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Effects of rolipram on histopathological outcome after controlled cortical impact injury in mice

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Abstract

Traumatic brain injury (TBI) pathology includes contusions, cavitation, cell death; all of which can be exacerbated by inflammation. We hypothesized that an anti-inflammatory drug, rolipram, may reduce pathology after TBI, since in several CNS injury models rolipram reduces inflammation and improves cell survival and functional recovery. Adult male C57BL/6 mice received a craniotomy over the right parietotemporal cortex. Vertically-directed controlled cortical impact (CCI) injury was delivered. Naïve controls were used for comparison. At 30 min post-surgery, animals were treated with vehicle or rolipram (1 mg/kg), and then once per day for 3 days. On day 3, the brains were systematically sectioned and stained to visualize the resulting pathology using hematoxylin and eosin (H&E) staining and NeuN immunocytochemistry. Total parietotemporal cortical contusion and cavity volume were significantly increased in rolipram-treated as compared to vehicle-treated CCI animals. Contusion areas at specific bregma levels indicated a significant effect of drug across bregma levels. Neuronal cell loss in the dentate hilus and area CA3 of the hippocampus were similar between vehicle and rolipram-treated animals. Although rolipram is well known to reduce pathology and inflammation in several other CNS injury models, the pathology resulting from CCI was worsened with rolipram at this particular dose and administration schedule. These studies suggest that consideration of the unique characteristics of TBI pathology is important in the extrapolation of promising therapeutic interventions from other CNS injury models.

Keywords

cyclic AMP; contusion; controlled cortical impact; mice; phosphodiesterase; rolipram

1. Introduction

Traumatic brain injury (TBI) is a serious health problem affecting approximately 1.7 million Americans annually [8, 43]. Experimental and clinical research has delineated the etiology of many pathomechanisms of TBI and several potential therapeutics have been identified [23, 25, 31]. However, limited clinical studies have reported beneficial effects of various pharmacological treatments [22]. Thus, there is a need to evaluate novel therapeutic interventions that target a range of cellular and molecular events and promote protection and repair in the complex, heterogeneous TBI patient population.

One of the major injury mechanisms investigated in animal models of brain injury is activation of inflammatory pathways [26]. After trauma, blood-brain barrier (BBB)

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dysfunction is acutely observed as well as activation of inflammatory cells including microglia, invading neutrophils, and macrophages/monocytes [21]. Activation and recruitment of inflammatory cells into the injured brain generates inflammatory cytokines, free radicals and other damaging molecules [16, 42]. Clinical studies have corroborated the increases in pro-inflammatory cytokines and chemokines using biomarkers [11, 39]. Thus, a major area of continued therapeutic development is the identification and testing of novel drugs that target excessive inflammatory events acutely after injury [25].

Previous brain and spinal cord injury studies have investigated drug candidates that modulate cyclic AMP (cAMP) signaling by targeting type 4 phosphodiesterase (PDE) isoforms found in inflammatory cells and neurons [1, 14, 33]. Inhibition of PDE4 with rolipram decreases inflammation after CNS injury [4, 12, 30]. Recently, our laboratories have reported that rolipram reduced levels of the proinflammatory cytokines TNF α and IL-1 β when given immediately prior to fluid-percussion brain injury [3]. In that study, pretreatment with rolipram significantly reduced histopathological damage by decreasing contusion volume, hippocampal cell death and axonal injury. To move this potential therapeutic to a more translational model of TBI, the effects of post-injury treatment with rolipram were next evaluated. Rolipram treatment initiated 30 minutes after fluid-percussion brain injury again reduced TNF α levels, but did not significantly reduce contusion volume [2]. Histopathological outcome was actually worsened and associated with an increase in cortical hemorrhage and BBB permeability. This outcome could not be explained by any rolipram-induced systemic effects including acute hypotension. These results suggest that caution is required when considering rolipram as a post-injury therapeutic and indicates a critical need to further clarify the neuroprotective and anti-inflammatory potential of PDE4 inhibitors.

TBI is a highly heterogeneous clinical problem with a wide range of pathologies and behavioral consequences [22]. To investigate the different types of TBI, models of diffuse and focal injury are used with several animal species [23, 38]. In previous TBI studies with rolipram, we utilized the parasagittal fluid-percussion brain injury model which produces a cortical contusion associated with selective neuronal vulnerability and diffuse axonal pathology [3]. This model is treatable with several pharmacological agents, environmental enrichment, and therapeutic hypothermia [24, 28, 41]. Another model of brain injury is controlled cortical impact (CCI) injury [6]. This model produces well-demarcated cortical infarcts that mimic the contusions commonly observed in severe TBI patients. Because pathophysiological injury cascades are known to be dependent on impact severity and location, it is important to determine whether post-injury rolipram treatment would alter histopathological outcome in a second TBI model with distinct histopathological characteristics. The overall purpose of this study was therefore to evaluate whether rolipram, at a dose previously found to improve outcome after CNS injury, would reduce histopathological damage when given 30 minutes after CCI injury in mice [4, 15, 19].

2. Materials and methods

All surgical 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. Adult C57BL/6 male mice (2–3 months, Charles River Laboratories, Wilmington, MA, USA) were anesthetized with ketamine and xylazine (intraperitoneal, i.p.). Body temperature was monitored with a rectal probe and maintained at 37°C. A 5 mm craniotomy was made over the right parietotemporal cortex (–2.5 mm posterior, 2.0 mm lateral from bregma). Injury was induced using the ECCI-6.3 device (Custom Design & Fabrication, Richmond, VA, USA) with a 3 mm impounder at 6 m/s velocity, 0.5 mm depth, 150 ms impact duration [6, 40].

Rolipram (Sigma-Aldrich, St. Louis, MO, USA) was administered at 1 mg/kg (i.p.) 30 minutes post-injury [2]. Rolipram or vehicle (6 ml/kg, 5% DMSO in saline) were given once per day for 3 days ($n=6$ mice/treatment).

At 3 days after TBI, animals were anesthetized and perfused transcardially with 4% paraformaldehyde and brains were paraffin embedded and sectioned at 10 μ m thick. Serial sections (150 μ m apart) were stained with hematoxylin and eosin (H&E). Contusion and cavity volumes were assessed by an investigator blinded to the treatments by tracing H&E stained sections using Neurolucida 10.50 (MicroBrightField, Williston, VT, USA) and an Olympus BX51TRF microscope (Olympus America, Center Valley, PA, USA) with a 4 $\times$ objective. Cavity boundaries were determined by tracing the contralateral cortex, and projecting the inverted contour over the ipsilateral cortex. Contusion boundaries were demarcated by pyknotic neurons, reactive astrocytes, hemorrhage, and edema. Contusion areas were calculated for four coronal levels at or around the epicenter (−1.1, −1.6, −2.7, −3.3 mm posterior from bregma). Images were taken at 20 $\times$.

To determine hippocampal neuronal survival, adjacent sections (150 μ m apart) were immunostained with NeuN (1:500, Millipore, Temecula, CA, USA). Immunostaining was developed with anti-mouse IgG (1:1000, Vector Laboratories, Burlingame, CA, USA), ABC elite (Vector Laboratories) and NiDAB (2.5%, nickel ammonium sulfate acetate-imidasole buffer, 0.05% DAB, 0.001% H₂O₂).

NeuN-positive cells were quantified using stereology and an Axiovert 200M microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). The dentate hilus and area CA3 of the hippocampus were contoured at 5 $\times$ magnification between bregma levels −1.6 to −2.7 mm (dentate hilus) or between bregma levels −1.9 to −2.1 mm (CA3) [3]. Using StereoInvestigator (10.50, MicroBrightField), a 40 $\times$ 65 μ m grid was placed over the hilus and a 55 $\times$ 110 μ m grid was placed over area CA3. Neurons were counted within a 35 $\times$ 35 μ m frame at 100 $\times$ (NA 1.30). Q values for the dentate hilus were 69–192 (mean 122 $\pm$ 6.6), the CE²/CV² ratio for vehicle-treated animals was 0.20 (ipsilateral) and 0.33 (contralateral), and the CE²/CV² ratio for rolipram-treated animals was 0.82 (ipsilateral) and 0.14 (contralateral). Q values for CA3 were 80–343 (mean 173 $\pm$ 10.4), the CE²/CV² ratio for vehicle-treated animals was 0.17 (ipsilateral) and 0.05 (contralateral), and the CE²/CV² ratio for rolipram-treated animals was 0.30 (ipsilateral) and 1.24 (contralateral). Images were taken at 20 $\times$.

Data are mean $\pm$ SEM. Contusion volumes were analyzed using Students t-test. Contusion areas were analyzed with a repeated measures two-way ANOVA (factors: group $\times$ bregma level), followed by Bonferroni t-tests. Dentate hilus and CA3 cell counts were analyzed using a two-way ANOVA with hemisphere (ipsilateral vs contralateral) and drug (vehicle vs rolipram) as the factors and post-hoc Bonferroni t-tests.

3. Results

At 3 days after CCI injury, a well demarcated contusion and cavity were observed in both treated and non-treated TBI animals. Rolipram increased contusion volume after CCI injury as compared to vehicle-treated controls (Fig. 1). In addition, contusion areas at specific bregma levels (−1.1, −1.6, −2.7, −3.3 mm) were analyzed to evaluate the treatment effects at multiple brain regions. A main effect of drug ($F_{(1,10)}=7.42$, $P=0.0214$) was observed. No main effect of bregma level and no significant interaction of bregma level and drug were detected.

A consistent pathology associated with CCI injury is the selective vulnerability of dentate hilar neurons. Quantitative assessment of dentate hilar cells demonstrated a significant

decrease in the number of NeuN-positive cells in the ipsilateral versus contralateral hemispheres (Fig. 2). There was a main effect of hemisphere ($F_{(1,20)}=22.48$, $P=0.0001$), but no main effect of drug and no significant interaction of drug and hemisphere. These results indicate that rolipram did not affect overall dentate hilus cell loss.

Area CA3 of the hippocampal region is another area of vulnerability after CCI injury. Small pockets of neuronal loss were observed with CCI injury on the ipsilateral side, and this was unaffected by rolipram treatment (Fig. 3). A main effect of hemisphere was observed ($F_{(1,20)}=17.85$, $P=0.0004$), and no significant main effect of drug or interaction of drug and hemisphere was detected. These results indicate that rolipram treatment after CCI injury has no significant effect on neuronal loss in area CA3 and dentate hilus in the hippocampus, but did exacerbate contusional size in the cortex.

4. Discussion

In this study, we report that the PDE4 inhibitor rolipram in a mouse model of CCI injury does not improve histopathological outcome when given posttrauma. This negative study is consistent with our previous report showing that posttraumatic rolipram treatment after moderate fluid-percussion brain injury also fails to protect against neuronal damage [2]. Together, these studies emphasize the continued need to examine the potential role of phosphodiesterase inhibition in preclinical models of TBI.

Previous studies in cerebral ischemia and spinal cord injury reported beneficial effects of rolipram even in the post-injury setting [4, 19, 27, 30]. However, with any pharmacological study, there could be side effects that abrogate the beneficial effects. In the case of rolipram, its effects on multiple PDE4 isoforms may have participated in the negative results of the present and previous TBI studies. For example, rolipram can potentially affect hemodynamic injury responses that complicate the usefulness of this drug [5, 36, 45]. In previous studies utilizing rats, rolipram did not significantly affect mean arterial blood pressure, blood pO_2 levels or cerebral blood flow [2, 3]. However, a previous study using mice found that rolipram decreased systemic arterial blood pressure [36]. Thus, it is possible rolipram may have exacerbated the cortical contusion by decreasing local cerebral blood flow to the vulnerable cortex [34]. Alternatively, rolipram may have contributed to progressive expansion of the hemorrhagic contusion. Upregulation of sulfonylurea receptor 1 (Sur1) in microvessels has been linked to hemorrhage expansion after TBI and experiments exploring the connection of PDE4 signaling and Sur1 may shed light on the molecular mechanisms of the increase in cortical contusion expansion observed with rolipram [29, 37]. More basic research is needed to understand which PDE4 isoforms target vascular integrity and how rolipram could produce adverse effects in neurotrauma that lead to vascular vulnerability [45].

Rolipram affects multiple PDE4 isoforms and therefore may target multiple cell types and biological processes [10]. The PDE4 family includes 20 isozymes encoded by four genes (PDE4A, B, C and D) [13]. Loss of specific PDE4 subtypes enhances memory and promotes neurogenesis [20]. The identification of novel PDE4 inhibitors that selectively inhibit specific isoforms may be more suitable for pharmacological protection after TBI and other CNS injuries [10]. For example, PDE4 inhibition is currently being investigated as a therapeutic for depression and age-related cognitive decline [32, 44]. Published data has emphasized that the PDE4A and B isoforms may be the most relevant for targeting these quality of life issues [10]. Because higher cortical functions including cognition and depression are important consequences of TBI, it will be important in future studies to test more selective PDE4 inhibitors to determine their efficacy in TBI [7, 9].

In this study, we utilized a standard model of TBI with differential characteristics compared to the fluid-percussion brain injury. CCI produces a well demarcated, focal contusion that extends from the pial surface down to deep cortical layers [6]. This is associated with severe perfusion deficits at the cortical contusion [18]. We also used mice in this study in contrast to our published work with rat fluid-percussion brain injury [2, 3]. The testing of various therapeutics in different injury models as well as species are important considerations for translation to the clinic [17]. The fact that posttraumatic treatment with rolipram in two models using different animal species produced negative effects emphasizes the need for additional work in this area.

In the present study, we used a dose and therapeutic route that we have found in our previous TBI study and has been shown in other CNS injury paradigms to improve histological outcome [4, 12, 15, 19]. A recent study in spinal cord injury assessed a wide range of rolipram doses for efficacy in improving pathology [35]. In that study, rolipram doses ranging from 0.1 to 5 mg/kg improved neuronal survival with 1 mg/kg being the most efficacious, which is the dose used in this study. In addition, it is possible that rolipram may be more effective in other therapeutic routes. In our previous TBI study and in other studies of cerebral ischemia, intraperitoneal administration of rolipram significantly improved histopathological outcome and behavioral recovery [3, 4, 12, 15]. However, a recent spinal cord injury study reported that although rolipram improved neuronal survival when given intravenously, orally, or subcutaneously, the most effective route was intravenous [35]. Additional studies are needed to test extensive dose responses and other administrative routes to maximize the chances of rolipram or other PDE4 inhibitors having a beneficial effect on their targeted pathomechanisms especially when given in the post-injury setting.

5. Conclusions

In summary, rolipram treatment failed to improve outcome after CCI injury in mice. The fact that the treatment actually increased some degree of histopathological damage in this model is important to consider as PDE4-related therapies move forward for CNS injury. The investigation of more selective PDE4 isoform-specific inhibitors is an important area for further studies. It is anticipated that as more information regarding the cellular and biochemical response to TBI is clarified, new selective blockers will be developed related to cAMP-related cell signaling cascades critical to cell survival and neuronal function.

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Abbreviations

BBB	blood-brain barrier
CCI	controlled cortical impact
cAMP	cyclic AMP
H&E	hematoxylin and eosin
i.p.	intraperitoneal
PDE	phosphodiesterase
Sur1	sulfonylurea receptor 1
TBI	traumatic brain injury

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Fig. 1. Cortical contusions after CCI injury in mice. Representative images of H&E stained sections at -2.4 mm posterior from bregma for vehicle-treated (A) and rolipram-treated (B) mice. Scale bar 500 μ m. Magnification of the contused cortex for vehicle (C) and rolipram (D) treatment. The contoured contusion and cavity areas are denoted by the dashed lines. Scale bar 250 μ m. Total contusion volume quantification (E) and contusion areas across bregma levels (F). Mean $\pm$ SEM, $n=6$ /group, ** $p<0.01$ for CCI+vehicle versus CCI+rolipram.

Fig. 2. Rolipram did not affect dentate hilar cell loss. Representative images of the dentate hilus immunostained with NeuN at bregma level -2.1 mm for vehicle-treated (**A**, **B**), and rolipram-treated (**C**, **D**) mice. Scale bar 100 μ m. Estimated numbers of dentate hilus NeuN-positive cells between bregma levels -1.6 to -2.7 mm (**E**). Mean $\pm$ SEM, $n=6$ /group.

Fig. 3.

Neuronal loss in area CA3 after CCI injury is not affected by rolipram treatment. Images taken at bregma level -1.9 mm for the ipsilateral side of vehicle-treated (**A**) and rolipram-treated animals (**B**) indicate small areas of neuronal loss (*arrows*). No significant cell loss was observed on the contralateral side with either vehicle (**C**) or rolipram treatment (**D**). Scale bar $100\ \mu\text{m}$. Quantification of CA3 cell loss between bregma levels -1.9 to -2.1 mm indicated a significant loss of neurons in both vehicle- and rolipram-treated animals on the ipsilateral side (**E**). Mean $\pm$ SEM, $n=6$ /group.