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Rolipram attenuates acute oligodendrocyte death in the adult rat ventrolateral funiculus following contusive cervical spinal cord injury

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ABSTRACT

Rolipram, an inhibitor of phosphodiesterase 4 (PDE4) proteins that hydrolyze cAMP, increases axonal regeneration following spinal cord injury (SCI). Recent evidence indicate that rolipram also protects against a multitude of apoptotic signals, many of which are implicated in secondary cell death post-SCI. In the present study, we used immunohistochemistry and morphometry to determine potential spinal cord targets of rolipram and to test its protective potential in rats undergoing cervical spinal cord contusive injury. We found that 3 PDE4 subtypes (PDE4A, B, D) were expressed by spinal cord oligodendrocytes. OX-42 immunopositive microglia only expressed the PDE4B subtype. Oligodendrocyte somata were quantified within the cervical ventrolateral funiculus, a white matter region critical for locomotion, at varying time points after SCI in rats receiving rolipram or vehicle treatments. We show that rolipram significantly attenuated oligodendrocyte death at 24 h post-SCI continuing through 72 h, the longest time point examined. These results demonstrate for the first time that spinal cord glial cells express PDE4 subtypes and that the PDE4 inhibitor rolipram protects oligodendrocytes from secondary cell death following contusive SCI. They also indicate that further investigations into neuroprotection and axonal regeneration with rolipram are warranted for treating SCI.

Spinal cord injury (SCI) consists of an irreversible primary injury followed by a secondary injury cascade that promotes additional cell death further reducing the chance for functional recovery. The secondary injury is a manifestation of many processes including excitotoxicity [42], calcium overload [51], oxidative stress [1], and inflammation [23], all which lead to apoptosis and the unnecessary death of potentially viable cells. Recent evidence suggests that inhibition of phosphodiesterase 4 (PDE4), a protein family responsible for cAMP hydrolysis [31], with the drug rolipram [55] provides protection against a multitude of apoptotic insults including reduction of caspase-3 activity, a downstream mediator of multiple apoptotic cascades [10]. While earlier studies targeting PDE4 inhibition with rolipram have demonstrated its success in aiding axonal regeneration following SCI [17,38,44], the protective effects of this treatment are largely unknown.

Spared axon demyelination occurs in human and experimental SCI [15,52]. A previous study using rolipram revealed increased numbers of oligodendrocyte-myelinated axons in the adult rat spinal cord white matter months after contusive SCI [44]. This could have been due to decreased oligodendrocyte death since they are highly vulnerable to secondary injury processes [11,12]. In particular, excitotoxicity [22,34] and tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine [26,50] are highly toxic to oligodendrocytes. Coincidentally, both have been implicated in augmenting PDE4 expression [16,32]. Furthermore, rolipram decreased TNF-α production [21,44,57] as well as protected a cell line of immortalized, O-2A derived oligodendrocyte-like cells from excitotoxicity [58,59], providing an additional benefit of rolipram treatment. Thus, in the present study we addressed whether rolipram prevents secondary death of oligodendrocytes in a rat model of contusive cervical SCI [39], the most frequent type of human SCI [6]. Using immunohistochemistry and morphometry, we show that oligodendrocytes and microglia co-express PDE4 subtypes providing

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two potential targets of rolipram. Moreover, we demonstrate the protective effect of rolipram on oligodendrocytes in the ventrolateral funiculus (VLF), a white matter region critical for locomotion [28,29].

All methods were approved by the Institutional Animal Care and Use Committee at the University of Louisville. They were conducted to minimize pain and discomfort as well as in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and with the Policies on the Use of Animals and Humans in Neuroscience Research. Twenty-eight (4 normal and 24 injured) adult (228 g to 267 g) female Sprague–Dawley rats (Charles River Laboratories, Inc., Wilmington, MA) were housed individually throughout the experiment and maintained on a 12-h light–dark cycle.

The rats were anesthetized with sodium pentobarbital (40–50 mg/kg, IP). The dorsal halves of the C3–C6 vertebrae were exposed and transverse vertebral process supports [40] were placed bilaterally at the C4–C5 vertebrae. Laminectomies were performed to expose the dura overlying the dorsal surfaces of the C5–C6 spinal cord segments between the C5 and C6 dorsal root entry zones. Contusive injuries of 180 ± 7 actual kilodynes were produced dorsal to ventral at the C5–C6 segments with a 3.7 mm diameter tip and an Infinite Horizon Impactor [39,49]. Two model 2002 (0.5 μl/h) ALZET® mini-osmotic pumps (DURECT Corp., Cupertino, CA) were inserted subcutaneously and bilaterally over the ribs adjacent to the vertebral column following the injury. Animals were randomly assigned prior to surgery to a group of 12 rats that received rolipram (0.5 mg/kg/day, Sigma, St. Louis, MO) [44], dissolved in DMSO (Sigma) or to a control group of 12 rats that received only DMSO. The treatments were administered for the duration of the experiment. Muscle and skin incisions were closed with silk sutures and wound clips, respectively. Post-operative care included Gentozen™ (10 mg/kg, IM, Schering-Plough Animal Health, Omaha, NE) antibiotic, topical Bacitracin Zinc Ointment USP (E. Fougera & Co., Melville, NY), and 5% dextrose in lactated Ringer's solution (5 ml, SC, Baxter Healthcare Corp., Deerfield, IL). All rats were largely immobile after SCI. For veterinary care, lactated Ringer's solution was injected subcutaneously plus Ensure® and cereal were provided to maintain hydration and attenuate body-weight loss. Bladders were emptied at least once daily with gentle pressure and gastrointestinal function was monitored daily. One rat was excluded from the rolipram-treated group due to morbidity following injury.

All rats were anesthetized with sodium pentobarbital (120 mg/kg, IP) 12, 24, or 72 h post-SCI. Transcardial perfusions were performed with heparinized, oxygenated, and calcium-free Tyrodes solution, followed by 0.1 M phosphate buffer, pH 7.4 (PB), containing 4% paraformaldehyde, and lastly with PB. Cervical spinal cords were removed and cryoprotected in PB containing 30% sucrose at 4 °C for 3–4 days. They were sectioned at 20 μm in the transverse plane with a cryostat. Sections mounted onto charged microscope slides were stained with 0.5% cresyl violet (Sigma) [39] to view the morphology of the SCI site or immunostained [33] to visualize oligodendrocytes, microglia, PDE4 expression, and cAMP-dependent phosphorylated PKA substrates. Following rinses in Tris-buffer with 0.9% saline, pH 7.4 (TBS), sections were blocked with TBS containing 0.05% Triton X-100 (TBST) and 10% normal donkey serum (NDS, Jackson Immuno, West Grove, PA) for 1 h at room temperature. They were next incubated overnight at 4 °C with TBST containing 10% NDS and combinations of mouse anti-adenomatus polyposis coli (APC, 1:150, Calbiochem, San Diego, CA) to identify mature oligodendrocytes, mouse anti-OX-42 (1:200, BD Biosciences, San Jose, CA) to identify microglia, as well as rabbit anti-PDE4A (1:100, FabGennix Inc., Frisco, TX), rabbit anti-PDE4B (1:150, FabGennix Inc.), rabbit anti-PDE4D (1:150, FabGennix Inc.), and rabbit anti-phospho-(Ser/Thr) PKA substrates (pPKA, 1:200, Cell Signaling Tech., Danvers, MA). Following rinses, sections were incubated for 1 h with TBST and 5% NDS containing combinations of species-specific donkey IgG antibodies (Jackson ImmunoResearch Laboratories Inc.) conjugated with flourescein isothiocyanate (FITC, 1:200) or cyanine 3 (Cy3, 1:200). Lastly, the sections were coverslipped with Mowiol mountant and stored at 4 °C.

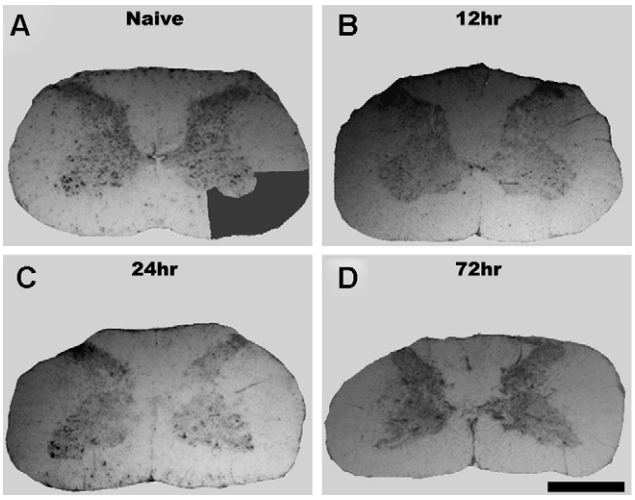

Fig. 1. Acute pathology of contusive cervical SCI. Representative cresyl violet stained, transverse sections of uninjured naïve (A), 12 (B), 24 (C), and 72 (D) h post-SCI. Shaded region in (A) indicates VLF. Scale bar = 1 mm.

Confocal images of the VLF at each C5-C6 SCI site and at a similar location in normal rats (Fig. 1A) were obtained using an Olympus laser confocal microscope and digitized with an Olympus Optical (Mellville, NY) laser Fluoview 500 software [33]. Adobe photoshop v9.02 (Adobe Systems Inc., San Jose, CA) was used to sharpen the images, adjust brightness and contrast, and compose the final images. Images of spinal cords from all rats were used to visualize PDE4 co-expression with oligodendrocytes. For PDE4 co-expression with microglia, images of sections from spinal cords at 3 days post-injury were used to ensure that activated microglia were present [24,46]. To quantify the numbers of oligodendrocyte somata in each rat, randomly selected left or right side VLF in 2 [36] APC and pPKA immunostained images that were 200 μm apart [7] at each C5–C6 SCI site and at a similar location in the normal rats were converted into black and white images then color inverted [27]. The total number of APC-immunopositive oligodendrocyte somata with pPKA-immunopositive nuclei in each section was quantified using ImageJ software (v.1.32j, National Institutes of Health) then converted to cells/cm^2. APC-immunopositive cells within the gray matter were excluded from quantification [4]. The oligodendrocyte numbers found in both sections of each rat were averaged. After Levene's test for equality of variances did not uncover significant differences, oligodendrocyte cell counts of the groups were compared using a 2x3 ANOVA followed by Tukey's HSD post hoc *t*-tests when appropriate with SPSS v.13.0 (SPSS, Chicago, IL) statistical software.

To locate potential spinal cord targets of rolipram treatment, we used double labeling with PDE4 sub-family-specific and glia-specific antibodies. Immunofluorescence revealed that APC-immunopositive oligodendrocytes throughout the cervical spinal cord white matter co-expressed all three PDE4 subtypes (PDE4A, B, D) (Fig. 2A–D). Additionally OX-42-immunopositive microglia at 3 days post-SCI, which are a major source of the pro-inflammatory cytokine TNF-α [2,45], expressed only the

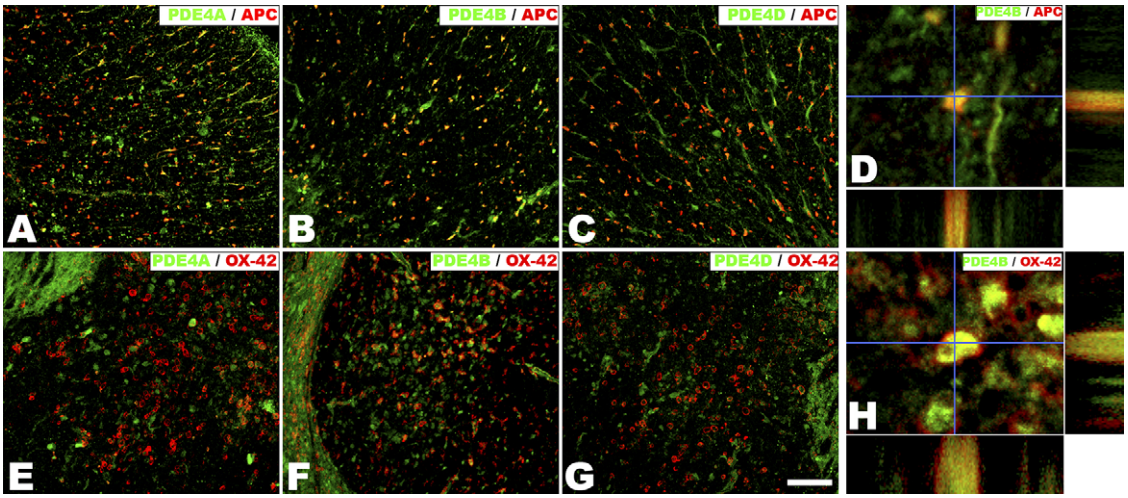

Fig. 2. Oligodendrocytes and microglia/macrophages in the adult rat cervical spinal cord VLF are co-labeled with PDE4 subtypes. Representative transverse sections of the 3-day injured adult rat C5-C6 spinal cord VLF immunostained in combination for PDE4A (A, E), PDE4B (B, F), or PDE4D (C, G) and APC (A–C) or OX-42 (E–G). APC-immunopositive oligodendrocyteswere co-labeled with all three PDE4s, whereas OX-42-immunopositive microglia/macrophages only were co-labeled with PDE4B. Colocalization is confirmed in orthogonal views of PDE4B with APC (D) and OX-42 (H). Scale bar = 100 m.

PDE4B subtype (Fig. 2E–H). Coincidentally, PDE4 knockout studies revealed that lipopolysaccharide induced TNF-α production and secretion were dependent upon PDE4B, not PDE4A or PDE4D [20,21]. By maintaining and elevating their cAMP levels after SCI, rolipram may exert its effect directly in oligodendrocytes to reduce intrinsic apoptotic signaling cascades and/or indirectly by attenuating the inflammatory response of adjacent microglia, particularly the reduction of pro-inflammatory cytokine, TNF-α.

To determine the protective potential of rolipram on oligodendrocytes, we assessed their survival in the cervical spinal cord VLF at 12, 24, and 72 h post-SCI (Figs. 1 and 3). At 12 h post-SCI, both rolipram-treated rats (156.6 ± 28.4) and DMSO-treated rats (160.3 ± 28.7) had similar numbers of oligodendrocytes to each other and to those of normal rats (167.1 ± 34.0). This provides evidence that these cells had survived for 12 h after the primary mechanical injury. There was a significant reduction of oligodendrocytes in both rolipram-treated rats (125.1 ± 12.6) and DMSO-treated rats (101.1 ± 13.1) at 24 h post-SCI. However, rolipram treatment significantly attenuated the oligodendrocyte death compared to DMSO-treated rats at this time point and through 72 h post-SCI (140.0 ± 24.1 vs. 98.6 ± 12.1). These data provide evidence that rolipram significantly protects oligodendrocytes from secondary injury following a contusive SCI.

Previous studies employing rolipram as a treatment have indicated its effectiveness in promoting regeneration following SCI through the inhibition of myelin associated glycoproteins [17,38,44]. The present study examined the use of rolipram treatment for oligodendrocyte protection following contusive cervical SCI. It demonstrates that (1) all PDE4 subtypes are co-expressed by oligodendrocytes, (2) OX-42 positive microglia co-express only the PDE4B subtype, and that (3) rolipram attenuates secondary oligodendrocyte death.

Similar to previous findings in rats after contusive SCI [9,11,13,25,35,47,53], we report significant oligodendrocyte death at 24 h post-SCI. Previous literature using a similar model reported drastic reduction in spinal cord cAMP to ∼60% that of normal levels at 24 h post-injury [44]. While the cause behind this reduction has yet to be fully delineated, it has been proposed that increased inflammation, particularly TNF-α-mediated [3,56], results in decreased cAMP [43]. This decrease could be due to changes in PDE4 expression or activity [20,21,32] since PDE4 expression levels were over 4-fold higher acutely post-SCI [8]. One possible mechanism of increased PDE4 expression could be through NF-κB activation after SCI [3,41], a known downstream target of TNF-α [5] and promoter of PDE4 transcription [54].

In vitro analysis of excitotoxic oligodendrocyte death reveals a protective role of maintaining and/or elevating cAMP levels with rolipram and/or cAMP analogues [58,59]. While it is unclear whether excitotoxicity has an effect on PDE4, it has been recently proposed that low concentrations of NMDA produce increased PDE4 protein expression and activity [16]. Consistent with this notion, experimental decreases in cAMP augment excitotoxic cell death [19,58]. Moreover, TNF-α also exacerbates excitotoxicity [37].

Thus, it could be hypothesized that the PDE4-mediated reduction in cAMP as a result of inflammation and/or excitotoxicity increases the vulnerability of oligodendrocytes. To test this hypothesis, we administered the PDE4 inhibitor rolipram after contusive cervical SCI at a dose previously demonstrated to maintain basal levels of cAMP [44]. We found increased numbers of oligodendrocytes at 24 h post-SCI persisting through 72 h (the longest time point examined). This suggests that in addition to facilitating axonal regeneration [17] there is also a protective effect of maintaining cAMP levels.

A myriad of events occur after SCI that lead to secondary cell death. In addition to inflammation and excitotoxicity, PDE4 may also be affected by other processes including increased oxidative stress [18] and $p75^{ntr}$ regulated cell death [48]. Likewise, oligodendrocytes are not the only cell types affected by apoptosis. Neurons are also vulnerable [14] and are thought to undergo similar events leading to apoptosis [13]. We observed expression of PDE4s by ventral horn motor neurons (data not shown). Whether rolipram plays a protective role on neurons following SCI warrants further investigation.

Our results demonstrate two potential targets of rolipram treatment, oligodendrocytes and microglia. Secondly, we provide evidence that rolipram treatment attenuates secondary death of oligodendrocytes within the VLF, a white matter region critical for locomotion. Additional protection might be obtained using larger doses of rolipram or combinatorial approaches, such as with neurotrophin-3 which when combined with cAMP elevating agents was shown to be beneficial in aiding axonal regeneration [30]. Also, further investigations into the mechanism(s) behind rolipram-mediated protection are essential for this, and newly developed PDE4 inhibitors, to effectively treat SCI.

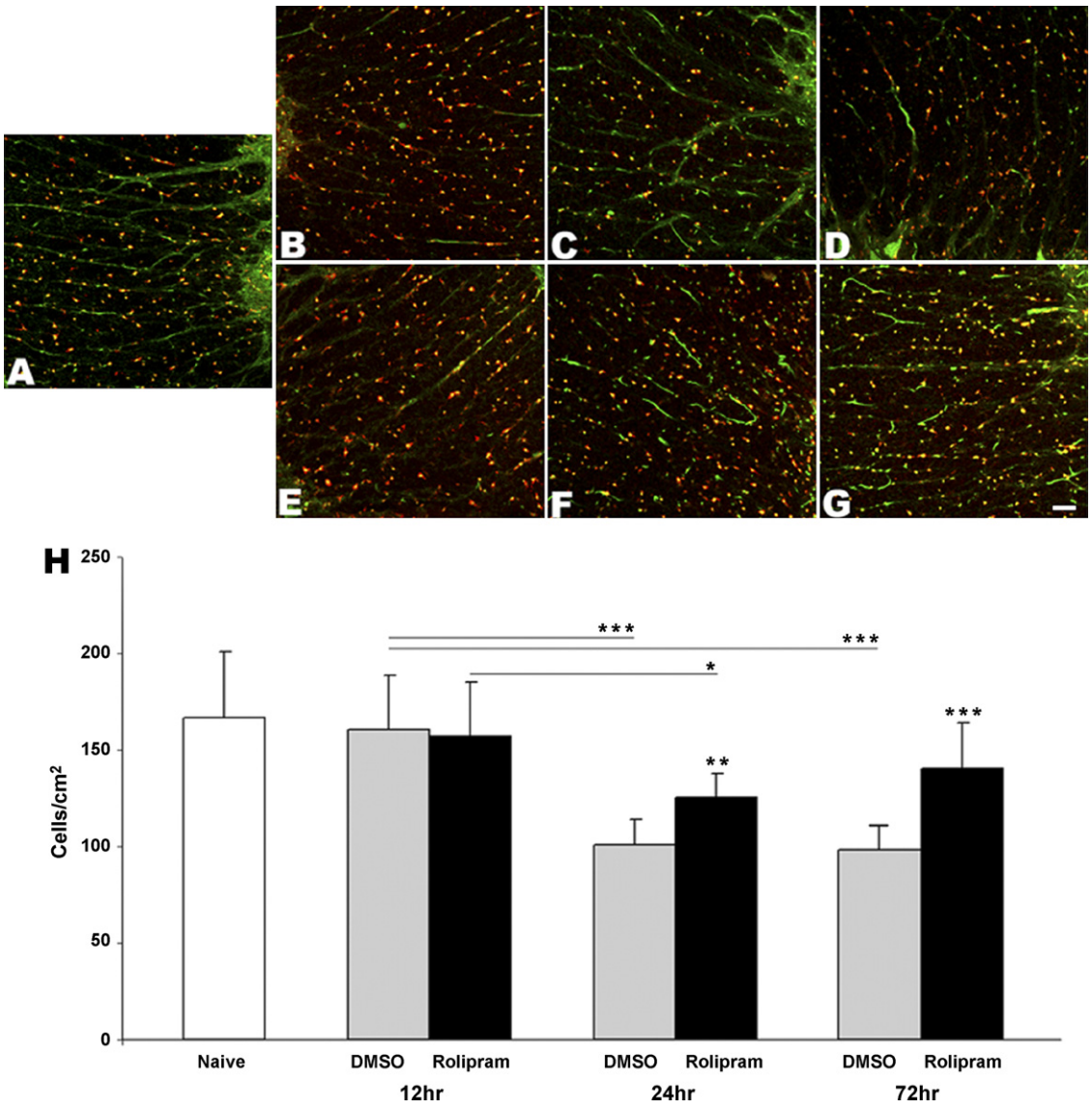

Fig. 3. Oligodendrocytes are spared after SCI by rolipram treatment. Representative transverse sections show APC (red)-and PKA (green)-immunopositive oligodendrocytes in the C5-C6 spinal cord VLF of a normal rat (A) and of both DMSO-treated rats (B-D), and rolipram-treated rats (E-G) 12 (B, E), 24 (C, F), and 72 (D, G) hours post-contusive cervical SCI. Comparisons between the numbers of APC- and pPKA-immunopositive oligodendrocyte somata in the C5-C6 spinal cord VLF of normal rats (n=4) and injured rats (n=4 for each group at each time point except that n=3 for rolipram-treated rats at 72 hours) revealed a significant reduction in both treated groups at 24 and 72, but not at 12, hours post-SCI (H). In contrast to DMSO, rolipram treatment significantly attenuated this loss. Significantly more oligodendrocyte somata continued to be seen in rolipram-treated rats' VLF at 72 hours post SCI compared to DMSO-treated rats. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Error bars represent standard deviations. Scale bar = 50 μm.

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