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Modulation of the cAMP signaling pathway after traumatic brain injury

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Abstract

Traumatic brain injury (TBI) results in both focal and diffuse brain pathologies that are exacerbated by the inflammatory response and progress from hours to days after the initial injury. Using a clinically relevant model of TBI, the parasagittal fluid-percussion brain injury (FPI) model, we found injury-induced impairments in the cyclic AMP (cAMP) signaling pathway. Levels of cAMP were depressed in the ipsilateral parietal cortex and hippocampus, as well as activation of its downstream target, protein kinase A, from 15 min to 48 hr after moderate FPI. To determine if preventing hydrolysis of cAMP by administration of a phosphodiesterase (PDE) IV inhibitor would improve outcome after TBI, we treated animals intraperitoneally with rolipram (0.3 or 3.0 mg/kg) 30 min prior to TBI, and then once per day for three days. Rolipram treatment restored cAMP to sham levels and significantly reduced cortical contusion volume and improved neuronal cell survival in the parietal cortex and CA3 region of the hippocampus. Traumatic axonal injury, characterized by β-amyloid precursor protein deposits in the external capsule, was also significantly reduced in rolipram-treated animals. Furthermore, levels of the pro-inflammatory cytokines, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), were significantly decreased with rolipram treatment. These results demonstrate that the cAMP-PKA signaling cascade is downregulated after TBI, and that treatment with a PDE IV inhibitor improves histopathological outcome and decreases inflammation after TBI.

Keywords

camp; Fluid-percussion; Inflammation; Interleukin-1β; PKA; Phosphodiesterase; Rolipram; TNF-α; Traumatic brain injury; TBI

Traumatic brain injury (TBI) is a prevalent, debilitating health problem, occurring in 1.4 million people each year and disabling 5 million people in the United States (Langlois et al., 2004). The subsequent progressive injury after brain trauma develops from hours to days after the initiating insult, providing an accessible time window for pharmacological therapies. Despite

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intense efforts, research in TBI has not yielded a therapy that has passed Phase III clinical trials (Doppenberg et al., 2004).

Brain trauma results in contusion formation, neuronal apoptosis, and axonal tract damage. These pathologies are worsened by the inflammatory cascade set into motion by the initial injury (Morganti-Kossmann et al., 2002, Dietrich et al., 2004). Two pro-inflammatory cytokines released after TBI are tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Numerous studies have documented rapid increases in TNF-α and IL-1β levels after TBI (Taupin et al., 1993, Shohami et al., 1994, Fan et al., 1996, Kinoshita et al., 2002, Vitarbo et al., 2004).

IL-1β synergistically acts with TNF-α to induce cell death after TBI. These pro-inflammatory cytokines stimulate inflammatory cells to release damaging reactive oxygen and nitrogen species, raise glutamate levels to excitotoxic levels, impair the ability of glia cells to buffer extracellular potassium, compromise the blood-brain barrier, and attract more inflammatory cells into the brain (Tanaka et al., 1994, Meda et al., 1995, Soares et al., 1995, Hu et al., 1997, Keeling et al., 2000). Once initiated, the inflammatory cascade becomes a toxic positive-feedback loop, further exacerbating brain pathology.

In other models of CNS injury, several studies have demonstrated that restoration of cyclic AMP (cAMP) levels improves outcome. In spinal cord injury, application of rolipram to inhibit the degradation of cAMP promotes axon sparing and results in locomotor improvements (Nikulina et al., 2004, Pearse et al., 2004). Similarly, in transient global ischemia rolipram improves neuronal survival in the hippocampus and hippocampal-dependent learning (Kato et al., 1995, Block et al., 1997, Imanishi et al., 1997, Block et al., 2001).

The effects of cAMP are short-lived because phosphodiesterases (PDEs) rapidly degrade cAMP (Manganiello et al., 1995). Of the ten classes of PDEs, two isoforms are highly selective for degrading cAMP, PDE IV and VII. Rolipram, a selective inhibitor of PDE IV, reduces inflammation in a number of diseases including asthma, multiple sclerosis, septic shock, rheumatoid arthritis, and inflammatory bowel disease (Dal Piaz and Giovannoni, 2000, Castro et al., 2005). Consequently, PDE IV inhibitors are widely-utilized by the pharmaceutical industry as anti-inflammatory drugs.

A primary action of cAMP is activation of protein kinase A (PKA). PKA phosphorylates transcription factors, including cAMP-responsive element binding (CREB) protein and nuclear factor-κB (NF-κB) p50 (Montminy and Bilezikjian, 1987, Hou et al., 2003). Phosphorylation of CREB stimulates transcription of cell survival genes (Mayr and Montminy, 2001). Phosphorylation of NF-κB p50 subunit suppresses transcription of genes with IκB elements in their promoters; this includes the pro-inflammatory cytokines TNF-α and IL-1β (Cogswell et al., 1994, Verghese et al., 1995, Hou et al., 2003). Thus, we hypothesized that rolipram treatment may improve TBI outcome by decreasing pro-inflammatory cytokine production.

Materials and methods

Traumatic brain injury

All experimental procedures were in compliance with the NIH *Guide for the Care and Use of Laboratory Animals* and approved by the University of Miami Animal Care and Use Committee. Male Sprague-Dawley rats (270–320 g; Charles River Laboratories, Raleigh, NC, USA) were anesthetized with 3% halothane, 70% N_2O, and 30% O_2, then intubated endotracheally and mechanically ventilated (Harvard Apparatus, Holliston, MA, USA) with 1.5% halothane, 70% N_2O, and 30% O_2. To immobilize the animals and facilitate mechanical ventilation, pancuronium bromide (0.5 mg/kg) was intravenously administered through the

femoral artery. On the day prior to TBI, animals received a 4.8 mm craniotomy (3.8 mm posterior to bregma, 2.5 mm lateral to the midline) and a modified plastic 18 gauge syringe hub (8 mm length, PrecisionGlide needle, Becton Dickinson, Franklin Lakes, NJ, USA) was secured over the right parietal cortex. The next day, animals were anesthetized, intubated, and then placed under a fluid-percussion brain injury (FPI) device. A moderate fluid-percussion pulse (2.0±0.2 atmospheres) was delivered to the right parietal cortex. Sham-operated rats received all surgical manipulations, but without the fluid-percussion pulse, and were monitored under anesthesia for 15–30 min after the sham injury. Rectal and temporalis muscle thermistors were used to maintain core and brain temperatures at 36.8–37.3°C using self-adjusting feedback warming lamps. Blood gases (pO_2 and pCO_2), blood pH, and mean arterial pressure were monitored 15 min before TBI and up to 4 hr after TBI and maintained within normal physiological ranges.

cAMP Assays

Six experimental groups (*n*=47) were used to measure cAMP levels by ELISA. Animals received either sham surgery (*n*=11) or moderate parasagittal FPI followed by recovery for 15 min (*n*=8), 1 hr (*n*=6), 4 hr (*n*=7), 24 hr (*n*=5), or 48 hr (*n*=10). The right (injured) parietal cortex, hippocampus, and thalamus were rapidly dissected at 4°C and frozen on liquid nitrogen. The tissue was briefly sonicated on ice (10 s, setting 2, Branson sonifier 450, Danbury, CT, USA) in 20 volumes of 0.1 N HCl and 500 μM 3-isobutyl-1-methylxanthine (IBMX). cAMP levels were quantified using a cAMP low pH ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol for the nonacetylated method. Each sample was assayed in duplicate.

Immunohistochemistry

At 5 min, 4 hr, and 24 hr after TBI, animals were perfused with saline (2 min, 4°C), then with 4% paraformaldehyde in phosphate-buffered saline (PBS; 30 min, 4°C). The brains were sectioned in PBS (50 μm thick) using a Leica vibratome (Leica Microsystems, Inc., Exton, PA, USA). Free-floating sections were blocked for 1 hr at RT in blocking buffer (PBS containing 5% normal goat serum, 0.2% fish skin gelatin, 0.3% TX-100). Sections were then incubated overnight at 4°C in blocking buffer with anti-NeuN (1:400, Chemicon, Temecula, CA, USA, MAB377) and anti-cAMP antibodies (1:1000, Chemicon, AB306). After incubation with the primary antibodies, the sections were rinsed with PBS, and incubated 2 hr at RT in blocking buffer with anti-mouse and anti-rabbit secondary antibodies labeled with Alexa 488 and 546 (Invitrogen, Carlsbad, CA, USA), respectively. The sections were then rinsed with PBS, and mounted using ProLong Gold antifade mounting medium (Invitrogen).

Images were obtained with a LSM510 laser scanning confocal microscope (Carl Zeiss, Inc., Thornwood, NY, USA) using 25X 0.8 NA and 63X 1.2 NA water-immersion lenses. At least 3 different sections were prepared from each animal; all animals in each group yielded similar results.

Western blot analysis

To assess for changes in PKA after TBI, six experimental groups (*n*=39) were used. Animals received either sham surgery (*n*=8) or moderate TBI followed by recovery for 15 min (*n*=7), 1 hr (*n*=7), 4 hr (*n*=8), 24 hr (*n*=4), or 48 hr (*n*=5). At various times after the TBI surgery, the ipsilateral parietal cortex and hippocampus were dissected at 4°C in saline and frozen on liquid nitrogen within 2 min of decapitation. To specifically determine biochemical changes in PKA that occurred at the synaptic membrane, the tissue was fractionated (Hu et al., 1999). The tissue was homogenized with a Dounce homogenizer (35 strokes, 4°C) in 1 ml of Lysis Buffer: 15 mM Tris pH 7.6, 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 1.25 μg/ml pepstatin A, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 0.5 mM PMSF, 0.1 mM Na_3VO_4, 50 mM NaF, 2

mM $Na_4P_2O_7$, and 1X phosphatase inhibitor cocktail set II (Calbiochem, San Diego, CA, USA). The samples were centrifuged (800xg, 10 min, 4°C). The supernatants were centrifuged again (10,000xg, 10 min 4°C) to generate a pellet containing synaptic membranes that was resuspended in lysis buffer with 0.1% Triton X-100. The samples were assayed for total protein using the Coomassie Plus assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Samples were boiled with 1X sample buffer for 7–9 min at 95°C. Equal amounts of protein (30 μg/lane) were electrophoresed (12.5% SDS-PAGE) and western blotted. The crude synaptic membrane fraction was western blotted for phospho-PKA Ser^{96} regulatory subunit II (RII; 1:1000, Upstate Cell Signaling Solutions, Lake Placid, NY, USA, 06-704), PKA RII (1:1000, Upstate Cell Signaling Solutions, 06-411), and β-tubulin (1:5000; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA, E7). For analysis of changes in phospho-CREB, total homogenates were western blotted and probed with antibodies against phospho-CREB Ser133 (1:1000, Cell Signaling Technology, 9191), anti-total CREB (1:1000, Cell Signaling Technology, 9192), and β-actin (1:5000, Sigma-Aldrich, AC-15). Epitopes were visualized with HRP-conjugated secondary antibodies (1:1000-1:5000; Cell Signaling Technology, Beverly, MA, USA) using the Phototope HRP Western blot detection system (Cell Signaling Technology) and developed on film (Phenix x-ray film BX; Phenix Research Products, Hayward, CA, USA). Films were developed to be in a linear range and densitized using LabWorks software (Ultra-Violet Products, Upland, CA, USA). Levels of phospho-protein immunoreactivity (e.g. phospho-PKA) were normalized to total protein immunoreactivity (e.g. PKA), then to β-tubulin immunoreactivity.

Rolipram administration

Rolipram (Sigma-Aldrich, St. Louis, MO, USA), was dissolved in 100% DMSO at 10 mg/ml, and then diluted with 0.9% NaCl for a final concentration of either 0.5 mg/ml or 0.05 mg/ml in 5% DMSO and 95% saline. The drug was administered intraperitoneally (i.p.) 30 min prior to TBI at 6 ml/kg. For each group, rolipram or vehicle (5% DMSO/95% saline) was administered once every 24 hr and on the final day of the experiment, 30 min prior to sacrifice.

Histopathological analysis

TBI- and sham-operated animals were anesthetized (3% halothane for 5 min) and perfused transcardially with isotonic saline for 2 min (75 mL) and then 30 min of 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (350 mL). The brains were embedded in paraffin and sectioned (10 μm thick). The sections were stained with hematoxylin and eosin (H&E) and alternative sections were immunostained with NeuN and β-APP. Cortical contusion volumes were determined by tracing the contused areas in H&E sections (150 μm apart) with a 20X objective on an Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) using the Neurolucida 7.50.1 software program (MicroBrightField Inc., Williston, VT, USA). The cortical contusion boundaries were well demarcated by pyknotic neurons, reactive astrocytes, hemorrhage, edema, and a shearing at the gray/white matter interface between the cortex and external capsule. Contusion areas were calculated for 5 coronal levels at and around the epicenter (-3.3, -4.3, -5.8, -6.8, and -7.3 mm posterior from bregma). To determine neuron survival and axonal tract pathology, serial sections (150 μm apart) from -3.3 to -5.8 mm posterior to bregma were incubated with NeuN antibody (1:500, Chemicon, MAB377) or β-APP antibody (1:500, Chemicon, MAB348), respectively. Immunostaining was developed with anti-mouse IgG (1:1000), ABC Elite (Vector Laboratories, Burlingame, CA, USA), and NiDAB (2.5% Nickle Ammonium Sulfate Acetate-Imidasole Buffer, 0.05% DAB, 0.001% H_2O_2, Vector Laboratories). NeuN-positive neurons were quantified in an unbiased, systematic manner using stereology with an Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.) by a blind observer (Suzuki et al., 2003, Suzuki et al., 2004). The parietal cortex overlying the contusion area and the CA3 region of the hippocampus were contoured at 20X, then a counting grid of 250×200 μm was placed in the parietal cortex or a grid of

140×70 μm was placed in the CA3 region. Using a 35×35 μm counting frame, NeuN-positive cells were counted in 25–40 randomly-placed sampling sites with Stereoinvestigator 7.50.1 software (MicroBrightField, Inc.) with a 63X, 1.4 NA objective. NeuN counts were measured from bregma levels -3.3 mm to -5.8 mm in sections spaced 150 μm apart. For cortical cell counts, Q values ranged from 446–715 and CE^2/CV^2 values were 0.04, 0.06, and 0.11 for the vehicle, 3.0 mg/kg rolipram and 0.3 mg/kg rolipram groups, respectively. For CA3 hippocampal cell counts, the Q range was 325–550 and CE^2/CV^2 values were 0.39, 0.11, and 0.11 for the vehicle, 3.0 mg/kg rolipram and 0.3 mg/kg rolipram groups, respectively. To quantify axonal pathology, the external capsule was traced at 20X magnification for 3 coronal levels, -3.3, -4.3, and -5.8 mm posterior to bregma. A counting grid of 120×300 μm (-3.3 mm bregma), 120×330 μm (-4.3 mm bregma), and 200×290 μm (-5.8 mm bregma) was placed over each tracing (Bramlett et al., 1997, Suzuki et al., 2004). Using a 35×35 μm counting frame, β-APP deposits in 40–50 randomly-placed sampling sites were counted in the external capsule at 63X magnification (NA 1.4) using Stereoinvestigator 7.50.1 software (MicroBrightField, Inc.). The Q values for β-APP counts ranged from 135–223 and the CE^2/CV^2 values were 0.10, 0.07, and 0.08 for the vehicle, 3.0 mg/kg rolipram and 0.3 mg/kg rolipram groups, respectively. The NeuN and β-APP counts at each bregma level were determined by averaging three consecutive sections at each specific bregma level.

Images were taken with a 40X objective on an Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc.) and montaged using the virtual slice module in the Neurolucida 7.50.1 software program (MicroBrightField, Inc.).

IL-1β and TNF-α ELISAs

Three experimental groups (*n*=22) were used to assess IL-1β and TNF-α levels by ELISA. Animals received either sham surgery (*n*=5) or moderate parasagittal FPI and treatment with vehicle (*n*=8) or 0.3 mg/kg rolipram (*n*=9) 30 min prior to FPI and 30 min prior to sacrifice. At 3 hr after FPI, the animals were sacrificed and the ipsilateral parietal cortex, hippocampus and thalamus were rapidly dissected on ice in saline. The tissue was briefly sonicated on ice (10 s, setting 2, Branson sonifier 450, Danbury, CT, USA) in 10 volumes/weight of Lysis Buffer supplemented with 0.1% Igepal CA-630 (Sigma-Aldrich). Total protein was measured using the Coomassie Plus assay kit (Bio-Rad Laboratories). Each sample was assayed in duplicate according to the manufacturer's protocol (R&D Systems, Inc.).

Statistical analysis

Data presented are mean±SEM. Statistical analyses are Student's *t* test or one-way ANOVAs with post-hoc Tukey HSD *t* tests.

Results

To ascertain if the cAMP-PKA pathway is a potential therapeutic target after TBI, we first determined if the cAMP-PKA pathway is modulated after TBI. At various times after sham or FPI surgery, the ipsilateral parietal cortex, hippocampus, and thalamus were assayed by ELISA for cAMP. Absolute levels of cAMP from cortices of sham animals were similar to levels previously reported in the literature (parietal cortex cAMP levels 184.1±5.6 pmol/mL, *n*=6) (Pearse et al., 2004). We found that cAMP levels decreased by 15 min after TBI in the ipsilateral hippocampus, and were depressed at 24 to 48 hr in the ipsilateral parietal cortex (Fig. 1). There were no changes in cAMP levels in the thalamus. To determine the cell types that were expressing cAMP, we performed immunohistochemistry of cAMP from animals after TBI. At 5 min after TBI, cAMP was predominantly localized in neurons, as identified by co-immunostaining with NeuN (Fig. 2). Similar results were obtained in animals at 4 and 24 hr after TBI.

cAMP primarily exerts its actions through PKA. When PKA is activated, the catalytic subunit autophosphorylates the regulatory subunit and this may facilitate dissociation and activation of the catalytic subunits (Keryer et al., 1998). To determine whether PKA activation is modulated after TBI, we performed western blot analysis with antibodies to phosphorylated, activated PKA regulatory subunit II. PKA autophosphorylation was downregulated in crude membrane fractions from the ipsilateral parietal cortex and hippocampus within 15 min after TBI, and this downregulation lasted for at least 48 hr (Fig. 3). Thus, the FPI model depresses both cAMP levels and PKA activation.

To determine whether inhibition of PDE IV would improve signaling through the cAMP-PKA pathway after TBI, we treated animals with vehicle (5% DMSO in saline, i.p.) or rolipram (0.3 mg/kg, i.p.), a selective PDE IV inhibitor, 30 min prior to moderate FPI, and 30 min prior to sacrifice at 24 hr after FPI, when cAMP levels are significantly depressed in the cortex and hippocampus. We found that rolipram restored cAMP levels in TBI animals to sham levels. Total CREB levels increased in the cortex and phosphorylated CREB levels increased in the hippocampus of TBI animals treated with rolipram as compared to TBI animals treated with vehicle (Fig. 4).

Next, to determine if rolipram would improve histopathology, we treated animals with vehicle or rolipram (0.3 mg/kg or 3 mg/kg) 30 min prior to moderate FPI, and then once per day for 3 days. We chose to pre-treat animals with rolipram to target the acute, rapid inflammatory signaling events initiated by trauma (Kinoshita et al., 2002, Vitarbo et al., 2004). Animals were assessed for histopathology at 3 days after TBI because there are reproducible, quantifiable histopathologies at this time point (Dietrich et al., 1994, Bramlett et al., 1997, Suzuki et al., 2003, Suzuki et al., 2004). Three days after moderate FPI and either vehicle or rolipram treatment, animals were perfused and the brains were stained with hematoxylin and eosin (H&E), a general nuclei and cytoplasmic stain to visualize the cortical contusions. We observed a significant decrease in cortical contusion size with 0.3 mg/kg rolipram treatment in comparison to vehicle treatment when quantified using unbiased stereology measurements (Fig. 5; contusion volume: vehicle 4.22±0.63 mm^3, n=9; 3.0 mg/kg rolipram 2.41±0.51 mm^3, n=8; 0.3 mg/kg rolipram 1.98±0.22 mm^3, n=6, $p<0.05$). A comparison of contusion volume at the epicenter of injury (-3.8 mm bregma) and the surrounding bregma levels illustrated that 0.3 mg/kg rolipram treatment reduced cortical contusion areas significantly at bregma levels -3.3 and -6.8 mm.

The parasagittal FPI model results in stereotypical neuronal death in the parietal cortex overlying the cortical contusion and in the CA3 region of the hippocampus (Grady et al., 2003, Witgen et al., 2005). Treatment of rolipram during TBI and for 3 days following moderate FPI improved neuronal survival in both the parietal cortex (Fig. 6) and the CA3 region of the hippocampus (Fig. 7) when assessed by counting cells positive for NeuN, a marker for neurons.

Another histopathological characteristic of TBI is traumatic axonal injury, which is exemplified by β-amyloid precursor protein (β-APP) deposits in white matter tracts (Bramlett et al., 1997, Ciallella et al., 2002, Suzuki et al., 2004). Traumatic axonal damage was assayed by quantifying the number of β-APP deposits in the external capsule the white matter tract between the hippocampus and parietal cortex. β-APP deposits were significantly reduced in animals treated with either 0.3 or 3.0 mg/kg rolipram at bregma level -3.3 mm, near the injury center (Fig. 8).

In other injury models, rolipram is well known to decrease the expression and release of the pro-inflammatory cytokines IL-1β and TNF-α (Prabhakar et al., 1994, Verghese et al., 1995, Griswold et al., 1998). To determine if rolipram treatment after TBI reduced the levels of IL-1β and TNF-α, animals were treated with vehicle or rolipram 30 min prior to moderate FPI,

then treated with rolipram 30 min prior to sacrifice. This treatment regime was designed to be similar to the previous experiments assessing histopathological outcome in which the animals received a final rolipram injection 30 min prior to sacrifice. The animals were assayed 3 hr after FPI for IL-1β and TNF-α; a time point when these cytokines are significantly elevated after brain injury (Kinoshita et al., 2002, Vitarbo et al., 2004). Injury-induced increases in IL-1β levels were significantly reduced with rolipram treatment in the hippocampus and thalamus (Fig. 9). The increase in TNF-α after TBI was also significantly reduced in the cortex and hippocampus with rolipram treatment. These results demonstrate that rolipram treatment during TBI reduces the inflammatory response in the brain.

Discussion

The parasagittal FPI model leads to reproducible histopathology in the brain, similar to the pathology typically seen in TBI patients (Dietrich et al., 1994, Gennarelli, 1994, Keane et al., 2001, Thompson et al., 2005). Accordingly, there are consistent, quantifiable focal and diffuse histopathologies that are all potential therapeutic targets (Dietrich et al., 1994, Bramlett et al., 1997, Ciallella et al., 2002, Grady et al., 2003, Suzuki et al., 2003, Suzuki et al., 2004, Witgen et al., 2005). In our studies, we found that rolipram, a selective PDE IV antagonist, improved histopathology at multiple levels. Rolipram treatment after TBI decreased cortical contusion size, neuronal death in the parietal cortex and CA3 region of the hippocampus, and β-APP deposits in the external capsule, the axonal tract between the hippocampus and parietal cortex. Of the two doses tested (0.3 and 3 mg/kg), we found that the lower dose trended towards more significance in reducing cortical contusion volume and β-APP deposits. These results are in accordance with previous results using rolipram as a therapeutic agent in spinal cord injury and transient global ischemia where lower doses were also more effective (Block et al., 1997, Nikulina et al., 2004). Rolipram has also been found to improve outcome in experimental allergic encephalomyelitis, Alzheimer disease, multiple sclerosis, ischemia, and striatal excitotoxicity (Genain et al., 1995, Navikas et al., 1998, Folcik et al., 1999, Gong et al., 2004, Demarch et al., 2007, Sasaki et al., 2007). Our results and the many studies assessing rolipram in models of neurological disorders suggest that use of a PDE IV antagonist may be a promising avenue of research as we search for a successful pharmacological therapy for TBI patients. However, the current studies utilized a pre-treatment paradigm to determine if rolipram would target relevant histopathology responses to brain trauma. These studies were proof of concept only to target the acute inflammatory response that occurs rapidly after TBI (Kinoshita et al., 2002, Vitarbo et al., 2004). It is important to determine the therapeutic time window of rolipram treatment after TBI to develop rolipram as a potential therapeutic intervention for TBI patients. Current studies are underway to determine if rolipram treatment attenuates histopathology and reduces inflammation when given after the TBI.

Initially, we had predicted that cAMP levels and PKA activation would rapidly and transiently increase after TBI. Type I and VIII adenylyl cyclases are activated by calcium, and there is a large influx of calcium into cells after TBI (Fineman et al., 1993, Cali et al., 1994, Matsushita et al., 2000, Osteen et al., 2004). Furthermore, in models of epilepsy and stroke, cAMP levels increase; thus it was surprising that we observed only a decrease in cAMP levels after brain injury (Ferrendelli et al., 1980, Prado et al., 1992). However, these results are in accordance with previous studies of TBI and spinal cord injury. In the FPI model of brain injury, cAMP levels have been found to decrease in the cortex, although in controlled cortical impact, one study has reported no change in cAMP levels after trauma (Dhillon et al., 1995, Armstead, 1997, Bell et al., 1998). In spinal cord injury, cAMP levels are chronically depressed from 1 day to 2 weeks (Pearse et al., 2004). Thus, unlike several other protein kinase cascades that are activated after TBI, the cAMP-PKA pathway is unique in that this signaling pathway is depressed after TBI (Hu et al., 2004, Atkins et al., 2006, Chen et al., 2006).

The decrease in cAMP levels after TBI could be due to either increased PDE activity or decreased adenylyl cyclase activity. In culture, PDE IV expression is upregulated when microglia, the endogenous inflammatory cells in the brain, are exposed to stimuli that induce their activation analogous to injury, such as lipopolysaccharide and the cytokine TNF-α (Jin and Conti, 2002, Sasaki and Manabe, 2004). Alternatively, one report has found that the pro-inflammatory cytokine TNF-α downregulates adenylyl cyclase activity in microglia (Patrizio, 2004). Further experiments are needed to determine whether the expression of PDE IV is upregulated after TBI and/or whether adenylyl cyclase activity is downregulated after TBI.

PDE IV selectively degrades cAMP (K_m 4 μM) as compared to cGMP (K_m >3000 μM), and is inhibited by the highly specific inhibitor, rolipram (Nemoz et al., 1985, Muller et al., 1996, Torphy, 1998). In the uninjured CNS, rolipram increases cAMP levels in the hippocampus (Barad et al., 1998, Van Staveren et al., 2001, Giorgi et al., 2004), and specifically in microglia and astrocytes as compared to neurons (Zhang et al., 2002). There are also very modest increases in cGMP levels with high concentrations of rolipram, suggesting that rolipram could work through cGMP although this is more unlikely (Van Staveren et al., 2001).

Activation of the classical cAMP-PKA signaling pathway by rolipram is a likely, though not only, mechanism that rolipram may have improved outcome after TBI. Rolipram can work through four mechanisms on PDE IV. First, rolipram inhibits cAMP hydrolysis by binding the cAMP catalytic site, the low affinity rolipram binding site. Secondly, rolipram also binds another region near the PDE IV catalytic site, the high affinity rolipram binding site, where it does not affect cAMP hydrolysis. The high affinity rolipram binding site is thought to elicit effects on PDE IV that are cAMP-independent and involve the MAPK signaling cascade (Souness and Rao, 1997, Martin et al., 2002, Zhao et al., 2003). Third, rolipram may inhibit PDE IV hydrolysis of cAMP and increase cAMP levels, but produce anti-inflammatory effects that occur independently of PKA, through Epac1, a cAMP-responsive guanine nucleotide exchange factor which activates the Ras family GTPases, or fourth, via the receptor for activated C kinase 1 and subsequent protein kinase C activation (De Rooij et al., 1998, Houslay and Adams, 2003). Further experiments are needed to determine the exact mechanism of how rolipram improved histopathology and reduced pro-inflammatory cytokine production after TBI.

Increasing activation of the cAMP-PKA pathway may improve the histopathology induced by TBI through a number of signaling pathways. Classically in neurons, PKA phosphorylates the transcription factor CREB to increase expression of cell survival genes such as BDNF and the anti-apoptotic protein bcl-2 (Freeland et al., 2001, Tabuchi et al., 2002, Deogracias et al., 2004, Meller et al., 2005). Previous studies have reported that CREB is activated after TBI and BDNF levels are elevated as well (Dash et al., 1995, Yang et al., 1996, Hicks et al., 1997, Truettner et al., 1999, Griesbach et al., 2004a, Griesbach et al., 2004b, Hu et al., 2004). Multiple protein kinases can phosphorylate CREB; a few of these include calcium/calmodulin-dependent protein kinase IV, ribosomal protein S6 kinase, mitogen- and stress-activated protein kinase, and MAP kinase-activated protein kinase-2 (Sun et al., 1994, Tan et al., 1996, Xing et al., 1996, Deak et al., 1998, Shaywitz and Greenberg, 1999, Bito and Takemoto-Kimura, 2003). Given the decreased levels of cAMP and PKA activation after TBI, it is likely that one of these other protein kinases phosphorylates CREB at 24 hr after TBI. Rolipram treatment increased CREB phosphorylation in the hippocampus and total CREB levels in the parietal cortex. The increase in total CREB levels may be a reflection of the increased neuronal survival with rolipram treatment. Together, these results suggest that the mechanism of rolipram's action may be through CREB. This is supported in other injury models as well where rolipram significantly increased CREB phosphorylation (Nagakura et al., 2002, Hosoi et al., 2003, Lee et al., 2004, Demarch et al., 2007).

Another signaling pathway regulated by cAMP-PKA is through the transcription factor NF-κB p50 to reduce expression of pro-inflammatory cytokines such as IL-1β and TNF-α (Montminy and Bilezikjian, 1987, Hou et al., 2003). Transcription of the *tnf-α* gene is suppressed by the NF-κB p50 subunit which constitutively binds the IκB element in the promoter region (Kuprash et al., 1995, Jimenez et al., 2001, Takahashi et al., 2002, Foey et al., 2003). PKA phosphorylation of Ser337 on the NF-κB p50 subunit increases its binding and repression of transcription of IκB-containing gene promoter of the *tnf-α* gene (Ollivier et al., 1996, Baer et al., 1998, Hou et al., 2003). How the cAMP pathway regulates IL-1β expression is an active area of investigation. Several studies have shown that raising cAMP levels with either neurotransmitters or phosphodiesterase inhibitors reduce IL-1β levels, but the exact mechanism is still unclear (Cogswell et al., 1994, Verghese et al., 1995, Si et al., 1998, Caggiano and Kraig, 1999, Cho et al., 2001, Dello Russo et al., 2004).

Reducing pro-inflammatory cytokine levels after TBI to improve outcome has met with varying success. Administration of an inhibitor of IL-1β receptors, IL-1 receptor antagonist (IL-1ra), reduces contusion volume and transgenic mice overexpressing IL-1ra have improved behavioral recovery after TBI (Sanderson et al., 1999, Tehranian et al., 2002). Similarly, knockout mice of *tnfα* have improved behavior recovery one week after TBI, but worsened histopathology and behavioral outcome 2–4 weeks after injury (Scherbel et al., 1999). These studies indicate that inflammation is a complex, evolving series of biochemical events that can be both detrimental and beneficial for functional outcome after injury. Thus, targeting the inflammatory cascade as a therapeutic intervention requires careful consideration of the optimal time window, dosage, and mechanism of action.

Although these studies demonstrate an improvement in histopathology after TBI, in consideration of the many failed clinical trials of other neuroprotective agents for the treatment of TBI, these preliminary studies are only proof of concept for the FPI model. It is important to extend these observations to a post-injury treatment paradigm and determine the therapeutic window for rolipram treatment after TBI. Furthermore, whether these improvements in histopathology are accompanied by an improvement in behavioral deficits remains to be determined. Another important consideration is to understand the consequences of decreased cAMP levels after TBI: what cell types exhibit decreases in cAMP-PKA signaling and whether this can be rescued with rolipram treatment. And finally, understanding the mechanism of how rolipram leads to an improvement in functional outcome, possibly by increasing CREB-regulated gene expression and decreasing the inflammatory response, is necessary to develop PDE IV inhibition into a potential therapy.

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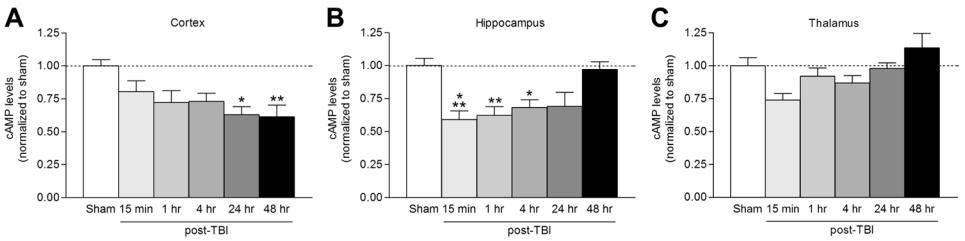

Fig 1.

cAMP levels decrease after TBI. The ipsilateral parietal cortex (A; n=4–10), hippocampus (B; n=4–11), and thalamus (C; n=5–10) were assayed by ELISA for cAMP levels after moderate parasagittal FPI. cAMP levels were significantly decreased in the cortex at 24 hr (n=4, *p<0.05) and 48 hr (n=6, **p<0.01) after TBI, and at 15 min (n=6, ***p<0.001), 1 hr (n=6, **p<0.01), and 4 hr (n=5, *p<0.05) after TBI in the hippocampus as compared to sham levels. Data represent mean ± SEM.

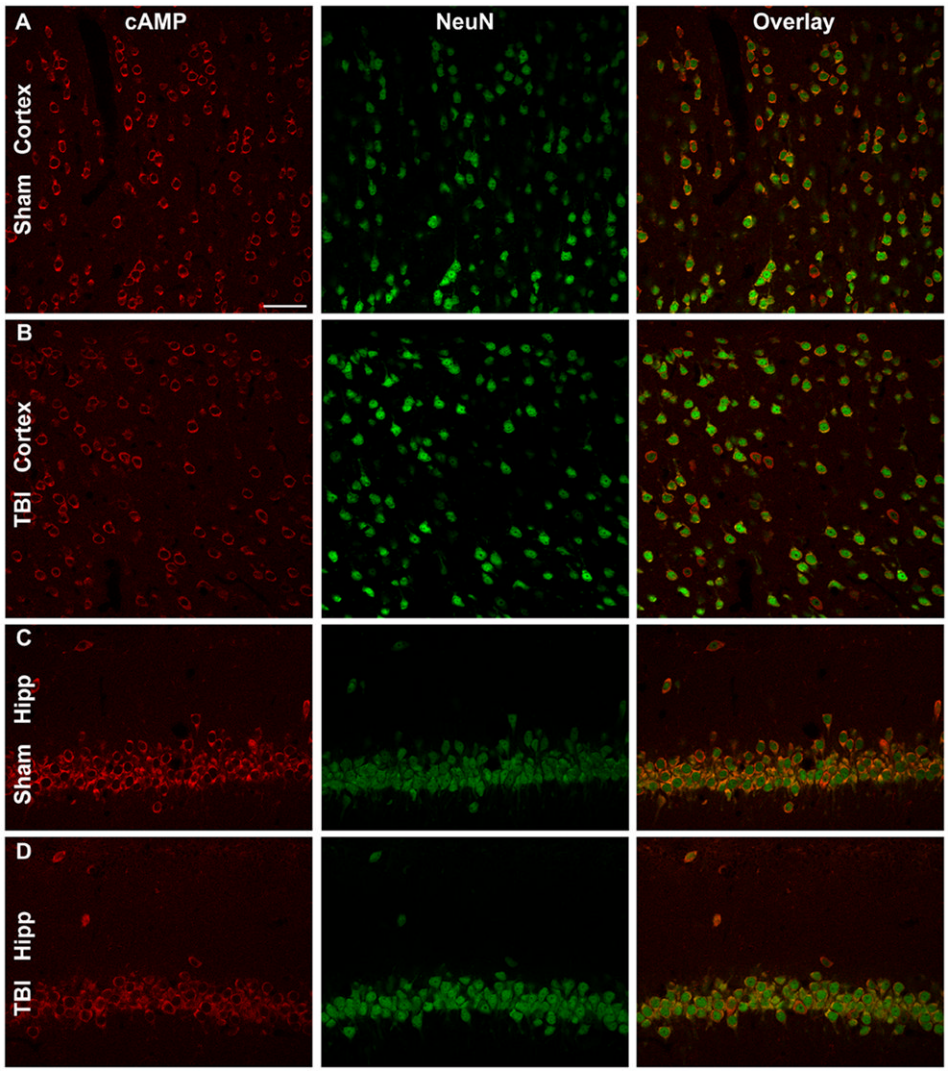

Fig 2.

cAMP immunostaining after TBI. The ipsilateral parietal cortex of sham surgery animals (A) and TBI animals (B) were immunostained with cAMP (*red*) and NeuN (*green*). Images were from animals perfused 5 min after surgery. There was co-localization of cAMP with NeuN. The CA1 region of the hippocampus of sham surgery animals (C) and TBI animals 5 min after trauma (D) were immunostained for cAMP and NeuN. In TBI animals, cAMP levels were modestly reduced in NeuN-positive cells. Images are representative of 3 animals in each group. Scale bar, 50 μm.

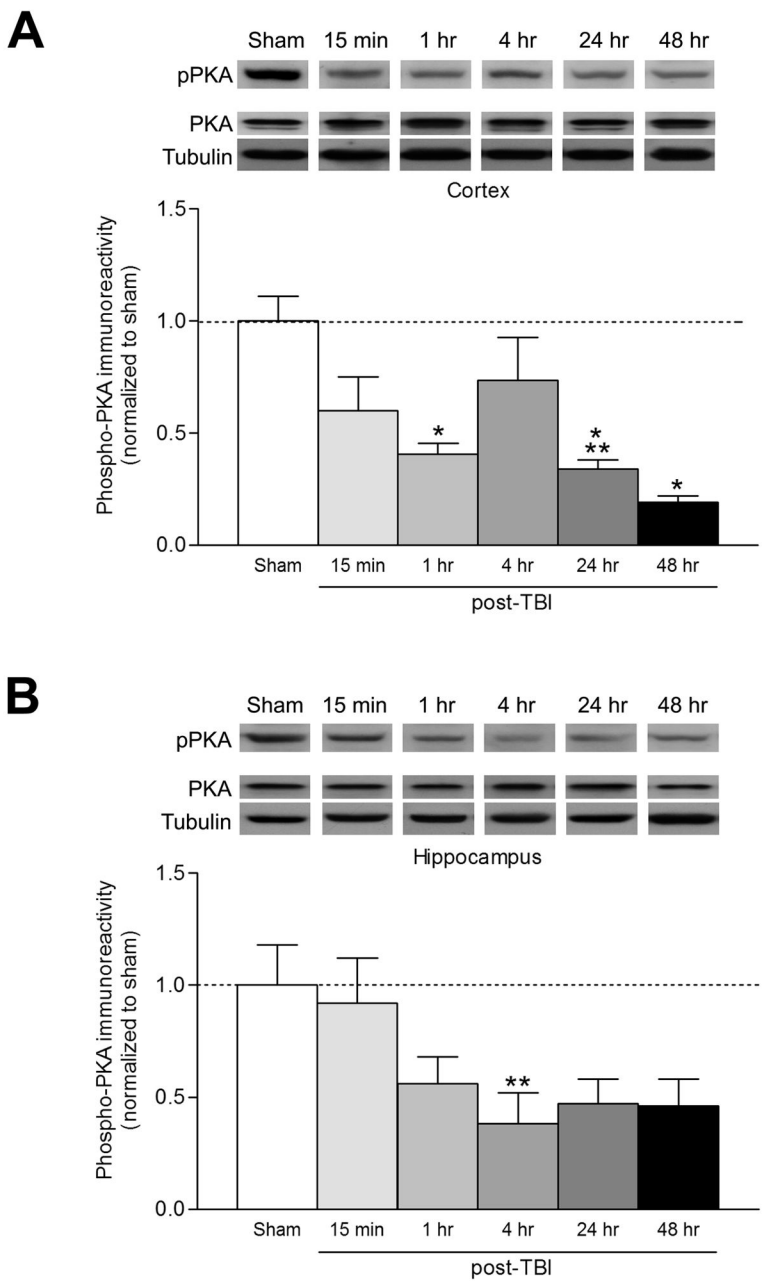

Fig 3.

PKA activation is downregulated after TBI. The ipsilateral parietal cortex (A; *n*=3–8) and hippocampus (B; *n*=3–6) were western blotted at the indicated times after TBI for activated, phosphorylated PKA (pPKA). PKA activation was decreased significantly in the cortex at 1 hr (*n*=5, **p*<0.05), 24 hr (*n*=3, ****p*<0.001), and 48 hr (*n*=5, **p*<0.05) after TBI as compared to sham animals. In the hippocampus, phosphorylated PKA levels were significantly decreased at 4 hr (*n*=7, ***p*<0.01) after TBI as compared to sham animals. Data represent mean ± SEM.

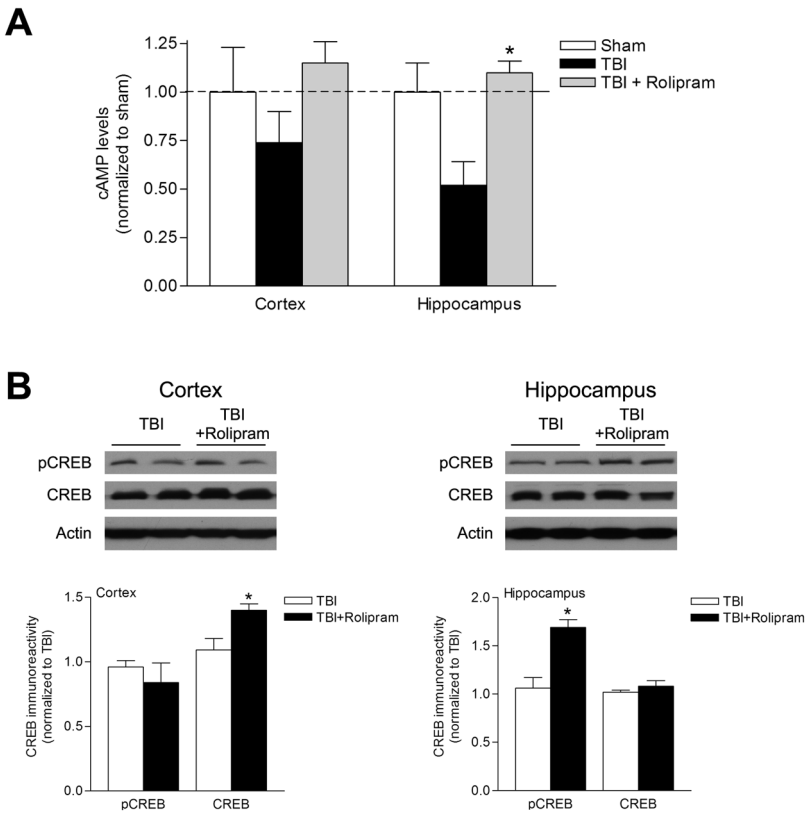

Fig. 4.

Rolipram treatment increased cAMP levels and phosphorylation of CREB. Animals were treated with vehicle (5% DMSO in saline) or rolipram (0.3 mg/kg, i.p.) 30 min prior to moderate parasagittal FPI. At 24 hr after injury, the animals were treated once more with vehicle or rolipram (0.3 mg/kg, i.p.), then sacrificed 30 min later. (A) cAMP levels were measured by ELISA in the ipsilateral parietal cortex and hippocampus. cAMP levels were increased to sham levels in the parietal cortex although this was not statistically significant (n=3 for each group). In the hippocampus, cAMP levels were significantly increased in TBI animals that received rolipram as compared to TBI animals that received vehicle (n=3 for each group, *p<0.05). (B) Phosphorylated CREB and total CREB levels were assayed by western blotting in the ipsilateral parietal cortex and hippocampus 24 hr after injury. Total CREB levels significantly increased in the parietal cortex in rolipram-treated animals as compared to vehicle-treated animals (n=3 for each group, *p<0.05). Phosphorylated CREB levels significantly increased in the hippocampus in rolipram-treated animals as compared to vehicle-treated animals (n=3 for each group, *p<0.05). Data represent mean ± SEM.

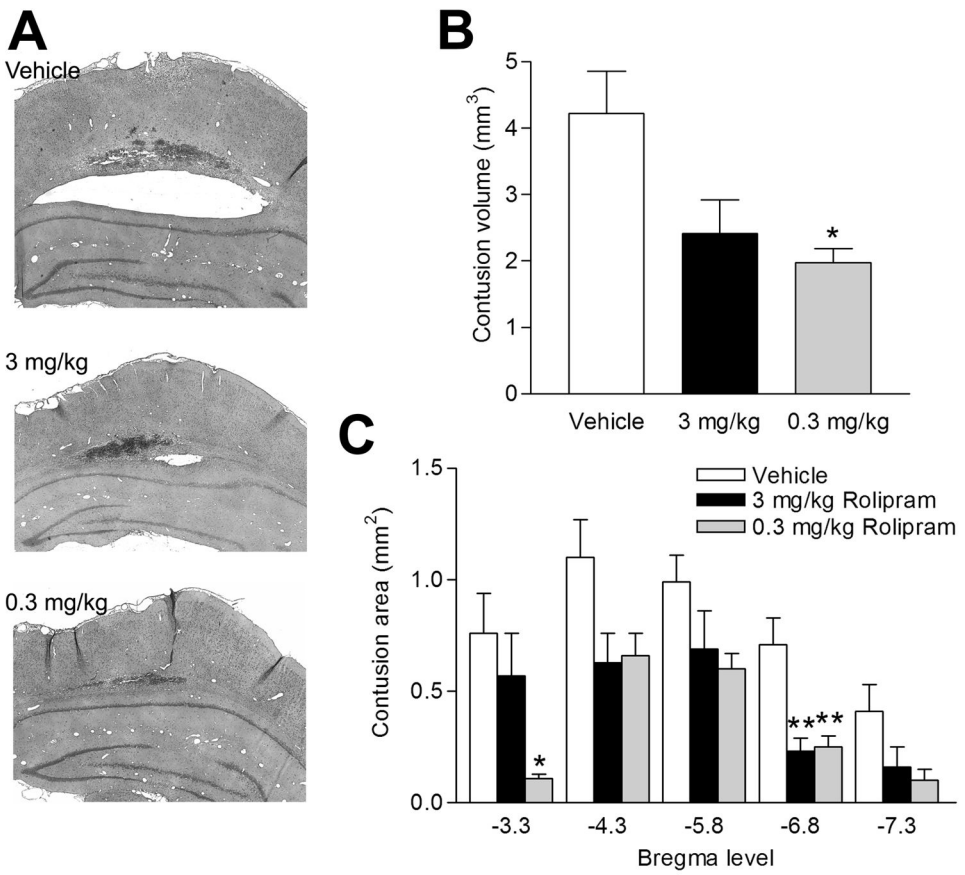

Fig 5.

Rolipram treatment decreased cortical contusions. Rats received vehicle (5% DMSO in saline) or rolipram i.p. 30 min prior to moderate parasagittal FPI. After TBI, the animals received vehicle or rolipram for 3 days and were then perfused for analysis at 30 min after their final injection. The brains were sectioned and stained with H&E, and the cortical contusion area was imaged. Representative sections at bregma level -5.8 mm are shown (A). Cortical contusion volume (B) and contusion areas at specific bregma levels (C) were quantified by stereology. The epicenter of the injury was at -3.8 mm bregma. The lower dose of rolipram, 0.3 mg/kg, significantly reduced total cortical contusion volume (n=6, *p<0.05) as compared to vehicle-treated animals and contusion area near the epicenter of the injury (bregma -3.3 mm, *p<0.05) as well as in the penumbra (bregma -6.8 mm, **p<0.01) as compared to vehicle-treated animals (n=9). Although the higher dose of 3.0 mg/kg rolipram reduced contusion volume as compared to vehicle-treated animals, this was not statistically significant (n=8). Data represent mean ± SEM.

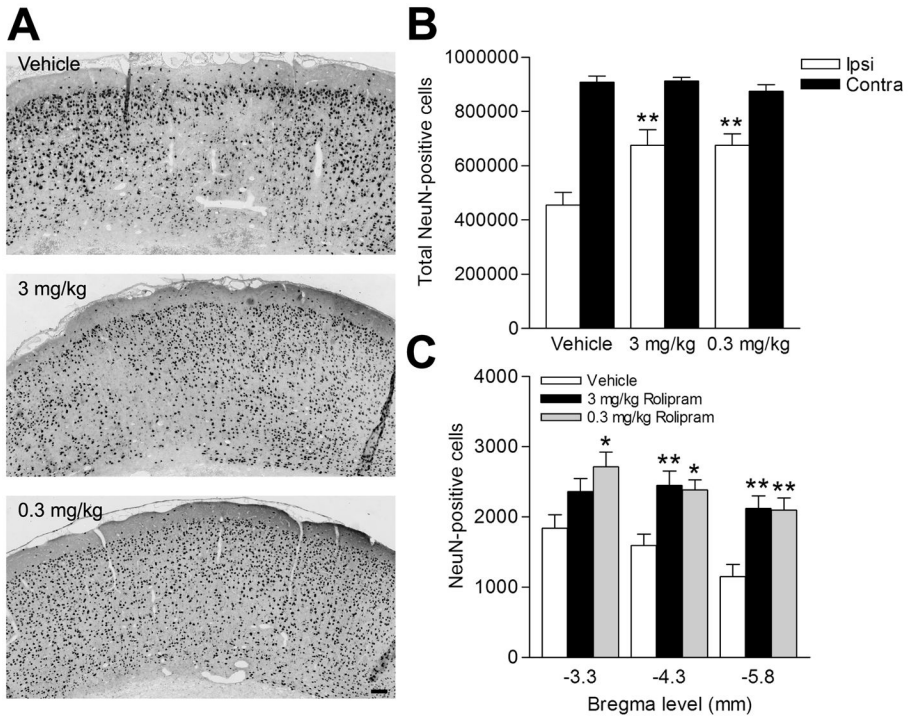

Fig 6.

Cortical neuron survival was improved with rolipram treatment. The parietal cortex overlying the contusion area between bregma levels -3.3 mm and -6.8 mm was immunostained with NeuN to identify surviving neurons. Animals were treated with vehicle, 0.3 mg/kg rolipram, or 3.0 mg/kg rolipram i.p. 30 min prior to injury, followed by once per day for 3 days. (A) Shown are representative images at bregma level -5.8 mm. (B) Total cortical neuron survival on the ipsilateral side (Ipsi) was significantly improved with both 0.3 mg/kg rolipram (n=5, $^{**}p<0.01$) and 3.0 mg/kg rolipram (n=8, $^{**}p<0.01$) as compared to vehicle-treated animals (n=9). There were no significant differences in total numbers of neurons on the contralateral side with rolipram treatment (Contra). (C) Quantification of neuronal survival at specific bregma levels illustrates that 3.0 mg/kg rolipram increased neuronal survival at bregma levels -4.3 mm and -5.8 mm (n=8, $^{**}p<0.01$) when compared to vehicle-treated animals (n=9). Rolipram at 0.3 mg/kg improved cortical neuron survival at all bregma levels tested (n=6; -3.3 mm, $^{*}p<0.05$; -4.3 mm, $^{*}p<0.05$; -5.8 mm, $^{**}p<0.01$). Data represent mean ± SEM. Scale bar, 50 μm.

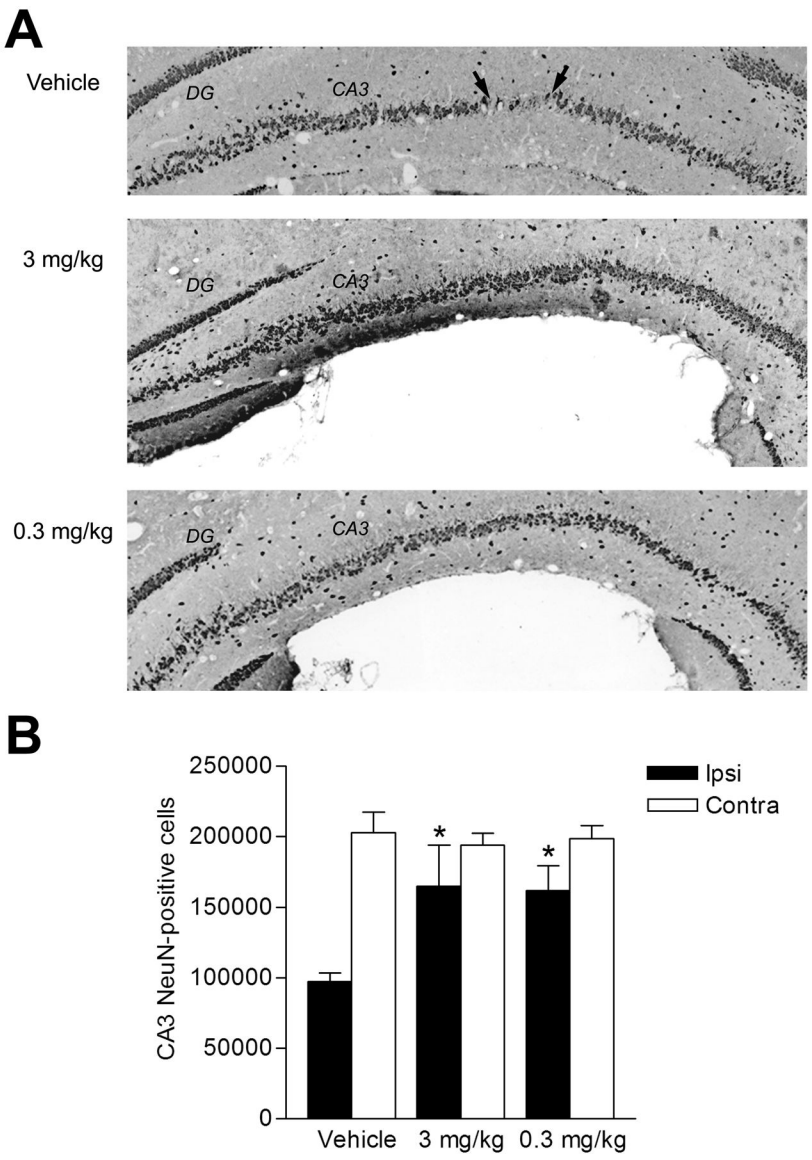

Fig 7.

Survival of CA3 hippocampal neurons with rolipram. (A) Animals received rolipram or vehicle 30 min prior to TBI, then once per day for 3 days. Sections were immunostained with NeuN. Bregma level -5.8 mm is shown. Arrows denote boundaries of an area of neuronal dropout. (B) Significant survival of CA3 neurons on the ipsilateral side (Ipsi) was seen with 0.3 mg/kg rolipram (n=4, *p<0.05) and 3.0 mg/kg rolipram (n=8, *p<0.05) as compared to vehicle-treated animals (n=9). There were no significant differences in neuronal survival on the contralateral side (Contra). Data represent mean ± SEM.

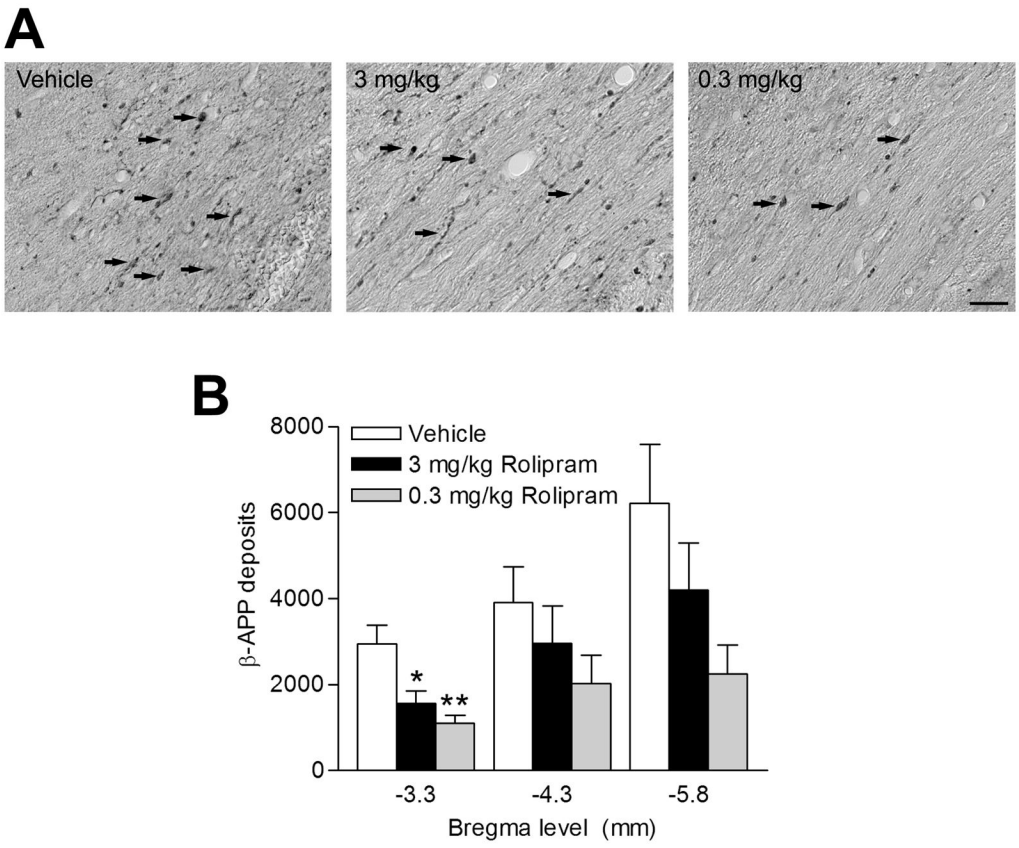

Fig 8.

The external capsule was stained with β-APP to assess axonal pathology. Animals were treated with vehicle, 0.3 mg/kg rolipram, or 3.0 mg/kg rolipram i.p. 30 min prior to injury and once per day for 3 days. (A) Shown is bregma level -5.8 mm. Arrows demarcate β-APP deposits in the external capsule. (B) Both 0.3 mg/kg (n=6, $^{**}p<0.01$) and 3.0 mg/kg rolipram (n=8, $^{*}p<0.05$) modestly reduced β-APP deposits at bregma level -3.3 mm as compared to vehicle-treated animals (n=8). Data represent mean ± SEM. Scale bar, 25 μm.

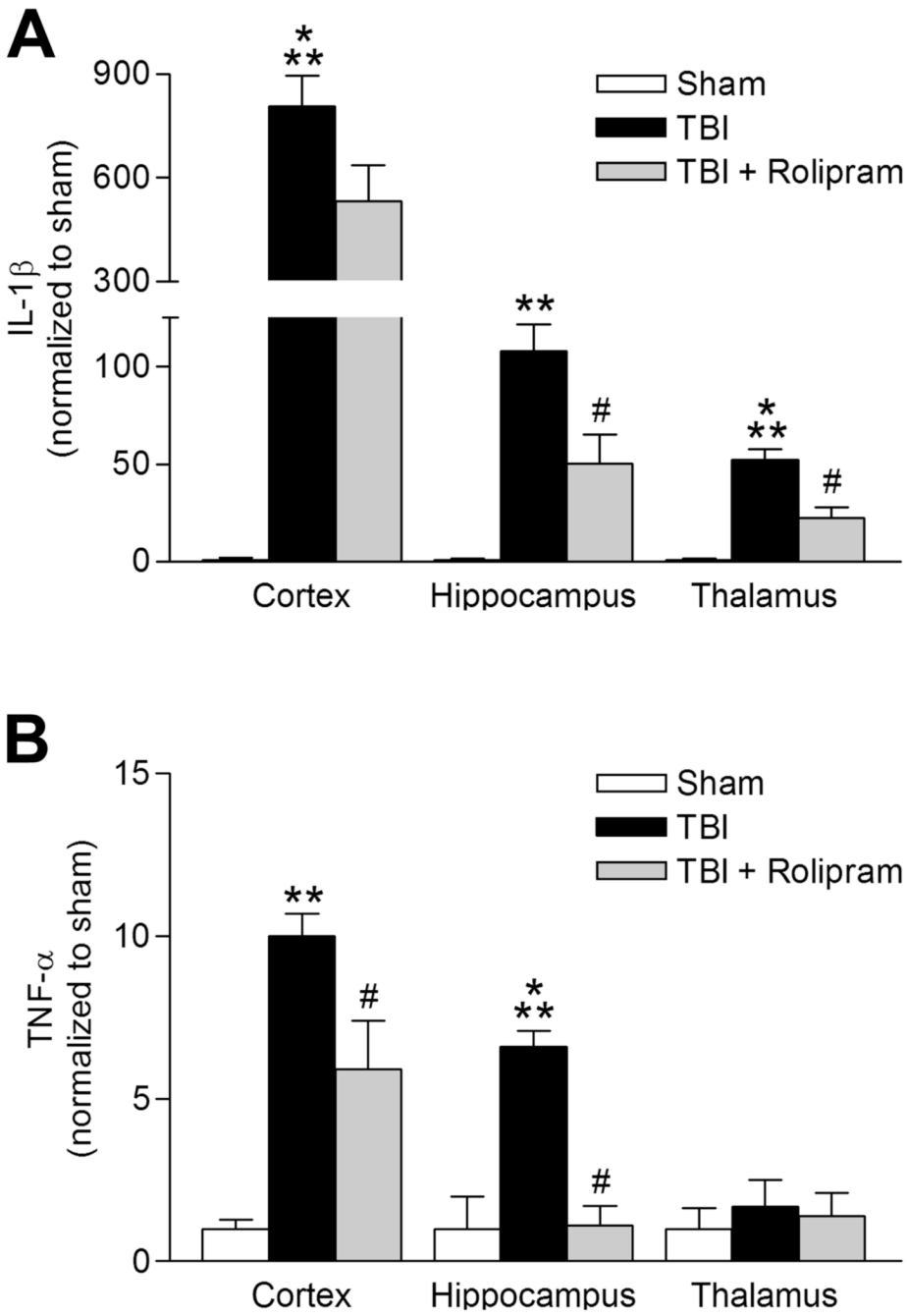

Fig 9.

Pro-inflammatory cytokine levels are attenuated with rolipram treatment. (A) IL-1β levels were assayed by ELISA at 3 hr after TBI. There was a significant increase in IL-1β levels in the ipsilateral parietal cortex (n=6, ***p<0.001), hippocampus (n=6, **p<0.01), and thalamus (n=8, ***p<0.001) as compared to sham animals (n=3–5). Rolipram treatment (0.3 mg/kg) 30 min prior to TBI and 30 min prior to sacrifice significantly reduced IL-1β levels in the hippocampus (n=7, #p<0.05) and thalamus (n=9, #p<0.001), but not in the parietal cortex (n=7), as compared to vehicle-treated TBI animals. (B) TNF-α levels significantly increased in the parietal cortex (n=6, **p<0.01) and hippocampus (n=6, ***p<0.001) at 3 hr after TBI as compared to sham animals. This increase in TNF-α was significantly reduced in rolipram-

treated TBI animals (parietal cortex n=7, #p<0.05; hippocampus n=6, #p<0.001) as compared to vehicle-treated TBI animals. Data represent mean ± SEM.