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The effects of cyclosporin-A on axonal conduction deficits following traumatic brain injury in adult rats

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Abstract

Immunophilin ligands, including cyclosporin-A (CsA), have been shown to be neuroprotective in experimental models of traumatic brain injury (TBI), and to attenuate the severity of traumatic axonal injury. Prior studies have documented CsA treatment to reduce essential components of posttraumatic axonal pathology, including impaired axoplasmic transport, spectrin proteolysis, and axonal swelling. However, the effects of CsA administration on axonal function, following TBI, have not been evaluated. The present study assessed the effects of CsA treatment on compound action potentials (CAPs) evoked in corpus callosum of adult rats following midline fluid percussion injury. Rats received a 20 mg/Kg bolus of CsA, or cremaphor vehicle, at either 15 m or 1 h postinjury, and at 24 h postinjury CAP recording was conducted in coronal brain slices. To elucidate how injury and CsA treatments affect specific populations of axons, CAP waveforms generated largely by myelinated axons (N1) were analyzed separately from the CAP signal which predominantly reflects activity in unmyelinated axons (N2). CsA administration at 15 m postinjury resulted in significant protection of CAP area, and this effect was more pronounced in N1, than in the N2, CAP component. This treatment also significantly protected against TBI-induced reductions in high frequency responding of the N1 CAP signal. In contrast, CsA treatment at 1 h did not significantly protect CAPs, but was associated with atypical waveforms in N1 CAPs, including decreased CAP duration and reduced refractoriness. The present findings also support growing evidence that myelinated and unmyelinated axons respond differentially to injury and neuroprotective compounds.

Keywords

Cyclosporin-A; compound action potentials; traumatic brain injury; neuroprotection; corpus callosum; rat

Introduction

The immunophilin ligand cyclosporin-A (CsA) has been shown to be neuroprotective in multiple experimental injury models, including traumatic brain injury (TBI), peripheral nerve damage, and both focal and global ischemia (reviewed by Kaminska, 2004). Diverse outcome

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measures have demonstrated CsA treatment to attenuate posttraumatic cognitive and motor dysfunction (Alessandri et al., 2002; Riess et al., 2001), and to reduce cellular and molecular abnormalities observed in the acute and delayed/secondary postinjury phases (Mbye et al., 2009; Okonkwo and Povlishock, 1999; Scheff and Sullivan, 1999; Sullivan et al., 1999; Sullivan et al., 2000a,b, and reviewed by Mazzeo et al., 2009a). Accumulating evidence indicates that CsA provides neuroprotection in TBI primarily by inhibiting mitochondrial permeability transition pore formation, thus preventing bioenergetic failure and downstream deleterious cascades.

Axonal injury is a consistent feature of TBI, occurring as focal or diffuse pathology, which evolves from local cytoskeletal disruption and impaired axonal transport, progressing to axonal swelling, and sometimes detachment and retraction bulb formation (Maxwell et al., 2003; Povlishock et al., 1992; Povlishock and Christman, 1995; Saatman et al., 1998; Smith et al., 1999). The immunophilin ligands, including CsA and FK506, have proven to be especially protective against axonal injury in experimental models (Buki et al., 1999; Marmarou and Povlishock, 2006; Mbye et al., 2009; Mobley and Agrawal, 2003; Okonkwo and Povlishock, 1999; Okonkwo et al., 1999; Reeves et al., 2007; Singleton et al., 2001). Investigations of CsA neuroprotective efficacy against TBI-induced axonal injury have focused largely on molecular markers of cytoskeletal damage and impaired axonal transport, or morphological assessments of axonal or mitochondrial swelling. However, a more comprehensive understanding of the therapeutic potential of CsA will require assessments of axonal function as well. Functional evaluations of CsA efficacy are also warranted because use of the compound has been associated with neurologic and epileptogenic effects noted clinically (Walker and Brochstein, 1988) and in experimental animal models (Famiglio et al., 1989; Racusen et al., 1990).

To address functional aspects of CsA neuroprotection, the present study examined how CsA treatments affect conduction properties of corpus callosum axons in brain slices prepared from adult rats following fluid percussion injury. The study obtained separate estimates of injury and treatment effects in myelinated and unmyelinated axon populations for several reasons. Studies of axonal injury have focused almost exclusively on the readily observable myelinated axons, while the histologically inconspicuous unmyelinated axons have tended to elude examination. However, electrophysiological findings from our laboratory indicated a differential vulnerability of unmyelinated and myelinated axons to TBI (Reeves et al., 2005). The value in accounting for a unique pathophysiological role, of the unmyelinated fibers, is underscored by stereological findings that unmyelinated axons comprise a numerical majority among fibers in subcortical white matter (Partadiredja et al., 2003). In addition, histopathological evidence also indicates that axonal injury is not uniformly expressed across axonal subpopulations. Specifically, in some injured axons, neurofilament compaction has been observed independently of impaired fast transport (Stone et al., 2001). The degree to which these two pathological components were conjointly expressed in individual axons was determined, in part, by axon caliber and anatomical location. In a morphological study of TAI within the corpus callosum of non-human primates, axolemmal tearing was observed to occur selectively at the nodes of small, thinly-myelinated axons within 35 min after injury (Maxwell et al., 1993).

To the extent that axon subtype influences susceptibility to injury, it would be expected that efficacy of neuroprotective compounds would depend on intrinsic properties of axons, including such parameters as caliber, myelination status, and anatomical location. In this context, we reported that a preinjury administration of FK506 produced different degrees of neuroprotection for the unmyelinated and myelinated axon populations (Reeves et al., 2007). The present study further pursues the issue of neuroprotection of immunophilin ligands, but now investigating CsA using a more clinically relevant postinjury administration paradigm. Findings revealed a preferential neuroprotection for CAP signals which are generated

predominantly by myelinated axons, when CsA was administered at 15 m, but not at 1 h, postinjury. However, CsA treatments at 1 h were associated with alterations of CAP duration and refractoriness. These effects were observed at 24 h postinjury, a survival time at which TBI-induced suppression of callosal CAPs has been shown to be at a maximum (Reeves et al., 2005), and a time at which multiple posttraumatic processes are well developed, including calpain-mediated spectrin breakdown, neurodegeneration, and amyloid precursor protein accumulation (Deng et al., 2007; Sato et al., 2001; Stone et al., 2000).

Methods

The procedures for this study followed all national guidelines for the care and use of experimental animals, and the experimental protocol was approved by the Medical College of Virginia Animal Research Committee. Male Sprague-Dawley rats (n = 64) weighing 300–350 g, at the start of the study, were used in these experiments. Animals were housed in individual cages in a temperature- (22° C) and humiditycontrolled (50% relative) animal facility on a 12-h light/dark cycle. Rat chow and water were continually available.

Fluid Percussion TBI Procedure, and CsA administration

The model used for injury was the same as that described by Dixon et al. (1987). Briefly, all animals were surgically prepared under nosecone isoflurane anesthesia (2% in carrier gas of 70% N₂O, 30% O₂), using sterile surgical conditions. Rats were positioned in a stereotaxic frame, and a 4.8 mm skull craniotomy was prepared over the midline, centered between bregma and lambda. A modified Leur-Loc syringe hub (2.6 mm i.d.) was then positioned over the exposed dura and bonded in place with cyanoacrylate adhesive, and the entire complex was anchored by two steel screws and dental acrylic. The open hub was packed with Gelfoam prior to scalp suturing and Bacitracin applied. Once animals had recovered from anesthesia, they were returned to their home cages.

Twenty-four hours following hub implantation, rats received fluid percussion injury or a sham injury. Rats were anesthetized with isoflurane (4% in carrier gas of 70% N₂O and 30% O₂), connected to a fluid percussion device through the exposed hub, and injured at 2.0 ± 0.2 atmospheres. The injury device consisted of a 60 × 4.5 cm Plexiglas water-filled cylinder, fitted at one end with a piston mounted on O-rings, with the opposite end housing a pressure transducer (Entran Devices, Inc.; EPN-0300A). At the time of injury, the Leur-Loc fitting, filled with 0.9% saline, was attached to the transducer housing. The injury was produced by a metal pendulum that struck the piston, injecting a small volume of saline into the cranial cavity, briefly deforming the brain tissue (20 ms pulse duration). The resulting pressure pulse was recorded extracranially by the transducer and expressed in atmospheres of pressure. Injury magnitude was controlled by setting the height from which the pendulum was released. Following injury all animals were promptly ventilated with room air until spontaneous breathing resumed. The duration of suppression of the righting reflex was used as an index of traumatic unconsciousness. For control rats administered sham injuries, TBI procedures were as above except the intracranial pressure pulse was not injected.

Rats received an intraperitoneal (i.p.) bolus of 20 mg/kg CsA (Sandimmune, Novartis Pharmaceuticals Inc.), based on the animal weight at the time of injury, or an equal volume of the commercial CsA vehicle consisting of polyethylene glycol/sterile saline/cremaphor oil. The dosage of CsA was selected based on previous studies showing this dose to provide neuroprotection following TBI, and to attenuate posttraumatic mitochondrial dysfunction (Albensi et al., 2000; Sullivan et al., 1999; Sullivan et al., 2000a). Each rat received a single i.p. bolus of either CsA or vehicle, at either 15 minutes (m) or 1 hour (h) postinjury, according to the following experimental groups (for each group n=8): TBI + vehicle (15 m); TBI + vehicle

(1h); TBI + CsA (15 m); TBI + CsA (1h); Sham + vehicle (15 m); Sham + vehicle (1h); Sham + CsA (15 m); Sham + CsA (1h).

Electrophysiological Recording Procedures

Following the fluid percussion injury, rats were allowed to survive for 24 hours prior to electrophysiological recording. Each rat was decapitated under isoflurane anesthetized and the brain rapidly removed. Coronal slices of 450 μm thickness were cut in ice cold (4 to 6°C) artificial cerebrospinal fluid (ACSF) with a vibrating-knife microtome (Electron Microscopic Sciences model OTS-3000-03). Those slices were saved which contained midline-crossing segments of the corpus callosum, overlying the mid-dorsal hippocampus, which yielded 3 to 5 slices per brain. Slices were then transferred to a holding chamber containing oxygenated ACSF at room temperature, and were allowed to equilibrate under these conditions for at least 1 h prior to recording. For recording, a slice was transferred to a submersion-type chamber, and perfused at a rate of 1–2 ml / min with ACSF at 23 degrees C. The ACSF contained (in mM): NaCl 124, KCl 5, NaH_2PO_4 1.25, NaHCO_3 26, MgSO_4 1.3, CaCl_2 2, glucose 10; pH 7.4; saturated with a 95% O_2 / 5% CO_2 gas mixture. All electrode probes were secured and positioned using micromanipulators (Model M3301, WPI, Sarasota, FL). A bipolar stimulating electrode (teflon-insulated tungsten; 0.3–0.4 mm intertip distance) was lowered into the corpus callosum, at approximately 0.5 mm lateral to midline, angled to ensure that both contacts were within the boundaries of the callosum. A recording electrode (ACSF-filled glass micropipette; resistance 6 – 8 $\text{M}\Omega$) was then placed in the corpus callosum of the contralateral hemisphere at a distance of approximately 1.0 mm from the stimulating electrode (Fig. 1). Both electrodes were initially lowered to approximately 200 μm below the slice surface, and fine adjustments were made in the depths of both electrodes to optimize the signal amplitude. For some slices the recording pipette was withdrawn and repositioned in the dorsoventral axis to improve the signal-to-noise ratio. Mediolateral adjustments were minimized to preserve the standard (~1.0 mm) electrode separation. Evoked callosal CAP field potentials were amplified (bandpass = DC to 10 kHz), and digitized at 25 kHz for offline analysis (Digidata 1322A data acquisition hardware and Clampfit 9.2 software, Molecular Devices Inc., Sunnyvale, CA).

The callosal CAP has previously been characterized as a biphasic waveform with an early component generated predominantly by relatively fast-conducting myelinated axons (N1), and a later occurring component reflecting mainly slower unmyelinated axons (N2) (Ai et al., 2007; Crawford et al., 2009; Preston et al., 1983; Reeves et al., 2005, 2007; Swanson et al., 1998). Ultrastructural and pharmacological evidence supports the identification of N1 and N2 with myelinated and unmyelinated fiber populations, respectively. The frequency distribution of visual callosal axon diameters is distinctly bimodal, corresponding to unmyelinated (mean diameter ~ 0.2 μm) and myelinated fiber populations (mean diameter ~ 0.7 μm) (Waxman and Swadlow, 1976), and this caliber range accords well with conduction velocities (0.3 – 12.9 m/s) of fibers in this same callosal region (Swadlow and Waxman, 1976). External application of the potassium blocking agent 4-AP, to the corpus callosum, potently increases the duration of N2, but does not significantly alter N1 (Preston et al., 1983; Reeves et al., 2005; Swanson et al., 1998), consistent with a submyelin distribution of potassium channels, and an accessibility of the channels along unmyelinated axons to the 4-AP.

Three parameters of the N1 and N2 waveforms were quantified: area, peak, and duration, as illustrated in Figure 1. Electrophysiological recording was conducted with slices at room-temperature (22–23 degrees C), based on previous findings that this range was optimal for separate quantification of the myelinated and unmyelinated CAP components (Reeves et al., 2005).

Stimulation used for evoked CAPs was constant current stimulus-isolated square wave pulses, with stimulus intensity and frequency adjusted for specific recording protocols. For analyses

of TBI-related changes in CAP amplitude, standardized input-output functions were generated, for each slice, by varying the intensity of stimulus pulses (200 μ s duration, delivered at 0.1 Hz) in ten equal steps from approximately threshold level to an asymptotic maximum for the N1 CAP component. This method of current standardization was adopted from our previous observation that the N2 CAP component required more current to reach asymptote than was necessary for N1 (Reeves et al., 2005), and this was consistent with the long-established principle that larger diameter axons have a lower stimulus-evoked threshold than smaller caliber axons (BeMent and Ranck, 1969; West and Wolstencroft, 1983). This protocol also results in conservative levels of stimulus currents, avoiding extensive stimulation at supramaximal levels for only one of the axonal subpopulations (i.e., the faster conducting N1 generating fibers), yet still permits measurement of the linear portion of the N2 input-output range. In refractoriness testing, pairs of pulses were presented with interpulse interval increased, in 0.5 ms steps, from 1.5 ms to 6.0 ms, using the maximum current level established during input-output testing for that slice. Also using the maximum current level, a high frequency train (100 Hz, duration=1 sec) was delivered to slices to test responsiveness to repetitive stimulation.

Injury and drug effects on input-output functions and refractoriness were assessed using CAP area. In these measurements, Clampfit v9.2 functions were used to separately quantify N1 and N2 areas, using a semi-automated process where each CAP was inspected to ensure accurate identification of the start and end of each wave component, and optimal normalization of measurement baselines. Measurements based on CAP area should best reflect total number of recruited fibers, a factor relevant to pathologies such as TBI, which conceivably may alter activation synchrony and conduction velocity. However, empirical results in our laboratory and others (Pryor and Shi, 2006), have indicted the use of CAP area and CAP peak to yield similar estimates of injury. In the present study, it was useful to use CAP peak to assess responses to 100 Hz stimulus trains, because baselines tended to be less stable during high frequency firing. Due to the large number of CAPs acquired in this protocol ($n=8000$), accuracy and efficiency were facilitated by the automated measurement of waveform peak (Clampfit v9.2).

Statistical analysis

For quantitative evaluation of injury or drug treatment effects, the unit of analysis was the rat, and not the slice. Accordingly, if more than one slice was used from a single rat, those multiple-slice data were averaged for that rat and used for the analysis. For input-output and refractoriness testing, quantitative analyses were conducted on waveforms which were the average of four successive sweeps, whereas waveforms captured during 100 Hz train stimulation were analyzed singly. Two categories of inferential statistical tests were used to evaluate the effects of injury and CsA on callosal CAPs. First, analyses conducted at single levels of stimulation were evaluated using ANOVA (SPSS v11.5) combined with Bonferroni posthoc tests. Secondly, the significance of shifts in curves, measured in input-output or refractory analyses, was evaluated with the Mann-Whitney U test. A nonparametric approach was selected in the latter case, because homogeneity of variance requirements were not met when ranges of stimulus intensities were applied. In analyses of refractoriness, ratios of CAP_2 / CAP_1 (obtained in paired stimulus presentations) were plotted as a function of interpulse interval. The significance level, $\alpha = 0.05$, was used for all inferential statistics, and averaged values are expressed as mean $\pm$ SEM.

Results

This study evaluated the efficacy of CsA to provide neuroprotection against midline fluid percussion TBI. Because we evaluated functional effects of CsA administration on axonal

injury, it was also essential to ensure that injury levels did not differ significantly between drug- and vehicle-treated groups. For this purpose, consistency in injury severity was assured by including only animals that received pressure pulse magnitudes in the range 2.0 ± 0.2 atmospheres. In addition, the duration of suppression of the righting reflex, used as an index of traumatic unconsciousness, was compared between injured groups receiving CsA and vehicle. The overall mean latency to regain the righting reflex, for all animals given fluid percussion injury, was 5.99 ± 0.33 minutes. None of the injury groups used in the study differed significantly from this overall mean righting latency ($F_{(2,29)}=1.14$; $p=0.336$).

The effects of injury on CAP amplitude

CAPs evoked in the corpus callosum of *in vitro* brain slices, in the present study, exhibited biphasic waveforms consistent with prior investigations (Ai et al., 2007; Preston et al., 1983; Reeves et al., 2005, 2007; Swanson et al., 1998), with the N1 peak occurring at a mean latency of 1.12 ± 0.08 ms, and the N2 peak at 2.77 ± 0.23 ms, in Sham+veh rats. Neither of the latency measures differed significantly between any of the analytic groups used in the study. Assuming the standard 1.0 mm distance between the stimulating and recording electrode, these latencies correspond to a conduction velocity of 0.89 m/s and 0.36 m/s for N1 and N2, respectively, as recorded at 23° C. Converted to physiological temperature, and assuming a similar temperature-velocity relationship as observed previously (Reeves et al., 2005), these estimates correspond to velocities of 1.67 m/s for N1 and 0.67 m/s for N2, at 37° C, which is within the range of *in vivo* fiber velocities previously reported for visual callosum in rabbit using precise antidromic stimulation (Swadlow and Waxman, 1976). Representative CAP waveforms from an input-output series, evoked using graded current intensities in a slice from a vehicle-treated sham rat, are shown in Figure 2A. Figure 2B shows CAPs collected at maximum stimulus intensities, representative of vehicle-treated sham and TBI rats, as well as TBI rats given CsA at 15 m and 1 h postinjury. All recordings were conducted at 24 h postinjury. These sample CAPs typify the injury-induced suppression observed in both N1 and N2 wave components (compare Sham+veh and TBI+veh waveforms), and the attenuation of this deficit in rats administered CsA at 15 m postinjury (compare TBI+veh and TBI+CsA(15m) waveforms).

Statistical comparisons of CAP amplitudes revealed that rats administered vehicle at 15 m postinjury did not significantly differ from those given vehicle at 1 h. Thus, comparing Sham+veh(15m) and Sham+veh(1h) showed that N1 Area did not differ between these groups ($p=0.462$), nor did N2 Area ($p=0.834$). Accordingly, for statistical purposes, data for these rats were pooled into a single analytic group referred to below as “Sham+veh.” In the same way, CAP amplitudes did not differ between TBI+veh(15m) and TBI+veh(1h), which were pooled as “TBI+veh.” Finally, the time of administration of CsA did not affect CAP amplitudes in sham operated rats, and these were combined as “Sham+CsA.”

Input-output curves are shown in Figure 2C, plotting mean CAP area as a function of normalized stimulus intensity, for each experimental group. Assessing the effects of injury in this way, across the full input-output range, revealed a greater injury deficit, and a larger degree of CsA neuroprotection, for the N1 CAP area than for the N2 CAP area. The input-output curve for N1 area in the TBI+veh group was shifted significantly downward relative to the Sham+veh group ($Z=3.02$, $p<0.01$), indicating an injury-induced suppression of evoked action potentials in the myelinated axon population. In TBI rats treated with CsA at 15 minutes postinjury the N1 CAP area was no longer suppressed below the Sham+veh level ($Z=1.81$, n.s.), and was significantly elevated above the TBI+veh group ($Z=2.87$, $p<0.01$), indicating an effective degree of neuroprotection for the myelinated fibers. Delaying CsA treatment until 1 h postinjury entirely eliminated the neuroprotection for the N1 CAP area, as the TBI+CsA(1h) input-output curve was significantly below that of the TBI+CsA(15m) group ($Z=3.25$, $p<0.01$) and actually fell below that of the TBI+veh group ($Z=2.50$, $p<0.05$). In sham rats, CsA

treatments had no significant effect on any CAP parameters in the study, including N1 area (comparing sham+veh vs. sham+CsA results).

In contrast to the significant injury and treatment effects seen for the N1 CAP area, the input-output curve measurements for the N2 CAP area did not reveal significant injury or treatment effects (right panel in Figure 2C). However, inspection of the N2 area input-output curves suggested that the TBI+CsA(15m) group tended to elevate above the TBI+veh group as stimulus intensity was increased. Thus, it is possible that the detection of a statistically significant degree of CsA neuroprotection, in the case of the N2 area, may depend in part on stimulus conditions. One factor underlying dissimilar N1 and N2 area results may be a difference in how myelinated and unmyelinated axons are recruited into the CAP response as stimulus current is increased from threshold to higher levels. To address this issue, the effects of injury and CsA treatment were also examined at a static level of stimulus intensity. Figure 3 shows mean N1 and N2 areas measured at the maximum current level used during input-output testing (100% level in Figure 2C). This approach continued to show a neuroprotection afforded to the myelinated axons when CsA was delivered at 15 m postinjury, as indicated by the fact that N1 area in TBI+veh rats fell below sham levels, while N1 area in TBI+CsA(15m) rats was not different from sham cases. Importantly, this analysis also revealed a significant increase in N2 CAP area in TBI rats administered CsA at 15 m after injury ($p<0.05$), which may reflect a beneficial effect of CsA, although the TBI+vehicle group narrowly failed to show a statistically significant injury effect. The N2 CAP area in TBI+CsA(15m) rats did not differ significantly from that in sham+veh or sham+CsA rats.

Analyses based on CAP areas, as presented above, should be optimal to assess CsA efficacy, if the drug affects either the total number of axons recruited into the CAP response, or the amplitudes of individual fiber potentials which summate to the full CAP waveform. This was the basis for the present emphasis on CAP area measurements, although an essentially identical pattern of injury and CsA treatment effects were obtained when CAP peak was analyzed (results not shown). However, there is fiber heterogeneity even within the population of myelinated axons, pertaining to caliber, myelin thickness, anatomical location, tortuosity, or possibly subtle molecular distinctions. Diversity would also be predicted within the unmyelinated axons. Measurement parameters which reflect the timing properties of CAP responses may reveal injury or drug effects not detected with amplitude (area or peak) measurements. For example, if TBI or CsA selectively affected the slowest fibers which normally comprise the N1 CAP, then the duration of that CAP component would decrease. Sample CAPs for vehicle- and CsA-treated TBI rats are shown in Figure 4A, with duration measurements indicated for N1 and N2 components. Statistical analysis of group data revealed the somewhat surprising result that the administration of CsA at 1 h postinjury was associated with significantly decreased durations of both the N1 and N2 CAP ($p<0.05$; Figure 4B), relative to sham levels. One explanation for this result would be a suppression of select populations of fibers, in response to CsA, conceivably comprised of fibers in a compromised or weakened status prevailing at 1 h after the fluid percussion injury.

Refractoriness Testing

To further address potential injury or CsA effects on the timing properties of callosal CAPs, the refractoriness of the N1 and N2 CAP areas were assessed. Figure 5A shows example paired responses acquired using interpulse intervals of 3, 4, 5, or 6 ms, from vehicle-treated sham and TBI rats, and from a TBI rat given CsA at 1 h. Group means are plotted in Figure 5B showing orderly increases in the second CAP, as a function of increasing interpulse interval, except for a divergence in the N1 CAP area of injured rats receiving CsA at 1 h that did reach significance ($p<0.05$). Although this effect was modest in magnitude, and may initially suggest an elevated responsiveness in the myelinated fiber population, it is more likely that CsA administered at

1 h may selectively suppress conduction in some fibers, and the refractoriness protocol demonstrated functioning in a subset of the myelinated axons.

High Frequency Responding

A final functional assessment was made of the ability of callosal axons to follow high frequency stimulation. Figure 6A shows example CAPs obtained during a 100 Hz stimulus train. To quantify the high frequency responses, each CAP peak was expressed as a percentage of the first response in the train, and the means of these ratios are plotted in Figure 6B. These scatterplots suggested that, for the N1 CAPs, TBI+veh responses were relatively suppressed towards the end of the train, and the CsA treatment provided some degree of protection against the suppression. To address these issues, the 100th CAP in the series (normalized to the first CAP in the train) was compared among the experimental groups. As seen in Figure 6C, peak amplitude of the N1 CAPs in the TBI+veh group were significantly reduced relative to Sham +veh levels ($p<0.05$). In addition, a significant protection against this postinjury suppression was noted in rats given CsA at 15 m after injury ($p<0.05$). In contrast, no significant injury- or CsA-related effects were noted for the N2 CAPs during the 100 Hz trains.

Discussion

The focus of this study was to evaluate functional characteristics of CsA neuroprotection in axons of the central nervous system. An increased understanding, of the electrophysiological features of the compound's effects, contributes to the growing knowledgebase regarding the therapeutic use of immunophilin ligands. The principal finding of the study suggested a significant neuroprotection for myelinated axons when CsA was administered at 15 m following fluid percussion injury. However, the drug effects were highly time-dependent, with some functional effects distinctive to the 1 h administration group. The present findings also support growing evidence that axon type, in this case myelinated vs. unmyelinated, exerts a potent influence on how nerve fibers respond to injury, and the degree to which they respond to neuroprotective compounds.

The corpus callosum is the largest white matter tract in the mammalian brain, making this structure an advantageous site for field potential measurements of fiber populations. Several laboratories have implemented studies which interpret N1 and N2 as useful measures of myelinated and unmyelinated function, respectively (Ai et al., 2007; Crawford et al., 2009; Preston et al., 1983; Reeves et al., 2005, 2007; Swanson et al., 1998), with the more recent reports focused on white matter pathologies. As detailed in Methods to the present report, correlated ultrastructural, electrophysiological, and pharmacological observations have led to the identification of the specific fiber populations which generate the N1 and N2 CAP components. It should be emphasized that, despite the distinctively bimodal distribution of fiber diameters and conduction velocities, there is also overlap in these parameters (Swadlow and Waxman, 1976; Waxman and Swadlow, 1976): some number of relatively faster-conducting unmyelinated axons are likely represented in the N1 CAP component, and some slower myelinated axons contribute to N2. However, consistent with the selective sensitivity of N2 to externally applied 4-AP, these waveform components are sufficiently enriched in their respective fiber types that N1 and N2 represent useful measures of myelinated and unmyelinated population activity, and are referred to as "myelinated CAP" and "unmyelinated CAP" below.

CsA Preservation of Myelinated (N1) CAP Amplitude

The major evidence for a neuroprotective benefit of CsA was the finding that a 15 m postinjury administration prevented suppression of the myelinated CAP area as measured at 24 h. The drug was less effective for the unmyelinated axons, and was contingent on the stimulus

conditions. In injured rats given CsA at 15 m postinjury, the unmyelinated CAP area was elevated over that in vehicle-treated injured rats when assessed at maximum stimulus levels, but not over the full input-output range. The injury-induced suppression of the unmyelinated CAP signal did not reach significance, in this series of rats, and this was likely a factor contributing to the mild CsA effect in this fiber population.

The mild injury effect, in the unmyelinated CAP response, contrasted with patterns of injury observed previously (Ai et al., 2007; Reeves et al., 2005), where unmyelinated deficits were equal to, or greater than, those in the myelinated axons. A less severe suppression of the unmyelinated CAP may have resulted from the neuroprotective effect reported recently for the cremaphor vehicle (Setkowicz and Guzik, 2007), and used in the present study. CsA is water insoluble, and the vehicle used here was the same pharmaceutical formulation used in clinical applications, consisting of ethanol and cremaphor (modified castor oil) dissolved in physiological saline, and was used in prior studies reporting CsA neuroprotection (Mbye et al., 2009; Okonkwo and Povlishock, 1999; Okonkwo et al., 1999; Scheff and Sullivan, 1999; Sullivan et al., 1999; Sullivan et al., 2000a,b). The goal of the present study was to document the functional effects of the clinically used pharmaceutical formulation, and a systematic evaluation of the vehicle effects were not pursued. Regarding the present results, it is possible that the cremaphor-based vehicle exerted a protection selective to the unmyelinated axons, underlying the mild injury and drug effects observed for these fibers. It is noteworthy that the same study finding neuroprotective benefits from the cremaphor vehicle also reported that CsA treatments could amplify negative consequences of a lesion injury (Setkowicz and Guzik, 2007). In this regard, some aspects of the present results also suggested modest detrimental effects when CsA was administered at 1 h postinjury. Specifically, the N1 area input-output function of the TBI+CsA(1h) group was shifted below that of the TBI+veh group. This result might be predicted if the drug action led to fewer fibers being recruited into the aggregate CAP response. In fact, certain results suggested that the 1 h CsA administration may have selectively suppressed the function of slower conducting myelinated axons. Specifically, durations of the N1 waveform component were significantly truncated, and the N1 CAPs exhibited an aberrantly shortened refractoriness. It is conceivable that the neuroprotection observed in rats given CsA at 15 m was based, in part, on a preservation of fibers that were suppressed in rats given the drug at 1 h postinjury. However, the observation that at 1 h CsA further depressed myelinated CAP amplitudes below the level seen in vehicle-treated TBI rats (see Fig. 2C), suggests a drug action on injured axons at that postinjury time point. Nevertheless, any CsA-induced functional suppression was limited, to the extent that it did not impact the benefits of CsA on high-frequency (100 Hz) responding of myelinated axons after the injury.

Morphological features of axons, including fiber caliber and myelination, along with the intrinsic structural properties of axonal membranes, may influence the responsiveness to injury and applied drugs. Considering the distinctively different surfaces presented to the extracellular milieu, by myelinated and unmyelinated axons, equally distinct responses to neuroprotective compounds (or vehicle formulations) would not be surprising. Moreover, there is reason to predict that the membrane structure, itself, may be different in myelinated and unmyelinated fiber populations. Specifically, freeze-fracture observations of developing optic nerve axons revealed distributions of intramembranous particles in premyelinated axons to be dissimilar to those in axons which had established a glial ensheathment (Black et al., 1982). As the glial-axon association progressed, the number and size of P-face particles increased, and aggregations of E-face particles developed at sites where nodes would presumably develop. Similar structural differences are likely to characterize myelinated and unmyelinated axolemma generally, including that in callosal axons, and may underlie neuropathological and treatment effects.

Therapeutic Temporal Window

Despite the alterations in CAP duration and refractoriness, associated with 1 h CsA administration, the drug was clearly protective when applied at 15 m postinjury. This was especially apparent for amplitude measures (CAP area and/or peak), which are well established outcome measures reflecting axonal integrity after neurotrauma (Ai et al., 2007; Baker et al., 2002; Crawford et al., 2009; Mobley and Agrawal, 2003; Nashmi and Fehlings, 2001; Reeves et al., 2005, 2007). It was also evident that the temporal window of effectiveness, for CsA protection of CAP amplitude measures used in this study, had elapsed by 1 h. The brevity of this window may be strongly influenced by the specific functional measures studied. By comparison, a study of CsA effectiveness in reducing lesion volume, induced by controlled cortical impact injury, found the drug to be effective when administered at 15 m or 1 h, but not at 6 h (Sullivan et al., 2000a). Interestingly, they also found that CsA treatment delayed until 24 h was again effective, attributed to fluctuations in blood-brain barrier penetration (BBB) by CsA. Because CsA does not freely cross the intact BBB, effective brain penetration depends on injury-induced compromise in the barrier (Uchino et al., 1995, 1998). Factors other than BBB fluctuations probably also contribute to the limited therapeutic window, for CsA to attenuate posttraumatic axonal conduction deficits. Much is currently unknown regarding how specific compounds interact with the injured white matter microenvironment, known to undergo rapid alterations in ionic milieu, axolemmal permeability, and deleterious molecular cascades in the initial minutes and hours following TBI (e.g., reviews by Maxwell et al., 1997; Povlishock and Katz, 2005; Smith and Meaney, 2000). The white matter response to injury is also conditioned by reactive changes in glia, which underscores the importance of reports that the immunosuppressants inhibit glial cell activation, and alter cytokine gene expression in astrocytes (Pyrzynska et al., 2001; Zawadzka and Kaminska, 2003). Agrawal and colleagues (2000) identified a critical role for glia in the pathogenesis of posttraumatic axonal conduction failure, noting that blockade of voltage-gated calcium channels, present on glia but not on axons, improved CAP amplitudes following a crush injury to excised dorsal columns.

Mitochondrial Pathology and Preferential Response of Myelinated Axons

The immunophilin ligands, CsA and FK506, achieve immunosuppressant efficacy mainly by inhibiting the phosphatase calcineurin (Liu et al., 1991). While FK506 neuroprotection is attributed to calcineurin inhibition, recent evidence indicates that CsA protects against TBI primarily by preventing mitochondrial permeability transition pore (MPTP) formation. The CsA derivative NIM811, which inhibits MPTP formation but not calcineurin activity, was found to retain the same neuroprotective potency as CsA in reducing posttraumatic spectrin proteolysis, neurologic dysfunction, and axonal degeneration (Mbye et al., 2008, 2009). Considering this evidence, it would seem likely that the neuroprotection observed in the current study was due largely to a preservation of mitochondrial integrity in callosal axons. The present observation of a disproportionate CsA benefit for the myelinated axons may reflect a more severe level of mitochondrial pathology in these larger fibers. In this context, it is notable that larger diameter axons have been reported to be more susceptible to oxygen deprivation than smaller fibers, in terms of impulse conduction (Peasley and Shi, 2002; Pryor and Shi, 2006), which was attributed to a relatively greater energy demand in the larger axons. Ultrastructural observations of rat optic nerve also support a preferential vulnerability of large myelinated axons to anoxic injury, associated with mitochondrial swelling and loss of normal cristae (Waxman et al., 1992). Similarly for the present study, the myelinated axons, larger in diameter than nearly all unmyelinated axons, may undergo a more critical level of posttraumatic bioenergetic failure, secondary to collapsing mitochondrial membrane potential, rendering them more responsive to CsA treatment. The selectivity of the larger myelinated fibers, to injury and CsA treatment, also suggests that if N2 CAPs are contaminated with small numbers

of myelinated action potentials, these would reflect slower and smaller diameter myelinated fibers injured to a much lesser degree.

While the above evidence is consistent with a CsA neuroprotection targeting mitochondrial pathology, it remains possible that calcineurin activity is involved in the injury response, and is susceptible to immunophilin ligand treatments. Evaluations of neuroprotective compounds are sensitive to numerous study variables, including injury model, anatomical region, and selection of outcome measures. Previous reports that FK506 failed to reduce posttraumatic cortical injury volume (Scheff and Sullivan, 1999), or cerebral edema (Stover et al., 2001), suggest limited benefits of calcineurin inhibition in certain models and measurement parameters. In contrast, FK506 was effective in traumatic axonal injury, attenuating impaired axonal transport (Marmarou and Povlishock, 2006; Singleton et al., 2001) and conduction deficits in callosal axons (Reeves et al., 2007). Both CsA and FK506 were observed to attenuate hypoxia-induced suppression of CAP amplitudes in spinal cord white matter, consistent with a protective role of calcineurin inhibition (Mobley and Agrawal, 2003). In view of this evident effectiveness in axonal pathology, it would be premature to rule out a calcineurin role in protection of posttraumatic axonal function afforded by CsA treatments.

Implications for CsA Neuroprotection

This study has both methodological and conceptual implications for evaluations of CsA-based neuroprotection. The present observations, based on axonal functionality, complement and extend previous investigations of CsA effects on posttraumatic cognitive function using the water maze (Alessandri et al., 2002) and hippocampal long-term potentiation (Albensi et al., 2000). Collectively, these studies point to significant functional neuroprotection that accords well with the larger body of research demonstrating histological and morphological preservation after CsA treatment. The present study also adds to growing evidence of a CsA neuroprotective efficacy, by documenting a protection of CAP amplitude and high-frequency responding when CsA was delivered at 15 m postinjury. However, among the secondary findings of this study were atypical CAP refractoriness and duration associated with 1 h CsA treatments. These effects would elude an analysis based only on conventional measurements of CAP amplitude, underscoring the advantages of multifaceted functional assessments in efforts to fully document neuroprotectant efficacy. Recent prospective clinical trials have shown CsA to have a good safety profile when treatments were initiated within the first 12 h postinjury to patients with severe head injuries (Mazzeo et al., 2009b). Increased understanding of the functional effects of CsA, including axonal conduction as studied here, should assist in the development of monitoring and assessment strategies to evaluate the full neuroprotective potential of the immunophilin ligands, in experimental and clinical investigations.

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Figure 1.

Electrode placement in corpus callosum, and waveform measurement parameters. Stimulating and recording electrodes were positioned to record evoked CAPs in midline-crossing callosal fibers. CAP waveforms display an early (N1) and late (N2) component, generated by myelinated and unmyelinated axons, respectively. Measurement technique for waveform area, peak, and duration are illustrated.

Figure 2.

Effects of injury and CsA treatment on CAP area. **A.** Input-output relationships were defined with graded stimuli spanning approximately threshold levels to the maximum for the N1 wave component. **B.** Representative CAPs evoked at the maximum stimulus level for vehicle treated sham and TBI rats, and for TBI rats given CsA at 15 m and 1 h. Time of stimulation marked with (●). Note preservation of the early N1 wave in the TBI+CsA(15m) condition. **C.** Input-output curves, showing N1 and N2 CAP area plotted as a function of normalized stimulus intensity. The average input-output function of the N1 CAP area was protected in TBI rats by treatment with CsA at 15 m, but not at 1 h, postinjury. Other significant curve shifts shown are: Sham+veh vs. TBI+veh; TBI+CsA(15m) vs. TBI+CsA(1h); TBI+veh vs. TBI+CsA(1h).

Average N2 area input-output functions were not significantly shifted by injury or CsA treatment. * $p < 0.05$. Symbols in (C) are slightly offset to avoid overlap of errorbars. CAP waveforms in (A) and (B) are averages of four sweeps. Group sizes: Sham+veh, $n=16$; Sham+CsA, $n=16$; TBI+veh, $n=16$; TBI+CsA(15m), $n=8$; TBI+CsA(1h), $n=8$.

Figure 3.

Effects of injury and CsA treatment on area of CAPs evoked with maximum stimulation. Mean N1 and N2 areas, evoked using the 100% stimulation intensity, are plotted for each analytic group. N1 areas in TBI+veh rats were significantly suppressed below sham levels, but N1 areas in TBI+CsA(15m) rats were not different from those of sham rats. For the N2 CAP areas evoked with maximum stimuli, there was not a significant injury effect (Sham+veh vs. TBI+veh), but CsA treatment administered at 15 m post-TBI significantly elevated the N2 area above vehicle-treated rats. This drug effect was not present when CsA was given at 1 h postinjury. Group sizes: Sham+veh, n=16; Sham+CsA, n=16; TBI+veh, n=16; TBI+CsA(15m), n=8; TBI+CsA(1h), n=8.

Figure 4.

Effects of injury and CsA treatment on duration of N1 and N2 waveform components. **A.** CAPs evoked at maximum stimulation levels, showing CAP durations typical of vehicle- and CsA-treated TBI rats. Waveforms are averages of four sweeps. **B.** Analysis of group mean durations revealed significantly decreased CAP durations in injured rats given CsA at 1 h postinjury. This effect was especially pronounced for the N1 CAP component. Group sizes: Sham+veh, n=16; Sham+CsA, n=16; TBI+veh, n=16; TBI+CsA(15m), n=8; TBI+CsA(1h), n=8.

Figure 5.

Effects of injury and CsA treatment on refractoriness of N1 and N2 waveform components.

A. Example paired CAP responses from Sham+veh, TBI+veh, and TBI+CsA(1h) cases, acquired using interstimulus intervals of 3, 4, 5, and 6 ms. Waveforms are averages of four sweeps. (●) marks response 1 stimulus, (▲) marks response 2 stimulus. Calibration: 1 mV × 5 ms. **B.** Analyses of group refractoriness revealed no differences among refractory curves, except an upward divergence in TBI+CsA(1h) rats over Sham+CsA rats ($p < 0.05$). No significant shifts in refractory curves were noted for N2 CAPs. Symbols in (B) are slightly offset to avoid overlap of errorbars. Group sizes: Sham+veh, $n=16$; Sham+CsA, $n=16$; TBI+veh, $n=16$; TBI+CsA(15m), $n=8$; TBI+CsA(1h), $n=8$.

Figure 6.

Effects of injury and CsA treatment on high frequency responding. **A.** Example evoked CAPs (single, non-averaged) at serial position 1, 25, 50, 75, and 100 in response to a 100 Hz stimulus train of 1 s duration. Calibration: 1 mV $\times$ 2 ms. **B.** Scatterplot showing mean of CAP peaks for each 100 responses in high frequency train, with each data point normalized to the first CAP in the series. In N1 scatterplot, note fatigue of TBI+veh responses and preservation of CsA-treated responses between CAP position 75–100. **C.** Group analysis of the N1 CAP number 100, expressed as a percentage of CAP number 1, showed group means showed TBI +veh to be significantly depressed by the injury, but CsA treatment at 15 m provided significant

protection of this function. Group sizes: Sham+veh, n=16; TBI+veh, n=16; TBI+CsA(15m), n=8; TBI+CsA(1h), n=8.