 2</td>
<td>5 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>3</td>
<td>4</td>
<td>37 ± 2</td>
<td>7 ± 3</td>
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<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>7</td>
<td>4</td>
<td>35 ± 1</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Neurofilament expression (Western blots)</td>
<td>Time course</td>
<td>Sham</td>
<td>1</td>
<td>5</td>
<td>34 ± 4</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sham</td>
<td>3</td>
<td>4</td>
<td>36 ± 6</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sham</td>
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<td>5</td>
<td>34 ± 3</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>1</td>
<td>7</td>
<td>38 ± 4</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>3</td>
<td>7</td>
<td>34 ± 4</td>
<td>7 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>7</td>
<td>7</td>
<td>36 ± 4</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Calcineurin activation</td>
<td>Time course</td>
<td>Sham</td>
<td>1 and 3</td>
<td>8</td>
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<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injured</td>
<td>1</td>
<td>10</td>
<td>35 ± 5</td>
<td>5 ± 2</td>
</tr>
</tbody>
</table>

The animals used to determine the pattern of neurofilament dephosphorylation were generated in an earlier study (19).

NA, not applicable.
component and from the second positive peak until the waveform reached baseline for the N2 component (Fig. 2C). Conduction velocity was calculated using the distance between the recording and stimulating electrodes as a function of the time from the stimulus artifact to the negative peak of N1 or N2. To analyze refractoriness (a measure of the time required to depolarize the membrane from a hyperpolarized state into a range where a second action potential can be generated), pairs of pulses were used, where the interpulse interval increased in 0.5-millisecond steps from 3 milliseconds through 12 milliseconds, then in 5-millisecond steps from 15 milliseconds through 35 milliseconds at the maximum current determined from the input-output testing for each slice. The amplitude of the peak from the second pulse, calculated as a percent of the peak from the first pulse, was plotted as a function of the interpulse interval. From this, the interval at which the amplitude recovered to 50% of the first peak was determined and compared across groups. All quantitative analysis of electrophysiology was performed on waveforms that were the average of 4 successive sweeps; 2 slices per animal were averaged to generate a single value for that animal.

**Immunohistochemistry and Histology**

Animals were perfused, and brains were processed as previously described (19). Sets of sections were stained for dephosphorylated 200-kDa neurofilament subunit (clone SMI-32, 1:5000; Sternberger Monoclonals, Baltimore, MD) or total

**TABLE 2. Animals Used in Postinjury FK506 Treatment Experiments**

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Experiment</th>
<th>Group</th>
<th>Time Point, days</th>
<th>n</th>
<th>Weight (mean ± SD), g</th>
<th>Apnea (mean ± SD), seconds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axonal conduction</td>
<td>FK506 treatment</td>
<td>Vehicle</td>
<td>3</td>
<td>6</td>
<td>36 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>5</td>
<td>35 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vehicle</td>
<td>7</td>
<td>5</td>
<td>33 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FK506-25</td>
<td>7</td>
<td>7</td>
<td>36 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Neurofilament dephosphorylation,</td>
<td>FK506 treatment</td>
<td>Vehicle</td>
<td>1</td>
<td>7</td>
<td>38 ± 5</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>IAT, axonal degeneration</td>
<td></td>
<td>FK506-10</td>
<td>1</td>
<td>7</td>
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<td>7 ± 2</td>
</tr>
<tr>
<td>(immunohistochemistry and histology)</td>
<td></td>
<td>FK506-25</td>
<td>1</td>
<td>6</td>
<td>37 ± 3</td>
<td>7 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vehicle</td>
<td>3</td>
<td>6</td>
<td>37 ± 4</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>6</td>
<td>34 ± 2</td>
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</tr>
<tr>
<td></td>
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<td>FK506-25</td>
<td>3</td>
<td>6</td>
<td>36 ± 3</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vehicle</td>
<td>7</td>
<td>6</td>
<td>35 ± 2</td>
<td>6 ± 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FK506-25</td>
<td>7</td>
<td>6</td>
<td>35 ± 2</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>Neurofilament expression</td>
<td>FK506 treatment</td>
<td>Vehicle, injured</td>
<td>3</td>
<td>4</td>
<td>34 ± 1</td>
<td>6 ± 0</td>
</tr>
<tr>
<td>(Western blots)</td>
<td></td>
<td>FK506-25, injured</td>
<td>3</td>
<td>4</td>
<td>34 ± 1</td>
<td>6 ± 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vehicle, sham</td>
<td>3</td>
<td>3</td>
<td>34 ± 1</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FK506-25, sham</td>
<td>3</td>
<td>4</td>
<td>36 ± 6</td>
<td>NA</td>
</tr>
</tbody>
</table>

FK506-10, 10 mg/kg FK506 per injection; FK506-25, 25 mg/kg FK506 per injection; IAT, impaired axonal transport measured using intra-axonal accumulation of amyloid precursor protein; vehicle, 20% methanol in PBS.

**FIGURE 1.** Measurement of compound action potential (CAP) in the corpus callosum. (A) Representative schematic diagram of the coronal slice containing the corpus callosum used for recording the CAP. (B) Stimulating and recording electrodes were placed approximately 0.5 mm on either side of the midline. (C) Representative trace of a typical CAP used to measure the amplitudes. The amplitude of the N1 component (myelinated fibers) was the difference in voltage from the first positive peak to the first negative trough; the amplitude of the N2 component (unmyelinated fibers) was measured by dropping a tangent from the baseline to the second negative trough. The duration of N1 was measured as the time between the first and second positive peaks; the duration of N2 was measured as the time between the second positive peak until the signal returned to baseline.
200-kDa neurofilament proteins (clone N52, 1:5000; Sigma-Aldrich, St. Louis, MO) and detected using biotinylated donkey anti-mouse immunoglobulin G secondary antibody (1:500; Jackson ImmunoResearch, West Grove, PA), with diaminobenzidine as the chromogen. Double label immunofluorescence for dephosphorylated neurofilament and APP was performed as previously described (16). Degeneration of axons was evaluated using Fluoro-Jade B (19).

SMI-32 and NF200 immunoreactivity were quantified at 3 levels (2.8, 3.6, and 4.3 mm posterior to bregma) using the grid method, as described (16). The area of APP immunoreactivity in the subcortical white matter regions was also
measured in these levels using ImageJ (National Institutes of Health) and expressed as a function of the total area of the coronal slice; each animal was represented by 1 value that was the average of the 3 sections. To quantify axonal degeneration, Matlab (version R010b) was used to calculate the density of Fluoro-Jade B–positive profiles in the corpus callosum in 3 sections taken from 2.8, 3.6, and 4.3 mm posterior to the bregma. Using the image toolbox, profiles were first identified based on staining intensity relative to background. Profile edges were defined based on a 40% increase from the immediate background, with profiles containing between 4 and 350 pixels deemed positive. The number of profiles in each section was determined as a function of the area of the corpus callosum in that section, and the final value represented the average profile density across the 3 sections.

**Immunoblot Analysis**

Lysates of subcortical white matter were prepared as described (13). Samples were run on denaturing 7.5% polyacrylamide gels, transferred to polyvinylidene fluoride membranes that were probed with anti-NF200 (clone N52; 1:10,000) and detected using donkey anti-mouse immunoglobulin G conjugated to horseradish peroxidase with enhanced chemiluminescence (Amersham, Arlington, IL). Equal loading of protein was confirmed by reprobing the blots with an antibody to actin (clone AC-40, 1:1000; Sigma-Aldrich). Densitometric analysis was performed by normalizing the intensity of the band of interest to that of actin (normalized relative optical density) using GeneSnap imaging and software (SynGene, Frederick, MD). Each sample was evaluated at least twice.

**Calcineurin Activity**

Lysates of subcortical white matter and cortex were prepared as described from sham- and brain-injured animals at 1 and 3 days postinjury (dpi) (13). Total phosphatase and calcineurin activities were assayed using a kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer’s instructions.

**Statistical Analysis**

All data are presented as means ± SD. For quantification of amplitudes of the CAP, a mixed-design analysis of variance was used, with injury and time as between-subject comparisons and normalized stimulus current as repeated measure factor using Statistica (version 7.1; Statsoft, Tulsa, OK). All other data were compared using a factorial analysis of variance with either injury status and time after injury or treatment and time after injury as between-subject comparisons.

**FIGURE 3.** Effect of diffuse brain injury on the electrophysiologic properties of the axons in the corpus callosum. (A–F) Graphs representing duration (A, B), conduction velocity (C, D), and refractoriness (E, F) of the N1 (A, C, E) and N2 components (B, D, F). Filled (sham-injured) and open (brain-injured) bars represent mean values; error bars represent SDs. All p values are significant compared with their respective sham-injured animals. *p < 0.05; **p < 0.005; #p < 0.01; ##p < 0.001; ###p < 0.0001.
Post hoc analysis was performed using the Newman-Keuls test. A value of $p < 0.05$ was considered significant in all analyses.

**RESULTS**

**Acute Neurologic Outcomes After Diffuse Brain Injury**

Closed head injury in 17-day-old rats resulted in skull fractures and hematomas that were accompanied by a brief period (4–8 seconds) of apnea (Tables 1, 2), similar to earlier observations (19). Importantly, no differences in body weights and posttraumatic apnea times were observed between the groups assigned for the various outcomes (Tables 1, 2).

**CAP After Diffuse Brain Injury**

Evoked CAPs in the corpus callosum of sham-injured immature (1, 7, and 14 dpi) rats resulted in a biphasic waveform similar to that observed in uninjured adult rats and mice (13, 23). The amplitude of the myelinated fibers (N1) of the CAP was consistently larger than that of the unmyelinated fibers (N2) (Fig. 2A). The amplitude of the N1 component remained constant over the 3 ages analyzed ($F_{2,38} = 0.601, p > 0.5$; Figs. 2B, C), whereas the amplitude of the N2 component

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Intra-axonal accumulation of dephosphorylated 200-kDa neurofilament subunit after diffuse brain injury in the immature rat. (A–F) Representative photomicrographs of SMI-32–labeled axons within the corpus callosum of sham (A) and brain-injured rats at 1 (B) and 3 days (C) after injury. (D) An example of SMI-32–labeled swollen contiguous axons. (E, F) Examples of terminal bulbs. (G–I) An example of double label immunofluorescence for intra-axonal amyloid precursor protein (APP) accumulation (red) and SMI-32 immunoreactivity (green) at 1 day after injury. Single-labeled profiles (either APP positive/SMI-32 negative or APP negative/SMI-32 positive) are denoted by arrows; double-labeled profiles (APP positive/SMI-32 positive) are denoted by arrowheads. Photomicrographs were obtained at $63 \times$ magnification. Scale bars in (C) (20 $\mu$m) and (F) (100 $\mu$m) represent 100 $\mu$m for (A–C) and (D–F), respectively.
decreased with age (TIME effect, \( F_{2,37} = 4.54, p < 0.02 \); Figs. 2D, E), suggestive of an increase in the degree of myelination. Diffuse brain injury resulted in a change to the shape of the waveform of CAPs, whereby both N1 and N2 components became temporally closer and the signal rarely returned to baseline between the 2 peaks (Fig. 2A). Quantitative analysis revealed a decrease in the amplitude of both N1 (INJURY effect, \( F_{1,38} = 71.83, p < 0.0001 \); Fig. 2B, C) and N2 components (INJURY effect, \( F_{1,37} = 5.16, p < 0.03 \); Fig. 2D, E). The absence of an interaction effect (TIME \( \times \) INJURY) for both N1 (\( F_{2,38} = 1.62, p > 0.05 \)) and N2 (\( F_{2,37} = 0.91, p > 0.05 \)) components indicate sustained deficits in the CAP amplitudes. Furthermore, slices from brain-injured animals required significantly more current to reach the maximum response versus slices from sham-injured animals (1 day, \(1.117 \pm 0.509 \) mA vs \(875 \pm 160 \) mA; 7 days, \(1.080 \pm 0.239 \) mA vs \(849 \pm 130 \) mA; 14 days, \(975 \pm 323 \) mA vs \(725 \pm 83 \) mA; INJURY effect, \( F_{1,37} = 8.37, p < 0.0007 \)).

Brain injury resulted in a decrease in the duration of both N1 (INJURY effect, \( F_{1,37} = 59.88, p < 0.0001 \); Fig. 3) and N2 components (INJURY effect, \( F_{1,37} = 17.41, p < 0.005 \); Fig. 3B) of the CAP during the 14 dpi. The conduction velocity of the N1 component increased with age (TIME effect, \( F_{2,34} = 3.43, p < 0.05 \); Fig. 3C) and was unaffected by injury (\( F_{2,34} = 3.23, p > 0.05 \); Fig. 3C). In contrast, the conduction velocity of the N2 component was independent of age (\( F_{2,342} = 0.18, p > 0.05 \); Fig. 3D) but was significantly increased in slices from brain-injured rats versus those from sham-injured animals (INJURY effect, \( F_{2,34} = 13.92, p < 0.001 \); Fig. 3D). Analysis of paired pulse recordings revealed that neither age nor injury affected the refractoriness of the N1 component (Fig. 3E). Although age did not affect the refractoriness of the N2 component (\( F_{2,32} = 154, p > 0.05 \); Fig. 3F), an INJURY effect was observed (\( F_{1,32} = 17.79, p < 0.001 \); Fig. 3F). The decrease in refractoriness of the N2 component was dependent on age after injury (TIME \( \times \) INJURY, \( F_{2,32} = 5.40, p < 0.05 \)) and was significant at 1 and 14 dpi (\( p < 0.05 \); Fig. 3F).

### Neurofilament Dephosphorylation After Diffuse Brain Injury

Immunoreactivity for the dephosphorylated 200-kDa neurofilament protein was not observed in white matter tracts...
of sham-injured animals (Fig. 4A). Diffuse brain injury resulted in swollen axons containing dephosphorylated neurofilaments in the corpus callosum, cingulum, and lateral white matter tracts between the cingulum and the rhinal fissure (Figs. 4B, C); injured axons were present between bregma and 5.6 mm posterior to bregma. Immunoreactivity for the SMI-32 antibody was extensive at 1 and 3 dpi in the corpus callosum (Figs. 4B, C), cingulum, and lateral white matter tracts (data not shown) and was present in swollen contiguous axons (Fig. 4D), some of which ended in terminal bulbs indicative of axonal disconnection (Figs. 4E, F). By 7 dpi, only a few injured axons were visible (data not shown). Axonal SMI-32 immunoreactivity was present in the areas of the white matter tracts in which APP-positive swellings (19) and neurofilament compaction (16) have been observed after brain injury. Although most axonal swellings that stained for SMI-32 did not stain for APP (Fig. 4G, arrows), there were sporadic axonal swellings that stained for both SMI-32 and APP (Fig. 4G, arrowheads). Sham-injured animals had no axons that stained for either APP or SMI-32 (data not shown).

Immunoreactivity for total NF200 was observed in the white matter tracts in sham-injured animals and appeared as diffuse staining within axons at all 3 ages evaluated (Fig. 5A). After injury, axonal accumulations of NF200 were observed in the corpus callosum, cingulum, and lateral white matter tracts at 1 dpi (Fig. 5B) and 3 dpi (Fig. 5C), predominantly as terminal bulbs; by 7 dpi, NF200-positive axonal swellings were minimally observed (data not shown). Interestingly, the overall NF200 immunoreactivity in contiguous axons seemed to decrease by 3 dpi (Fig. 5C), which was confirmed using immunoblot analysis of white matter tissue lysates (Fig. 5D). Total NF200 appeared as doublet ranging in size between 180 and 200 kDa (Fig. 5D), as previously observed (52), and quantification of the relative optical density of both bands representing total NF200 protein revealed an increase in NF200 expression as a function of age in the sham animals (TIME effect, $F_{2,29} = 6.92, p < 0.005$; Fig. 5E) and a decrease as a function of INJURY ($F_{1,29} = 11.99, p < 0.005$; Fig. 5E).

**Calcineurin Activity After TBI**

Diffuse brain trauma resulted in a decrease in total phosphatase activity in lysates of white matter at 1 dpi but not 3 dpi ($F_{2,10} = 5.74, p < 0.05$; Fig. 6A); however, there was no change in calcineurin activity ($F_{2,10} = 2.49, p > 0.05$; Fig. 6A). Neither total phosphatase nor calcineurin activity in cortical lysates was affected by brain trauma (Fig. 6B).

**Effect of FK506 on Posttraumatic Neurofilament Dephosphorylation**

Brain trauma in the rats given vehicle resulted in intraxonal SMI-32–positive swellings at 1 and 3 dpi in the corpus callosum (Figs. 7A, B) and cingulum (not shown) to a similar extent as in untreated brain-injured animals. Administration of 10 or 25 mg/kg of the calcineurin inhibitor FK506 had no

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**Figure 6.** Phosphatase activity after diffuse brain injury in the immature rat. (A, B) Total and EGTA-sensitive (calcineurin) phosphatase activities were measured using lysates of the subcortical white matter (A) or cortex (B). Shaded bars represent average calcineurin activity; the unfilled part represents average EGTA-insensitive activity. Error bars = SD.

**Figure 7.** Effect of FK506 on dephosphorylation and expression of 200-kDa neurofilament subunit after diffuse brain injury in the immature rat. (A–D) Representative SMI-32 photomicrographs of vehicle-treated animals at 1 (A) and 3 (B) days after injury and FK506-treated animals at 1 (C) and 3 (D) days after injury. (E) Quantification of SMI-32–positive profiles in subcortical white matter tracts using the grid method. IHC, immunohistochemistry. (F, G) Representative NF200 photomicrographs of vehicle (F) and FK506 (G)-treated rats at 3 days after injury. (H) Quantification of NF200-positive profiles in subcortical white matter tracts. (I) Representative immunoblots of NF200 and actin (loading control) of lysates from subcortical white matter tracts at 3 days after injury. (J) Quantification of optical density of NF200 relative to that of actin. The increase in the NF200 expression in the FK506-treated injured animals was not significant. * $p < 0.05$; ## $p < 0.001$. Scale bar = 100 μm.
Effect of FK506 on Posttraumatic Axonal Degeneration

Diffuse brain injury resulted in intra-axonal accumulation of APP within multiple white matter tracts at 1 and 3 dpi (Figs. 8A, C). In contrast to observations in adult brain-injured rats, posttraumatic administration of FK506 did not affect the extent of impaired axonal transport in the corpus callosum at any time after injury (Figs. 8B, D). These qualitative observations were confirmed by quantification of the area positive for APP immunoreactivity in the subcortical white matter region. Factorial analysis of variance revealed a TIME effect indicative of decreased APP immunoreactivity at 3 dpi (F1,13 = 48.84, p < 0.0001) but not a TREATMENT effect, suggesting that administration of neither dose of FK506 reduced axonal injury at either time point (F2,132 = 1.30, p = 0.27; Fig. 8E). As reported previously (19), areas immunoreactive for axonal APP at 1 and 3 dpi demonstrated evidence of axonal degeneration (Fluoro-Jade B reactivity) at 3 and 7 dpi (Figs. 8F, H). Posttraumatic administration of FK506 reduced the extent of Fluoro-Jade B reactivity in the corpus callosum (Figs. 8G, I). Quantification of the density of Fluoro-Jade B-positive profiles revealed a treatment effect (F1,10 = 8.21, p = 0.001; Fig. 8J). Administration of FK506 to sham-injured animals did not result in intra-axonal APP accumulation or axonal degeneration (data not shown).

Effect of FK506 on CAP

At 3 and 7 dpi, the amplitudes of both N1 and N2 components of the CAP were decreased in vehicle-treated (20% methanol in 1 × PBS) brain-injured rats (Figs. 8K, L) to a similar extent as previously observed (Figs. 2C, E). Although administration of FK506 (25 mg/kg) was observed to reduce the extent of neurofilament dephosphorylation and axonal degeneration (vide supra), it decreased the amplitude of the N1 component compared with that in vehicle-treated animals (TREATMENT effect, F1,18 = 10.33, p < 0.005; Fig. 8K). In addition, an interaction effect (TREATMENT × TIME, F1,18 = 5.97, p < 0.05) revealed that the FK506-induced decrease in amplitude was restricted to the 7-day time point (Fig. 8K). No effect of posttraumatic administration of FK506 was observed on the amplitude of the N2 component of the CAP at either 3 or 7 dpi, although the amplitude did increase between 3 and 7 dpi independent of treatment status (TIME effect, F1,18 = 9.21, p < 0.01). Treatment of brain-injured animals with FK506 did not affect any of the other parameters of the CAP such as duration and conduction velocity (data not shown). Administration of FK506 to sham-injured rats did not affect axonal conductance (data not shown).

DISCUSSION

This is the first study characterizing deficits in CAPs after TBI in the immature rat. The decrease in CAP amplitude of both the myelinated and unmyelinated fiber populations was observed as early as 1 dpi and persisted up to 2 weeks. These functional deficits were accompanied by intra-axonal dephosphorylation of NF200, a decrease in total NF200 protein, and axonal degeneration. Treatment with the calcineurin inhibitor FK506 significantly attenuated intra-axonal accumulations of total and dephosphorylated NF200 and axonal degeneration. FK506 had no effect on NF200 protein expression or CAP deficits at 3 dpi, but it exacerbated the CAP deficit in only the myelinated fibers at 7 dpi. These data do not seem to provide a causal link between posttraumatic neurofilament alterations and axonal conductance and underscore the importance of using multiple outcome measures to evaluate the efficacy of a treatment paradigm. Furthermore, our observations highlight the concept that age at injury may determine functional alterations and response to treatment.

Similar to what has been observed in kittens and in contrast to adult rats, the CAP of the uninjured immature rat was slower, had longer latencies, and required more current (23, 53). Diffuse brain injury in the immature rat resulted in decreased amplitude of the CAP by 1 dpi in both unmyelinated and myelinated fibers, as has been reported for the...
brain-injured adult rat (23, 24). However, in contrast to the adult brain-injured rat, where the amplitude of the myelinated fibers recovered to near control levels by 7 dpi (23), we observed a sustained deficit up to 2 weeks, which may suggest differences in injury severity between the 2 ages. Brain injury resulted in sustained deficits in the unmyelinated fibers up to 7 dpi in the adult rat and up to 14 dpi in the immature rat (23, 24), suggesting that the unmyelinated axons at either age may be more vulnerable to trauma despite potential differences in injury severity. It is important to note that midline fluid percussion injury to the adult rat brain does not result in skull fractures or hematomas, which may affect the outcome. The decrease in both the amplitude and the duration of the CAP suggests that myelinated and unmyelinated axons are either dysfunctional or that fewer axons are contributing to the CAP. In the acute posttraumatic period, the injury-induced CAP amplitude decrease may be the result of dysfunctional sodium channels, which are proteolyzed by calpain after mechanical trauma in vitro or in vivo (54, 55). The evidence of axonal degeneration during the first week after injury suggests that the sustained deficits observed in the CAP may reflect a decreased number of axons.

Further evidence that fewer axons contribute to the CAP comes from the analyses of the conduction velocity and refractoriness. Neither the conduction velocity nor the refractoriness of the myelinated fibers was changed, suggesting that injury does not interfere with the functional properties of individual axons. In contrast, injury to the adult brain decreased the conduction velocity and increased the refractoriness of the myelinated axons (23, 27), indicating an age-at-injury response. Injury to the immature animals resulted in an increase in conduction velocity of the unmyelinated fibers that was accompanied by a decrease in the refractoriness, which may simply be the result of the greater susceptibility of the smaller axons compared with the larger axons, leading to a shift in the population of unmyelinated axons. In contrast, the unmyelinated fibers in the injured adult brain exhibited decreased conduction velocity and increased refractoriness, which led the authors to conclude that the individual axons were functionally deficient (23, 27, 32, 33, 56). Structurally, axons of varying diameter exhibit different pathologic alterations after injury, suggesting that axon caliber may contribute to traumatic susceptibility (10, 14, 57–59).

The mechanisms underlying CAP deficits after injury have not been completely elucidated. When the rat optic nerve was exposed to axonic conditions ex vivo, the CAP disappeared and recovered to only 28% in the presence of Ca++ and 100% in Ca++-free artificial cerebrospinal fluid (30). Anoxia in the presence of a calcium channel antagonist, such as verapamil, diltiazem, or nifedipine, resulted in greater recovery of CAP of rat optic nerves (31). Although the effect of calcium channel blockers on axonal structure and function has not been tested after TBI, the calpain inhibitor MDL28170 and the calcineurin inhibitors FK506 and cyclosporin A were effective in ameliorating CAP deficits (27, 32, 33). In addition, these studies provided evidence that the calpain and calcineurin inhibitors reduced intra-axonal APP accumulation, suggesting that posttraumatic calcium entry in the adult may be associated with generalized axonal dysfunc-

In contrast, we did not observe an effect of FK506 (10 and 25 mg/kg, this report, or 1 and 5 mg/kg, data not shown) on impaired axonal transport. The doses of FK506 cannot explain this lack of an effect because lower doses were neuroprotective after CNS injury in either the adult or the immature rat (33, 46, 47, 51, 60, 61). Rather, this may reflect that, in contrast to the adult rats (42–45), diffuse brain injury in the immature rat did not appear to activate calcineurin.

Calcineurin has been shown to dephosphorylate neurofilament in vitro and in vivo (40, 41). We observed intra-axonal accumulation of dephosphorylated neurofilament, lending support to the idea that neurofilament compaction in the immature brain (16) may result from dephosphorylation of the side-arm domains (34–36). Although calcineurin activity did not increase after TBI, total phosphatase activity in the white matter decreased and led us to postulate that the relative contribution of calcineurin activity to the total phosphatase activity increased. This hypothesis was validated by the partial reduction of dephosphorylated neurofilament accumulations in brains of rats treated with FK506. Furthermore, the coupling of neurofilament dephosphorylation to the intra-axonal accumulation of total neurofilament suggests that disruption of axon structure may accompany axon degeneration. This was borne out by the observation that FK506 treatment reversed the posttraumatic accumulation of total neurofilament within axons and reduced axonal degeneration; the effect of FK506 and cyclosporin A on axonal degeneration in the adult rat has not been evaluated. Interestingly, the dose of FK506 that reduced axonal damage and degeneration in the immature brain-injured rat exacerbated CAP deficits at 7 dpi, suggesting that the health of the remaining axons was negatively affected by the treatment and providing additional evidence that age at injury also determines therapeutic efficacy. Alternatively, the decrease in intra-axonal SMI-32 and NF200 accumulations may suggest that FK506 treatment accelerates axonal degeneration, which may explain the decrease in the number of Fluoro-Jade B–labeled profiles.

The data in the present study provide evidence of structural and functional deficits in axons of the immature brain after traumatic injury. These studies highlight the importance of using multiple outcome measures to determine efficacy of therapeutic strategies. Our observations indicate the importance of mechanistic and therapeutic studies in age-appropriate animal models of TBI.

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