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The fatty acid amide hydrolase inhibitor PF-3845 promotes neuronal survival, attenuates inflammation and improves functional recovery in mice with traumatic brain injury

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Abstract

Traumatic brain injury (TBI) is the leading cause of death in young adults in the United States, but there is still no effective agent for treatment. N-arachidonylethanolamine (anandamide, AEA) is a major endocannabinoid in the brain. Its increase after brain injury is believed to be protective. However, the compensatory role of AEA is transient due to its rapid hydrolysis by the fatty acid amide hydrolase (FAAH). Thus, inhibition of FAAH can boost the endogenous levels of AEA and prolong its protective effect. Using a TBI mouse model, we found that post-injury chronic treatment with PF3845, a selective and potent FAAH inhibitor, reversed TBI-induced impairments in fine motor movement, hippocampus dependent working memory and anxiety-like behavior. Treatment with PF3845 inactivated FAAH activity and enhanced the AEA levels in the brain. It reduced neurodegeneration in the dentate gyrus, and up-regulated the expression of Bcl-2 and Hsp70/72 in both cortex and hippocampus. PF3845 also suppressed the increased production of amyloid precursor protein, prevented dendritic loss and restored the levels of synaptophysin in the ipsilateral dentate gyrus. Furthermore, PF3845 suppressed the expression of inducible nitric oxide synthase and cyclooxygenase-2 and enhanced the expression of arginase-1 post-TBI, suggesting a shift of microglia/macrophages from M1 to M2 phenotype. The effects of PF3845 on TBI-induced behavioral deficits and neurodegeneration were mediated by activation of cannabinoid type 1 and 2 receptors and might be attributable to the phosphorylation of ERK1/2 and AKT. These results suggest that selective inhibition of FAAH is likely to be beneficial for TBI treatment.

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All the authors declare no conflict of interest.

Keywords

TBI; FAAH inhibition; AEA; inflammation; neuroprotection

1. Introduction

Traumatic brain injury (TBI) is a worldwide public health problem. Despite the fact that approximately 2% of Americans are currently living with TBI-related disabilities and the cost associated with their care annually valued at \$60.0 billion (Faul et al., 2010; Loane et al., 2009), finding an effective therapeutic strategy for TBI is still an unmet medical need. Current failure to have an FDA approved agent to treat TBI is likely due to the complexity of its pathophysiology, which encompasses several pathological conditions including excitotoxicity, inflammation, oxidative stress and neuronal death (Loane and Faden, 2010). Therefore, an effective treatment for TBI might require an agent that possesses multipotent properties, capable of interacting with multiple pathways to ameliorate TBI-associated pathological changes.

For more than a decade, the endocannabinoid system has emerged as a therapeutic target for several neurological disorders, including TBI (Shohami et al., 2011). Several studies have reported that cannabinoids can alleviate blood-brain barrier dysfunction and brain edema, reduce lesion volume and neuronal death, and improve behavioral performance in rodent models of TBI (Panikashvili et al., 2006; Panikashvili et al., 2001). The protective effects are attributable to their anti-oxidative, anti-inflammatory and anti-excitotoxic properties and are likely mediated by activation of cannabinoid type 1 and type 2 receptors (CB1R and CB2R), which are expressed by neurons and inflammatory cells (Cohen-Yeshurun et al., 2011; van der Stelt et al., 2001). However, the use of cannabinoids as therapeutic agents is limited by the potential psychotropic side effects caused by CB1 receptor activation. Endocannabinoids are endogenous ligands that activate cannabinoid receptors and are produced in a site- and event- specific manner. This “on demand” synthesis enables activation of cannabinoid receptors locally and therefore can avoid the undesirable side effects elicited by the non-selective, global CB1 receptor activation in neurons (Hwang et al., 2010; Naidoo et al., 2012).

N-arachidonylethanolamine (AEA) and 2-arachidonoylglycerol (2-AG) are two major endocannabinoids in the brain. AEA is mainly hydrolyzed by the fatty acid amide hydrolase (FAAH), whereas 2-AG is degraded primarily by monoacylglycerol lipase (MAGL) and to a lesser extent by alpha, beta-hydrolase domain 6 (ABHD6) (Khan et al., 2009; Savinainen et al., 2012). AEA and 2-AG are reportedly increased after brain injury and believed to be protective (Shohami et al., 2011). However, the compensatory protective effect of AEA and 2-AG is limited due to their rapid re-uptake and degradation by respective hydrolytic enzymes. Therefore, inhibiting the degradation of endocannabinoids could sustain their brain levels and extend their therapeutic efficacy (Savinainen et al., 2012). Indeed, inhibition of MAGL provided neuroprotection and improved cognitive function in a transgenic mouse model of Alzheimer’s disease (Chen et al., 2012). This is also consistent with our recent findings that selective inhibition of ABHD6, which causes a moderate increase of 2-AG in

neurons (Marrs et al., 2010), protects against TBI via activation of both CB1R and CB2R (Tchantchou and Zhang, 2013). Several studies have shown that boosting endogenous levels of AEA using FAAH inhibitors, such as OL-135 or URB597, is effective in various animal models of acute and chronic pain, cholestasis, gastrointestinal inflammation and anxiety (Ahn et al., 2008; Kinsey et al., 2009; Sagar et al., 2008). Although these compounds selectively increase endogenous levels of AEA, their short half-lives *in vivo* and inhibitory action on several carboxylesterases in the liver (Lichtman et al., 2004; Zhang et al., 2007) make them unsuitable for clinical application. Recently, a novel FAAH inhibitor, PF-3845, has been developed and shown to have higher selectivity and longer duration of FAAH inhibition; as such this agent is ideal for studying the role of FAAH in various model systems (Ahn et al., 2009; Booker et al., 2012).

In this study, we investigated the therapeutic properties of PF3845 on TBI-induced impairments in behavioral performance, neuroinflammation and neurodegeneration, using a mouse model of TBI. The involvement of CB1R and CB2R and the potential mechanisms of the action of PF3845 were also examined.

2. Materials and Methods

2.1. Reagents

A FAAH inhibitor PF3845, the CB1R antagonist AM 281 and the CB2R antagonist AM 630 were purchased from Tocris Bioscience (Ellisville, MO). All other chemicals and reagents were purchased from Sigma (St. Louis, MO), unless stated otherwise.

2.2. Animals

Eight-week-old, male C57BL/6 mice weighing 25–30 g (Jackson Laboratory, Bar Harbor, ME) were used in this study. Animals were maintained under a controlled environment with a temperature of $23 \pm 2^\circ\text{C}$, a 12 h light/dark cycle and continuous access to food and water *ad libitum*. The study was performed in accordance to the guidelines of the Animal Use and Care Committee of the Uniformed Services University of the Health Sciences. Each experimental group contained 10 to 17 mice for behavioral studies, in which 4 to 7 mice were used for histological and biochemical analyses.

2.3. TBI model

Animals were subjected to TBI by controlled cortical impact (CCI) as described previously (Tchantchou and Zhang, 2013). Briefly, surgical procedures were performed under general anesthesia with isoflurane (2%) while the body temperature was maintained at 37°C using a heating pad coupled to a rectal probe. CCI was performed after craniotomy over the left parietal cortex using a stereotactically positioned 3 mm diameter stainless steel-tipped piston centered at 2 mm posterior and 2.5 mm lateral to bregma (Myer et al., 2006). Impact was induced using an electromagnetically controlled stereotaxic impactor (Leica, Wetzlar, Germany) with piston velocity set at 5 m/s and a depth penetration of 1.5 mm. Sham-operated mice were anesthetized with isoflurane, followed by craniotomy without trauma induction.

2.4. Drug administration and brain tissue collection

The FAAH inhibitor PF3845 (2 mg/kg, 5 mg/kg or 10 mg/kg) dissolved in 1% DMSO in normal saline or an equal volume of 1% DMSO in saline (10 ml/kg) was injected intraperitoneally (i.p.) 30 min after TBI, and then once daily for 3 or 14 days depending on the experimental design (Fig. 1A). To determine the cannabinoid receptor dependency, the CB1 receptor antagonist AM 281 (3 mg/kg) and CB2 receptor antagonist AM 630 (3 mg/kg) were administered to additional groups of animals 30 min prior to PF3845 injection and then once daily for 3 or 14 days (Di Filippo et al., 2004). During the 14-day treatment regimen, animals were subjected to a battery of behavioral tests at different time points (Fig. 1A). Two hours after the last injection on day 3 or 14 post-injury, animals were anesthetized using isoflurane and then transcardially perfused. The brain tissues were embedded and sectioned for histological analysis. In some groups of animals, mice were sacrificed by cervical dislocation, and the brain tissues were collected and used for the following assays: The frontal cortex and hippocampus were dissected from the ipsilateral hemisphere and used for western blot analysis and ELISA; in other groups of animals, the ipsilateral and contralateral hemispheres were separated and used for FAAH activity assay or endocannabinoid quantitation (Fig. 1A).

2.5. Assessment of functional recovery

Several behavioral tests were performed to assess functional recovery: Motor coordination was evaluated using a beam-walk balance test; a spontaneous alternation Y-maze test was used to measure working memory; and TBI-induced anxiety was assessed using Zero maze.

2.5.1. Beam-walk balance test—The beam-walk balance test was performed to assess fine motor movements as previously described (Loane et al., 2009; Tchantchou and Zhang, 2013). The beam-walk apparatus consists of a wooden beam measuring 6 mm in width, 120 cm in length, and suspended 30 cm above a table. The beam-walk balance test was performed at different time points (Fig. 1A), and results interpreted as we previously reported (Tchantchou and Zhang, 2013).

2.5.2. Spontaneous alternation Y-maze test—The spontaneous alternation Y-maze test measures hippocampus-based working memory (Tanaka et al., 2009), and was used to evaluate the effect of PF3845 treatment on TBI-induced deficits. The Y-maze device (Stoeling Co, Wood Dale, IL) has a symmetrical plastic Y shape with arms measuring 25 cm long, 8 cm wide and 15 cm high. The spontaneous alternation Y-maze test was performed on day 10 post-injury (Fig. 1A) and results interpreted as previously described (Tchantchou and Zhang, 2013).

2.5.3. Elevated zero maze test—The elevated zero maze is used to test anxiety-like behavior in rodents. The device (Stoeling Co) is an elevated circular platform measuring 60 cm in diameter with a 5 cm path comprising two open and two closed regions of equal size, opposite to each other. Testing was performed on days 6 and 14 post-TBI (Fig. 1A). At the start of each test session, each mouse was placed on the same open arm facing the center of the maze, and allowed to explore the maze for 5 min. The animal movement was videotaped and the time spent in open zones and the number of zone entries were scored (Lehmann and

Herkenham, 2011). Anxiety behavior was determined by the time spent in the open zone versus the number of entries to the open zone.

2.6. FAAH activity assay

FAAH activity was measured as previously described (Gunduz-Cinar et al., 2013). In brief, ipsilateral and contralateral cerebral hemispheres were collected from experimental mice treated with vehicle or PF3845 (5 mg/kg). Homogenates were prepared in a buffer containing 10 mM Tris-HCL (pH 7.6) and 1mM EDTA, centrifuged (1000 g, 10 min), and the supernatants containing 175 µg of total protein were mixed in silanized glass tubes with [³H]anandamide (American Radiolabeled Chemicals, St Louis, MO; specific activity, 60 mCi/mmol; 2 µM final concentration) and fatty acid free bovine serum albumin (10 mg/ml) to a final volume of 200 µl. Tubes were incubated at 37°C for 10 min and the reactions were stopped by addition of 400 µl chloroform/methanol (1:1) and tube incubation on ice. Samples were vortexed and centrifuged (700 g, 5 min), and 200 µl of the aqueous phase were taken to measure the radioactivity using liquid scintillation counter. The results were expressed in disintegrations per minute (dpm).

2.7. Mass Spectrometry Lipid Analysis

At 3 days after vehicle or drug injection, animals were sacrificed by cervical dislocation and the ipsilateral and contralateral cortices were dissected, flash frozen in liquid nitrogen and stored at -80°C. Lipids from cortical tissue were extracted via homogenization and sonication in 500 µl of methanol containing 1000 pmol AA-d₈, 5 pmol AEA-d₈ and 600 pmol 2-AG-d₈ (all from Cayman Chemical, Ann Arbor, MI). The supernatant was removed and dried under nitrogen. Samples were then resuspended in 100 µl of methanol: water (70:30). Analytes were quantified using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) on a Quantum triple-quadrupole mass spectrometer in positive-ion mode using selected reaction monitoring. Detection of eicosanoids was performed as previously described (Hermanson et al., 2013; Patel et al., 2009). For lipid analysis the mobile phases used were 80 µM AgOAc with 0.1% (v/v) acetic acid in H₂O (solvent A) and 120 µM AgOAc with 0.1% (v/v) acetic acid in MeOH (solvent B). The analytes were eluted using a gradient from 20% A to 99% B over 5 min. The transitions used were *m/z* 434→416 for oleoylethanolamine (OEA), *m/z* 456→438 for AEA, *m/z* 464→446 for AEA-d₈, *m/z* 463→389 for 2-oleoylglycerol (2-OG), *m/z* 485→411 for 2-AG, *m/z* 493→419 for 2-AG-d₈. Peak areas for the analytes were normalized to the appropriate internal standard and then normalized to tissue mass.

2.8. Histology

Histological analysis was performed on frozen brain sections that were stained with hematoxylin and eosin (H&E) for the measurement of the lesion volume and Fluoro-Jade B (FJ-B) to determine the number of degenerating cells. The sections were also immunostained to detect the expression of inflammatory markers or amyloid precursor protein.

2.8.1. Fluoro-Jade B staining—One out of every eight serial sections was stained by FJ-B as previously described (Schmued and Hopkins, 2000; Tchantchou and Zhang, 2013).

Stained sections were dried, mounted with DPX, and the FJ-B positive cells in the dentate gyri of these sections were counted using 20x objective. The number of FJ-B positive cells from these sections was multiplied by 8 to determine the total number of FJ-B positive cells in the whole dentate gyrus.

2.8.2. Hematoxylin and eosin staining—At 14 days after CCI injury, animals were deeply anesthetized and then transcardially perfused with heparin saline followed by 4% formaldehyde. Brains were collected and 30 µm thick sections were stained with H&E and scanned with an Epson scanner. The lesion volume was determined as we previously described (Tchantchou and Zhang, 2013; Yu et al., 2012).

2.8.3. Immunohistochemistry—To assess the expression of microtubule-associated protein 2 (MAP-2), amyloid precursor protein (APP), F4/80 (a marker for microglia/macrophages), cyclooxygenase 2 (COX-2), inducible nitric oxide synthase (iNOS) and arginase-1 (Arg-1), 30 µm thick frozen brain sections were immunostained with respective antibodies. In brief, sections were blocked with 5% normal donkey serum, then incubated overnight at 4°C with a mixture containing a monoclonal mouse anti-MAP-2 antibody (1:500; Chemicon International, Temecula, CA) and a polyclonal rabbit anti-APP antibody (1:250, Cell Signaling Technology, Boston, MA); a mixture containing a monoclonal rat anti-mouse F4/80 antibody (1:200; eBioscience, San Diego, CA) and a polyclonal rabbit anti-COX-2 antibody (1:400; Cayman Chemical, Ann Arbor, MI); a mixture containing a monoclonal rat anti-mouse F4/80 antibody (1:200; eBioscience) and a polyclonal rabbit anti-iNOS antibody (1:300; Millipore, Billerica, MA) or a mixture containing a monoclonal rat anti-mouse F4/80 antibody (1:200; eBioscience) and a polyclonal goat anti-Arg-1 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). Sections were rinsed and incubated for 1 h at room temperature with a Texas Red-conjugated rabbit anti-goat antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) to detect APP, COX-2, iNOS and Arg-1, a FITC-conjugated goat anti-mouse antibody (1:500; Jackson ImmunoResearch Laboratories) to detect MAP-2 or a FITC-conjugated goat anti-rat antibody (1:500; Jackson ImmunoResearch Laboratories) to detect F4/80. The sections were then transferred into a 1 µg/ml DAPI solution for 5 min. After DAPI staining, all sections were mounted, examined under the microscope and the overlap fraction between COX-2, iNOS or Arg-1 immunoreactive cells and F4/80 immunopositive cells was determined using the NIS Element software.

2.9. Western blotting analysis

Western blotting was performed to determine the expression of synaptophysin, PSD-95, Bcl-2, heat shock proteins 70/72 (Hsp70/72), COX-2, COX-1, ERK1/2, phosphorylated ERK1/2 (pERK1/2), AKT, pAKT and beta-actin. The ipsilateral cerebral cortices from the experimental animals were manually homogenized in ice-cold lysis buffer containing 50 mM HEPES, pH 7.5, 6 mM MgCl₂, 1 mM EDTA, 75 mM sucrose, 2.5 mM benzamidine, 1 mM dithiothreitol, 1% Triton X-100 and the protease inhibitor cocktail and centrifuged at 10,000 rpm for 25 min. Proteins (30–40 µg) from each sample supernatant were separated by electrophoresis on 4–12% SDS-polyacrylamide gels (Invitrogen, Carlsbad, CA), then transferred to a nitrocellulose membrane. The membranes were blocked with 5% fat free

milk for 1 h and incubated overnight at 4°C with rabbit polyclonal antibodies against synaptophysin (1:500; Cell signalling Technology, MA), PSD-95 (1:300; Cell signaling), Bcl-2 (1/400), Hsp70/72 (1/200) (both from Santa Cruz), COX-2 (1:500), COX-1 (1:500) (both from Cayman Chemical), pERK1/2 (1:1000), ERK1/2 (1:1000), pAKT (1:1000), AKT (1:1000) (all from Cell Signaling) or a mouse anti- β -actin monoclonal antibody (1:4000; Sigma). The blots were washed in TBS-Tween and further incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1: 5000; Bio-Rad Life Sciences, Hercules, CA). Reactive proteins were visualized by enhanced chemiluminescence according to the manufacturer's protocol (Thermo Scientific, Rockford, IL). Band signal intensity was determined using Image J software (NIH).

2.10. Caspase-3 activity assay

Caspase-3 activity in ipsilateral cortices and hippocampi of TBI mice was examined using a colorimetric kit (BioVision kit; Mountain View, CA). The optical density of the cleaved caspase 3 products at 405 nm wavelength was determined following the manufacturer's instructions and results were interpreted as previously reported (Cohen-Yeshurun et al., 2011).

2.11. Statistical analysis

Statistical analyses were performed using GraphPad InStat 3 software (GraphPad Software, Inc., La Jolla, CA). One way and repeated analysis of variance together with Bonferroni's multiple comparison post-test was used to compare differences among the multiple groups, and Student's t-test was used to compare two groups. Results were quantified and expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

3. Results

3.1. PF3845 restored hippocampus-dependent memory performance post-TBI and the improvement was mainly blocked by CB1 receptor antagonist

The effect of PF3845 on hippocampus-dependent memory was assessed using the spontaneous alternation Y-maze test. Compared to the performance observed in sham-operated animals, TBI significantly impaired the mouse's ability to continuously alternate their entrance into Y-maze arms during exploration. The percent alternations in TBI and sham operated groups were $49 \pm 2\%$ and $69 \pm 2\%$, respectively ($p < 0.001$; Fig. 1B). Although PF3845 treatments with 2 mg/kg and 10 mg/kg did not have any effect on the deficits in CCI-injured mice ($51 \pm 2\%$ and $52 \pm 3\%$ alternations, respectively), treatment with 5 mg/kg completely restored the ability of TBI mice to successfully alternate arms during maze exploration ($71 \pm 2\%$; Fig. 1B). This improvement was completely blocked by the CB1R antagonist AM 281 ($45 \pm 4\%$; $p < 0.001$; Fig. 1C). Co-administration with the CB2R antagonist, AM 630, partially but also significantly reversed the effect of PF3845 on the working memory deficit ($55 \pm 2\%$; $p < 0.05$; Fig. 1C).

3.2. PF3845 attenuated TBI-induced anxiety and the effect was not mediated by cannabinoid receptors

The zero maze test was used to evaluate the effect of PF3845 on TBI-induced anxiety. On day 6 post-injury, across all of the experimental treatments, there were no differences in the time spent in the open zone. However, by day 14 TBI significantly reduced the average amount of time on the open regions (CCI/Veh group, 2.27 ± 0.33) compared to the performance in the sham-operated group (5.35 ± 0.48 ; $p < 0.001$; Fig. 1D), and treatment with 5 mg/kg and 10 mg/kg of PF3845 significantly attenuated anxiogenic behavior. Compared to the vehicle-treated TBI animals, the time spent in the open zone per visit in these treatment groups on day 14 post-injury was 4.56 ± 0.25 ($p < 0.001$) and 4.19 ± 0.39 ($p < 0.05$), respectively (Fig. 1D). However, the effect of PF3845 on TBI-induced anxiety was not affected by the CB1R or CB2R antagonists (Fig. 1E).

3.3. PF3845 reduced TBI-induced deficits in fine motor movement and the improvement was mainly mediated by CB2 receptor activation

To determine the effect of PF3845 on TBI-induced deficits in fine motor movement, the beam-walk balance test was performed and the number of foot faults over a total of 50 steps was recorded. Injury to the left parietal cortex severely altered the animals' ability to move on a fine beam, where the numbers of foot faults in vehicle-treated TBI animals were 49 ± 1 and 31 ± 3 at 7 and 14 days post-injury, respectively. Although 2 mg/kg PF3845 did not improve injury-induced motor deficits, treatment with 5 mg/kg and 10 mg/kg of PF3845 significantly reduced the number of foot faults to 22 ± 4 and 12 ± 4 on day 7, and 13 ± 2 and 4 ± 1 on day 14 after TBI ($p < 0.001$; Fig. 1F). To determine whether the action of PF3845 is mediated by activation of CB1R and CB2R, TBI mice were co-administered CB1 or CB2 receptor antagonist with PF3845. The improvement by PF3845 was completely blocked by co-administration with the CB2R antagonist on days 7 and 14 post-TBI ($p < 0.001$; Fig. 1G), but was only partially reversed by co-treatment with the CB1R antagonist ($p < 0.05$; Fig. 1G).

Because better functional recovery was generally achieved with PF3845 at 5 mg/kg when compared to 2 mg/kg and 10 mg/kg, this concentration was used to determine the effects of PF3845 treatment on FAAH inhibition, the alteration of endocannabinoid levels in the brain, and the histological and molecular changes after TBI.

3.4. PF3845 treatment inhibited FAAH and increased AEA levels in TBI mouse brain

To determine whether PF3845 treatment could effectively inactivate FAAH activity in mouse brain, animals were sacrificed at 3 days post-TBI with/without drug treatment. Compared to the TBI-vehicle group, treatment with PF3845 (5 mg/kg once daily for 3 days) almost completely blocked the FAAH activity as demonstrated by the reduction of [^3H]-AEA hydrolysis. In the ipsilateral and contralateral TBI mouse brain, only $3.54 \pm 1.08\%$ and $3.22 \pm 0.66\%$ of FAAH activity remains in the drug treated groups ($p < 0.001$ compared to the sham and TBI-vehicle groups). Notably, there was no difference in the AEA hydrolytic activity in sham and TBI animals without drug treatment (Fig. 2A). Consistently, PF3845 induced-FAAH inactivation resulted in an eight-fold increase of AEA in the ipsilateral and contralateral cerebral hemispheres of TBI mice compared to the sham and TBI mice without

drug treatment ($p < 0.001$). Moreover, there was a significant increase in AEA levels in the ipsilateral hemispheres of vehicle treated TBI mice compared to the sham-operated animals ($p < 0.05$; Fig. 2B). PF3845 treatment also caused a significant increase of 2-AG levels in the ipsilateral, but not in the contralateral hemispheres of TBI mice brains when compared to the sham and TBI-vehicle groups (1.4-fold increase, $p < 0.001$; Fig. 2C). In addition, PF3845 also significantly increased the endogenous levels of 2-OG and OEA in both ipsilateral and contralateral TBI mouse brain (data not shown).

3.5. PF3845 reduced lesion volume in the cortex and neuronal injury in the dentate gyrus and the effect on neurodegeneration was partially blocked by CB1 and CB2 receptor antagonists

FJ-B staining was used to detect degenerating cells in the dentate gyri of vehicle and PF3845 treated mice and to determine the possible involvement of cannabinoid receptors on day 3 post-injury. TBI caused a dramatic increase in degenerating neuronal cells in the dentate gyri ($2146 \pm 71/\text{DG}$; Fig. 3A, B) and PF3845 treatment significantly reduced the number of degenerating neurons compared to vehicle-treated mice ($688 \pm 40/\text{DG}$; $p < 0.001$; Fig. 3A, B). No FJ-B positive cells were found in the dentate gyri of sham-operated animals or in the contralateral hemispheres of the vehicle treated TBI mice (data not shown). The protective effect of PF3845 on hippocampal neuronal loss was significantly blocked by CB1R and CB2R antagonists. The average number of degenerative cells per dentate gyrus in TBI mouse co-treated with PF3845 and CB1R or CB2R antagonists was 1414 ± 68 and 1203 ± 63 , respectively ($p < 0.001$; Fig. 3A, B). H&E staining was performed to assess the effect of PF3845 on TBI-induced lesion volume. PF3845 treatment at 5 mg/kg significantly reduced the lesion volume from $4.58 \pm 0.27\text{mm}^3$ (in vehicle treated TBI mice brains) to $2.44 \pm 0.33\text{mm}^3$ ($p < 0.01$; Fig. 3C).

3.6. PF3845 suppressed the expression of TBI-induced amyloid precursor protein, dendritic loss, and increased the expression of synaptophysin in the mouse hippocampus

Amyloid precursor protein (APP) is overexpressed in the perikaryon and neurites of hippocampal neurons after TBI (Itoh et al., 2009). This is accompanied by acute hippocampal dendritic loss and synaptic degeneration (Gao et al., 2011). Using histo-immunostaining, we observed that most cells in the APP-positive area had condensed nuclei, suggesting that APP expression is associated with neuronal cell loss (Fig. 4A). The administration of PF3845 significantly reduced APP expression in the dentate gyrus on day 3 post-injury; the average fluorescence signal intensity in vehicle and PF3845 treated TBI mice dentate gyri was 3866 ± 325 and 2661 ± 359 , respectively ($p < 0.05$, Fig. 4B). PF3845 treatment also prevented TBI-induced dendritic damage (Fig. 4A), evaluated by MAP-2 immunostaining, and increased the expression of synaptophysin (a pre-synaptic marker) by 40% on days 3 and 14 post-TBI when compared to vehicle-treated animals (Fig. 4C, D). However, treatment with PF3845 did not reduce the loss of the post-synaptic marker PSD95 on days 3 and 14 post-TBI (Fig. 4C, D). The expression of APP and changes in dendritic density were not observed in the contralateral side of the vehicle administered TBI mouse brain (data not shown).

3.7. PF3845 increased the levels of Bcl-2 and Hsp70/72 in TBI mouse cortex and hippocampus

Since PF3845 reduced the number of degenerating cells in TBI mice (Fig. 3), we sought to examine its effect on the expression of the anti-apoptotic protein, Bcl-2, and the anti-oxidant heat shock proteins (Hsp) 70 and 72. On day 3 post-TBI, treatment with PF3845 increased Bcl-2 expression by 40% and 49% in the ipsilateral cerebral cortex ($p < 0.05$) and the hippocampus ($p < 0.01$), respectively (Fig. 5A, B). PF3845 also increased the levels of Hsp72 and Hsp70 both in the cortex and hippocampus on days 3 and 14 post-TBI. Compared to the levels seen in brain samples from the vehicle-treated TBI mice, Hsp72 levels were up-regulated by 62% ($p < 0.001$) and 34% ($p < 0.05$) in the cortex and by 59% ($p < 0.001$) and 72% ($p < 0.001$) in the hippocampus on days 3 and 14 post-injury. Similarly, the expression of Hsp70 was increased in the cortex by 68% ($p < 0.001$) and 30% ($p < 0.05$), and in the hippocampus by 57% ($p < 0.01$) and 62% ($p < 0.01$) on days 3 and 14 post-injury (Fig. 5A, C). The effect of PF3845 on the activity of a pro-apoptotic protein, caspase-3 was also evaluated. Caspase-3 activity was significantly increased both in the ipsilateral cortices and hippocampi of TBI mice compared to the sham-operated mice ($p < 0.05$; Fig. 5D). PF3845 appeared to reduce the caspase-3 activity in the cortex and hippocampus, but statistical significance was not reached (Fig. 5D).

3.8. PF3845 suppressed COX-2 expression in TBI mouse cortex and hippocampus

COX-2 is an inflammatory molecule that is significantly induced after TBI (Dash et al., 2000). We found that COX-2 expression was increased in the cortex and hippocampus. Compared to the sham operated animals, COX-2 levels increased in the ipsilateral cortical tissues by 89% ($p < 0.001$) on day 3 post-injury (Fig. 6A, B), and returned to baseline levels on day 14 (data not shown). There were no differences in the expression levels of COX-1 in sham, vehicle and PF3845 treated groups (Fig. 6A). In the ipsilateral hippocampus, histo-immunostaining showed that an average of 25% of the area fraction was COX-2 positive and was mainly expressed in microglia cells (Fig. 6C, D). There was no COX-2 expression in the hippocampus of the sham operated mouse (data not shown). PF3845 suppressed COX-2 expression by 68% in the cortices and by 80% in the hippocampi of TBI mice ($p < 0.001$, Fig. 6A-D).

3.9. PF3845 reduced iNOS expression and increased the expression of Arg-1 in microglia of the TBI mouse brain

Inducible nitric oxide synthase (iNOS) is expressed in microglia and macrophages under inflammatory conditions, including TBI (Wada et al., 1998). We found a dramatic induction of iNOS in microglia/macrophages in the ipsilateral cortices of vehicle-treated mice on days 3 and 14 post-injury. The fraction of microglia/macrophages expressing iNOS was $74 \pm 6\%$ and $64 \pm 3\%$ on days 3 and 14 post-injury, and was reduced to $29 \pm 6\%$ and $21 \pm 4\%$ by PF3845 treatment ($p < 0.001$; Fig. 7A, B). No iNOS expression was observed in the cortices of sham control and vehicle treated TBI animals (data not shown). However, the density of microglia/macrophages in TBI mouse brain was not affected by PF3845 treatment (458.5 ± 47.3 cells/mm² and 477 ± 60.5 cells/mm² on day 3 post-TBI with/without PF3845 treatment; 424.5 ± 37.8 cells/mm² and 453 ± 50.7 cells/mm² on day 14 with/without PF3845

treatment). We tested the hypothesis that PF3845 treatment may cause a shift of microglia phenotype from pro-inflammatory to anti-inflammatory by examining the effect of PF3845 on the expression of Arg-1, a marker of alternatively activated microglia. The average fraction of F4/80-positive cells expressing Arg-1 in vehicle-treated TBI mice ipsilateral cortices was $24 \pm 5\%$ and $15 \pm 3\%$ on days 3 and 14 post-injury, respectively; and PF3845 significantly increased that fraction to $77 \pm 4\%$ and $66 \pm 6\%$ ($p < 0.001$, Fig. 8A, B). These results indicate that PF3845 caused a shift of microglia/macrophages from pro-inflammatory to anti-inflammatory phenotypes.

3.10. PF3845 increased ERK1/2 and AKT phosphorylation in TBI mouse brain

Since treatment with PF3845 improved functional recovery and attenuated neurodegeneration through CB1R and CB2R (Figs. 1 and 3), and knowing that activation of these receptors results in the enhanced expression of extracellular signal regulated kinase 1/2 (ERK1/2) and serine/threonine protein kinase AKT, which are critical for cell survival (Galve-Roperh et al., 2008; Tchantchou and Zhang, 2013), we sought to examine the effect of PF3845 treatment on ERK and AKT phosphorylation in the cortex and hippocampus using western blot analysis. Although there were no differences between sham-operated and vehicle-treated TBI animals, PF3845 significantly increased ERK1/2 and AKT phosphorylation in ipsilateral cortex on day 14 post-TBI (Fig. 9A-C). Compared to the vehicle-treated TBI mice on days 3 and 14 post-injury, PF3845 treatment increased ERK1/2 phosphorylation by 90% ($p < 0.001$) and 68% ($p < 0.01$) (Fig. 9A, B), and the AKT phosphorylation by more than 2-fold ($p < 0.05$, Fig. 9A, C). Similarly, PF3845 treatment increased ERK1/2 phosphorylation by 41% ($p < 0.01$) and 26% ($p < 0.05$) in the ipsilateral hippocampus on day 14 post-TBI when compared to the untreated animals at 3 and 14 days post-injury (Fig. 9D, E). AKT phosphorylation decreased significantly in the hippocampi of TBI mice on day 3 post-injury when compared to the sham-operated animals ($57.9 \pm 9\%$ versus $100 \pm 1.14\%$, $p < 0.01$) and phosphorylation levels were restored by PF3845 treatment ($102.01 \pm 5.23\%$, $p < 0.001$; Fig. 9D, F). Notably, AKT phosphorylation in the hippocampus was equal to control levels on day 14 post-injury and was not affected by PF3845 treatment at this later time point (Fig. 9D, F).

4. Discussion

This is the first study to report on the beneficial effects of FAAH inhibition in an animal model of TBI. Our results present clear evidence that selective inhibition of FAAH with PF3845 produced a broad-spectrum therapeutic effect in mice with TBI. Post-injury treatment with PF3845 improved the functional recovery of fine motor movement, hippocampus-dependent memory performance and anxiety-like behavior. PF3845 treatment effectively inactivated FAAH and resulted in enhanced AEA levels in TBI mouse brain, which subsequently reduced cortical lesion volume and neurodegeneration in the dentate gyrus, inhibited APP expression, and increased the levels of the anti-apoptotic protein Bcl-2 and the anti-oxidant proteins Hsp70 and Hsp72. PF3845 treatment also prevented dendritic damage and attenuated TBI-induced decreases in synaptophysin expression, it suppressed COX-2 and iNOS induction, and increased the expression of Arg-1. Furthermore, we

demonstrated that the beneficial effects of PF3845 are at least in part mediated by CB1R and CB2R and the enhanced phosphorylation of cell survival kinases ERK1/2 and AKT.

The CCI method used to induce moderate TBI in this study produced several behavioural deficits, including impairments in fine motor coordination, working memory and anxiety. PF3845 at 5 mg/kg appeared to be the most suitable dose for behavioral improvement when compared to doses at 2 mg/kg and 10 mg/kg. At this concentration, PF3845 most effectively reversed TBI-induced deficits in memory performance and anxiety-like behavior and it produced the same effect as 10 mg/kg in improving motor function. These results are consistent with a recent report showing that PF3845 reduces inflammatory pain in a dose-dependent manner with a minimum effective dose of 3 mg/kg (i.p.), albeit the fact that PF3845 treatment at 1 mg/kg resulted in a seven-fold increase of AEA levels in the brain, compared to the 10-fold increase with administration of PF3845 at 10 mg/kg. These results suggest that complete blockade of FAAH might be required to see its maximum therapeutic effect (Ahn et al., 2009). Consistent with this report, we found that PF3845 treatment at 5 mg/kg almost completely inhibited FAAH activity, and resulted in at least an 8-fold increase of AEA levels in the brain. However, FAAH inhibition also elevated levels of several non-cannabinoid fatty acid amides, such as OEA and N-palmitoyl ethanolamine (PEA), to activate non-cannabinoid receptors, such as TRPV1 and PPAR α (Ahn et al., 2009; Panlilio et al., 2013). Indeed, we found that the effects of PF3845 on fine motor movement and working memory performance were mediated primarily by activation of CB2 and CB1 receptors, respectively, but the anxiolytic effect of PF3845 seemed to be caused by cannabinoid receptor independent mechanisms.

Several studies have demonstrated that FAAH inhibition produces memory enhancing effects in rodents subjected to aversive tasks such as escaping from deep water or avoiding a foot shock-associated area (Mazzola et al., 2009; Panlilio et al., 2013). For example, FAAH inhibition with URB597 was shown to enhance memory acquisition through CB1 receptor and PPAR α (Mazzola et al., 2009). Moreover, targeted gene deletion or pharmacological inactivation of FAAH with the reversible inhibitor, OL-135, accelerated acquisition and extinction rates in a spatial memory task in a CB1 receptor dependent manner (Varvel et al., 2007). FAAH inhibition with AM3506 was also found to facilitate extinction learning via CB1 receptor activation (Gunduz-Cinar et al., 2013). In contrast to our observations and those of other investigators showing the beneficial effects of FAAH inhibition on memory performance in animals subjected to aversive conditions and the animal models of diseases, a few studies reported that boosting AEA brain levels in rodents subjected to reward-motivated tasks, such as appetitively motivated tasks, did not have memory enhancing effects (Wise et al., 2009); or could even be disruptive to memory acquisition (Goonawardena et al., 2011). This bimodal effect of FAAH inhibition on memory performance suggests that boosting AEA levels in the brain may have memory enhancing properties in animal models involved in aversive situations due to a modulatory response to stressful conditions (Panlilio et al., 2013), and could as well support the role of PF3845 on TBI-induced memory impairment. In addition to increasing the brain levels of AEA and OEA, treatment with PF3845 also caused a 2-fold increase of 2-OG levels in both ipsilateral and contralateral TBI mouse brain. Interestingly, a significant increase of 2-AG was also found in the ipsilateral TBI mouse brain, but not in the contralateral side. One possible

explanation for this difference is that the COX-2 reduction by PF4845 treatment in the ipsilateral TBI mouse brain leads to accumulation of free arachidonic acid which may be directed towards the production of 2-AG (Bisogno et al., 1997; Gopez et al., 2005). Another possible explanation is that inhibition of COX-2 can prevent 2-AG oxygenation and therefore results in an accumulation of 2-AG (Duggan et al., 2011).

There is mounting evidence suggesting that TBI associated dysfunctions in brain regions such as the prefrontal cortex, hippocampus and amygdala lead to a greater incidence of anxiety-related disorders (Meyer et al., 2012; Moore et al., 2006). We found that PF3845 attenuated TBI-induced anxiety-like behavior in mice. This observation is in agreement with previous findings indicating that both genetic deletion and pharmacological inhibition of FAAH with URB597 produced anxiolytic properties in mice involving CB1 receptor activation (Busquets-Garcia et al., 2011; Moreira et al., 2008). However, different from those reports, our findings suggest that non-cannabinoid receptors, such as TRPV-1 and PPAR α , might be involved in the anxiolytic effect of PF3845 on TBI (Ahn et al., 2009; Panlilio et al., 2013).

Inflammatory pain in arthritic models is generally associated with deficits in motor function (Amdekar et al., 2012). We demonstrated that PF3845 reduced TBI-induced impairment in fine motor movement, an improvement that was completely blocked by a CB2 receptor antagonist, and partially by CB1 receptor antagonist. This effect might be attributed to its demonstrated anti-nociceptive and anti-inflammatory properties (Ahn et al., 2009). Consistent with our findings, PF3845 administration in a rodent CFA model of inflammatory pain produced significant and sustained analgesic effects mediated by CB1 and CB2 receptors (Ahn et al., 2009). Moreover, genetic deletion of FAAH or its pharmacological inhibition with PF3845 both alleviates LPS-induced pain response in mice (Booker et al., 2012). Furthermore, administration of a single dose of the AEA analog, N-arachidonoyl-L-serine, improved several head injury-induced neurological disorders, including fine motor movement over 14 days post-TBI (Cohen-Yeshurun et al., 2011).

TBI induces significant hippocampal neuronal loss (Tchantchou and Zhang, 2013; Yu et al., 2012), which is associated with an overexpression of APP in neuronal perikaryon (Itoh et al., 2009), as well as dendritic damage and synaptic degeneration (Gao et al., 2011). Considering that the hippocampus is the epicenter for cognitive and memory functions, injury to the hippocampus could contribute to TBI-induced memory impairment. Our findings showed that PF3845 significantly reduced hippocampal neuronal loss, suppressed APP expression, prevented dendritic damage and attenuated the TBI-induced decrease in synaptophysin expression. These effects may contribute, at least in part, to the therapeutic actions of PF3845 on TBI-induced deficits in working memory. Furthermore, PF3845 increased the expression of the anti-apoptotic protein, Bcl-2, and the anti-oxidant proteins, Hsp70/72. Increased expression of these proteins was found to provide neuroprotection after brain injury (Oddi et al., 2012) and to prevent surgery-induced memory decline (Vizcaychipi et al., 2011). Consistent with those findings, a recent report demonstrated that AEA enhances the expression of Hsp72, which contributes to the protection against ischemic injury (Li et al., 2013).

TBI-induced neuronal cell death is often accompanied by microglial activation and the release of pro-inflammatory mediators (Lenzlinger et al., 2001). The pro-inflammatory mediators, such as COX-2 and iNOS, are inducible and markedly up-regulated after TBI and are believed to play an important role in the secondary process of injury (Khan et al., 2009). We found that PF3845 suppressed the expression of COX-2 and iNOS in brain homogenates and inflammatory cells, consistent with the reports that inhibition of COX-2 and iNOS may improve the functional outcome in rodent models of TBI (Khan et al., 2009; Thau-Zuchman et al., 2012). Despite a significant reduction of iNOS expression in the TBI mouse brain after PF3845 treatment, the density of microglia cells was unaffected. On the other hand, the expression of Arg-1 was dramatically increased in microglia. These results suggest that PF3845 treatment induced a switch of microglia from a pro-inflammatory phenotype (M1) to an anti-inflammatory phenotype (M2) (Loane and Byrnes, 2010). It has been reported that Arg-1 is up-regulated in M2 phenotypic microglia/macrophages and is also implicated in the repair process following injury (Rojo et al., 2010). Recently, we and others have also shown that inhibiting the hydrolysis of the endocannabinoid 2-AG, and the administration of exogenous 2-AG can shift microglia/macrophages phenotype from M1 to M2 in TBI (Tchantchou and Zhang, 2013) and in experimental autoimmune encephalomyelitis (EAE) mouse models (Lourbopoulos et al., 2011).

Phosphorylation of ERK1/2 and PI3K/AKT is a common signaling event that occurs downstream of cannabinoid receptor activation (Galve-Roperh et al., 2008), and is involved in the control of inflammatory response and neuronal survival (Molina-Holgado et al., 2007). In this study, we found that the effects of PF3845 on working memory, motor function and neuronal degeneration were mediated by CB1R and CB2R and were associated with the activation of ERK1/2 and AKT in TBI mouse brain. Similarly, administration of N-arachidonoyl-L-serine to mice subjected to closed head injury reduced the lesion volume, reversed the reduction of ERK1/2 at 2 hours, and led to an increase in AKT phosphorylation in cortical tissues at 2 and 4 hours after brain injury (Cohen-Yeshurun et al., 2011).

This study indicates that the therapeutic effects of PF3845 are mediated by activation of CB1R and CB2R, although activation of non-cannabinoid receptors such as TRPV-1 and PPAR α might be also involved. Importantly, the broad spectrum therapeutic effects of PF3845 on several TBI associated pathological markers suggest that selective inhibition of FAAH may be a useful strategy for treatment.

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References

- Ahn K, Johnson DS, Mileni M, Beidler D, Long JZ, McKinney MK, Weerapana E, Sadagopan N, Liimatta M, Smith SE, Lazerwith S, Stiff C, Kamtekar S, Bhattacharya K, Zhang Y, Swaney S, Van Becelaere K, Stevens RC, Cravatt BF. Discovery and characterization of a highly selective FAAH inhibitor that reduces inflammatory pain. *Chem Biol.* 2009; 16:411–420. [PubMed: 19389627]
- Ahn K, McKinney MK, Cravatt BF. Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. *Chem Rev.* 2008; 108:1687–1707. [PubMed: 18429637]
- Amdekar S, Roy P, Singh V, Kumar A, Singh R, Sharma P. Anti-inflammatory activity of lactobacillus on carrageenan-induced paw edema in male wistar rats. *Int J Inflamm.* 2012; 2012:752015. [PubMed: 22518342]
- Bisogno T, Sepe N, De Petrocellis L, Di Marzo V. Biosynthesis of 2-arachidonoyl-glycerol, a novel cannabimimetic eicosanoid, in mouse neuroblastoma cells. *Adv Exp Med Biol.* 1997; 433:201–204. [PubMed: 9561135]
- Booker L, Kinsey SG, Abdullah RA, Blankman JL, Long JZ, Ezzili C, Boger DL, Cravatt BF, Lichtman AH. The fatty acid amide hydrolase (FAAH) inhibitor PF-3845 acts in the nervous system to reverse LPS-induced tactile allodynia in mice. *Br J Pharmacol.* 2012; 165:2485–2496. [PubMed: 21506952]
- Busquets-Garcia A, Puighermanal E, Pastor A, de la Torre R, Maldonado R, Ozaita A. Differential role of anandamide and 2-arachidonoylglycerol in memory and anxiety-like responses. *Biol Psychiatry.* 2011; 70:479–486. [PubMed: 21684528]
- Chen R, Zhang J, Wu Y, Wang D, Feng G, Tang YP, Teng Z, Chen C. Monoacylglycerol lipase is a therapeutic target for Alzheimer's disease. *Cell Rep.* 2012; 2:1329–1339. [PubMed: 23122958]
- Cohen-Yeshurun A, Trembovler V, Alexandrovich A, Ryberg E, Greasley PJ, Mechoulam R, Shohami E, Leker RR. N-arachidonoyl-L-serine is neuroprotective after traumatic brain injury by reducing apoptosis. *J Cereb Blood Flow Metab.* 2011; 31:1768–1777. [PubMed: 21505478]
- Dash PK, Mach SA, Moore AN. Regional expression and role of cyclooxygenase-2 following experimental traumatic brain injury. *J Neurotrauma.* 2000; 17:69–81. [PubMed: 10674759]
- Di Filippo C, Rossi F, Rossi S, D'Amico M. Cannabinoid CB2 receptor activation reduces mouse myocardial ischemia-reperfusion injury: involvement of cytokine/chemokines and PMN. *J Leukoc Biol.* 2004; 75:453–459. [PubMed: 14657208]
- Duggan KC, Hermanson DJ, Musee J, Prusakiewicz JJ, Scheib JL, Carter BD, Banerjee S, Oates JA, Marnett LJ. (R)-Profens are substrate-selective inhibitors of endocannabinoid oxygenation by COX-2. *Nat Chem Biol.* 2011; 7:803–809. [PubMed: 22053353]
- Faul, M.; Xu, L.; Wald, M.; Coronado, V. Traumatic brain injury in the United States: Emergency department visits, hospitalizations and deaths 2002–2006. Atlanta, GA: Centers for Disease Control and Prevention, National Center for Injury Prevention and Control; 2010.
- Galve-Roperh I, Aguado T, Palazuelos J, Guzman M. Mechanisms of control of neuron survival by the endocannabinoid system. *Curr Pharm Des.* 2008; 14:2279–2288. [PubMed: 18781978]
- Gao X, Deng P, Xu ZC, Chen J. Moderate traumatic brain injury causes acute dendritic and synaptic degeneration in the hippocampal dentate gyrus. *PLoS One.* 2011; 6:e24566. [PubMed: 21931758]
- Goonawardena AV, Sesay J, Sexton CA, Riedel G, Hampson RE. Pharmacological elevation of anandamide impairs short-term memory by altering the neurophysiology in the hippocampus. *Neuropharmacology.* 2011; 61:1016–1025. [PubMed: 21767554]
- Gopez JJ, Yue H, Vasudevan R, Malik AS, Fogelsanger LN, Lewis S, Panikashvili D, Shohami E, Jansen SA, Narayan RK, Strauss KI. Cyclooxygenase-2-specific inhibitor improves functional outcomes, provides neuroprotection, and reduces inflammation in a rat model of traumatic brain injury. *Neurosurgery.* 2005; 56:590–604. [PubMed: 15730585]
- Gunduz-Cinar O, MacPherson KP, Cinar R, Gamble-George J, Sugden K, Williams B, Godlewski G, Ramikie TS, Gorka AX, Alapafuja SO, Nikas SP, Makriyannis A, Poulton R, Patel S, Hariri AR, Caspi A, Moffitt TE, Kunos G, Holmes A. Convergent translational evidence of a role for anandamide in amygdala-mediated fear extinction, threat processing and stress-reactivity. *Mol Psychiatry.* 2013; 18:813–823. [PubMed: 22688188]

- Hermanson DJ, Hartley ND, Gamble-George J, Brown N, Shonesy BC, Kingsley PJ, Colbran RJ, Reese J, Marnett LJ, Patel S. Substrate-selective COX-2 inhibition decreases anxiety via endocannabinoid activation. *Nat Neurosci.* 2013; 16:1291–1298. [PubMed: 23912944]
- Hwang J, Adamson C, Butler D, Janero DR, Makriyannis A, Bahr BA. Enhancement of endocannabinoid signaling by fatty acid amide hydrolase inhibition: a neuroprotective therapeutic modality. *Life Sci.* 2010; 86:615–623. [PubMed: 19527737]
- Itoh T, Satou T, Nishida S, Tsubaki M, Hashimoto S, Ito H. Expression of amyloid precursor protein after rat traumatic brain injury. *Neurol Res.* 2009; 31:103–109. [PubMed: 19228461]
- Khan M, Im YB, Shunmugavel A, Gilg AG, Dhindsa RK, Singh AK, Singh I. Administration of S-nitrosoglutathione after traumatic brain injury protects the neurovascular unit and reduces secondary injury in a rat model of controlled cortical impact. *J Neuroinflammation.* 2009; 6:32. [PubMed: 19889224]
- Kinsey SG, Long JZ, O'Neal ST, Abdullah RA, Poklis JL, Boger DL, Cravatt BF, Lichtman AH. Blockade of endocannabinoid-degrading enzymes attenuates neuropathic pain. *J Pharmacol Exp Ther.* 2009; 330:902–910. [PubMed: 19502530]
- Lehmann ML, Herkenham M. Environmental enrichment confers stress resiliency to social defeat through an infralimbic cortex-dependent neuroanatomical pathway. *J Neurosci.* 2011; 31:6159–6173. [PubMed: 21508240]
- Lenzlinger PM, Morganti-Kossmann MC, Laurer HL, McIntosh TK. The duality of the inflammatory response to traumatic brain injury. *Mol Neurobiol.* 2001; 24:169–181. [PubMed: 11831551]
- Li Q, Shi M, Li B. Anandamide enhances expression of heat shock protein 72 to protect against ischemia-reperfusion injury in rat heart. *J Physiol Sci.* 2013; 63:47–53. [PubMed: 23007622]
- Lichtman AH, Leung D, Shelton CC, Saghatelian A, Hardouin C, Boger DL, Cravatt BF. Reversible inhibitors of fatty acid amide hydrolase that promote analgesia: evidence for an unprecedented combination of potency and selectivity. *J Pharmacol Exp Ther.* 2004; 311:441–448. [PubMed: 15229230]
- Loane DJ, Byrnes KR. Role of microglia in neurotrauma. *Neurotherapeutics.* 2010; 7:366–377. [PubMed: 20880501]
- Loane DJ, Faden AI. Neuroprotection for traumatic brain injury: translational challenges and emerging therapeutic strategies. *Trends Pharmacol Sci.* 2010; 31:596–604. [PubMed: 21035878]
- Loane DJ, Pocivavsek A, Moussa CE, Thompson R, Matsuoka Y, Faden AI, Rebeck GW, Burns MP. Amyloid precursor protein secretases as therapeutic targets for traumatic brain injury. *Nat Med.* 2009; 15:377–379. [PubMed: 19287391]
- Lourbopoulos A, Grigoriadis N, Lagoudaki R, Touloumi O, Polyzoidou E, Mavromatis I, Tascos N, Breuer A, Ovadia H, Karussis D, Shohami E, Mechoulam R, Simeonidou C. Administration of 2-arachidonoylglycerol ameliorates both acute and chronic experimental autoimmune encephalomyelitis. *Brain Res.* 2011; 1390:126–141. [PubMed: 21406188]
- Marrs WR, Blankman JL, Horne EA, Thomazeau A, Lin YH, Coy J, Bodor AL, Muccioli GG, Hu SS, Woodruff G, Fung S, Lafourcade M, Alexander JP, Long JZ, Li W, Xu C, Moller T, Mackie K, Manzoni OJ, Cravatt BF, Stella N. The serine hydrolase ABHD6 controls the accumulation and efficacy of 2-AG at cannabinoid receptors. *Nat Neurosci.* 2010; 13:951–957. [PubMed: 20657592]
- Mazzola C, Medalie J, Scherma M, Panlilio LV, Solinas M, Tanda G, Drago F, Cadet JL, Goldberg SR, Yasar S. Fatty acid amide hydrolase (FAAH) inhibition enhances memory acquisition through activation of PPAR- α nuclear receptors. *Learn Mem.* 2009; 16:332–337. [PubMed: 19403796]
- Meyer DL, Davies DR, Barr JL, Manzerra P, Forster GL. Mild traumatic brain injury in the rat alters neuronal number in the limbic system and increases conditioned fear and anxiety-like behaviors. *Exp Neurol.* 2012; 235:574–587. [PubMed: 22498103]
- Molina-Holgado F, Rubio-Araiz A, Garcia-Ovejero D, Williams RJ, Moore JD, Arevalo-Martin A, Gomez-Torres O, Molina-Holgado E. CB2 cannabinoid receptors promote mouse neural stem cell proliferation. *Eur J Neurosci.* 2007; 25:629–634. [PubMed: 17328768]
- Moore EL, Terryberry-Spohr L, Hope DA. Mild traumatic brain injury and anxiety sequelae: a review of the literature. *Brain Inj.* 2006; 20:117–132. [PubMed: 16421060]
- Moreira FA, Kaiser N, Monory K, Lutz B. Reduced anxiety-like behaviour induced by genetic and pharmacological inhibition of the endocannabinoid-degrading enzyme fatty acid amide hydrolase

(FAAH) is mediated by CB1 receptors. *Neuropharmacology*. 2008; 54:141–150. [PubMed: 17709120]

- Myer DJ, Gurkoff GG, Lee SM, Hovda DA, Sofroniew MV. Essential protective roles of reactive astrocytes in traumatic brain injury. *Brain*. 2006; 129:2761–2772. [PubMed: 16825202]
- Naidoo V, Karanian DA, Vadivel SK, Locklear JR, Wood JT, Nasr M, Quizon PM, Graves EE, Shukla V, Makriyannis A, Bahr BA. Equipotent inhibition of fatty acid amide hydrolase and monoacylglycerol lipase - dual targets of the endocannabinoid system to protect against seizure pathology. *Neurotherapeutics*. 2012; 9:801–813. [PubMed: 22270809]
- Oddi S, Latini L, Viscomi MT, Bisicchia E, Molinari M, Maccarrone M. Distinct regulation of nNOS and iNOS by CB2 receptor in remote delayed neurodegeneration. *J Mol Med (Berl)*. 2012; 90:371–387. [PubMed: 22198001]
- Panikashvili D, Shein NA, Mechoulam R, Trembovler V, Kohen R, Alexandrovich A, Shohami E. The endocannabinoid 2-AG protects the blood-brain barrier after closed head injury and inhibits mRNA expression of proinflammatory cytokines. *Neurobiol Dis*. 2006; 22:257–264. [PubMed: 16364651]
- Panikashvili D, Simeonidou C, Ben-Shabat S, Hanus L, Breuer A, Mechoulam R, Shohami E. An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature*. 2001; 413:527–531. [PubMed: 11586361]
- Panlilio LV, Justinova Z, Goldberg SR. Inhibition of FAAH and activation of PPAR: new approaches to the treatment of cognitive dysfunction and drug addiction. *Pharmacol Ther*. 2013; 138:84–102. [PubMed: 23333350]
- Patel S, Kingsley PJ, Mackie K, Marnett LJ, Winder DG. Repeated homotypic stress elevates 2-arachidonoylglycerol levels and enhances short-term endocannabinoid signaling at inhibitory synapses in basolateral amygdala. *Neuropsychopharmacology*. 2009; 34:2699–2709. [PubMed: 19675536]
- Rojo AI, Innamorato NG, Martin-Moreno AM, De Ceballos ML, Yamamoto M, Cuadrado A. Nrf2 regulates microglial dynamics and neuroinflammation in experimental Parkinson's disease. *Glia*. 2010; 58:588–598. [PubMed: 19908287]
- Sagar DR, Kendall DA, Chapman V. Inhibition of fatty acid amide hydrolase produces PPAR- α -mediated analgesia in a rat model of inflammatory pain. *Br J Pharmacol*. 2008; 155:1297–1306. [PubMed: 18724387]
- Savinainen JR, Saario SM, Laitinen JT. The serine hydrolases MAGL, ABHD6 and ABHD12 as guardians of 2-arachidonoylglycerol signalling through cannabinoid receptors. *Acta Physiol (Oxf)*. 2012; 204:267–276. [PubMed: 21418147]
- Schmued LC, Hopkins KJ. Fluoro-Jade B: a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res*. 2000; 874:123–130. [PubMed: 10960596]
- Shohami E, Cohen-Yeshurun A, Magid L, Algali M, Mechoulam R. Endocannabinoids and traumatic brain injury. *Br J Pharmacol*. 2011; 163:1402–1410. [PubMed: 21418185]
- Tanaka K, Yagi T, Shimakoshi R, Azuma K, Nanba T, Ogo H, Tamura A, Asanuma M. Effects of galantamine on L-NAME-induced behavioral impairment in Y-maze task in mice. *Neurosci Lett*. 2009; 462:235–238. [PubMed: 19615429]
- Tchantchou F, Zhang Y. Selective inhibition of α/β -hydrolase domain 6 attenuates neurodegeneration, alleviates blood brain barrier breakdown, and improves functional recovery in a mouse model of traumatic brain injury. *J Neurotrauma*. 2013; 30:565–579. [PubMed: 23151067]
- Thau-Zuchman O, Shohami E, Alexandrovich AG, Trembovler V, Leker RR. The anti-inflammatory drug carprofen improves long-term outcome and induces gliogenesis after traumatic brain injury. *J Neurotrauma*. 2012; 29:375–384. [PubMed: 21561314]
- van der Stelt M, Veldhuis WB, van Haften GW, Fezza F, Bisogno T, Bar PR, Veldink GA, Vliegthart JF, Di Marzo V, Nicolay K. Exogenous anandamide protects rat brain against acute neuronal injury in vivo. *J Neurosci*. 2001; 21:8765–8771. [PubMed: 11698588]
- Varvel SA, Wise LE, Niyuhire F, Cravatt BF, Lichtman AH. Inhibition of fatty-acid amide hydrolase accelerates acquisition and extinction rates in a spatial memory task. *Neuropsychopharmacology*. 2007; 32:1032–1041. [PubMed: 17047668]

- Vizcaychipi MP, Xu L, Barreto GE, Ma D, Maze M, Giffard RG. Heat shock protein 72 overexpression prevents early postoperative memory decline after orthopedic surgery under general anesthesia in mice. *Anesthesiology*. 2011; 114:891–900. [PubMed: 21317632]
- Wada K, Chatzipanteli K, Kraydieh S, Busto R, Dietrich WD. Inducible nitric oxide synthase expression after traumatic brain injury and neuroprotection with aminoguanidine treatment in rats. *Neurosurgery*. 1998; 43:1427–1436. [PubMed: 9848857]
- Wise LE, Harloe JP, Lichtman AH. Fatty acid amide hydrolase (FAAH) knockout mice exhibit enhanced acquisition of an aversive, but not of an appetitive, Barnes maze task. *Neurobiol Learn Mem*. 2009; 92:597–601. [PubMed: 19524055]
- Yu F, Wang Z, Tchantchou F, Chiu CT, Zhang Y, Chuang DM. Lithium ameliorates neurodegeneration, suppresses neuroinflammation, and improves behavioural performance in a mouse model of traumatic brain injury. *J Neurotrauma*. 2012; 29:362–374. [PubMed: 21895523]
- Zhang D, Saraf A, Kolasa T, Bhatia P, Zheng GZ, Patel M, Lannoye GS, Richardson P, Stewart A, Rogers JC, Brioni JD, Surowy CS. Fatty acid amide hydrolase inhibitors display broad selectivity and inhibit multiple carboxylesterases as off-targets. *Neuropharmacology*. 2007; 52:1095–1105. [PubMed: 17217969]

Highlights

- PF3845 improves motor function, working memory and anxiety behavior after TBI.
- PF3845 suppresses neuroinflammation and shifts microglia phenotype from M1 to M2.
- PF3845 attenuates neurodegeneration and reduces lesion volume.
- The action of PF3845 is mediated by CB1/CB2 receptor activation.
- The therapeutic mechanisms of PF3845 are associated with AKT and ERK phosphorylation.

Figure 1. PF3845 attenuated TBI-induced anxiety, reduced deficits in hippocampus-dependent memory performance and in motor coordination via activation of CB1 and CB2 receptors

(A) Detailed timeline of all behavioral tests and the end points of experimental procedures starting on day 3 prior to surgery (study day -3). BW, beam walk test; CCI, controlled cortical impact; ZM, zero maze; YM, Y- maze test; TC, tissue collection; HT, histology; WB, western blot; ELISA, enzyme-linked immunosorbent assay. (B) Deficits in hippocampus-dependent working memory performance were determined by the number of alternations achieved during five minutes of maze exploration. TBI reduced the number of

alternations and treatment with PF3845 at 5 mg/kg reversed injury-induced memory deficits ($***p < 0.001$; Mean $\pm$ SEM; $n = 17/\text{group}$). **(C)** The effect of PF3845 on hippocampus-dependent working memory deficits was completely blocked by the CB1R antagonist AM 281, but only partially, although significantly reversed by the CB2R antagonist AM 630 ($***, ###p < 0.001$; $\#p < 0.05$; Mean $\pm$ SEM; $n = 10$ for the antagonist groups and 17 for Control, TBI and PF3845 at 5 mg/kg groups). **(D)** The Zero maze test was performed on days 6 and 14 post-injury to determine the effect of PF3845 on TBI-induced anxiety. On day 6 after injury, TBI slightly reduced the time spent in the open zone per visit, which was restored by PF-384 treatment. On day 14 post-TBI, the time spent in the open zones per visit was dramatically reduced but significantly reversed by treatment with PF3845 at 5 mg/kg and 10 mg/kg PF3845 ($***p < 0.001$; $**p < 0.01$; $*p < 0.05$; Mean $\pm$ SEM; $n = 12/\text{group}$). **(E)** The effect of PF3845 effect on TBI-induced anxiety was not affected by CBR1 and CB2R antagonists. **(F)** The number of foot faults during the beam-walk test was drastically increased on days 3 and 7 after injury, followed by a partial recovery observed on day 14 post-TBI. PF3845 treatment dramatically reduced the number of foot faults in a dose-dependent manner on days 7 and 14 post-TBI ($***p < 0.001$; Mean $\pm$ SEM; $n = 12$). **(G)** Motor improvements after treatment with PF3845 (5 mg/kg) was completely reversed by the CB2R antagonist AM-630 on days 7 and 14 post-injury. On the other hand, the CB1R antagonist AM-281 only partially but significantly blocked the effect of PF3845 on fine motor movement deficits at the same time points. Notably, the blocking effect of AM 630 on PF3845-induced improvement of fine motor movement was significantly greater than that of AM 281 ($***, \&\&p < 0.001$; $**p < 0.01$; $\&\#p < 0.05$; Mean $\pm$ SEM; $n = 10$).

Figure 2. PF3845 treatment inhibited FAAH activity and increased anandamide levels in TBI mouse brain

(A) Treatment with PF3845 significantly inactivated FAAH activity both in the ipsilateral and contralateral hemispheres of TBI mouse brain (** $p < 0.001$, Mean $\pm$ SEM; $n = 6$). (B) Endocannabinoid quantitation in brain tissues found that PF3845 treatment at 5 mg/kg significantly enhanced AEA levels in both ipsilateral and contralateral hemispheres of TBI mouse brain; AEA levels were also upregulated by TBI without treatment (* $p < 0.05$ and *** $p < 0.001$, Mean $\pm$ SEM; $n = 7$). (C) A significant increase in 2-AG levels was found

in the ipsilateral, but not in the contralateral hemisphere of TBI mouse brain after PF3845 treatment (** $p < 0.01$ and *** $p < 0.001$, Mean $\pm$ SEM; $n = 7$).

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Figure 3. PF3845 reduced lesion volume in the cortex and neurodegeneration in the dentate gyrus of TBI mouse brain and the effect on neurodegeneration was partially blocked by CB1 and CB2 receptor antagonists

(A) Representative micrographs showing FJB positive cells in the hippocampal dentate gyri of TBI mice treated with vehicle, PF3845 (5 mg/kg) alone or in combination with CB1 and CB2 receptor antagonists AM 281 and AM 630 three days post-TBI (degenerating cells shown in green and DAPI positive cells in blue). Scale bar = 50 μ m. (B) PF3845 treatment significantly reduced the number of degenerating cells in the dentate gyrus and that effect was partially but significantly blocked by both CB1R and CB2R antagonists ($^{***}p < 0.001$, $^{###}p < 0.001$; $^{\&}p < 0.05$; Mean $\pm$ SEM; $n = 4$). (C) The lesion volume in TBI mouse brain was significantly reduced by PF3845 treatment ($^{**}p < 0.01$; $n = 5$).

Figure 4. PF3845 suppressed the expression of TBI-induced amyloid precursor protein, dendritic damage and up-regulated the expression of synaptophysin in the mouse hippocampus

(A) Representative microphotographs showing double immunostaining of MAP-2 and APP in ipsilateral cerebral cortices from both vehicle and PF3845 treated groups on day 3 post-TBI. MAP-2 is shown in green, APP in red and DAPI in grey. The arrow in the insert points to the condensed nuclei. Scale bar = 50 μ m. (B) TBI induced a robust expression of APP, mainly in the perikaryon of hippocampal neuronal cells, which was significantly reduced by PF3845 treatment (* $p < 0.05$; Mean $\pm$ SEM; $n = 5$). (C) Representative immunoblots showing the expression of synaptophysin and PSD-95 in the ipsilateral cortices of both vehicle and PF3845 treated animals on days 3 and 14 post-TBI. Actin was used as a loading control. (D) Quantification of synaptophysin and PSD-95 signal intensity indicated that synaptophysin expression is suppressed on days 3 and 14 after injury, but was significantly up-regulated by PF3845 treatment. PSD-95 expression was also significantly reduced by TBI, but was not affected by PF3845 treatment (** $p < 0.001$; **, $\#p < 0.01$; and *, $\&p < 0.05$; Mean $\pm$ SEM; $n = 5-6$).

Figure 5. PF3845 up-regulated the expression Bcl-2 and Hsp70&72 proteins and decreased caspase-3 activity

(A) Representative immunoblots showing the expression of Bcl-2 and Hsp72&70 in the ipsilateral cortices and hippocampi of vehicle and PF3845 treated animals on days 3 and 14 after TBI. Actin was used as a loading control. (B) Bcl-2 protein was significantly increased by PF3845 in the ipsilateral cortex and hippocampus on day 3 post-TBI (** $p < 0.01$; * $p < 0.05$; Mean $\pm$ SEM; $n = 5$). (C) Quantification of Hsp72&70 indicating their expression was significantly up-regulated by PF3845 in the ipsilateral cortex and hippocampus 3 and 14 days post-TBI (***, ### $p < 0.001$; **, ## $p < 0.01$; *, # $p < 0.05$; Mean $\pm$ SEM; $n = 5$). (D) Caspase-3 activity examined by ELISA showed a significant increase in the ipsilateral cortices and hippocampi of TBI mice on day 3 post-injury (* $p < 0.05$; Mean $\pm$ SEM; $n = 5$), which was relatively reduced by PF3845 treatment. (* $p > 0.05$; Mean $\pm$ SEM; $n = 5$).

Figure 6. PF3845 suppressed COX-2 expression in TBI mouse cortex and hippocampus
(A) Representative immunoblots showing the expression of COX-2 and COX-1 in the ipsilateral cortices of vehicle and PF3845 treated animals on day 3 post-TBI. Actin was used as a loading control. **(B)** Quantification of band intensity indicates that COX-2 expression is significantly upregulated on day 3 post-TBI and reduced by PF3845 (** $p < 0.001$; Mean $\pm$ SEM; $n = 5$). **(C)** Representative microphotographs of double immunostaining of COX-2 and F4/80 in ipsilateral hippocampi of vehicle and PF3845 treated TBI mice on day 3 post-insult. F4/80, a microglia/macrophage marker, is shown in green, COX-2 in red and DAPI in blue. The yellow color indicates co-localization of COX-2 and F4/80. Scale bar = 50 μ m. **(D)** COX-2 positive area fraction in ipsilateral hippocampus was significantly increased by TBI on day 3 post-injury and was dramatically reduced by PF3845 treatment (** $p < 0.001$; Mean $\pm$ SEM; $n = 4-6$).

Figure 7. PF3845 decreased the number of iNOS expressing microglia cells

(A) Representative microphotographs of double immunostaining of iNOS and F4/80 in ipsilateral cortices from both vehicle- and PF3845-treated mice on days 3 and 14 post-TBI. Scale bar = 50 μ m. (B) Quantification of the overlap fraction of iNOS/F4/80 immunopositive cells indicating that PF3845 treatment significantly suppressed iNOS expression in inflammatory cells on days 3 and 14 post-injury (***,###P < 0.001; Mean $\pm$ SEM; n = 4).

Figure 8. PF3845 enhanced Arg-1 expression

(A) Representative microphotographs of double immunostaining of iNOS and Arg-1 in ipsilateral cortices from both vehicle and PF3845 treated mice on days 3 and 14 post-TBI. Scale bar = 50 μ m. (B) Quantification of the overlap fraction of Arg-1/F4/80 immunopositive cells indicates that PF3845 treatment significantly enhanced Arg-1 expression in inflammatory cells on days 3 and 14 post-injury (***, ###P < 0.001; Mean $\pm$ SEM; n = 4).

Figure 9. PF3845 increased ERK and AKT phosphorylation in TBI mouse brain

(A) Representative immunoblots showing the expression of ERK, pERK, AKT and pAKT in the ipsilateral cortices of both vehicle and PF3845 treated animals in 3 and 14 days post-TBI. Actin was used as a loading control. (B, C) The ratios of pERK/ERK and pAKT/AKT show a significant increase in ERK and AKT phosphorylation by PF3845 on day 14 post-injury, when compared to vehicle-treated animals (** $p < 0.001$; * $p < 0.01$; * $p < 0.05$; Mean $\pm$ SEM; $n = 6$). (D) Representative immunoblots showing the expression of ERK, pERK, AKT and pAKT in the ipsilateral hippocampi of both vehicle and PF3845 treated animals in 3 and 14 days post-TBI. Actin was used as a loading control. (E) pERK/ERK ratio shows a significant increase in ERK phosphorylation by PF3845 on day 14 post-injury, when compared to vehicle-treated animals (** $p < 0.01$; * $p < 0.05$; Mean $\pm$ SEM; $n = 6$). (F) Quantification of pAKT/AKT ratio showing a significant increase in AKT phosphorylation by PF3845 on day 3 post-injury when compared to the vehicle-treated TBI group (** $p < 0.001$; Mean $\pm$ SEM; $n = 6$).