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Combined inhibition of cell death induced by apoptosis inducing factor and caspases provides additive neuroprotection in experimental traumatic brain injury

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Abstract

Neuronal programmed cell death (PCD) contributes to delayed tissue damage after traumatic brain injury (TBI). Both caspase-dependent and caspase-independent mechanisms have been implicated, with the latter including apoptosis inducing factor (AIF). The peptidyl-propyl isomerase Cyclophilin A (CypA) transports AIF from the cytosol to the nucleus, a key step for AIF-dependent cell death. We compared the effects of single versus combined inhibition of caspase and AIF pathways in a mouse controlled cortical impact (CCI) model, by examining the effects of CypA gene knockout (CypA^{-/-}), caspase inhibition with a pan-caspase inhibitor (boc-aspartyl(OMe)-fluoromethylketone, BAF), or combined modulation. TBI caused caspase activation as well as translocation of AIF to the nucleus. Markers of caspase activation including caspase-specific fodrin cleavage fragments and number of FLIVO positive cells were reduced in BAF-treated CypA^{+/+} mice, whereas markers of AIF activation including AIF/H2AX interaction and AIF translocation to the nucleus were attenuated in CypA^{-/-} mice. Each single intervention, (CypA^{-/-} or BAF-treated CypA^{+/+}) reduced the number of apoptotic cells (TUNEL-positive) in the cortex and improved long-term sensorimotor function; CypA^{-/-} also attenuated microglial activation after injury. Importantly, BAF-treated CypA^{-/-} mice, showed greater effects than either intervention alone on multiple outcomes including: reduction in TUNEL-positive cells, decrease in neuroinflammation, improved motor and cognitive recovery, and attenuation of lesion volume and neuronal loss in the hippocampus. Using two *in vitro* neuronal cell death models known to induce AIF-mediated PCD, we also showed that neurons from CypA^{-/-} animals were protected and that effects were unrelated to caspase activation. These data indicate that AIF-mediated and caspase-dependent pathways contribute independently and in parallel to secondary injury after

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TBI, and suggest that combined therapeutic strategies directed at multiple PCD pathways may provide superior neuroprotection than those directed at single mechanisms.

Keywords

Traumatic Brain Injury; Apoptosis Inducing Factor; Caspase; Cyclophilin A

Introduction

Traumatic brain injury (TBI) is a major public health problem in the United States, with more than 1.7 million new cases every year (Faul M, 2010) and accounting for 60% of all trauma deaths (Dutton et al., 2010). In spite of significant effort there is still no effective treatment for clinical TBI (Faden and Stoica, 2007; Stoica et al., 2009a). TBI causes cell death and neurological dysfunction through both direct physical disruption of tissue or pathways (primary injury), and through subsequent biochemical changes (secondary injury); the latter represents delayed and potentially reversible molecular and cellular pathophysiological mechanisms (Stoica and Faden, 2010). Delayed neuronal cell death is a key element of the secondary injury, reflecting various types of neuronal programmed cell death (PCD) and contributes significantly to posttraumatic neurological deficits in experimental TBI (Stoica and Faden, 2010; Yakovlev et al., 2001). Both caspase-dependent and caspase-independent types of PCD contribute to such neuronal loss (Stoica and Faden, 2010). Caspase mediated apoptosis was initially proposed as an important PCD pathway involved, based upon the demonstration of caspase activation in experimental TBI models and neuroprotection shown with selective caspase inhibitors (Knoblauch et al., 2002; Yakovlev et al., 1997). Subsequent studies suggested involvement of additional potential PCD mechanisms, such as the translocation of apoptosis inducing factor (AIF) (Slemmer et al., 2008a; Tomita et al., 2006; Tweedie et al., 2007; Zhang et al., 2002).

AIF is a flavoprotein that is normally localized within the mitochondrial inter-membrane space (Susin et al., 1999; Joza et al., 2001). When released into the cytosol, it translocates to the nucleus, where it binds chromatin and causes peripheral chromatin condensation and DNA fragmentation (Slemmer et al., 2008a; Suzuki et al., 2009; Zhang et al., 2002; Zhu et al., 2007b). In contrast to other apoptosis associated genes whose expression decreases with age (Yakovlev et al., 2001), AIF expression does not decrease during development (Stoica et al., 2009b; Zhu et al., 2003). This suggests that AIF may play a more major role in apoptosis in the adult brain. Cyclophilin A (CypA) has been identified as an obligate co-factor for AIF nuclear translocation (Cande et al., 2004). AIF physically interacts with CypA and the AIF/CypA complex has been shown to translocate to the nucleus in neurons following neonatal hypoxia-ischemia (Zhu et al., 2003; Zhu et al., 2007a). Notably, CypA knockout (CypA^{-/-}) confers significant neuroprotection in this model. Neurons from AIF hypomorphic mutant (Harlequin; Hq) mice are resistant to NMDA or glutamate neurotoxicity *in vitro* (Culmsee et al., 2005; Wang et al., 2004), and Hq mutant mice have reduced brain damage after cerebral ischemia *in vivo* (Culmsee et al., 2005; Zhu et al., 2007b). Studies using the Hq mouse model suggested that AIF and caspases may act through parallel pathways, with caspase inhibition and low-level AIF expression having potentially additive neuroprotective effects (Zhu et al., 2007b). Unlike Hq mutant mice, CypA^{-/-} mice are phenotypically normal, and therefore provide a better model for examining the role of AIF mediated PCD pathways after injury. We hypothesized that both caspase-dependent and AIF-mediated PCD pathways contribute to neuronal apoptosis after TBI, and that combined inhibition of these pathways results in additive neuroprotection. Here, we used a clinically relevant experimental TBI model in mice, the controlled cortical impact (CCI) injury (Fox et al., 1998), which causes many physiological, neurological and pathological changes that parallel those in human TBI,

including widespread acute and chronic neuronal injury, and delayed secondary injury at a distance from the trauma site (Cernak, 2005; Povlishock et al., 1994). The CCI model produces a moderately severe injury that is characterized by a significant cortical lesion as well as neuronal cell loss in the hippocampus and related functional (sensorimotor and cognitive) deficits. Significant correlations are obtained between lesion volume and deficits in fine-motor co-ordination, and hippocampal cell loss and cognitive impairments in this model (Kabadi et al., 2012a; Kabadi et al., 2011; Kabadi et al., 2012b).

We compared post-traumatic cell loss and neurological dysfunction in CypA^{+/+} versus CypA^{-/-} mice, with or without treatment of the pan-caspase inhibitor boc-aspartyl(OMe)-fluoromethylketone (BAF), to elucidate the relative roles of AIF and caspase mediated PCD pathways in secondary injury following TBI, and to determine whether combined inhibition provides additive neuroprotection.

Materials and Methods

Controlled Cortical Impact Injury

All surgical procedures were carried out in accordance with protocols approved by University of Maryland School of Medicine Institutional Animal Care and Use Committee. 129S6/SvEv Ppia/Cyclophilin A knockout (CypA^{-/-}) mice were provided by Dr. Jeremy Luban, University of Geneva. CypA^{-/-} mice are phenotypically normal and breeding and genotyping was performed as previously described (Colgan et al., 2000). Our custom-designed controlled cortical impact (CCI) injury device (Fox et al., 1998) consists of a microprocessor-controlled pneumatic impactor with a 3.5 mm diameter tip. Ten to twelve week-old, male CypA^{+/+} and CypA^{-/-} mice (20–25g) were anesthetized with isoflurane evaporated in a gas mixture containing 70% N₂O and 30% O₂ and administered through a nose mask (induction at 4% and maintenance at 2%). Monitoring respiration rate and pedal withdrawal reflexes assessed depth of anesthesia. The mouse was placed on a heated pad, and a core body temperature was maintained at 37°C. The head was mounted in a stereotaxic frame, and a 4-mm craniotomy was made on the central aspect of the left parietal bone. The impounder tip of the injury device was then extended to its full stroke distance (44 mm), positioned to the surface of the exposed dura, and reset to impact the cortical surface. Moderate injury was induced using an impactor velocity of 6 m/s and deformation depth of 2 mm as previously described (Loane et al., 2009). After injury, the incision was closed with interrupted 6-0 silk sutures, anesthesia was terminated, and the animal was placed into a heated cage to maintain normal core temperature for 45 minutes post-injury. All animals were monitored carefully for at least 4 hours after surgery and then daily. Sham animals underwent the same procedure as injured mice except for the impact.

Drug administration

At 30 minutes after CCI, CypA^{+/+} or CypA^{-/-} mice received a single intracerebroventricular (ICV) injection of boc-aspartyl(OMe)-fluoromethylketone (BAF; BioVision, Mountain View, CA) into the left lateral ventricle (coordinates from bregma = A: -0.5, L: -1.0, V: -2.0) using a 30 gauge needle attached to a Hamilton syringe at a rate of 0.5 µl/min, with a final volume of 5 µl, 300 nmols of BAF. Vehicle treated mice received injections of saline/DMSO.

Western Blot

At 24 hours after CCI, mice were anesthetized (100 mg/kg sodium pentobarbital, *i.p.*), transcardially perfused with ice-cold saline and decapitated. A 5 mm area surrounding the lesion epicenter on the ipsilateral cortex was rapidly dissected and immediately frozen on dry ice. Cortical tissue was homogenized in RIPA homogenization buffer which contains 50 mM

Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X100, and proteinase inhibitor cocktail (Sigma), and centrifuged at 15,000 rpm for 15 minutes at 4 °C to isolate proteins and protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL). Twenty-five µg of protein was run on SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The blot was then probed with antibodies against alpha-Fodrin (1:1000, cat. no. EPR3017; Biomol GmbH, Germany), AIF (1:1000, cat. no. 4642 Millipore, Billerica, MA), H2AX (1:1000, cat. no. NB100-383 Novus, Littleton, CO), CypA (1:1000, cat. no. ab41684, Abcam, Cambridge, MA). Beta-actin (1:5000, Sigma-Aldrich, St. Louis, MO) was used as an endogenous control. Immune complexes were detected with the appropriate HRP-conjugated secondary antibodies and visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). Chemiluminescence was captured on a Kodak Image Station 4000R station and protein bands were quantified by densitometric analysis using Carestream Molecular Imaging Software (Carestream Health, Rochester, NY). The data presented represents the density of target protein divided by the density of the beta-actin endogenous control in each sample, and are expressed in arbitrary units.

Co-immunoprecipitation

At 24 hours after CCI, cortical tissue from the ipsilateral hemisphere was rapidly dissected and homogenized in IP lysis buffer contain 50 mM Tris-HCl buffer (pH7.6), 150 mM NaCl, 2 mM EDTA, 0.5% NP-40 supplement with protease inhibitor cocktail (Sigma). Co-immunoprecipitation was performed using Pierce Crosslink IP Kit (Thermo Scientific). One milligram of tissue homogenate was pre-cleared with agarose beads by end-over-end rotation for 30 minutes at 4°C. Five micrograms of anti-CypA (cat. no. ab41684, Abcam) or anti-H2AX (cat. no. NB100-383, Novus) antibodies were incubated overnight at 4°C to form immunocomplexes. Protein A/G agarose beads were added and incubated for 2 hours at 4°C to precipitate immunocomplexes. Beads were washed three times with IP lysis buffer and eluted with 50 µl of elution buffer for 15 minutes at room temperature. The elution buffer eluted immunoprecipitated proteins from the antibody, which remained bound to the beads. The supernatant containing the co-precipitated proteins was carefully aspirated and boiled with sample buffer, separated on 4~12% bis-tris gradient gel (Invitrogen) and transferred onto nitrocellulose membrane. Membranes were probed with antibodies against AIF (cat. no. 4642, Millipore), H2AX, CypA as described previously. Normal rabbit IgG (Santa Cruz Biotechnology) was used as a negative control in co-immunoprecipitation studies.

Immunohistochemistry

Immunohistochemistry was performed on 20 µm sections from CypA^{+/+} and CypA^{-/-} mice at 24 hours post-CCI as previously described (Belanger et al., 2009; Piao et al., 2009)). The following primary antibodies were used: mouse anti-AIF (1:300, cat. no. 13116, Santa Cruz Biotechnology), rabbit anti-CypA (1:500; cat. no. ab41684, Abcam), rabbit anti-H2AX (1:500, cat. no. NB100-383, Novus), mouse anti-NeuN (1:500, cat. no. MAB377, Millipore), and standard immunostaining techniques were employed. Counterstaining was performed with 4', 6-diamidino-2-phenylindole (DAPI) (1 µg/ml; Sigma). Sections to which the primary antibodies were not added served as a negative control. Fluorescence microscopy was performed using a LEICA (TCS SP5 II) confocal microscope system (Leica Microsystems, Exton, PA).

Semi-quantitative analysis of immunolabelled cells was performed by an investigator blinded to treatment group using ImageJ software, version 1.4 (NIH) as previously described (Hilton et al., 2008). Briefly, immunofluorescent images from independent sections taken by the channel of interest were converted to 8-bit grayscale. The background was set as indicated using threshold command and cells were counted using the analyze particles

command. All images in the same series were processed using the same analysis parameters and the entire image was used for counting. The numbers shown in the graphs represent the average number of cells per image for each treatment. Unlike stereology, which offers a quantitative assessment of the total cell number throughout the entire lesion volume, this method provides a semi-quantitative representation of cell number per representative field (the multi-tiled images presented) of three to five sections.

In Situ Detection of Caspase Activity

“FLuorescence *in vivo*” (FLIVO; Immunohistochemistry Technologies LLC, AbCys SA, Paris) is a fluorescent-labeled pan-caspase inhibitor, which binds preferentially to active caspases thereby enabling *in situ* visualization of caspase activity in the brain. FAM-FLIVO was injected *ICV* into the left ventricle of CypA^{+/+} and CypA^{-/-} mice 24 hours after CCI. Each mouse received 1.5 µg of FAM-FLIVO diluted in 5 µl of PBS containing 30% DMSO. After 30 min, mice were deeply anesthetized and trans-cardially perfused with 4% PFA and coronal sections (60 µm and 20 µm) mounted on a slide and serially collected throughout the injured brain starting at +1.78 mm from bregma. Standard immunocytochemistry was combined with *in situ* FLIVO to visualize caspase activity in the brain.

Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling [TUNEL] analysis

TUNEL analysis was performed on 20 µm sections from CypA^{+/+} and CypA^{-/-} mice at 24 hours post-CCI using an ApopTag® Fluorescein/Red detection kit (Millipore) as described in the manufacturer’s instructions. Counterstaining was performed with 4', 6-diamidino-2-phenylindole (DAPI) (1 µg/ml; Sigma).

Primary Cell Culture

Cerebellar granule cells (CGC) cultures were prepared as previously described (Eldadah et al., 1997). Briefly, cerebella from postnatal day 7 CypA^{+/+} and CypA^{-/-} pups were cleaned from their meninges in Krebs-Ringer’s bicarbonate buffer containing 0.3% bovine serum albumin (BSA, Invitrogen). The cerebella were minced and dissociated in the same buffer with 1800 U/ml trypsin (Sigma) at 37°C for 10 minutes. Following the addition of 200 U/ml DNase I (Sigma) and 3600 U/ml trypsin inhibitor (Sigma) to the suspension, cells were triturated, and the cell suspension were pass through a cell strainer with 100 µm pore size. The cells were plated on 96-well plates coated with 10 µg/ml poly-D-lysine at a density of 2×10^5 cells/cm² in seeding medium, which consisted of Basal Medium Eagle (BME, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Scientific), 2 mM glutamine, 100 µM gentamicin sulphate and 25 mM potassium chloride. After 16 hours, 10µM cytosine arabino-furanoside (Sigma) was added to prevent glial proliferation. AIF-mediated apoptosis was induced in CGC by incubation with PARP activators, H₂O₂ (20 µM, Sigma) or MNNG (25 µM, TCI America, Portland, OR) in conditioned medium.

Assessment of Cell Viability

Cell viability was measured by retention and deesterification of calcein AM (Invitrogen) (Eldadah et al., 1997). Briefly, culture medium in 96-well plates was replaced with 5 µM calcein AM in Locke’s buffer containing 154 mM NaCl, 5.6 mM glucose, and 5 mM HEPES (pH 7.4). After incubation at 37°C for 30 minutes, fluorescence was measured using a Biotek Synergy HT multi-detection microplate reader at 485 nm excitation and 560 emission wavelengths.

Assessment of Caspase-3 like Activity

The assay for caspase-3-like activity was performed as described (Eldadah et al., 1997). Cytosolic proteins were extracted with lysis buffer, which containing 10 mM HEPES-KOH, pH 7.2, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol and protease inhibitors (Sigma). Twenty μ g of protein was incubated with equal volumes of 40 μ M fluorescent tetrapeptide substrate Ac-DEVD-AMC (Alexis Biomedicals, San Diego, CA). Free AMC accumulation, which resulted from cleavage of the aspartate-AMC bond, was monitored continuously in each sample over 10 in 96-well microtiter plates using a Biotek Synergy HT multi-detection microplate reader at 360 nm excitation and 460 nm emission wavelengths. The emission from each well was plotted against time. Linear regression analysis of the initial velocity (slope) of each curve yielded an activity for each sample. Data were expressed as percentages of caspase activity in samples from control cells.

Motor and Cognitive Function Testing

Motor performance was assessed at 1, 3, 7, 14, and 21 days post-injury using the beam walk task, as previously described (Fox et al., 1998). Briefly, mice were trained to cross a narrow wooden beam 6 mm wide and 120 mm in length, which was suspended 300 mm above a 60 mm thick foam rubber pad. The number of foot-faults for the right hind-limb was recorded over 50 steps and a basal level of competence at this task was established before surgery with an acceptance level of <10 faults per 50 steps. Spatial learning and memory was assessed using an acquisition paradigm of the Morris Water Maze (MWM) test on post-injury days (PID) 14, 15, 16 and 17, as described previously (Fox et al., 1998; Loane et al., 2009). A white circular pool was divided into four quadrants using computer-based AnyMaze video tracking system (Stoelting Co., Wood Dale, IL) and the platform was hidden in one quadrant (south-west) 14 inches from the wall. Spatial learning and working memory performance was assessed by determining the latency (seconds) to locate the submerged hidden platform with a 90 second limit per trial (4 trials per day for 4 consecutive days; PID 14–17). Reference spatial memory was assessed by a probe trial carried out on post-injury day 18, as the time spent (seconds) with a 60 second limit in the quadrant where the platform had been hidden during acquisition phase. A visual cue test was subsequently performed on post-injury day 18 using a flagged platform placed on the platform in one of the quadrants (with a 90 second limit per trial) and latency (seconds) to locate the flagged platform was recorded. In addition, the Water maze search strategies employed by each animal during the acquisition trials on day 4 of the MWM test were analyzed as previously described (Brody and Holtzman, 2006). Briefly, three strategies were identified and categorized: spatial strategy was defined as swimming directly to platform no more than 1 loop or swimming directly to the correct target quadrant and searching; systematic strategy was defined as searching the interior portion of or entire tank without spatial bias, and searching incorrect target quadrant; and looping strategy was defined as circular swimming around the tank, swimming in tight circle, and/or swimming around the wall of tank.

Stereological Counting of Surviving Hippocampal Neurons

At 21 days post-injury, mice were anesthetized (100 mg/kg sodium pentobarbital, *i.p.*) and trans-cardially perfused with 4% paraformaldehyde (10% buffered formalin solution, Fisher Scientific). The brain was removed and post-fixed in 4% paraformaldehyde overnight and cryoprotected in 30% sucrose. Sixty and twenty μ m coronal sections were cut ($3 \times 60\mu$ m followed by $3 \times 20\mu$ m sections) and serially collected throughout the injured brain starting at +1.78mm from bregma. Sections were mounted onto glass slides for histological or immunohistochemical analysis. Stereoinvestigator software (MBF Biosciences, Williston, VT) was used to count the total number of surviving neurons in the CA2/3 sub-field of the hippocampus using the optical fractionator method of unbiased stereology. Every fourth 60 μ m section was analyzed beginning from a random start point. Sections were analyzed

using a Leica DM4000B microscope. The optical dissector had a size of 50 μ m by 50 μ m in the x and y-axis with a height of 10 μ m and guard zone of 4 μ m from the top of the section. Dissectors were positioned every 150 μ m in the x and y-axis. The sampled region for each hippocampal subfield was demarcated in the injured hemisphere and cresyl-violet neuronal cell bodies were counted. The volume of the hippocampal subfield was measured using a Cavalieri estimator method. The estimated number of surviving neurons in each field was divided by the volume of the region of interest to obtain the cellular density expressed in cells/mm³.

In vivo MRI

At 21 days after CCI, brain lesion volume was assessed using T2-weighted MRI as previously described (Loane et al., 2009). Briefly, anesthetized animals were placed in a heated plexiglass holder and a respiratory motion detector positioned over the thorax to facilitate respiratory gating. The plexiglass holder was then placed in the center of the 7 Tesla magnet bore (Bruker Medical Inc., Billerica, MA) where a 72 mm proton-tuned birdcage coil was positioned. Field homogeneity across the brain was optimized and a sagittal scout image was acquired (RARE, i.e. rapid acquisition relaxation enhancement pulse sequence) image, field of vision, 4 $\times$ 4 cm; 128 $\times$ 128 resolution; repetition time (TR) to echo time (TE), 1,500/10 milliseconds with a RARE factor of 8, making the effective TE 40 milliseconds). Multi-slice, multi-echo T2-weighted images were acquired using the following parameters: 3 $\times$ 3 cm FOV, 256 $\times$ 256 resolution, 1,500/10 msec TR:TE, 8 RARE factor, TE 40msec, 10 $\times$ slices, 0.75mm slice thickness. Lesion volume was quantified from the summation of areas of hyperintensity on each slice, multiplied by slice thickness, for both the ipsilateral and contralateral hemispheres. Contralateral volumes were subtracted from ipsilateral volumes to obtain TBI-induced lesion volumes.

Statistical Analysis

Examiners blinded to treatment group performed lesion volume, functional data, Western blot, and unbiased stereological analysis. Quantitative data are presented as mean $\pm$ standard error of the mean. Functional data were analyzed with repeated measures one-way analysis of variance (ANOVA) and post-hoc adjustments using Tukey's test. Search strategy analysis was analyzed using a Chi-square analysis. Remaining data were analyzed using Student's *t*-test or one-way ANOVA, where appropriate. All statistical tests were performed using the GraphPad Prism Program, Version 3.02 for Mac (GraphPad Software, San Diego, CA). A *p* value < 0.05 was considered statistically significant. Unless otherwise mentioned we specifically describe all statistical significant intergroup comparisons; the mention of trend reflects observable differences that do not reach statistical significance.

Results

Combined interventions that target caspase-dependent and AIF-mediated PCD pathways result in additive neuroprotection and improved functional recovery after TBI

To explore the therapeutic potential of targeting caspase-dependent and AIF-mediated PCD, we randomized moderate-level CCI-injured CypA^{-/-} or CypA^{+/+} mice into two treatment groups (n=15/group), and administered a pan-caspase inhibitor, BAF (300 nmols) or vehicle by ICV injection at 30 minutes post-injury. The CCI model induces traumatic injuries on the left parietal cortex and sub-cortical regions including the hippocampus, thereby producing sensorimotor and learning and memory deficits. We assessed long-term motor and cognitive functional recovery through 21 days post-injury using beam walk and Morris water maze (MWM) tasks respectively. BAF-treated CypA^{+/+} and vehicle-treated CypA^{-/-} TBI mice exhibited significant improvement in fine-motor co-ordination over time (29 $\pm$ 3 and 18 $\pm$ 4 foot-faults respectively, *p* <0.05) and the combined intervention of BAF-treated CypA^{-/-}

TBI mice resulted in the most significant improvement in fine motor co-ordination (16 ± 2 , $p < 0.01$) when compared to vehicle-treated CypA^{+/+} TBI mice (39 ± 2 foot-faults; Fig 1A).

During the acquisition phase of the MWM test only BAF-treated CypA^{-/-} TBI mice (28.6 ± 3.7 seconds) had significantly reduced latency to reach the submerged hidden platform when compared to vehicle-treated CypA^{+/+} TBI mice (Fig. 1B, 52.5 ± 4.5 seconds; $p < 0.05$), suggesting significant improvement in spatial learning and working memory. BAF-treated CypA^{+/+} (41.3 ± 4.0 seconds) and vehicle-treated CypA^{-/-} TBI mice (34.0 ± 3.7 seconds) showed improved performance in this task, but failed to reach statistical significance when compared to vehicle-treated CypA^{+/+} TBI mice. All TBI mice performed well in the visual cue test and swim speeds did not differ across groups (data not shown). Reference memory was assessed using the MWM probe trial on post-injury day 18 (Fig. 1C). Similarly, BAF-treated CypA^{-/-} TBI mice spent significantly more time in the target quadrant during the probe trial when compared to vehicle-treated CypA^{+/+} TBI mice ($p < 0.05$), and BAF-treated CypA^{+/+} and vehicle-treated CypA^{-/-} TBI mice showed a trend toward increased time spent in the target quadrant, but did not reach statistical significance. Analysis of the search strategy used by each group of mice to locate the submerged hidden platform during the final day of the MWM acquisition phase revealed that CCI-injured vehicle-treated CypA^{+/+} mice used diminished spatial and systematic search strategies and increased looping strategy compared to the CCI-injured BAF-treated CypA^{-/-} mice (Fig 1D, $p < 0.05$, $\chi^2 = 15.53$). The single intervention groups showed more modest differences in search strategy behaviors.

TBI-induced lesion volume was measured using T2-weighted MRI at 21 days post-injury. Representative T2-weighted MRI images for each group of mice are shown in Fig 2A. BAF-treated CypA^{+/+} (5.40 ± 0.86 mm³) and vehicle-treated CypA^{-/-} (5.06 ± 0.77 mm³) TBI mice had reduced lesion size when compared to vehicle-treated CypA^{+/+} TBI mice (7.03 ± 0.74 mm³), but did not reach statistical significance (Fig. 2B). In contrast, BAF-treated CypA^{-/-} TBI mice (3.59 ± 0.87 mm³) had significantly reduced lesion size when compared to vehicle-treated CypA^{+/+} TBI mice ($p < 0.05$). Regression analysis revealed a positive correlation between improved motor function performance and reduced lesion volumes due to the combined interventions ($r^2 = 0.8328$, $p < 0.01$; Fig. 2C). Unbiased stereological assessment of surviving neurons was performed in CA2/3 and DG sub-regions of the hippocampus at 21-day post injury. BAF-treated CypA^{-/-} TBI mice demonstrated a significant increase in CA2/3 neuronal survival when compared to vehicle-treated CypA^{+/+} TBI mice (Fig. 2D, $p < 0.05$). BAF-treated CypA^{+/+} and vehicle-treated CypA^{-/-} TBI mice showed non-significant increases in neuronal survival in this region. Neuronal survival in DG showed similar trends as CA2/3 but failed to reach statistical significance (data not shown). There was a positive correlation between improved cognitive performance and reduced CA2/3 neuronal cell loss due to the combined interventions ($r^2 = 0.8857$, $p < 0.01$; Fig. 2E).

Coincident activation of AIF- and caspase- dependent cell death after TBI

To better characterize the role of these neuronal PCD pathways after TBI, post-traumatic caspase activity was visualized using FAM-FLIVO, an *in situ* active caspase detection fluorescent marker, and AIF nuclear translocation was visualized by immunohistochemistry (Fig. 3A). At 24 hours after injury, AIF immunoreactivity was robustly increased throughout the contused cortex, whereas caspase activity was up-regulated in more conserved sub-cortical regions (regions away from the site of impact). It is speculated that the increased AIF staining reflects accessibility of the AIF protein to the antibody after release of AIF from the mitochondria (Slemmer et al., 2008b). AIF immunoreactivity and caspase activity was also detected in the CA2/3 and DG regions of the hippocampus (data not shown). Regions that showed either single AIF- or FAM-FLIVO-positive cells were interspersed and partially overlapped with areas that showed AIF/FAM-FLIVO double-positive cells.

Notably, higher magnification of DAPI staining demonstrated that AIF-positive cells (AIF+/FLIVO-) with a neuronal morphology displayed nuclear peripheral chromatin condensation, a feature of AIF-mediated PCD, whereas FAM-FLIVO positive cells (AIF-/FLIVO+) displayed advanced nuclear condensation, a feature of caspase-dependent cell death (Fig 3B). No FAM-FLIVO or AIF-positive cells were observed in sham-injured controls.

Cyp A facilitates the interaction of AIF with H2AX following TBI and promotes AIF-mediated neuronal cell death in vitro

To further explore the role of CypA in AIF-mediated cell death, subcellular localization and protein-protein interactions were investigated at 24 hours post-injury. CypA was predominantly expressed in the cytoplasm of neurons of sham-injured controls, but following TBI it translocated to the nucleus in neurons displaying chromatin condensation (Fig. 4A). Notably, CypA co-localized with AIF in the cytoplasm of dying neurons prior to translocation of AIF to the nucleus (Fig. 4B). Further analysis revealed that AIF co-localized with histone H2AX in the nucleus (Fig. 4C); this physical interaction has been shown to regulate chromatinolysis in MEF cells and cell-free systems (Artus et al., 2010; McMillan et al., 2011). Co-immunoprecipitation studies demonstrated a physical interaction of AIF with both CypA and histone H2AX following TBI. AIF co-immunoprecipitated with CypA and histone H2AX in TBI samples but failed to physically interact with them in sham-injured controls (Fig. 4D; $p=0.042$ for AIF/CypA interaction, $p=0.019$ for AIF/H2AX interaction). Normal IgG antibodies were used as negative controls and the physical interaction of AIF/CypA and AIF/H2AX were absent in these samples (data not shown). These data demonstrate for the first time the AIF-CypA physical interaction in the cytoplasm and the AIF-H2AX physical interaction in the nucleus of neurons after TBI. In addition, the interaction between AIF and the nuclear protein histone H2AX supports the nuclear translocation of AIF following trauma. We repeated these studies in cortical homogenates from CypA^{+/+} and CypA^{-/-} TBI mice, and found that the physical interaction of AIF with histone H2AX was significantly attenuated in CypA^{-/-} TBI samples when compared to CypA^{+/+} TBI samples (Fig. 4E, $p<0.05$), thereby demonstrating that CypA is essential for the interaction of AIF with H2AX and for AIF-mediated PCD after TBI.

To confirm the role of CypA in AIF-mediated PCD we used primary cerebellar granule cells (CGC) generated from CypA^{+/+} and CypA^{-/-} mice and exposed them to hydrogen peroxide (H₂O₂) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to induce AIF-mediated cell death (Yu et al., 2002; Wang et al., 2009; Artus et al., 2010). Exposure to 20 μ M H₂O₂ or 25 μ M MNNG for 24 hours induced significant neuronal death, whereas CypA^{-/-} neurons were significantly protected in both models (Fig. 5A and B). We did not detect caspase-3 activity after H₂O₂ or MNNG exposure (Fig. 5C and D), and BAF treatment (100 μ M) failed to block H₂O₂ or MNNG-induced neurotoxicity (data not shown) supporting the caspase-independent characteristics of these models.

Caspase activity is not required for CypA/H2AX and AIF interactions

To investigate the potential link between caspase-dependent and AIF-mediated PCD pathways following TBI we treated CypA^{+/+} TBI mice with vehicle or BAF (300 nmols; ICV) at 30 minutes post-injury and measured α -fodrin cleavage as a marker of post-traumatic cell death. Alpha-fodrin undergoes both calpain-dependent cleavage (150/145 kD) and caspase-dependent cleavage (150/120 kD) after TBI (Siman et al., 1984; Cryns et al., 1996; Siman et al., 2004), and in these studies BAF treatment significantly reduced the 120 kD caspase-dependent cleavage fragment of α -fodrin after TBI (Fig. 6A; $p<0.05$ versus CCI + vehicle), but only modestly reduced the 150/145 kDa cleavage fragment. There were no significant differences in either calpain-dependent or caspase-dependent cleavage of α -fodrin between vehicle-treated CypA^{+/+} and CypA^{-/-} TBI mice (data not shown). Notably,

treatment with BAF did not interfere with AIF-mediated pathways after TBI as BAF did not disrupt the physical interactions between AIF/CypA or AIF/H2AX (Fig. 6B), suggesting significant independence between caspase-dependent and AIF-mediated signaling pathways after TBI.

Both caspase-dependent and AIF-mediated cell death mechanisms contribute to post-traumatic neuronal cell death

CypA^{+/+} and CypA^{-/-} TBI mice were treated with vehicle or BAF (300nmols; ICV) at 30 minutes post-injury and the number of cells undergoing caspase-dependent and AIF-mediated cell death at 24 hours after TBI was evaluated (Fig. 7A). The CypA^{-/-} intervention significantly and preferentially attenuated the number of AIF-positive cells after TBI, whereas BAF-treatment significantly and preferentially attenuated FLIVO-positive caspase activation (Fig. 7B and C). Both interventions resulted in a significant, albeit partial, attenuation of the number of dying cells (TUNEL-positive) after TBI (Fig. 7D). The combined intervention of BAF-treatment in CypA^{-/-} TBI mice consistently showed greatest reduction in FLIVO-positive, AIF-positive and TUNEL-positive cells. Moreover, BAF-treated CypA^{-/-} TBI mice demonstrated a significant reduction in apoptotic (TUNEL-positive) cells in comparison to single intervention, BAF-treated CypA^{+/+} or vehicle-treated CypA^{-/-} TBI mice. Thus, our data suggest the additive neuroprotective effects of combined inhibition that targets both caspase-dependent and AIF-mediated cell death mechanisms following TBI.

Post-traumatic microglial activation is significantly attenuated in CypA^{-/-} TBI mice

We assessed whether caspase inhibition (BAF treatment) and/or CypA^{-/-} could reduce post-traumatic inflammation and oxidative stress. ED1 (CD68), a marker of reactive and phagocytic microglia, was significantly up-regulated in microglia that displayed a hypertrophic/bushy cellular morphology and co-expressed gp91^{phox}, the membrane bound component of the NADPH oxidase enzyme, in the vehicle-treated CypA^{+/+} TBI group (Fig. 8A). In addition, there was also evidence of increased oxidative stress in this group as 3NT-positive cells surrounded reactive microglia. Notably, vehicle-treated CypA^{-/-} and BAF-treated CypA^{-/-} TBI groups had significantly reduced ED1 expression when compared to the vehicle-treated CypA^{+/+} TBI group (Fig. 8B; $p < 0.01$). BAF-treated CypA^{+/+} TBI samples had reduced ED1 expression when compared to vehicle-treated TBI samples, but did not reach significance. These data suggest that CypA plays a significant role in TBI-initiated neuroinflammation whereas caspases appear to have a more limited role.

Discussion

We demonstrate here, for the first time, that CypA^{-/-} provides neuroprotection in a clinically relevant experimental model of TBI. Our data show the coincident activation of caspase-dependent and AIF-dependent cell death after CCI; the latter involves the physical interactions of AIF/CypA and translocation of AIF to the nucleus. In addition, we show that translocated AIF physically interacts with histone H2AX in the nucleus following TBI and that CypA is required for AIF-mediated chromatinolysis in neurons after injury. Inhibition of either AIF (CypA^{-/-}) or caspase (BAF treatment) pathways is neuroprotective. Importantly, our studies demonstrate that combined treatment that targets both the caspase-dependent and AIF-mediated PCD pathways - CypA^{-/-} mice treated with the pan-caspase inhibitor BAF - after TBI results in superior neuroprotection compared with either single intervention alone.

In the mid 1990s several groups showed that neuronal cell death consistent with caspase-dependent apoptosis occurs after experimental TBI (Conti et al., 1998; Rink et al., 1995).

We and others have used peptide-based caspase inhibitors to demonstrate that caspases play a role in neuronal death *in vitro* (Eldadah et al., 1997) and in post-traumatic apoptosis and associated neurological dysfunction in various TBI models *in vivo* (Clark et al., 2000a; Knoblach et al., 2004; Knoblach et al., 2002; Sullivan et al., 2002; Yakovlev et al., 1997). In the lateral fluid percussion (LFP) model of TBI in rats we have shown that the somewhat selective caspase-3 inhibitor zDEVD-fmk attenuated neuronal apoptosis and improved early motor recovery (Yakovlev et al., 1997) and that treatment with the pan-caspase inhibitor zVAD-fmk improved both motor recovery and cognitive function (Knoblach et al., 2002). Furthermore, treatment with zDEVD-fmk also improved motor scores and cognitive function and attenuated lesion volume in a mouse CCI model (Knoblach et al., 2004). In contrast, in a rat model of CCI plus moderate hypoxemia, treatment with zDEVD-fmk had no effect on functional outcomes although it attenuated contusion volume and neuronal loss in the hippocampus (Clark et al., 2000b). A limitation of zDEVD-fmk and zVAD-fmk is that both compounds can inhibit not only caspases but also other proteases such as calpains (Knoblach et al., 2004; Knoblach et al., 2005). BAF may provide greater specificity, as it is more selective than many used peptide-based caspase inhibitors (Knoblach et al., 2005). However, in rat CCI BAF treatment did not attenuate the lesion volume (Sullivan et al., 2002); nor did it improve neurological outcomes, lesion volume or hippocampal neuronal loss (Clark et al., 2006). Thus, the beneficial effects of caspase inhibitors following TBI have not been consistently demonstrated across laboratories or models. Possible explanations for the observed inconsistencies include differences in experimental TBI models and species used, the diversity of caspase inhibitors and doses employed, and the different types of functional outcomes used. Our current data show that as single treatment BAF is mechanistically effective (significant decrease in FLIVO+ cells and fodrin cleavage) in reducing caspase activity but only modestly effective in improving motor function and attenuating the number of TUNEL+ cells. In all other functional (cognitive outcomes) and structural (lesion volume, hippocampal cell loss and neuroinflammation) measures of injury, BAF treatment showed only non-significant trends for improvement. Our data suggest that caspase-dependent mechanisms play a relatively minor role in secondary injury after CCI in adult mice, and/or that the multiplicity of secondary PCD injury mechanisms minimize the therapeutic effectiveness of targeting caspase mechanisms in isolation.

AIF has also been implicated in neuronal cell death after CNS injury (Osato et al., 2010; Slemmer et al., 2008a; Stoica and Faden, 2010; Zhang et al., 2002; Zhu et al., 2003). Cao et al. showed that following transient global ischemia AIF translocates from the mitochondria to the nucleus in injured hippocampal neurons in a caspase-independent manner and induces large-scale DNA degradation producing 50 kbp fragments (Cao et al., 2003). In a neonatal hypoxia-ischemia model, BAF treatment did not affect AIF translocation to the nucleus in injured brain neurons, suggesting the relative independence between caspase- and AIF-mediated pathways (Zhu et al., 2003). The earliest support for the role of AIF pathways in neuronal cell death in the brain used a model of transient focal cerebral ischemia in Hq mice, demonstrating that lower levels of AIF expression correlated with decreased infarct volumes and attenuation of neuronal cell death in the ischemic penumbra (Culmsee, 2005). In the same model, the AIF hypomorphic mutation and caspase inhibition showed additive neuroprotective effects (Zhu et al., 2006). More recently, in an adult mouse model of global ischemia, significant attenuation in neuronal loss in the hippocampus was observed in Hq animals compared to wild type (Thal et al., 2011). Fewer studies have examined the role of AIF in TBI models. Zhang and colleagues used rat CCI plus hypoxia to show that AIF translocation to the nucleus in injured neurons of the cortex and hippocampus coincides with large-scale DNA fragmentation (Zhang et al., 2002), whereas Slemmer and colleagues reported that Hq mice showed attenuation in secondary contusion expansion after CCI (Slemmer et al., 2008b).

Much of the prior work examining the potential role of AIF-dependent cell death in CNS injury used the Hq model, as knockout of this gene is fatal *in utero*. However, the Hq model suffers from significant limitations- including phenotypic abnormalities and increased basal oxidative stress (Zhu et al., 2006). Furthermore, studies have shown that AIF deficiency results in early mitochondrial degeneration followed by progressive neurodegeneration throughout the brain (El Ghouzzi et al., 2007). Therefore, a better genetic alternative for targeting AIF pathways utilizes knockout of CypA, which appears a required element for AIF-mediated cell death; it physiologically interacts with AIF and promotes AIF translocation to the nucleus (Cande et al., 2004; Zhu et al., 2007a). Zhu and colleagues, using the neonatal hypoxia-ischemia model, showed that CypA^{-/-} mice showed reduced nuclear pyknosis and attenuated infarct volume as compared to CypA^{+/+} controls (Zhu et al., 2007a).

Nuclear translocation and co-localization of AIF with CypA in neurons was demonstrated by co-immunolabeling in the post-traumatic brain; this co-localization pattern occurred mainly in cells whose nucleus displayed chromatin condensation patterns similar to stage I apoptosis, which is characteristic of AIF-mediated cell death (Cande et al., 2004; Susin et al., 2000). We used co-immunoprecipitation to present the first confirmation of the physical interaction between AIF and CypA after TBI. Recent studies have described the role of histone H2AX in PCD using *in vitro* models. Upon MNNG exposure, AIF translocates to the nucleus and interacts with H2AX to promote chromatinolysis in cooperation with CypA (Artus et al., 2010). Here, using co-immunolabeling and co-immunoprecipitation we demonstrate for the first time that AIF and H2AX interact after TBI, providing further support for AIF translocation to the nucleus where H2AX is located. Some of the nuclei exhibiting co-localized histone H2AX and AIF at 24 hour after TBI demonstrated features of stage I apoptosis, which may reflect AIF-mediated chromatin degradation (Artus et al., 2010; Baritaud et al., 2010; Celeste et al., 2003; Rodriguez-Paez et al., 2005; Stoica et al., 2009c). Notably, in CypA^{-/-} mice the histone H2AX and AIF interaction was significantly reduced, thereby preventing a key step in AIF-mediated PCD after TBI. These data suggest that CypA is a required co-factor in caspase-independent and AIF-mediated PCD pathway following brain trauma.

The *in vitro* studies demonstrate that CGCs from CypA^{-/-} mice are highly resistant to AIF-mediated neuronal cell death induced by H₂O₂ or MNNG treatment; these models show minimal caspase-dependent cell death. Our *in vivo* data also shed light on the relationship between caspase-dependent and -independent pathways in neuronal death after TBI. BAF-treatment significantly reduced FLIVO-positive cells and attenuated the caspase-3-dependent cleavage of α -fodrin, without significantly altering the AIF nuclear translocation or AIF-CypA and AIF-H2AX interactions. In addition, CypA^{-/-} mice did not show a significant reduction in the number of FLIVO-positive cells after TBI. These findings are consistent with prior work demonstrating that caspase-3-like activity is similar in CypA^{-/-} and Hq mutant mice after hypoxia-ischemia (Zhu et al., 2007b).

A notable finding from these studies is that combined inhibition of caspase-dependent and caspase-independent PCD mechanisms after TBI provides additive neuroprotection. This conclusion is based on the following observations: 1. the combined intervention (BAF-treated CypA^{-/-}) shows the most significant improvements in fine-motor co-ordination over time as well as reduction in TUNEL-positive cells and activated microglia when compared to the vehicle-treated CypA^{+/+} group; 2. it is the only intervention demonstrating significant improvements in spatial learning and reference memory performance in the MWM and probe test as well as significantly reduced lesion volume and attenuation of hippocampal cell loss.

Both caspases and CypA are also known to participate in cellular processes relevant for secondary injury but not directly related to PCD. For example, CypA is implicated as a mediator of oxidative stress and inflammation in non-neuronal cells (Kim et al., 2005; Satoh et al., 2008 & 2009) including monocytes (Seizer et al., 2011; Yuan et al., 2010). Also, caspases may play a role promoting microglial activation (Burguillos et al., 2011). Activated microglia produce various pro-inflammatory molecules - such as IL-1 β , nitric oxide, complement components, MCP-1, osteopontin, MHC class I and II and ROS - which have been implicated in secondary injury (Loane and Byrnes, 2010). Thus, inhibition of CypA or caspase pathways could also serve a neuroprotective role by limiting inflammatory responses. Our data show that CypA^{-/-} and especially the CypA^{-/-} + BAF combined intervention significantly reduce microglial activation and oxidative stress following TBI, but it is not clear whether this reflects a primary effect on neuroinflammation or an indirect effect in response to reduced neuronal death. The complexity of the CypA function is also illustrated by recent studies suggesting that CypA may protect neurons against *in vitro* oxidative and ischemic injury (Boulos et al., 2007) and attenuate blood-brain barrier permeability and tissue damage in a stab wound model of brain injury (Redell et al., 2007). Thus, other methods such as cell specific knockout of CypA are needed to clarify more precisely the role(s) of CypA in brain trauma.

In contrast to previously published studies, our investigation of AIF in secondary injury focused not only on mechanistic aspects of neuronal cell death but also examined long-term functional outcomes and associated neuronal loss. This approach offers a more critical assessment of the pathobiological role of AIF in CNS injury and for the therapeutic potential of interventions that target it. Following CCI, CypA^{-/-} mice showed significantly better recovery of motor function as well as attenuation of TUNEL+ cells and degree of microglial activation in comparison to wild type animals. Yet with all other functional (cognitive outcomes) and structural (lesion volume and hippocampal cell loss) measures of injury CypA^{-/-} showed only non-significant trends for improvement. We speculate that inhibition of AIF-dependent PCD may unveil alternative activation of other cell death mechanisms, such as caspase-dependent apoptosis. Thus, interventions directed toward AIF-mediated cell death alone may be insufficient to have a major impact on longer term functional outcomes.

In conclusion, single intervention with either BAF treatment or CypA^{-/-} reduced cell death and improved neurological outcomes following TBI, suggesting that both caspase-dependent and AIF-mediated PCD pathways contribute to secondary injury after TBI. These PCD mechanisms appear to be largely independent and occur in parallel. The key finding of these studies was that the combined interventions targeting both caspases and AIF showed the largest and most significant neuroprotective effects after TBI. That combined interventions targeting multiple PCD mechanisms provides superior neuroprotection in this rodent CCI model suggests that multi-drug approaches or single agents targeting both pathways (e.g. HSP 70 activators, (Stoica et al., 2009a)) may be a preferred therapeutic strategy for the treatment of human brain trauma.

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Abbreviations

AIF	apoptosis inducing factor
BAF	boc-aspartyl(OMe)-fluoromethylketone

CCI	controlled cortical impact
CypA	Cyclophilin A
Hq	harlequin
ICV	intracerebroventricular
PCD	programmed cell death
TBI	traumatic brain injury

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Highlights

1. AIF- and caspase-dependent cell death pathways occur concurrently after TBI
2. Inhibiting AIF- and caspase-dependent cell death provide additive neuroprotection
3. Cyclophilin A is required for AIF nuclear translocation after TBI
4. Histone H2AX interacts with AIF in the nucleus following TBI

Figure 1. Combined inhibition results in additive benefits in neurological recovery after TBI
A. TBI-induced fine motor co-ordination deficits were assessed using a beam walk test. TBI-injured BAF-treated CypA^{+/+} mice and vehicle-treated CypA^{-/-} mice showed significant improvements in fine motor co-ordination over time compared to vehicle-treated CypA^{+/+} mice (* $p < 0.05$ versus CypA^{+/+} + Veh). Injured BAF-treated CypA^{-/-} mice showed the most significant improvements in fine motor co-ordination compared to vehicle-treated CypA^{+/+} mice (** $p < 0.01$ versus CypA^{+/+} + Veh; repeated measures one-way ANOVA, Tukey's post hoc test, $n = 13-15$ /group, $F = 11.17$). **B.** Spatial learning and working memory was assessed using a Morris water maze test. Injured BAF-treated CypA^{-/-} mice had significantly reduced latency to find the submerged hidden platform when compared to vehicle-treated CypA^{+/+} mice (** $p < 0.01$ versus CypA^{+/+} + Veh; $n = 10-12$ /group, repeated measures one-way ANOVA, Tukey's post hoc test, $F = 7.146$). **C.** Reference memory was assessed using the MWM probe test on day 18 post injury. There was a significant improvement in reference memory in injured BAF-treated CypA^{-/-} mice when compared to vehicle-treated CypA^{+/+} mice (* $p < 0.05$ versus CypA^{+/+} + Veh; $n = 10-12$ /group, One-way ANOVA, Tukey's post-hoc test). **D.** Search strategy to locate the submerged hidden platform during final day of MWM acquisition phase was analyzed in each group of mice.

Injured BAF-treated CypA^{-/-} mice primarily used a spatial search strategy with limited random looping search strategy (*p<0.05; $\chi^2=15.53$, Chi-squared analysis).

Figure 2. Combined inhibition reduces the lesion size and hippocampal neuron loss after TBI
A. Representative T2-weighted MRI images from CCI-injured vehicle- and BAF-treated CypA^{+/+} and CypA^{-/-} mice at 21 days post injury. **B.** T2-weighted MRI lesion volume quantification. Lesion volume was significantly reduced in the injured BAF-treated CypA^{-/-} group (* $p<0.05$ versus CypA^{+/+} + Veh; $n=10-13$ /group, Mean $\pm$ SEM, One-way ANOVA, Tukey's post-hoc test). **C.** Linear regression analysis comparing TBI-induced lesion size with footfalls in the beam walk test at 21 days post injury ($r^2=0.8328$, $n=6-7$, $p<0.01$, one-tailed Pearson's correlation). **D.** Unbiased stereological assessment of surviving neurons in the CA2/3 region of the hippocampus. BAF-treated CypA^{-/-} mice had significantly

improved CA2/3 neuronal survival after TBI (* $p < 0.05$ versus CypA^{+/+} + Veh; $n = 5-7$ /group, Mean $\pm$ SEM, One-way ANOVA, Tukey's post-hoc test). **E.** Linear regression analysis comparing TBI-induced neuronal loss in the CA2/3 with latency to reach the submerged platform on day 17 of MWM ($r^2 = 0.8857$, $n = 4-5$, $p < 0.01$, one-tailed Pearson's correlation).

Figure 3. AIF-mediated and caspase-dependent cell death pathways are activated after TBI

A. Representative images from ipsilateral cortex centered on the lesion area of CCI-injured CypA^{+/+} mice at 24 hours after TBI. Increased AIF-immunoreactivity (red) was detected throughout the contused cortex, whereas increased caspase activity (FLIVO; green) was detected in discrete regions of the cortex including deep-lying subcortical regions. AIF- or FLIVO-positive cells were interspersed throughout the cortex and partially overlapped with areas containing AIF/FLIVO double-positive cells. Bar: 500 μ m. **B.** High magnification images show no FLIVO-activity and very low AIF-immunoreactivity in the cytoplasm of sham-injured control cells (upper panels). The lower panels illustrate regions of AIF-predominant (AIF+/FLIVO-; red arrows), caspase-predominant (AIF-/FLIVO+; green arrows) and concurrent AIF- and caspase-mediated (AIF+/FLIVO+; yellow arrow) cell death in the contused cortex 24 hours after TBI. DAPI staining demonstrates chromatin condensation and nuclear changes in dying neurons. Bar: 10 μ m.

Figure 4. CypA-AIF interacts in the cytoplasm and AIF-H2AX interacts in the nucleus of neurons following TBI

A. CypA (red) is predominantly expressed in the cytoplasm of neurons (NeuN, green) in sham-injured controls and translocated to the nucleus at 24 hours after TBI. **B.** AIF staining (red) increases in intensity and is co-localized with CypA (green) in the cytoplasm following TBI. **C.** AIF staining (red) increases in intensity and is co-localized with histone H2AX (green) in the nucleus following TBI. Histone H2AX staining illustrates nuclear morphological features typical of stage I chromatinolysis. Bar: 10 μ m. Orthogonal projections demonstrate co-localized immunostained markers in A – C. **D.** Co-immunoprecipitation of CypA-AIF and H2AX-AIF was performed using cortical tissue extracts from sham-injured and CCI-injured mouse brain to demonstrate the physical interaction of CypA-AIF and H2AX-AIF after TBI. Bar graphs represent the fold-change in AIF band intensity in co-immunoprecipitation studies (* $p < 0.05$ versus sham; $n = 3$, Mean $\pm$ SEM, Student's t -test). **E.** Co-immunoprecipitation of CypA-AIF and H2AX-AIF was performed using cortical tissue extracts from sham- and CCI-injured CypA^{+/+} and CypA^{-/-} mice to demonstrate that CypA is required for the physical interaction of AIF-H2AX. Bar graphs represent the fold-change in AIF band intensity in co-immunoprecipitation studies (* $p < 0.05$, versus CypA^{+/+} CCI; ++ $p < 0.05$, versus CypA^{-/-} CCI; $n = 3$, Mean $\pm$ SEM, One-way ANOVA, Tukey's post-hoc test).

Figure 5. CypA deficiency blocked AIF-mediated cell death *in vitro*

A. H₂O₂-treatment (20 μ M) induced significant AIF-mediated neuronal cell death in CypA^{+/+} primary cerebellar granular cells (CGCs) while cells from CypA^{-/-} mice were completely protected (*p<0.05 versus control CypA^{+/+}, ++p<0.01 versus H₂O₂-treated CypA^{+/+}; One-way ANOVA Kruskal-Wallis test, Dunn's multiple comparison test). **B.** MNNG-treatment (25 μ M) induced significant neuronal cell death in CypA^{+/+} CGCs while cells from CypA^{-/-} mice were completely protected (*p<0.05 versus control CypA^{+/+}, ++p<0.01 versus MNNG-treated CypA^{+/+}; One-way ANOVA Kruskal-Wallis test, Dunn's multiple comparison test). **C.** Caspase-3 activity was not detected in H₂O₂-treated CGCs. **D.** Caspase-3 activity was not detected in MNNG-treated cells.

Figure 6. Caspase inhibition does not block the interactions between CypA/H2AX and AIF
A. Western blot for α-fodrin cleavage products in sham-injured, CCI-injured vehicle and BAF-treated CypA^{+/+} mice at 24 hours post TBI. CCI resulted in significant calpain-dependent (150/145 kD) and caspase-dependent (120 kD) cleavage of α-fodrin in vehicle-treated CypA^{+/+} mice (* $p < 0.05$ versus sham). BAF-treatment significantly reduced CCI-induced caspase-dependent cleavage of α-fodrin (120 kD; ** $p < 0.05$ versus CCI+Veh; $n = 3$, Mean $\pm$ SEM, One-way ANOVA, Tukey's post-hoc test). **B.** Co-immunoprecipitation of CypA-AIF and H2AX-AIF was performed using cortical extracts from CCI-injured vehicle- and BAF-treated CypA^{+/+} mice. Caspase inhibition by BAF treatment failed to disrupt the physical interaction of CypA-AIF and H2AX-AIF after TBI. Bar graphs represent the fold-change in AIF band intensity in co-immunoprecipitation studies (* $p < 0.05$ versus sham; $n = 3$, Mean $\pm$ SEM, One-way ANOVA, Tukey's post-hoc test).

Figure 7. Combined inhibition targeting AIF- and caspase-mediated cell death mechanisms results in an additive reduction of TBI-induced apoptosis

A. Representative images of the ipsilateral cortex centered on the lesion area from CCI-injured vehicle- and BAF-treated CypA^{+/+} and CypA^{-/-} mice at 24 hours after TBI. Bar: 500 μ m. **B – D.** Semi-quantitative analysis of caspase-positive (FLIVO+; green), AIF-positive (AIF+; red) and apoptotic (TUNEL-positive; blue) cells following TBI. Caspase-positive cells were significantly reduced in BAF-treated CypA^{+/+} and BAF-treated CypA^{-/-} samples but not in vehicle-treated CypA^{-/-} compared to vehicle-treated CypA^{+/+} samples (* p <0.05, ** p <0.01 versus CypA^{+/+} + Veh). AIF-positive cells were significantly reduced in vehicle- and BAF-treated CypA^{-/-} but not in BAF-treated CypA^{+/+} samples compared to vehicle-treated CypA^{+/+} samples (+++ p <0.001 versus CypA^{+/+} + Veh). Apoptosis was significantly reduced by BAF-treatment (CypA^{+/+} + BAF) or CypA^{-/-} (CypA^{-/-} + Veh) when compared to vehicle-treated CypA^{+/+} samples (^^^ p <0.001 versus CypA^{+/+} + Veh). The combined intervention (CypA^{-/-} + BAF) resulted in an additive reduction in apoptosis following TBI (# p <0.05 versus CypA^{-/-} + Veh). n =3–4/group, Mean $\pm$ SEM, One-way ANOVA, Tukey's post-hoc test.

Figure 8. TBI-induced microglial activation was significantly reduced in CypA^{-/-} mice
A. Representative images from CCI-injured vehicle- and BAF-treated CypA^{+/+} and CypA^{-/-} mice contused cortex at 21 days post-injury. TBI induced chronic microglial activation (ED1-positive cells, purple) in vehicle-treated CypA^{+/+} mice, which colocalized with a membrane bind form of NADPH subunit, gp91^{phox} (red). Surrounding those hypertrophic bushy microglia an oxidative stress marker, 3NT (green) are robustly upregulated. ED1, gp91^{phox} and 3NT expression was dramatically downregulated in vehicle- and BAF-treated CypA^{-/-} mice. Bar: 50 μ m. **B.** Semi-quantification analysis of ED1-positive cells following TBI in each group. ED1-positive cells were significantly reduced in vehicle- and BAF-treated Cyp^{-/-} samples (**p<0.01 versus vehicle-treated CypA^{+/+}). N=3–4/group, Mean $\pm$ SEM, One-way ANOVA, Tukey's post-hoc test.