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Targeting Kv1.3 channels to reduce white matter pathology after traumatic brain injury

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Abstract

Axonal injury is present in essentially all clinically significant cases of traumatic brain injury (TBI). While no effective treatment has been identified to date, experimental TBI models have shown promising axonal protection using immunosuppressants FK506 and Cyclosporine-A, with treatment benefits attributed to calcineurin inhibition or protection of mitochondrial function. However, growing evidence suggests neuroprotective efficacy of these compounds may also involve direct modulation of ion channels, and in particular Kv1.3. The present study tested whether blockade of Kv1.3 channels, using Clofazimine (CFZ), would alleviate TBI-induced white matter pathology in rodents. Postinjury CFZ administration prevented suppression of compound action potential (CAP) amplitude in the corpus callosum of adult rats following midline fluid percussion TBI, with injury and treatment effects primarily expressed in unmyelinated CAPs. Kv1.3 protein levels in callosal tissue extracts were significantly reduced postinjury, but this loss was prevented by CFZ treatment. In parallel, CFZ also attenuated the injury-induced elevation in pro-inflammatory cytokine IL1- β . The effects of CFZ on glial function were further studied using mixed microglia/astrocyte cell cultures derived from P3-5 mouse corpus callosum. Cultures of callosal glia challenged with lipopolysaccharide exhibited a dramatic increase in IL1- β levels, accompanied by reactive morphological changes in microglia, both of which were attenuated by CFZ treatment. These results support a cell specific role for Kv1.3 signaling in white matter pathology after TBI, and suggest a treatment approach based on the blockade of these channels. This therapeutic strategy may be especially efficacious for normalizing neuro-glial interactions affecting unmyelinated axons after TBI.

Keywords

microglia; inflammation; white matter injury; recovery of function; compound action potentials; glial cell culture

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Introduction

Traumatic brain injury (TBI) is an enduring public health problem, with over 1.7 million new TBIs occurring annually in the United States alone (Rutland-Brown et al., 2006). Because of their extended cytoarchitecture and high membrane content, axons are a subcellular compartment which is highly vulnerable to TBI. Axonal injury is a concomitant of most TBIs requiring hospitalization, where diffuse forms of this pathology were observed to occur in 72% of patients with moderate or severe TBI, and were associated with worse outcome (Skandsen et al., 2010). To date, no effective treatment for TBI-induced axonal injury has been demonstrated for head injured patients. However, studies using rodent models of TBI have shown promising reductions in axonal pathology using treatment with immunosuppressant drugs, especially FK506 and Cyclosporine-A (CsA). These compounds reduce axonal damage in multiple ways following TBI: attenuating cytoskeletal compaction (Büki et al., 1999; Okonkwo et al., 1999), alleviating impairments to axonal transport (Marmarou et al., 2006; Singleton et al., 2001), and improving axonal conduction properties (Reeves et al., 2007; Colley et al., 2010).

Despite these promising experimental results, the usefulness of immunosuppressants in a neuroprotective strategy is diminished by significant side effects, including nephrotoxicity and hypertension (Fung and Starzl, 1995; Staats and Tett, 2004). FK506 and CsA inhibit calcineurin, a Ca^{2+} -calmodulin activated protein phosphatase, preventing subsequent calcineurin-dependent interleukin-2 transcription and T lymphocyte activation (Marks, 1996; Sigal and Dumont, 1992; Thomson et al., 1995), depressing the acute inflammatory response. Because the nephrotoxicity of FK506 and CsA shares a common molecular basis with the immunosuppressive effect (Sigal et al., 1991), and the neuroprotective efficacy of these drugs does not depend on their immunosuppressant activity (Mbye et al., 2008), it is reasonable to investigate alternative neuroprotective strategies which target other calcineurin-independent mechanism(s) of these compounds.

Accumulating evidence shows FK506 and CsA exert powerful effects on ion channel functioning, which may play key roles in their neuroprotective efficacy. Several types of voltage-gated potassium (Kv) channels are directly modulated by FK506 independently of calcineurin. Studies in rat hippocampal neurons showed FK506 directly modulated the activity of the Ca^{2+} -dependent K^{+} channel (Terashima et al., 1998), and inhibited the delayed rectifier K^{+} channel current (Yu et al., 2007). Of particular interest, FK506 inhibited Kv1.3 channels by mechanisms not involving the suppression of calcineurin phosphatase activity, but by a direct interaction with the channel protein (Ahn et al., 2007). Kv1.3 channels have been most thoroughly characterized in T lymphocytes (reviewed by Cahalan and Chandy, 2009), but are widely distributed in CNS (Mourre, 1999), and are integral to microglial function (Kotecha and Schlichter, 1999; Pannasch et al., 2006). Increasing evidence points to a contribution of Kv1.3 channels in diverse neuropathologies, including Alzheimer's disease (Rangaraju et al., 2014), multiple sclerosis and other autoimmune diseases (Lam et al., 2011; Rus et al., 2005), and apoptosis (Szabo et al., 2008). We predict that Kv1.3 channels will prove useful therapeutic targets in TBI as well.

There is evidence to suggest that pharmacologic blockade of Kv1.3 channels would exert a neuroprotective effect by reducing acute TBI-induced microglial activation. Results from cell culture experiments showed that activated microglia can induce cell death of hippocampal neurons through a process requiring microglial Kv1.3 channel activity (Fordyce et al., 2005), and that blockade of Kv1.3 channels markedly diminished microglial migration to cellular debris (Natile-McMenemy et al., 2007). Accordingly, in this study we investigated the extent to which pharmacologic inhibition of Kv1.3 channel activity would provide a neuroprotective effect in white matter, and whether any treatment benefit is related to reduced posttraumatic neuroinflammation and microglial reactivity. There is extensive evidence that neuroinflammatory events are a major determinant of injury progression in the secondary phase of TBI pathology (reviewed by Morganti-Kossmann et al., 2007; Woodcock and Morganti-Kossmann, 2013), and there is a growing recognition of the importance of glial activation after TBI, with its impact on axons serving as a major factor in the balance of pathology vs. recovery after white matter injury (reviewed by Kou et al., 2014).

The present investigation tested the efficacy of blocking Kv1.3 channel activity to alleviate axonal conduction deficits in a well-established rodent model of TBI. For this purpose, Clofazimine (CFZ) was administered following fluid percussion injury. CFZ is an FDA-approved antibiotic that has been used in humans since the early 1960s. Apart from its anti-mycobacterial activity, CFZ was demonstrated to be a potent and highly-selective inhibitor of Kv1.3 channels, without affecting the enzymatic activity of calcineurin (Ren et al., 2008). Here, the effects of injury and CFZ treatment on axonal function were first evaluated in adult rats using compound action potentials (CAPs) evoked in the corpus callosum. Analyses of CAPs were focused on separate evaluations of myelinated vs. unmyelinated axons. Previous results, obtained with the same injury parameters as used presently, revealed these two axon populations to have distinct functional impairments after TBI (Reeves et al., 2005), different injury-induced structural alterations (Reeves et al., 2012), and dissimilar responses to neuroprotective treatment with FK506 (Reeves et al., 2007) and CsA (Colley et al., 2010). Because myelinated and unmyelinated axons present markedly different interfaces to surrounding glial cells, it was expected that the greater axolemmal exposure of unmyelinated fibers to the extracellular milieu would influence the response to CFZ treatment. Based upon our preliminary immunohistochemical (IHC) observations that corpus callosum axons and glia express both Kv1.3 and Kv1.2 channel proteins, we next examined the effect of CFZ treatment on expression of these proteins using callosal tissue extracts and IHC.

In a complementary set of experiments, we determined how CFZ blockade of Kv1.3 affects inducible reactivity of mixed microglial and astrocyte cultures. Given that prior studies pointed to Kv1.3 mediation of reactive glial response (Natile-McMenemy et al., 2007), and differences likely exist between gray and white matter glia (Anderson et al., 2014; Gertig et al., 2014), we used corpus callosum-derived glial cultures to test the efficacy of CFZ to protect against pro-inflammatory cytokine induction after lipopolysaccharide (LPS) treatment. While LPS does not directly model TBI-induced inflammation, it is a common approach used for the analysis of Kv1.3 role in microglia of other tissue injury models (Charolidi et al., 2015; Moussaud et al., 2009; Pannasch et al., 2006; Villalonga et al., 2010). Since high levels of inflammatory cytokines generated around axon damage are capable of signaling microglial activation within minutes postinjury (Woodcock, Morganti-Kossmann,

2013), and Kv1.3 signaling is well documented for CNS microglia, we reasoned that LPS exposure would provide ample acute signal to initially test CFZ/callosal glia interaction in vitro. Once confirmed, future studies would be feasible, applying TBI-specific molecular activators of inflammation, such as reactive oxygen species, heat shock proteins, IL-1 cytokines and lysed axonal proteins (Walko III et al., 2014). Using morphological phenotype and IL1- β cytokine expression as metrics for reactivity, we predicted that CFZ would attenuate prior LPS-induction of activated microglial morphology, and reduce the associated glial increase in pro-inflammatory IL1- β .

Materials and Methods

Experimental Animals

All procedures met national guidelines for care and use of laboratory animals, and all experimental protocols were approved by the VCU Institutional Animal Care and Use Committee. Both rat and mouse subjects were utilized. Male Sprague Dawley rats (300-350 g; Harlan Laboratories) were housed in pairs under a temperature (22°C) and humidity controlled environment with food and water *ad libitum*, and subjected to a 12 hour dark-light cycle. Rats (n=34) were used in electrophysiological experiments to determine the efficacy of CFZ to protect against axonal conduction deficits induced by midline fluid percussion injury (FPI). For *in vitro* experiments to assay the effect of CFZ on glial inflammatory response, corpus callosa were harvested from litters of C57BL/6 mouse pups (P3-5), to generate mixed glial cultures of microglia and astrocytes. All *in vitro* experiments were repeated in four separate cultures.

Fluid Percussion TBI Procedure, and CFZ administration

The model used for injury was the same as that described by Dixon et al. (1987). Briefly, all animals were surgically prepared under nose-cone isoflurane anesthesia (2% in carrier gas of 70% N₂O, 30% O₂). 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 (Model 01-B, Custom Design and Fabrication, Inc., Richmond, VA) consisted of a 60 $\times$ 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. 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 that the intracranial pressure pulse was not injected.

At 15 minutes following TBI, rats received an intraperitoneal bolus of 50 mg/kg CFZ (Sigma-Aldrich, Inc.) in vehicle (100% dimethyl sulfoxide), or vehicle alone, injected at a volume of 0.1 ml / 100 g body weight. The following experimental groups were used: TBI + vehicle (n=7); TBI + CFZ (n=9); Sham + vehicle (n=11); Sham + CFZ (n=7).

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 4% isoflurane anesthesia, and the brain rapidly removed. Coronal slices of 450 μ m thickness were cut in ice cold (4 to 6°C) artificial cerebrospinal fluid (ACSF) with a vibrating-knife microtome (model OTS-3000-03, Electron Microscopy Sciences, Hatfield, PA). All slices were saved which contained midline-crossing segments of the corpus callosum, including genu, callosal midbody, and splenium regions. 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. 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. For recording, a slice was transferred to a submersion-type chamber, and perfused at a rate of 1-2 ml / min., and recordings conducted with slices at room-temperature (22-23 degrees C), based on our previous finding that this temperature range was optimal for separate quantification of the myelinated and unmyelinated CAP components (Reeves et al., 2005). 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, and a recording electrode (ACSF-filled glass micropipette; resistance 4 - 8 M Ω) was also placed in the corpus callosum of the contralateral hemisphere at a distance of approximately 1.0 mm from the stimulating electrode (Fig. 1A). Fine adjustments were made in the depths of both stimulating and recording electrodes to optimize the signal amplitude. Evoked compound action potentials (CAPs) were amplified (bandpass = DC to 10 kHz), and digitized at 25 kHz for offline analysis.

The callosal CAP has been extensively 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) (Preston et al., 1982; Swanson et al., 1998; Ai et al., 2007; Reeves et al., 2005, 2007; Crawford et al., 2009; Colley et al., 2010) [Fig. 1B]. CAP amplitudes, for N1 and N2 waveforms, were calculated as shown in Fig. 1C, by averaging the vertical distances of the negative peak to the preceding and following positivities [$\text{N1} = (a+b)/2$; $\text{N2} = (c+d)/2$]. 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. We previously demonstrated this method of current standardization results in conservative levels of stimulus currents, avoiding extensive stimulation at supramaximal levels for N1 fibers, and captures the linear portion of the N2 signal (Reeves et al., 2005). In refractoriness testing, pairs of pulses were presented with interpulse interval increased, in 1.0 ms steps, from 4.0 ms to 15.0 ms, using the maximum current level established during input-output testing for that slice. In analyses of refractoriness, mean ratios of CAP2/CAP1 (obtained in paired stimulus presentations) were plotted as a function of interpulse interval, and fitted to Boltzmann sigmoid curves. Conduction velocity of callosal axons was measured by holding the stimulating electrode in a constant position, while systematically moving the recording pipette to acquire CAPs at inter-electrode distances between 0.75 mm and 3.0 mm, as determined using a motorized micromanipulator (model MPC-385-2, Sutter Instrument Co., Novato, CA). Conduction velocity was computed as the inverse of the slope in linear regressions relating response latency to inter-electrode distance (Crawford et al., 2009).

Immunohistochemistry and Western blot of Corpus Callosum

In order to document cellular localization of callosal Kv1.3 and Kv1.2, immunohistochemistry (IHC) was first performed in a subset of TBI and Sham injured rats (n=3/group) surviving for 24h after injury. Animals were perfused with 4% paraformaldehyde, 30-40 μ m coronal brain sections cut by vibratome and tissues processed for IHC (Warren et al., 2012) using antibodies for Kv1.3 (*Alomone*, *Neuromab*; 1:50), Kv1.2 (*Alomone*; 1:150) IBA1 (*Wako*, *Abcam*; 1:300, 1:400), and GFAP (*Dako*; *Encor Biotechnology*; 1:20,000, 1:2,000). Images were collected on a Zeiss 700 confocal microscope for qualitative tissue localization of each protein. A second subset of rats was then subjected to TBI, Sham injury or TBI+CFZ treatment and at 24h after injury, animals were either decapitated under 4% isoflurane anesthesia and corpus callosum dissected for Western blot (WB; n=7/group) analysis of protein extracts (Reeves et al., 2010), or perfused with aldehyde as described above to perform Kv1.3 IHC (n=3/group). For WB, tissue was homogenized in 1X RIPA Lysis Buffer (*Millipore*) containing Complete Protease Inhibitor Cocktail (*Roche*), 0.1% SDS and 2mM EGTA. After centrifugation (14,000xg at 4°C) supernatants were collected, assayed for protein content and aliquots of 20 μ g separated on 4-12% SDS-PAGE prior to transfer to PVDF membranes (*Invitrogen*). Post blot membranes were subjected to immunobinding with either Kv1.3 or Kv1.2 antibodies (*Alomone*; 1:100, 1:150) or IL1- β (*R&D Systems*; 1:200) after published methods (Reeves et al., 2010). Immunoreactive bands were visualized with Gene Snap (*Syngene*) instrumentation using ECLTM Western Blotting Analysis System (*Amersham*), relative optical density (ROD) measured with Gene Tools programs and results reported as percent of untreated control values run on the same blot.

Culture of Enriched Corpus Callosum Glia

Mixed primary glial cultures, enriched for callosal microglia and astrocytes, were generated from P3-5 C57BL/6 mouse pups and prepared for culture as previously described (McCarthy and de Vellis, 1980; Trimmer et al., 1991; Kozlova and Takenaga, 2005). Cells were plated in 25cm²(T25) flasks in a culture medium (CM) consisting of Dulbecco's modified Eagle's medium, 10% fetal calf serum (Hyclone), pyruvate (1mM) and antimycotic-antibiotic (*Invitrogen*) at a density of 5 x 10⁵ cells. Cultures were replated (4-8 X 10⁵ cells/T25 flask) and used for protein extraction and WB analysis when confluent (total days in vitro: 19-20). MatTek coverslip dishes (coated with polylysine) were seeded from the same cell suspension at subconfluent density for immunocytochemistry (ICC). At 14 DIV, a subset of dishes and confluent flasks were exposed to LPS (1ug/ml CM) in order to mimic postinjury inflammation (Zhang and Gottschall, 1997; Rosenberg, 2001; Liu et al., 2007). After 24h, LPS containing media was removed from dishes and flasks and replaced with fresh CM for a 1h wash out period. Following LPS wash out, CFZ (10μM in CM) was added to dishes and flasks for an additional 24h period according to published protocols (Steel et al., 1999; Ren et al., 2008). In addition to LPS + CFZ, each study included dishes and flasks that were exposed to LPS (1ug/ml in CM) or CFZ (10μM in CM) or left untreated (CM alone) for 24h. At the end of treatment intervals, flasks were photographed with phase microscopy to document cell morphology, then media was removed, flasks rinsed with Dulbecco's PBS (+Ca²⁺, Mg²⁺) and cells scraped free (in Dulbecco's PBS (-Ca²⁺, Mg²⁺) for protein extraction. As for tissue extracts, protein aliquots were separated by 4-12% SDS-PAGE and transferred to PVDF membrane (Reeves et al., 2010; Chan et al., 2014) prior to WB probe of IL1-β (*R&D Systems*; 1:200) expression. Immunopositive bands were also visualized with Gene Snap (*Syngene*), relative optical density (ROD) measured with Gene Tools and results reported as percent of untreated control values run on the same blot. Parallel MatTek dishes in each treatment group were fixed with 4% paraformaldehyde and stored in PBS. The PBS was removed and replaced with Vectashield mounting media plus DAPI in order to collect confocal fluorescent images and assess the co-localization of signals from IL1-β (*R&D Systems*; 1:100) or Kv1.3 (*Neuromab*; 1:50) in IBA1+ (*Wako, Abcam*; 1:300, 1:400) microglia or GFAP+ (*Dako, Encor Biotechnology*; 1:20,000, 1:2,000) astrocytes, stained as described for IHC. Images were collected using an Olympus FV1000 confocal microscope. Experiments were repeated in four separate cultures.

Statistical analysis

For input-output and refractoriness testing, quantitative analyses were conducted on waveforms which were the average of four successive sweeps. In cases where more than one slice was used from a single rat, those multiple-slice data were averaged for that rat and used for the analysis. Two categories of inferential statistical tests were used to evaluate the effects of injury and CFZ on callosal CAPs. First, analyses conducted at single levels of stimulation were evaluated using ANOVA (SPSS v21) combined with Bonferroni posthoc tests, and where appropriate, effect size was estimated using the Cohen's D statistic. 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. Statistical analysis of WB data was also performed using SPSS v21. Raw relative optical density measures were first tested for normality (Kolmogorov-Smirnov statistic) and homogeneity of variance. Appropriate data were then analyzed with ANOVA, and Dunnett post hoc tests. The significance level, $\alpha = 0.05$, was used for all inferential statistics, and averaged values are expressed as mean $\pm$ SEM.

Results

Because this study evaluated functional effects of CFZ 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 with injury 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 CFZ and vehicle. Mean latency to regain the righting reflex in TBI+CFZ rats was 6.81 ± 0.92 minutes, which did not differ from the mean latency of 7.10 ± 0.92 minutes measured in TBI+vehicle rats ($F_{(1,15)}=0.091$; $p=0.767$).

CFZ crosses the blood-brain barrier following TBI

Previous work has indicated that, in normal uninjured animals, CFZ crosses the blood-brain barrier (BBB), but only in very small amounts (O'Connor et al., 1995). However, breakdown of the BBB is a well established component of TBI pathophysiology (reviewed by Shlosberg et al., 2010). For the purposes of the present investigation, it was important to establish the degree to which CFZ gains parenchymal access when applied systemically following the fluid percussion injury. To address this issue, we made use of the autofluorescence properties of CFZ which will fluoresce under 488 nm illumination (Baik and Rosania, 2011). Following CFZ administration at 15 min postinjury, and histological assessment at 24 h, we observed only limited CFZ in brains of sham-injured rats (Fig. 2A). However, when administered after TBI, CFZ fluorescence was abundant, showing increased diffuse localization and cellular accumulations morphologically consistent with callosal glia (Fig. 2B).

The effects of injury and CFZ treatment on CAP amplitude

The effects of injury and drug treatment on CAP amplitude were analyzed in two ways. First, waveform measurements obtained from the three callosal regions (genu, middle, splenium) were averaged into a single “whole callosum” estimate of CAP amplitude. Because midline fluid percussion induces a diffuse injury (Kelley et al., 2007; Reeves et al, 2012), without the focal contusions of lateral fluid percussion or controlled cortical impact (reviewed by Bondi et al, 2015), it was expected that aggregating CAP measurements from all three regions would provide a stable estimate of global axonal functioning throughout the callosum structure, appropriate for a diffuse pathology. All 34 rats undergoing CAP recording were included in the whole callosum analysis of CAP amplitude, with group sizes ranging from 7 to 11 for the various treatment groups. A second approach conducted analyses separately for CAPs acquired in the genu, middle callosum, or splenium, with

group sizes ranging from 4 to 9 for each combination of treatment group and callosal region. It was anticipated these region-specific results would reveal a functional correlate to our prior observations of a rostro-caudal gradient in unmyelinated axon density, where we found unmyelinated axons to be most abundant in the splenium, and to decrease to a minimum in the genu (Reeves et al., 2012).

Representative CAP waveforms from input-output series, evoked using graded current intensities, are shown for each analytic group in Figure 3A. These sample CAPs typify the injury-induced suppression observed in the signal component generated primarily by unmyelinated axons (N2), seen by comparing Sham+vehicle waves with TBI+vehicle. Administration of CFZ after injury protected against this functional suppression (TBI+CFZ waveforms in Figure 3A), but the drug treatment did not alter CAPs evoked in sham-injured rats (Sham+CFZ). Input-output curves from each group, representing whole-callosum averages, were examined to test for significant injury-induced suppression (downward curve shifts) and for neuroprotective treatment efficacy (upward shifts). N2 CAP input-output amplitudes were severely suppressed by the fluid percussion injury [Sham+vehicle vs. TBI+vehicle: $Z=8.93$; $p<0.001$] (Fig. 3C). However, N2 input-output curves measured in slices from rats treated with CFZ after injury were significantly elevated over injured rats given vehicle alone [TBI+CFZ vs. TBI+vehicle: $Z=8.100$; $p<0.001$] (Fig. 3C), indicating a significant neuroprotective effect of CFZ for the unmyelinated axon population. Input-output recordings for the myelinated axons (N1) also showed curve shifts indicating a more modest, but still statistically significant, injury effect [Sham+vehicle vs. TBI+vehicle: $Z=4.026$; $p<0.001$], and a significant effect of CFZ treatment [TBI+CFZ vs. TBI+vehicle: $Z=4.15$; $p<0.001$] (Fig. 3B).

Although the input-output analysis indicated significant effects of TBI and CFZ treatment, for both N1 and N2, visual inspection of Figs. 3B,C suggested that TBI induced a more severe axonal dysfunction as measured with the N2 signal. In addition, the functional restoration associated with CFZ treatment appeared more potent for N2. In order to directly compare the magnitude of injury and drug treatment effects on the myelinated vs. unmyelinated fiber populations, ANOVA analyses were conducted on CAPs evoked at a single normalized stimulus intensity (the 100% level, as shown in Figs. 3B,C). At the 100% stimulation level, the mean N2 CAP amplitude measured in Sham+vehicle rats was 1.88 ± 0.30 mV. This evoked response was reduced by an average of 76% in TBI+vehicle rats to 0.44 ± 0.06 mV ($p<0.01$). In the case of N1 CAPs, despite significant curve shifts seen in the N1 input-output functions (Fig. 3B), ANOVA analyses restricted to the single 100% stimulation level did not show a significant effect of TBI (Sham+vehicle vs. TBI+vehicle; $p=0.490$). ANOVA results at 100% stimulation also showed a sharp contrast in CFZ neuroprotective potency for these axonal populations, and these comparisons are shown in Figs. 3D,E (“Whole CC” bars). The CFZ treatment significantly increased the mean N2 CAP amplitude from 0.44 ± 0.06 mV in TBI+vehicle to 1.61 ± 0.23 mV in TBI+CFZ rats ($p<0.01$), whereas the drug did not improve N1 CAP amplitude ($p=0.146$). An analysis of magnitude of effects, using Cohen D coefficients, for the N2 CAP showed $D=1.85$ (injury effect) and $D=2.28$ (drug treatment effect), which were markedly larger than those for N1 CAPs ($D=0.35$, injury; and, $D=0.67$, drug treatment).

The more severe injury-induced suppression of unmyelinated (N2) CAP amplitude over that of myelinated (N1) CAPs is consistent with previous studies using midline fluid percussion (Ai et al., 2007; Reeves et al., 2005, 2007; Colley et al., 2010). In the present study, maximum N2 CAP amplitudes post-TBI were only $23.56\% \pm 2.95\%$ of sham control values, which was a significantly greater deficit than for the postinjury N1 CAPs, which decreased to $85.20\% \pm 22.27\%$ of sham levels ($F_{1,12}=7.53$; $p<0.05$). However, our recent evidence of a striking genu-to-splenium gradient of increasing unmyelinated fiber density motivated us to investigate if these varying densities would impact the effects of injury or drug treatment on unmyelinated CAPs. Prior to considering the injury effects, however, we first asked if these regional differences in fiber density correlated with evoked CAP amplitudes in uninjured (Sham+vehicle) rats. Consistent with the highest abundance of unmyelinated fibers in the splenium, we observed the largest maximum CAP magnitudes were observed in this region (2.36 ± 0.45 mV). However, this response level was not significantly different from maximum CAPs in the middle callosum (1.31 ± 0.34 mV) or the genu (1.51 ± 0.31 mV) ($F_{2,15}=2.18$; $p=0.148$). In addition, these amplitudes did not exhibit the uniform splenium-to-genu gradient predicted from measurements of fiber density based on stereological ultrastructure (Reeves et al., 2012). Moreover, following TBI, there were no significant differences in the maximum N2 CAPs among the three callosal subregions (compare TBI+vehicle bars in Fig 3E).

Although regional differences in the density of unmyelinated axons did not predict the severity of TBI-induced suppression of CAP amplitude, some features of unmyelinated axon cytoarchitecture may strongly influence drug efficacy in white matter. Unmyelinated axons present an uninterrupted axolemmal exposure to the extracellular environment, including matrix molecules and dissolved compounds in the interstitial compartment. Importantly, the distinctive morphology of these small fibers is also likely to affect how they interact with surrounding glial cells, a critical factor in drug strategies targeting post-traumatic inflammation. Present results indicated CFZ treatment was most effective in the splenium, where unmyelinated axons are most abundant. In Sham+vehicle rats, the maximum N2 CAP amplitude measured in the splenium was 2.36 ± 0.45 mV. Following TBI, this parameter was significantly reduced to 0.62 ± 0.14 mV (TBI+vehicle) ($p<0.01$), but measured 2.12 ± 0.32 mV in TBI+CFZ rats, indicating CFZ treatment significantly prevented CAP suppression in this region ($p<0.01$). The efficacy of CFZ to prevent TBI-induced N2 CAP suppression is illustrated in Fig. 3E, for each of the callosal subregions, comparing TBI+vehicle to TBI+CFZ. In the splenium, CFZ produced a mean 1.50 ± 0.32 mV increase in maximum N2 CAP amplitude over that of vehicle-treated injured rats ($p<0.01$). Mean improvement of N2 signal amplitude, related to CFZ treatment, for middle callosum was 0.75 ± 0.23 mV ($p<0.05$), and for the genu was 0.38 ± 0.30 mV (not significant). These results for a neuroprotective effect of CFZ are in register with a gradient of decreasing unmyelinated axon density from the splenium to the genu, as are the Cohen D magnitudes of effect: splenium ($D=1.97$), middle callosum ($D=1.76$), and genu ($D=1.65$). Measurements of maximum N1 CAP amplitudes did not reveal any significant effects of CFZ in any callosal region (Fig. 3D).

The effects of injury and CFZ treatment on refractoriness and conduction velocity

The above analyses of CAP amplitudes suggest the TBI model reduced the numbers of intact axons recruited into the evoked CAP field potential, or reduced the mean amplitude of fields generated by individual axons. CFZ administration provided significant protection against this deficit for the unmyelinated fiber population. Complementary to the CAP amplitudes, it was also useful to assess timing properties of the fiber pathway, specifically refractoriness and conduction velocity, which reflect intrinsic axonal properties, and are less sensitive to the absolute numbers of recruited fibers than is the amplitude parameter. Due to practical limitations imposed by brain slice viability, it was not always possible to complete timing recordings from all three callosal regions for every rat. Accordingly, data were aggregated into whole callosum assessments for refractoriness (n=21 rats) and conduction velocity (n=27 rats), with analytic group sizes ranging from 4 to 8.

Figure 4A shows representative CAP waveforms evoked by the second pulse of paired stimulus presentations, using interpulse intervals ranging from 4 to 15 ms. Refractoriness was quantified as the ratio of the second CAP amplitude to the first, expressed as a percent ($C2 / C1 \times 100$). Group means are plotted in Fig. 4B, showing orderly increases of this ratio as a function of interpulse interval. Analyses of the N1 (myelinated axons) curves revealed a downward shift indicating some loss of function due to TBI: Sham+vehicle vs. TBI+vehicle, $Z=3.23$, $p<0.01$. Further, this deficit in refractoriness was significantly prevented by CFZ treatment (TBI+vehicle vs. TBI+CFZ, $Z=1.98$, $p<0.05$), although this neuroprotective effect was modest in size. No other curve shifts were significant for the N1 signal, and neither TBI or CFZ treatment conditions resulted in any significant shifts in refractoriness curves for the unmyelinated (N2) fibers.

Axonal conduction velocity was not significantly altered by the injury or drug treatment. Figures 5A,B show representative waveforms and regression plots used to calculate velocity, for a control (Sham+vehicle) animal. Group means are plotted in Fig. 5C. None of the experimental groups significantly differed from the overall mean of $0.72 \pm 0.28 \text{ m s}^{-1}$ for the myelinated fibers ($F_{(3,26)}=0.37$; $p=0.777$), or $0.32 \pm 0.01 \text{ m s}^{-1}$ for the unmyelinated fibers ($F_{(3,26)}=1.35$; $p=0.282$). Although this is a negative finding, it is useful by providing evidence that injury and CFZ treatment do not impact basic mechanisms of action potential propagation. Instead, the lack of effects on CAP conduction velocity, taken together with significant TBI and CFZ alterations to CAP amplitude, suggest injury and treatment modulated either the number of axons recruited into the evoked field potential or the average amplitude of action potential discharges in individual fibers.

Kv1.3, Kv1.2 distribution after TBI; effect of CFZ on inflammatory response

IHC of rat corpus callosum showed that both Kv1.3 and Kv1.2 were localized within axons and glia of the white matter tract (Fig. 6A). At 24h after TBI, confocal imaging showed significant signal for each channel within process bearing glia surrounding callosal vessels, as well as intermittent Kv1.3 and Kv1.2 signal along axon bundles. Under higher magnification, axon labeling (Fig. 6A, inset) shows a 'paired' distribution indicating paranodal clustering of K⁺ channel proteins (Rasband 2004; Utsunomiya et al., 2007), with some nodes showing co-localization of the two Kv channels. This overall pattern indicated

that Kv1.3 expression occurs in both the axons of the corpus callosum, as well as the commonly reported distribution of the channel within CNS glia (Kotecha and Schlichter, 1999; Pannasch et al., 2006). Additional co-localization experiments combining Kv1.3 with microglial marker IBA1 or astrocyte marker GFAP, revealed that each glial cell type expressed the channel (Fig. 6B). WB probe of total callosal protein extracts showed that TBI significantly reduced Kv1.3 by 32.9% relative to Sham levels ($p < 0.05$) (Fig. 6C). The mean Kv1.3 level in the TBI+CFZ group was significantly elevated by 102.7% over the TBI group ($p < 0.05$). Although Kv1.3 protein levels in TBI+CFZ rats were elevated by 36.0% over Sham rats, this difference was not significant ($p = 0.217$). No difference in Kv1.3 protein expression was found between sham injured control and sham injured+CFZ groups ($p = 0.617$). Neither TBI or CFZ treatment significantly altered the level of callosal Kv1.2 protein (Fig. 6D). When postinjury Kv1.3 channel expression was probed by IHC, with and without CFZ treatment, group differences consistent with WB results were observed (Fig. 6E). In these cases, single channel 594 WL confocal imaging was employed to avoid CFZ 488 WL autofluorescence. This pattern of results, a CFZ treatment effect on Kv1.3, but not Kv1.2 protein, is consistent with the prior finding that CFZ is highly selective for Kv1.3, relative to Kv1.2 channels. Specifically, CFZ was shown to strongly suppress Kv1.3 currents with an IC_{50} of 470 nM, which was lower by a factor of at least 20 compared to other Kv family members tested (Kv1.1, Kv1.2, Kv1.5) (Ren et al., 2008). In parallel, we also observed a significant injury-induced increase in callosal IL1- β (33 and 17 kD; both $p < 0.05$), reflecting elevation of pro- and activated forms of the cytokine (Auron et al., 1984; March et al., 1985). In the presence of CFZ, this inflammatory response was blocked, IL1- β protein returning to sham control levels ($p < 0.05$) (Fig. 7). While these data support a cell based Kv1.3-mediated inflammatory mechanism for CFZ influence on CAP recovery, the mixed nature of corpus callosum tissue extracts does not permit discrimination as to whether this neuroprotective effect is generated through axonal or glial pathways. Given that corpus callosum glia can be harvested and tested for CFZ effects in vitro, we next used callosal derived mixed microglial/astrocyte cultures to explore glial response after induction of inflammatory pathology.

Effect of CFZ on LPS induced inflammatory response in callosal glia

Using morphological phenotype as an index of glial reactivity, we first examined the effects of LPS, CFZ and LPS+CFZ on microglial and astrocyte structure under controlled in vitro conditions. Phase microscopy of flasks prior to protein extraction revealed that the predominant LPS effect was a shift in microglial morphology relative to untreated controls, changing from a resting, ramified state to an activated form, including larger cell bodies, multiple lobular processes and evident vacuoles (Figs. 8A, B). While astrocyte response to LPS was less, these bed layer cells did show evidence of membrane retraction. Cells exposed to CFZ alone showed uptake of the drug in the form of dense granular inclusions, consistent with other reports of in vitro CFZ exposure (Baik and Rosania, 2011), however, this was not accompanied by a significant shift in glial phenotype relative to untreated controls (Fig. 8D). By contrast, LPS+CFZ treated cells showed extreme cytoplasmic retraction of microglia, often appearing as highly rounded cells with short, spikey cytoplasmic processes (Fig. 8C). These results show that callosal microglia respond to LPS inflammatory stimulation with

significant shift toward reactive phenotype and suggest that CFZ has potential to blunt the response.

We next examined glial protein extracts generated from untreated, LPS, CFZ and LPS+CFZ treated flasks for pro-inflammatory cytokine IL1- β expression (Fig. 9). The goal was to determine if CFZ blockade of Kv1.3 channels affects acute pro-inflammatory cytokine signaling within callosal glia. WB assay of confluent flasks from four separate culture experiments revealed low control cellular expression of two major IL1- β bands (33, 28 kD), reflecting pro-form of the cytokine. LPS treatment significantly increased each pro-IL1- β signal approximately five fold over untreated controls ($p < 0.05$ for both 33 and 28 kD; 33kD illustrated in Fig. 9). We also found that LPS inflammatory stimulus induced significant upregulated expression of two low kD IL1- β signals (19, 17 kD), which we interpret as representing cleaved, active forms of the cytokine (Black et al., 1989; $p < 0.05$ for both 19 and 17 kD; 17kD illustrated in Fig. 9). Notably, these low molecular weight forms of IL1- β were not expressed above background detection level in the untreated controls. When LPS treated flasks were washed and exposed to CFZ, we observed a reversal of LPS-induced IL1- β expression; LPS+CFZ samples were not different from untreated controls for the 33kD pro-IL1- β ($p = 0.995$), and reduced to approximately 5% of LPS signal for the 17kD active IL1- β , a level not significantly different from untreated controls ($p = 0.935$). In flasks exposed to CFZ alone, IL1- β expression was not different from untreated cultures, and similarly showed a significantly lower level of cytokine when compared with LPS treated flasks. Taking IL1- β expression as a metric of pro-inflammatory signaling, these results support our hypothesis that CFZ neuroprotection of TBI-induced corpus callosum axon damage includes attenuation of white matter glial cytokine production and activation.

Since flask analysis of molecular expression cannot discriminate cell type, we applied fluorescent ICC methods to MatTek dishes set up in parallel with the flask groups in order to probe for individual microglial and astrocyte response to LPS and CFZ (Figs. 10-12). Antibodies to IBA1 and GFAP proteins were paired with the same Kv1.3 and IL1- β antibodies used for WB analysis. First, we paired each marker with Kv1.3 antibody to confirm channel expression in callosal glia and determine the effects of LPS on Kv1.3 distribution (Fig. 10). Because CFZ selectively inhibits Kv1.3 function (Ren et al., 2008), and Kv1.3 channels are considered principal regulators of microglial cell reactivity (Rangaraju et al, 2015), we focused on microglial labeling differences. In normal untreated callosal cultures, Kv1.3 expression was evident for each microglial morphology present, even in cells with lower IBA1 signal (Fig. 10A). Interestingly, we also observed a shift in Kv1.3 protein localization from central perinuclear sites to peripheral membrane proximal regions after LPS inflammatory stimulation. This intracellular shift is consistent with the reported movement of Kv1.3 protein within CNS microglia exposed to direct current stimulation during proliferation in vitro (Kotecha and Schlichter, 1999). As predicted from the literature (Zhu et al., 2014), we also found that callosal astrocytes express Kv1.3, albeit at a lower level than microglia (Fig. 10B). Further, callosal astrocyte Kv1.3 signal was less affected by LPS stimulation. While different glial types may contribute to change in Kv1.3 expression, these data support a prominent microglial role in mediating Kv1.3 channel response during injury-induced white matter inflammation.

With the Kv1.3 expression and reactivity to inflammatory stimulus confirmed for callosal glia, we turned to probing for cell-specific changes in cytokine expression/distribution with CFZ treatment. In the untreated dishes we found baseline IL1- β expression for both glial cell types to be more concentrated in cytoplasm around nuclei (Figs. 11-12). LPS induced an increase of IL1- β staining that was prominent in reactive microglia, but not in astrocytes. IL1- β + microglia exhibited retracted, rounded profiles, with increased lobular processes, consistent with the morphological changes seen by phase microscopy (Fig. 8). When LPS treated dishes were subsequently exposed to CFZ, the rise in pro-inflammatory IL1- β within microglia was no longer evident (Fig. 11), an effect supported by prior studies showing a reduced cytokine induction within CNS microglia when Kv1.3 signaling is blocked with margatoxin (Charolidi et al., 2015). Interestingly, although astrocyte IL1- β signal was detectable after LPS, it was not qualitatively different from untreated controls (Fig. 12). CFZ alone did show reduced IL1- β signal in each glial cell type, but had the greatest effect on microglia. These ICC observations mirror the results with WB studies of IL1- β in flasks. They suggest that callosal microglia are major contributors to elevation of IL1- β after LPS exposure, and that when CFZ is added under conditions of inflammation, attenuation of pro-inflammatory cytokines occurs predominantly within reactive microglia.

Discussion

In the present study, postinjury functional changes in the corpus callosum were primarily observed in the unmyelinated axon population, with only minor alterations in the myelinated fibers. This was consistent with our prior findings using the moderate midline fluid percussion model, which produces a diffuse pathology, without significant contusion (Reeves et al., 2005, 2007, 2010, 2012; Colley et al., 2010). Measurements of evoked CAP amplitude showed a marked suppression after TBI. The pattern of electrophysiological changes observed, impaired CAP amplitudes with largely spared conduction velocity and refractoriness, suggests the injury reduced the number of responsive axons recruited into the evoked response, or attenuated mean amplitude of fields surrounding discharging axons. Postinjury administration of CFZ resulted in a potent protection against this deficit. The present investigation explored one component of TBI pathology which we predict is fundamental to this deficit and neuroprotection: postinjury neuroinflammation and its reduction by the blockade of Kv1.3 channels. In the corpus callosum, Kv1.3 channels were localized within both endogenous glia, and at sites marking myelinated fiber paranodes, suggesting a complex role for the channel in TBI white matter pathology. Kv1.3 assay in whole callosal extracts showed a rapid (<24h) reduction in channel protein after TBI. Acute postinjury CFZ treatment in vivo significantly elevated channel protein over this injury-induced reduction, approaching sham injured control levels. This result suggests that CFZ not only blocks Kv1.3, but also signals change in channel expression in the callosum. While we did not assess Kv1.3 mRNA level after TBI or with CFZ treatment, it is possible that altered Kv1.3 protein expression after CNS injury, or with CFZ exposure, is mediated at the transcriptional level (Villalonga et al., 2010; Peng et al., 2014). Indeed, peripheral nerve axotomy was reported to reduce Kv1.3 mRNA expression by approximately 40% (Yang et al., 2004), a finding consistent with the postinjury Kv1.3 protein loss in the present study. Further examination of the TBI effect on Kv1.3 transcript will be necessary to clarify how

the observed Kv1.3 response is regulated. Nevertheless, our WB observations were matched by loss of callosal Kv1.3 tissue staining with TBI and also showed a notable elevation of Kv1.3 antibody signal throughout the callosum for TBI+CFZ samples. In this regard, the absence of injury or drug treatment effects to Kv1.2 protein levels supports a key role for Kv1.3 signaling in this white matter pathology, and responsiveness to CFZ. Kv1.2 and Kv1.3 are both members of the Shaker-related family of Kv channels, and the Kv1.3-specific CFZ effects suggests the treatment did not broadly affect Kv channels; instead, this outcome is consistent with the established role of Kv1.3 channels in TBI-induced microglial activation. Injury-induced expression of pro-inflammatory cytokine IL1- β was also significantly reversed with CFZ. These results suggested that, in a mixed sample of callosal axons and glia subjected to injury, both the increase in Kv1.3 protein expression and attenuation of inflammation was correlated with CAP recovery. Our next experiments with glial cultures were designed to explore how the individual callosal microglia and astrocytes contribute to this inflammatory signaling mechanism. We found that LPS-induced inflammation caused a surge in glial IL1- β production, which was reversed by the presence of CFZ, however, CFZ alone failed to change IL1- β expression in untreated callosal glia. The cellular response to LPS was mainly within the microglial population, and coupled with a shift in localization of Kv1.3 from perinuclear clusters to a more diffuse, membrane-associated distribution. Since both axons and glia express Kv1.3, and cytokine production is principally glial, these results suggested that white matter CFZ neuroprotection is likely multifaceted, attenuating pro-inflammatory glial signals, while also increasing expression of Kv1.3 channels in both cell types.

Although this is the first investigation of the therapeutic potential of Kv1.3 inhibition in TBI pathobiology, these channels are emerging as promising neuroprotective targets in diverse pathologies. Our observations that postinjury axonal function can be altered by Kv1.3 manipulation, and that white matter microglia potentially mediate this effect through Kv1.3 regulation of inflammatory molecules, are supported by Kv1.3 studies using models of neurodegeneration and epilepsy. Postmortem analysis of Alzheimer's disease patients revealed significantly higher levels of Kv1.3 channels co-localized with microglial cells, but not with astrocytes (Rangaraju et al., 2015). Kv1.3 was shown to be highly expressed on inflammatory leukocyte infiltrates in brains from multiple sclerosis patients, and Kv1.3 was elevated in both gray and white matter (Rus et al., 2005). Pre-clinical studies have also implicated Kv1.3 channels in apoptosis and status epilepticus. In addition to their localization on the plasma membrane, Kv1.3 channels present on the inner mitochondrial membrane can target Bax and facilitate the induction of apoptosis in T lymphocytes (Szabo et al., 2008). Activation of Kv1.3 and Kv1.1 channels can cause apoptotic death of retinal ganglion cells after optic nerve transection (Koeberle et al., 2009). In rodent status epilepticus, Kv1.3 channels play a pivotal role in generation of large K⁺ currents induced in hippocampal microglial cells, which were activated following a systemic injection of kainate (Menteyne et al., 2009). Together with the present results, these data support a major Kv1.3 role in the evolution of axonal pathology, mediated principally through microglial pro-inflammatory signaling which selectively targets the more vulnerable unmyelinated fiber populations.

CFZ inhibition of Kv1.3 suppresses post-traumatic microglial reactivity

The microglial cell population was a focus for this study because their activation is a common and persistent feature of TBI pathobiology, and the regulatory roles of microglial Kv1.3 are expressed in the activated phenotype. Kelley et al (2007) studied the relationship between axonal injury and microglial activation, using the same midline fluid percussion model as used in the electrophysiological arm of the present study. In gray matter regions, they observed this injury to induce a diffuse parenchymal pathology, which provoked microglial activation in the absence of focal contusion or hemorrhage. Traumatic axonal injury was co-localized with activated microglia in mediodorsal neocortex, dentate gyrus, and dorsolateral thalamus. Their results are consistent with a surveilling role of microglia which detects and responds to axonal perturbations following injury. Because unmyelinated axons present a greater axolemmal exposure to glial surveillance and pro-inflammatory molecules, we hypothesized they will be more amenable to pharmacologic strategies suppressing microglial activation. Due to their high membrane-to-cytoplasm ratio, and their small diameter, the unmyelinated axon population is especially at risk to the membrane-targeting pathology of TBI (Reeves et al., 2005, 2007, 2012). Thus, the permissive role of Kv1.3 activity in generating microglial activation, migration, and expression of pro-inflammatory molecules would exacerbate unmyelinated axon pathology. Indeed, the CAP recordings in this study showed the unmyelinated signal underwent the greater degree of post-traumatic suppression, and was most protected by CFZ treatment, relative to the myelinated signal. Kv1.3 is a component of the outward rectifier currents in microglia (Khanna et al., 2001; Pannasch et al., 2006), and these channels are key regulators of microglial function (Rangaraju et al., 2015). Prior experiments with primary cortical microglia showed that blocking Kv1.3 channels markedly diminished microglial migration to areas of cellular debris (Nutile-McMenemy et al., 2007). Here we show that neuroprotective CFZ also shifts callosal glial phenotype, however, the effect occurred only after high inflammatory activation with LPS, and did not occur in response to CFZ alone. Further, callosal microglial phenotype change was predominant relative to astrocyte response, which consisted of significant cytoplasmic retraction and attenuated process extension. We also found that predicted CFZ binding to glial Kv1.3 caused its internalization into cytoplasmic granules, often localized at sites adjacent to the nucleus and associated organelles. This pattern of CFZ processing matches that of autophagosomal-like inclusions in liver or kidney cells, where soluble CFZ was shown to target mitochondria, endoplasmic reticulum and Golgi Kv1.3 channels, potentially attenuating ATP production and protein processing (Baik and Rosania, 2011, 2012; Yoon et al., 2015). It has also been reported that peripheral monocyte derived macrophages become apoptotic following CFZ administration (Fukutomi et al., 2011), thereby reducing the number of activated inflammatory macrophages available to infiltrate injured white matter. Such intracellular processing of CFZ would also be consistent with the reduction in cytoplasmic area and process retraction that we observed in callosal glia exposed to the drug.

In activated and proliferating hippocampal microglial cells, Kv1.3 channels migrate to the cell surface (Kotecha and Schlichter, 1999). This translocation of Kv1.3 from a perinuclear/cytosolic compartment into the membrane was consistent with the present observations of Kv1.3 redistribution in response to LPS treatment. Interestingly, this shift occurred

principally in callosal microglia. Fordyce et al (2005) also showed that *in vitro* LPS activation of cortical microglia increased membrane distribution of Kv1.3, without overall change in Kv1.3 protein level, and had the capacity to promote peroxynitrite release, capable of inducing cell death of adjacent hippocampal neurons. Kv1.3 protein mobilization during microglial activation not only demonstrates the functional relevance of Kv1.3 channels, but also suggests that their susceptibility to selective channel blockers could alter microglial reactivity after white matter injury. In this context, we also probed for Kv1.3 by WB in callosal mixed glial cultures (*data not shown*), where we observed a single 55kD Kv1.3 species, smaller than the predicted 68-70 kD monomer or the 160 kD dimerized channel protein (Kotecha 1999; Fadool 1997; Zhu, 2014). Since higher kD tyrosine phosphorylated forms of the channel show functional suppression (Colley and Fadool, 2004), we speculate that the 55kD in callosal glial cultures may represent an active, less phosphorylated Kv1.3 form. Overall, we conclude that Kv1.3 channel alteration contributes to deficits in axon functional activity after TBI and mediates white matter microglial reactivity during the evolution of axonal pathology.

CFZ neuroprotection includes the reduction of glial pro-inflammatory signals

Microglial phenotype and Kv1.3 distribution observed in our study suggest that CFZ neuroprotection of corpus callosum CAPs after TBI can be mediated by anti-inflammatory effects on resident microglia. Kv1.3 is expressed in numerous CNS cell types including not only microglia (Khanna et al., 2001; Panasch et al., 2006; Rangaraju et al., 2015) but also astrocytes (Zhu et al., 2014), oligodendrocytes (Chittajallu et al., 2002), olfactory bulb neurons (Fadool et al., 2004), Purkinje cells (Mckay et al., 2005), and cortical interneurons (Duque et al., 2013). Kv1.3 signaling mechanism has been characterized in most detail for T lymphocytes (reviewed by Cahalan and Chandy, 2009). During T cell activation, alternating efflux through Kv1.3 channels and influx through calcium release-activated calcium (CRAC) channels establish critical calcium oscillations, and sufficient cytosolic calcium levels required to sustain the activated state. Experiments with peptide and small-molecule inhibitors of Kv1.3 have shown that blocking these channels suppresses gene expression and, notably, cytokine production in T cells, suggesting a promising therapeutic strategy against disorders inducing significant immunological response (Chandy et al., 2004). Thus, our observed CFZ neuroprotection appears associated with blockade of Kv1.3, and may share overlapping mechanisms with other therapeutic immunosuppressants.

FK506 and CsA are immunosuppressants shown to be neuroprotective in some models of experimental TBI (Marmarou and Povlishock, 2006; Okonkwo et al., 1999; Reeves et al., 2007; Scheff and Sullivan, 1999; Singleton et al., 2001), but current thinking suggests that this treatment benefit may occur independently of their immunosuppressive action (Mbye et al., 2008). These drugs act through multiple molecular pathways, for example not only can CsA suppress mitochondrial transition permeability through cyclophilin-D, but it can also inhibit calcineurin dephosphorylation (Crompton, 1999; Friberg et al., 1998; Friberg and Wieloch, 2002). Because manipulation of these pathways could produce negative off-target effects, it is desirable to define more specific targets of these anti-inflammatory compounds in order to optimize neuroprotective activity, but avoid potential toxicity. In this regard, published cell culture experiments point to Kv1.3 as one such target, showing that FK506

inhibited Kv1.3 in a concentration-dependent manner without suppression of calcineurin protein phosphatase activity. Ahn and colleagues (2007) suggested FK506 may allosterically modulate Kv1.3 channel kinetics, facilitated by the lipophilic properties of FK506, which readily partitions into the plasma membrane. Similar to our observations of CFZ effect on callosal microglia, results from experimental stroke models have implicated glia as direct targets of FK506, with modulation of cell reactivity and inflammation the likely mechanism of its neuroprotection (Kaminska et al., 2004; Zawadzka et al., 2012).

Using callosal glial cultures we further confirmed that CFZ neuroprotection is linked to inflammatory signaling, finding significant upregulation of pro-inflammatory cytokine IL1- β (5 fold over that of controls) after LPS stimulation, and that CFZ essentially blocks this induction. As with phenotype assessment, CFZ alone had no effect on IL1- β induction, further supporting the interpretation that CFZ Kv1.3 channel interaction occurs during *active* microglial pro-inflammatory cell signaling within the injured corpus callosum. These results are consistent with demonstrated pro-inflammatory stimulation of CNS glia by LPS (Lund et al., 2006; Rhol et al., 2007; Charolidi et al., 2015). We also found that LPS upregulation of both pro- and active IL1- β are affected by CFZ, indicating that blockade of Kv1.3 second messenger signaling pathways may affect both intracellular processing and gene expression of the cytokine in white matter glia. Taken together, these findings suggest that immunosuppressant neuroprotection in TBI may arise, at least in part, through Kv1.3 inhibition, which points to these channels as viable and specific therapeutic targets. The present study directly supports this idea, showing effective CAP protection in damaged white matter tracts with the application of anti-inflammatory drug and Kv1.3 inhibitor CFZ.

Alternatively, other reports of therapeutic CFZ application suggest separate anti-inflammatory pathways through which the drug might also contribute to TBI neuroprotection. These involve CFZ induction of leukocyte cell death (O'Connor et al., 1995; Arbiser and Moschella, 1995) and the form of CFZ intracellular uptake to alter cell function (Yoon et al., 2015). Each pathway could affect the extent of callosal microglial reactivity and pro-inflammatory cytokine induction after TBI. For example, we observed reduced expression of callosal IL-1 β after *in vivo* systemic CFZ treatment, a response which may be influenced by the number of activated peripheral monocytes entering the injured brain to drive local microglial activation. In this context, it has been reported that peripheral monocyte derived macrophages become apoptotic following CFZ administration (Fukutomi et al., 2011), which, with TBI, could reduce the number of activated inflammatory macrophages available to infiltrate injured white matter (Semple et al., 2009; Szmydynger-Chodobska et al., 2011; Hsieh et al., 2014; Muccigrosso MM et al., 2016). Such monocyte cell death may occur due to internalization of soluble CFZ as membrane aggregates, which targets mitochondria, endoplasmic reticulum and Golgi, potentially attenuating ATP production and protein processing (Baik and Rosania, 2011, 2012; Leanza et al., 2012, 2014). A second route for CFZ uptake is in the form of phagocytosed bio crystals (Yoon et al., 2015). Interestingly, our culture studies confirmed glial CFZ internalization into cytoplasmic crystal-like granules, often localized at sites adjacent to the nucleus and associated organelles. This pattern of CFZ processing has been posited to act through Toll-like receptor pathways to reduce gene transcription of pro-inflammatory TNF α and enhance anti-inflammatory IL1-RA signals (Yoon et al., 2015), also potentially attenuating postinjury

immune cell activation. Reduced expression of pro-inflammatory transcripts would be consistent with the recovery of CAPs, attenuated microglial reactivity and reversal of glial IL1- β induction we found associated with CFZ neuroprotection of injured axons. These types of CFZ intracellular processing would also be in line with the reduction in cytoplasmic area and process retraction that we observed for callosal glia exposed to the drug. While intriguing possibilities, these alternative CFZ pathways remain to be tested within callosal glia after TBI. Notably, some of these pathways have already been linked to Kv1.3 signaling in other cell models (Leanza et al., 2012; Wang et al., 2012).

Kv1.3 channels as novel, safe therapeutic targets after TBI

Our results showing CFZ neuroprotection against white matter damage strongly suggest that targeting Kv1.3 channel pathways may be a potentially valuable therapeutic approach after TBI. The growing recognition of a Kv1.3 role in the neuropathologies of Alzheimer's Disease, multiple sclerosis, and other autoimmune disorders, has led to the development of both peptide (e.g., Shk-L5) and non-peptide (e.g., PAP-1) blockers of Kv1.3 as experimental therapeutics. Accumulating evidence suggests Kv1.3 is a relatively safe therapeutic target, and toxicology studies with PAP-1 and ShK-L5 reported no histopathology, and no hematological or serum chemistry changes (Azam et al., 2007; Beeton et al., 2006). Further, the potential side-effects of Kv1.3 blockers are likely to be minimal, and these compounds have shown a good safety profile in rodents and primates (Rangaraju et al., 2009). The only CNS deficit observed in Kv1.3-deficient mice was hypersensitivity to odorants caused by altered development of the olfactory bulb (Fadool et al., 2004). Thus, a safe Kv1.3 targeted compound, which can mediate both glial inflammation and axonal functional activity, could be administered in a time-dependent manner after TBI to foster neuronal stability and potential circuit regeneration.

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Highlights

- Clofazimine (CFZ) improved corpus callosum function after traumatic brain injury.
- CFZ prevented postinjury loss of Kv1.3 protein, and IL1- β elevations, in callosum.
- Lipopolysaccharide (LPS) induced reactive changes in cultured callosal glial cells.
- CFZ normalized LPS-induced structural change, and IL1- β elevation, in cultured glia.
- Kv1.3 has a role in traumatic white matter injury, and is a target for therapy.

Figure 1. Electrode placement for CAP recording and waveform quantification

A. Stimulating (Stim) and recording (Rec) electrodes were positioned to acquire CAPs evoked in midline-crossing corpus callosum axons. **B.,C.** Representative CAP waveform showing N1 component, generated primarily by myelinated axons, and slower N2 wave generated mainly by unmyelinated axons. CAP peak amplitudes were quantified as: $N1 = (a + b)/2$; $N2 = (c + d)/2$.

Figure 2. CFZ penetration through the blood-brain barrier following TBI

A. CFZ administered after sham injury showed limited access to brain tissue, accumulating in vascular walls (arrows). [Dashed line shows corpus callosum 'cc' borders]. **B.** CFZ given after TBI showed abundant penetration, with increased diffuse localization and concentration in glial cells.

Figure 3. Effects of TBI and CFZ treatment on callosal CAP amplitude

A. Representative CAPs, from each study group, evoked with a graded series of stimulus intensities ('input-output' series). **B.,C.** Mean CAP amplitudes measured in input-output recording for each analytic group. ‡ p<0.001, Mann-Whitney U test of input-output curve shift. **D.,E.** Evaluation of injury and CFZ treatment at maximal stimulus intensity (100% level in B., C.), for whole cc, and for specific callosal regions: genu, middle, and splenium. At this single stimulus level, only the unmyelinated CAPs (N2 in panel E.) showed a significant TBI-induced reduction in amplitude (ANOVA, ***p<0.001, **p<0.01, *p<0.05, 'TBI+vehicle' vs. 'Sham+vehicle' [Sham groups not shown in panels D.,E.]). Similarly, assessments of CFZ efficacy to prevent post-TBI suppression of CAPs, at the 100% stimulus intensity, showed significant protection only for the unmyelinated N2 signal. Analyses at each callosal subregion showed the drug treatment was most protective in the splenium, where unmyelinated axons are most abundant (ANOVA, §§p<0.01, §p<0.05, 'TBI+vehicle' vs. 'TBI+CFZ').

Figure 4. Effect of TBI and CFZ treatment on callosal CAP refractoriness

A. Example waveforms show second potentials, in paired responses, evoked using inter-pulse intervals of 4-15 msec. A representative series of waveforms are shown for each analytic group. **B.** Plots of mean CAP amplitude elicited by the second pulse in each paired stimulation (C_2) divided by the CAP amplitude to single pulse stimulation (C_1), for each study group. Statistical analyses showed small, but significant, shifts in the refractoriness curves only for the myelinated N1 signal ($\ddagger p < 0.01$, $*p < 0.05$, Mann-Whitney U).

Figure 5. Effect of TBI and CRZ treatment on axonal conduction velocity in the corpus callosum
A. Representative CAP waveforms acquired in a Sham+Vehicle rat with distance between stimulating and recording electrodes varied between 0.73mm and 2.08mm. **B.** Linear regression analysis of same waveforms in panel A. relating response latency to inter-electrode distance. **C.** Analyses of mean conduction velocities, derived from regression analyses, did not show significant effects of TBI or CFZ treatment for either N1 or N2 CAP waveforms.

Figure 6. Callosal Kv1.3 channel protein in axons and glia is altered after injury and with CFZ treatment

A. Confocal overlay showing Kv1.3 (red) and Kv1.2 (green) in rat corpus callosum 24h following midline fluid percussion TBI. Low magnification shows that each channel protein is found in reactive glia around callosal vessels (arrowheads) and along axon bundles (arrows). Inset shows paired paranodal distribution of Kv1.3 and Kv1.2 channels, some nodes with co-localization (yellow arrow), others with single channel expression (green, red arrows). **B.** Confocal overlays showing Kv1.3 in callosal astrocytes of sham injured (GFAP +, left-arrows; inset shows cell body and perivascular co-localization) and microglia of 24h postinjury cases (IBA1+, right-arrows). **C.** Western blot (WB) of protein extracts from 24h postinjury corpus callosum revealed that TBI reduced 67kD Kv1.3 levels and that CFZ treatment reversed loss of Kv1.3 expression. Data expressed as percent of paired untreated sham controls run on same blot. Lanes representative of group effects are shown in each panel. (ANOVA, * $p < 0.05$). **D.** When the same extracts were probed by WB for 75kD Kv1.2 changes after injury and with CFZ, no differences in protein level were found. **E.** Parallel IHC of callosum with single channel Kv1.3 labeling. Sham cases show mixed axonal and glial labeling (arrows). TBI reduced Kv1.3 cell body staining relative to sham at 24h, with elevated Kv1.3 label in TBI+CFZ cases. Bars= 20 μ m (inset A Bar=8 μ m; inset B Bar=10 μ m).

Figure 7. Callosal IL1- β protein in axons and glia is altered after injury and with CFZ treatment Western blot (WB) of protein extracts from 24h postinjury corpus callosum probed for IL1- β changes after injury and with CFZ showed that both pro- and active forms of the cytokine (33,17 kD) were elevated in TBI samples, with reduction to control levels after CFZ treatment. Data expressed as percent of paired untreated sham controls run on same blot. Lanes representative of group effects are shown in each panel. (ANOVA, * $p < 0.05$).

Figure 8. Phase microscopy of corpus callosum mixed glial cultures (14DIV) illustrating the effects of LPS, LPS+CFZ, or CFZ treatment on astrocytes and microglia

A. Untreated control cultures show confluent flat bed astrocytes (arrowhead) with ramified microglia (arrows) growing on their surface. **B.** Induction of inflammation with LPS resulted in a prominent microglial shift from ramified phenotype to reactive state, with larger cell bodies, multiple lobular processes and evident vacuoles. While astrocyte response to LPS was minor, these bed layer cells did show some evidence of membrane retraction. **C.** LPS cultures treated with CFZ show extreme cytoplasm retraction in both astrocytes (arrowhead) and microglia (arrowheads). **D.** Notably, CFZ alone, without inflammatory stimulation, shows astrocyte (arrowhead) and microglial (arrows) morphology more similar to that of untreated controls. Bar= 20 μ m.

Figure 9. WB analysis of pro-inflammatory cytokine IL1-β protein in corpus callosum mixed glial cultures (14DIV) subjected to LPS, LPS+CFZ or CFZ treatment

Protein extracts from confluent 25mm² glial flasks probed with IL1-β antibody revealed four major bands (*pro form*, 33&28 kD; *active form* 19&17 kD). Densitometric analysis showed significant elevation of each form with LPS inflammation induction and significant reduction of IL1-β after CFZ was added to the LPS culture (33kD and 17kD shown in figure). CFZ IL1-β expression was not different from that of untreated control flasks. Data expressed as percent of paired untreated control cultures run on same blot. Lanes representative of group effects are shown in each panel. (ANOVA, *p<0.05).

Figure 10. Corpus callosum mixed glial cultures grown in MatTek dishes and subjected to confocal dual labeling with antibodies to Kv1.3 (green) and microglial marker protein IBA1 (red) or astrocyte marker protein GFAP (red)

A. Microglia are predominantly ramified in untreated cultures (arrowhead), shifting to reactive rounded cells with lobular processes after LPS (arrows). Kv1.3 protein is localized within the majority of microglia in each field and shifts from a normal concentration around nuclei (inset, UNT), to a more uniform distribution after inflammatory stimulation (inset, LPS). **B.** Astrocytes show mixed flat and fibrous morphologies. Kv1.3 signal is much reduced in astrocytes relative to surrounding microglia (not stained in these images) and predominantly found in small aggregates around cell nuclei (arrow). Astrocytes do not show LPS group differences in Kv1.3 expression. Bars= 20 μ m.

Figure 11. Corpus callosum mixed glial cultures from each group assayed in Figures 8 and 9, grown in MatTek dishes and subjected to confocal dual labeling with antibodies to IL1- β (green) and microglial marker protein IBA1 (red)

Microglial morphology ranges from predominantly ramified in untreated and CFZ groups, to reactive rounded cells with lobular processes after LPS and LPS+CFZ. Reactive microglia induced by LPS contain the largest IL1- β signal (arrows), and when CFZ is added to LPS, microglial IL1- β expression is reduced to near control levels. Similar to blot data, CFZ alone treatment does not increase IL1- β signal over controls. Notably, level of IL1- β expression can vary significantly among different microglia. Bar= 20 μ m.

Figure 12. Corpus callosum mixed glial cultures from each group assayed in Figures 8 and 9, grown in MatTek dishes and subjected to confocal dual labeling with antibodies to IL1- β (green) and microglial marker protein GFAP (red)

Astrocytes present mixed flat and process bearing morphologies across groups, with modest IL1- β labeling, primarily within the glial cell body (arrows). In contrast to microglia, astrocytes do not show LPS-induced reactivity, nor do they exhibit prominent group differences in IL1- β expression relative to CFZ exposure, suggesting they contribute less to group shifts in flask IL1- β protein determined by WB analysis. Bar= 20 μ m.