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Rolipram promotes functional recovery after contusive thoracic spinal cord injury in rats

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Abstract

Numerous animal model studies in the past decade have demonstrated that pharmacological elevation of cyclic AMP (cAMP) alone, or in combination with other treatments, can promote axonal regeneration after spinal cord injury. Elevation of cAMP via the phosphodiesterase 4 (PDE4) inhibitor, rolipram, decreases neuronal sensitivity to myelin inhibitors, increases growth potential and is neuroprotective. Rolipram's ability to cross the blood–brain barrier makes it a practical and promising treatment for CNS regeneration. However, several studies have questioned the efficacy of rolipram when given alone. The purpose of this investigation was to determine the effects of continuous administration of rolipram, given alone for 2 weeks, following a moderate T10 contusion injury in rat. Functional recovery was evaluated using the 21-point Basso, Beattie and Bresnahan (BBB) locomotor recovery scale and the beam walk. We used three-dimensional (3D) instrumented gait analysis to allow detailed assessment and quantification of hindlimb motion. The amount of the damaged tissue and spared white matter was estimated stereologically. Our results show that administration of rolipram following acute spinal cord contusion results in improved motor performance at each time-point. Dynamic assessment of foot motion during treadmill walking revealed a significantly decreased external rotation during the entire step cycle after 8 weeks in rolipram-treated animals. Stereological analysis revealed no significant differences in lesion volume and length. By contrast, spared white matter was significantly higher in the group treated with rolipram. Our results suggest a therapeutic role for rolipram delivered alone following acute SCI.

1. Introduction

The annual incidence of acute spinal cord injury (SCI) world-wide is 20–40 cases per million [1]. Spinal cord injury remains one of the leading causes of lasting disability in young adults and poses limited prospects for functional recovery. Currently, intravenous methylprednisolone sodium succinate (MPSS) is the therapeutic mainstay following acute SCI. However, many clinicians question use of the MPSS protocol due to conflicting experimental study results [2–4] combined with relatively small neurological improve-ment in humans [5,6]. Thus, there is a pressing need to develop effective and clinically relevant therapies to promote recovery after spinal cord injury.

Following SCI, neurons respond with an initial period of growth followed by growth cone collapse and failure of significant axon regeneration [7]. Two major factors contributing to the inhibitory milieu of the injured central nervous system (CNS) are myelin-associated proteins and the glial scar [8,9]. To overcome myelin protein inhibition, one avenue of research has focused on increas-ing the level of cyclic adenosine monophosphate (cAMP) in injured spinal cord by either local application of cAMP analogues or inhibiting the phosphodiesterases (PDE) that degrade cAMP [10]. Inhibition of PDE4 to produce elevated levels of intracellular cAMP is one of the most promising treatments for promoting axonal regeneration. Rolipram, a specific inhibitor of PDE4 that crosses the blood–brain barrier, delivered alone or in combination with other therapies, is a therapeutic candidate for SCI treatment [11,12].

There is considerable experimental evidence demonstrating that rolipram is capable of influencing neuronal and glial cell function through the elevation of intracellular cAMP levels and PKA/CREB activation. It promotes axonal regeneration, is neuron and glial-protective and improves locomotor function after exper-imental SCI [11–15]. However, significant controversy remains regarding the benefits of rolipram delivered as a single treatment after SCI [16,17]. In the present study, we investigated whether the continuous administration of rolipram, given alone for 2 weeks following contusion injury, could improve locomotor recovery and alter the extent of tissue damage.

It is critical to adequately evaluate the efficacy of therapeutic intervention after spinal injury and to distinguish among sponta-neous recovery, compensatory strategies and recovery of motor function in response to the therapeutic measure [18]. It is well accepted that while the 21-point Basso, Beattie and Bresnahan (BBB) locomotor recovery scale covers a broad range of functional recovery [19], it can be less sensitive at specific levels of recov-ery. This is due to the ordinal nature of the scale and the difficulty of correctly assessing forelimb–hindlimb coordination [20]. There-fore, in addition to BBB scoring, we also included the beam walk in our analysis.

One limitation of these endpoint measures is that they fail to provide information about how the task is being performed. To address this, we obtained continuous kinematic measures to gain information regarding dynamic function of the hindlimb. Recently, our group used four charged-couple device (CCD) cameras to esti-mate the three-dimensional (3D) position of the hindlimb in the sagittal, coronal and transverse planes in uninjured rats [21]. In this study, we applied these same measures to injured rats. To our knowledge, this is the first detailed kinematic description of all three planes performed after experimental spinal cord injury in rats. Using unbiased stereology we also estimated lesion volume, lesion length and the extent of spared white matter.

2. Materials and methods

2.1. Experimental animals

Forty Wistar adult female rats (Harlan, Barcelona, Spain) weighing approxi-mately 200 g were used in this study. Data were collected from a total of 34 animals as per the training protocol (see training procedure). All animals were kept in venti-lated, humidity and temperature-controlled rooms with a 12/12-h light/dark cycle. The animals were housed on sawdust and received food pellets and water ad lib. All procedures were performed with the approval of the Veterinary Authorities

of Portugal in accordance with the European Communities Council Directive of 24 November, 1986 (86/609/EEC).

2.2. Surgical procedure

Animals were randomly assigned and blindly divided into 2 groups. Using an osmotic mini-pump (model 2ML2, Durect Corp., Cupertino, CA, USA), one group (n = 15) received rolipram (3.18 mg/kg/day) dissolved in dimethyl sulphox-ide (DMSO) (Rolipram group). A control group (n = 15) received DMSO only (DMSO group). The pumps were implanted subcutaneously and removed after 2 weeks with animals under isoflurane anaesthesia (5 min). A third group (n = 4), subject to iden-tical surgical procedures but without impaction, served as sham-operated controls (Sham group).

Rats were anaesthetized with ketamine (80 mg/kg) and medetomidine (0.2 mg/kg). A midline incision exposed the spinal column at the T8–T11 level, and the paravertebral muscles were dissected bilaterally to visualize the trans-verse apophyses. Care was taken to perform a laminectomy at the T10 vertebral level that was only slightly larger than the 2.5 mm impactor tip. Animals in the Rolipram and DMSO groups then received a 200-kdyn contusion with the Infinite Horizon spinal cord injury device (Precision Systems & Instrumentation, Lexington, KY, USA). The surgical procedure was performed with the aid of an operating micro-scope (M680, Leica, Wetzlar, Germany). Subsequently, the wound was irrigated with saline solution and the muscle, fascia and skin were reapproximated with absorbable sutures. Body temperature was maintained with a heating pad at 37 ± 0.5 ° C during the surgery and postoperative periods. An ophthalmic gel (Lacryvisc, Alcon, Lisbon, Portugal) was applied to prevent drying of the eyes.

After surgery, 10 ml of 0.9% sodium chloride and 30 mg/kg of sulphadiazine and trimethoprim were injected subcutaneously. Access to food was facilitated by pla-cing softened food pellets directly in the bottom of each cage. The state of hydration and gastrointestinal function were monitored daily. Lactated Ringer's solution was injected subcutaneously when necessary. Rats were weighed daily for the first 7 days post-surgery and then weighed weekly. Postoperative care included the man-ual expression of bladders twice a day until bladder function returned, as well as injections of sulphadiazine and trimethoprim twice a day for up to 1 week.

2.3. Training procedure

Two weeks before the collection of data, rats were pre-trained to walk on a hori-zontal runway 1-m long and elevated 1 m above the floor. Additionally, each animal was first trained daily to walk consistently on a treadmill at a speed of 40 cm/s, within a Plexiglas enclosure (53 cm × 10 cm × 14 cm) with a removable top (Letica, Scien-tific Instrument, Barcelona, Spain). Ten minute long training sessions were given once a day with low intensity footshock used initially as negative reinforcement to improve performance. In our training protocol when the rats failed to walk regularly and spontaneously after three sessions, they were eliminated from further study. We attempted to reduce animal stress by minimizing noise levels and handling the rats gently to obtain locomotion under normal conditions.

2.4. Functional assessment

All tests were performed before SCI and for 8 weeks post-injury. Using the BBB locomotor rating scale, injured rats were assessed 2 days after surgery, and then once a week. For analysis of the beam walk, animals were assessed 2 weeks post-operatively and then once a week. Kinematic data was collected before spinal injury and then at the end of the experiment.

The locomotor activity of individual animals was judged according to the 21-point open-field BBB scale [19]. A score of 0 was given for no spontaneous hindlimb movement while a score of 21 indicated normal locomotion. Animals were placed in an open field, a transparent plexiglas box (100 cm × 100 cm) and locomotor scoring was performed for four minutes by two experienced examiners. Observers were unaware of the treatment received by each animal.

The beam walk test was performed according to the descriptions of von Euler et al. [22]. Seven beams of different widths were used as narrow pathways, with the following score: (1.7-cm = 7; 2.7-cm = 6; 3.7-cm = 5; 4.7-cm = 4; 5.7-cm = 3; 6.7-cm = 2; 7.7-cm = 1). Rats were placed in the widest beam and the ability to

cross the horizontal beam without foot slips within two trials was observed. The procedure was repeated on successively narrower beams. The narrowest beam a rat could walk was recorded.

To collect the 3D kinematic data, hair was clipped around both hindlimbs to improve the visual image obtained for analysis. Hemispheric green markers with a diameter of 2 mm were placed on the skin over five anatomic landmarks on the lateral side of the left hindlimb: the iliac crest, the greater trochanter, the knee joint, the lateral malleolus, and fifth metatarsal head. Additionally, a marker was placed on the iliac crest of the right hindlimb. The same operator performed all marker placements to avoid inter-tester variability. The colour passive markers were used together with appropriate illumination produced by four fluorescent high frequency lamps (StockerYale 13W, 25 kHz, Prophotonix, Inc, Salem, USA) mounted around the capture volume.

3D video recordings were made with four CMOS cameras (PhotonFocus MV-D640C, Lachen, Switzerland) oriented at 45° with respect to the direction of the

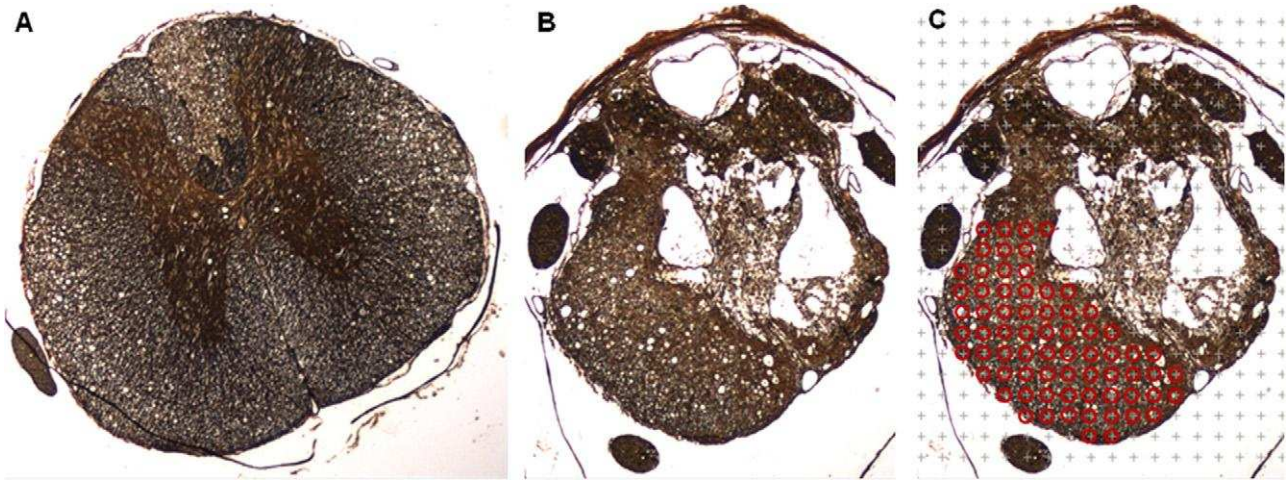


Fig. 1. Histological appearance of spinal cord transverse section after silver stain. Panel A shows a picture taken at a level where the spinal cord is not lesioned where white and grey matter can be easily recognized. Panel B shows a picture taken at a level of the lesion epicentre where silver stain allows to clearly detect the damaged and spared areas. In panel C, the point counting frame is used to estimate spared white matter area. Original magnification 40×.

locomotion (the rat sagittal plane) on both sides. Kinematic data were collected at a sampling rate of 144 Hz. Camera magnification was calibrated to cover a 20 cm length of the treadmill apparatus, and allowed the recording of ten consecutive steps. Data were recorded while rats walked at 40 cm/s, which is within normal walking velocity, where they typically utilize a lateral sequence walk. Images were acquired using Video Savant 4 software (IO Industries Inc, Ontario, Canada) with colour images at 640 × 480 pixel resolution. Cameras were strategically placed around the animal to minimize marker occlusion, maximize resolution and to improve the accuracy of the 3D reconstruction process. A new calibration object was designed that allowed simultaneous calibration of all four cameras. Use of the calibration object ensured that the projection matrixes were all related to the same coordinate system and allowed all points to be used to compute the kinematic parameters without transformation. The camera calibration, the 3D reconstruction process for each side and indirect computation of the knee position was performed as described by Couto et al. [23].

Kinematic plots of the pelvis, hip, knee and ankle were generated by computer tracking of retroflective joint markers representing the sagittal, coronal and trans-verse planes. The hip, knee, and ankle joint angles were measured at the flexor side of each joint in the sagittal plane. Pelvic obliquity and hip ad–abduction were examined in the coronal plane whereas pelvic and foot rotation were studied in the transverse plane. Pelvic obliquity was measured as the angle formed between a line joining the two iliac crests and the horizon. Hip ad–abduction was measured as the angle formed between the femur and a line perpendicular to the line joining the two iliac crests. Pelvic rotation was measured as the angle formed

between a line joining the two iliac crests and a line perpendicular to the direction of progression. Foot rotation was measured as the angle formed between the foot segment (refer-ence line from the lateral malleolus to the fifth metatarsal head) and the direction of progression.

2.5. Stereological analysis

Eight weeks post-injury, under deep anaesthesia with ketamine (80 mg/kg) and medetomidine (0.2 mg/kg), animals (n = 8 for each experimental group) were per-fused through the heart with 300 ml of phosphate buffered solution (PBS) followed by 300 ml of 4% paraformaldehyde in cold 0.1 M PBS, pH 7.4. Spinal cords were exposed at the T10 level from a dorsal approach and a 2-cm-long segment (1 cm upstream and 1 cm downstream to the lesion point) was removed and postfixed in 4% paraformaldehyde for 4 h. Samples were then washed in PBS, embedded in paraffin and serially sectioned (at 10- m nominal thickness) transversely to the main spinal cord axis, recording the progressive number of each section.

For each spinal cord, 20 sections (with a distance of 1 mm between them) were selected by systematic random sampling [24]. After sectioning the entire spinal cord segment and recording the progressive number of each section, a starting section was randomly selected along the more rostral 1-mm-long segment. Sections to be analysed were systematically selected by jumping 1 mm caudally (i.e. 100 sections ahead). All selected sections were processed for silver staining (Fig. 1A and B) (Bio-Optica, Milan, Italy). Although eriochrome cyanine staining is more commonly used for spinal cord sections staining and evaluation of the white matter sparing [15,25], we demonstrated previously that silver staining allows clear detection of spinal cord histology as well [4]. Stained sections were then analysed on a stereological work-station equipped with a DM400 microscope with DFC320 digital camera and IM50 image manager system (Leica Microsystems, Wetzlar, Germany). In each section, a point frame was overlaid on the picture of the SC (Fig. 1C) and the lesion area was estimated by counting the number of points hitting the lesion area. The point-count values were then multiplied by the inter-point squared distance to obtain the area estimation in that section. Then, in order to obtain estimation of the total volume of the lesion, the Cavalieri method was used [26]. Cavalieri method allows to estimate the volume of an object by multiplying the sum of the areas measured on systematic random parallel sections by the distance between the sections [27].

In addition, besides total lesion volume, we estimated the lesion length as the distance between the most rostral and the most caudal section in which the lesion could be identified. The area of spared white matter at the lesion epicentre is acquiring growing attention among researchers as a morphological indicator of the severity of the SC injury and as a tool for evaluation of regenerative treatments [28–31]. It has been reported that the white matter sparing is the parameter cor-relating the most with functional outcome [16,32–35]. We estimated the area of spared white matter using the point counting frame method (Fig. 1C). The epicentre of the lesion was established as the section where the smallest area of spare white matter was detected.

2.6. Numerical and statistical analysis

The step cycle, which is the basic unit of measurement in gait analysis, was split into two parts, designated the stance and the swing phases. The stance phase was defined as the part of the step cycle that begins when the foot contacts the tread-mill belt, and terminates when the foot starts its forward movement. In accordance with our previous work, we subdivided the stance phase in the rat into three major components: (1) weight acceptance, occupying the first 20% of the stance phase;
(2) mid-stance, occurring between 20 and 40% of the stance phase; and (3) push-off, for the last 60% of the stance phase [36]. The swing phase was considered to begin at the onset of forward foot movement and to terminate as the foot strikes the treadmill belt. For each step, the duration of the stance and swing phases was normalized. Cubic spline interpolation was applied to the original data regarding the angular position of the pelvis, hip, knee and ankle to obtain 101 samples per step cycle regardless of their duration. This numerical treatment was performed with Matlab computational software (The MathWorks Inc., Natick, MA, USA).

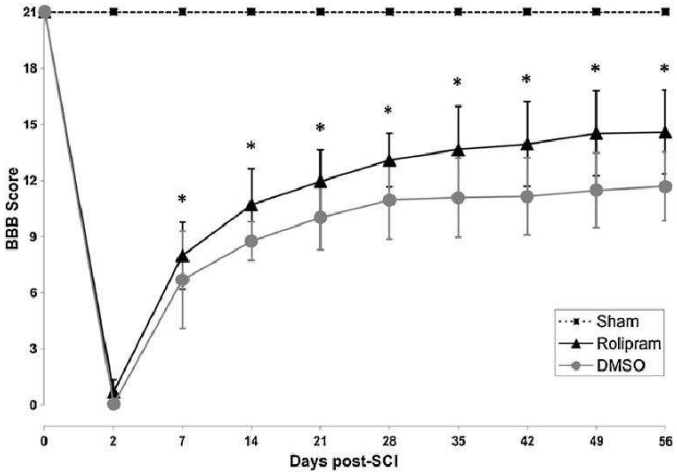
Mean ± standard deviation (S.D.) values for all measured variables were reported. For categorical data (i.e. BBB scores and Beam walk results) statistical comparisons between treatment groups were made

using the Mann–Whitney U test. Comparisons between treatment groups and between pre and post injury parameters describing hindlimb joints' kinematics during treadmill gait were car-ried out by 2-way repeated measures ANOVA (General Linear Model). Sphericity was confirmed using Mauchly's test and Greenhouse–Geisser epsilon correction for cor-rection of the degrees of freedom when appropriate. Statistical tests were performed using SPSS computational software (Statistical Package for the Social Sciences Inc., Chicago, USA).

Stereological estimates were subject to one-way analysis of variance (ANOVA) test using "Statistica per discipline bio-mediche" software (McGraw-Hill, Milano, Italia). Statistical significance was accepted at $P < 0.05$.

3. Results

The mean force at the impact site recorded by the Infinite Hori-zon Impactor device was 201.6 ± 2.1 kdyn and 200.9 ± 1.7 kdyn for the rolipram and DMSO groups, respectively. There was no



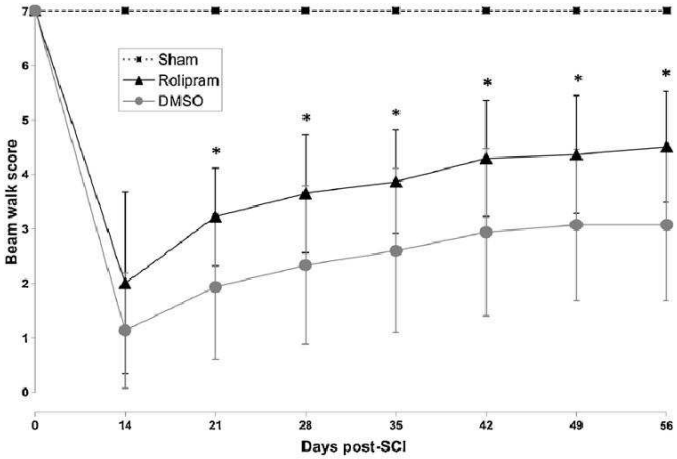


Fig. 2. Time course of motor recovery showing Basso, Beattie, and Bresnahan (BBB) scores for Rolipram (black trace) vs. DMSO (grey trace) groups. All values are given as mean ± standard deviations (n = 15 in each group). * $P < 0.05$.

Fig. 3. Time course of motor recovery showing beam walk performance, using seven wooden planks of different widths (7.7–1.7 cm) which were 1 m long, for Rolipram (black trace) vs. DMSO (grey trace) groups. All values are given as mean ± standard deviations (n = 15 in each group). * $P < 0.05$.

significant difference in impact force between the treatment groups. All contused animals exhibited signs of paraplegia fol-lowed by significant improvement over the subsequent 8 weeks. Bladder voiding capability returned within the first post-surgical week. Laminectomy alone (Sham group) did not affect neurologic function as per our assessment criteria.

3.1. BBB locomotor score

All rats had BBB scores of 21 prior to injury and BBB scores of 0.7 ± 0.6 and 0 ± 0.1 for the rolipram and DMSO groups, respectively, on the second day after SCI (Fig. 2) BBB scores of rolipram treated rats were significantly better compared to DMSO controls starting at the earliest timepoint, 7 days after injury ($P = 0.001$). Moreover, at each later timepoint rolipram treated animals scored significantly better on the BBB scale ($P < 0.05$). After 35 days, both groups demonstrated a characteristic plateau in recovery. At the end of the experiment, the average BBB score of the Rolipram group reached 14.6 ± 2.2. The animals dis-played consistent coordinated plantar stepping and consistent forelimb–hindlimb coordination. In contrast, the average BBB score of the DMSO group remained lower throughout the experiment and reached 11.7 ± 1.8 ($P = 0.001$), reflecting frequent to consistent weight-supported steps and occasional forelimb–hindlimb coordi-nation.

3.2. Beam walk

Deficits in the descending fine motor system were examined using the beam walk test. Prior to injury, rats were able to cross the narrower beam with no errors in foot placement (Fig. 3). Follow-ing injury, both experimental groups exhibited severe deficits. Two weeks after injury, when beam walk testing was started, rolipram-treated animals performed better (2.0 ± 1.7) than control animals (1.1 ± 1.1), although this difference did not reach the level of sta-tistical significance ($P = 0.148$). After 3 weeks of recovery period rolipram treated animals had significantly higher beam walk scores than control group ($P = 0.012$). Similar to the results of BBB scoring, both groups exhibited improved coordination over 8 weeks of test-ing with rolipram treated animals performing consistently better throughout the testing period compared to DMSO-treated animals ($P < 0.05$).

3.3. 3D hindlimb kinematics

To characterize 3D hindlimb kinematics during treadmill loco-motion, a total of 150 step cycles from each experimental group ($n = 15$) were obtained before spinal cord contusion injury. The average toe-off (TO) time was calculated to be 67% into the step cycle. Records of treadmill performance produced by all pre-surgical animals were similar to our previous study estimating the 3D position of the hindlimb in the sagittal, coronal, and transverse planes (Fig. 4) [21]. Statistical analysis of differences in joint angu-lar positions between the Rolipram and DMSO groups revealed no significant differences with respect to angle magnitude.

Eight weeks following injury, both experimental groups dis-played severe locomotor deficits in all three planes of motion; 8 animals, in each experimental group, satisfactorily completed 10 consecutive step cycles that could be analysed. Fig. 5 (first column) shows the sagittal plane kinematics for hip, knee and ankle motion for Rolipram (black trace) and DMSO (grey trace) groups, respec-tively. Both groups exhibited increased hip extension along the step cycle. The knee and ankle joints showed reduced extension at ini-tial contact (IC) and all joints exhibited a substantial increment in the extension at TO. In contrast to the uninjured animals, max-imum extension in the knee angle trajectory in injured animals occurred during the stance–swing transition. In addition, injured animals lost the double peak pattern of the ankle angle. The analy-sis of the gait changes following SCI demonstrated that all sagittal joint kinematics parameters were significantly affected by spinal contusion ($P < 0.05$), with the exceptions of minimum knee angle during the stance phase ($P = 0.629$) and hip angle at IC ($P = 0.325$). Eight weeks after lesion, we found identical changes in hindlimb kinematics for the sagittal plane, with no significant differences between Rolipram and DMSO groups.

Hip ad–abduction and pelvic obliquity for Rolipram (black trace) and DMSO (grey trace) groups were

analysed in the coronal plane (Fig. 5, second column). We observed that the peak abduction of the hip during push-off was considerably less in injured animals (the significant main effect of time for hip abduction at TO is P = 0.015). Following spinal injury, pelvic obliquity was more subtle than in uninjured animals (the significant main effect of time for maxi-mum and minimum pelvic obliquity during the stance phase are, respectively P = 0.002 and P = 0.000). At the end of the experiment, we found identical changes in hindlimb kinematics for the coronal plane, with no significant differences between Rolipram and DMSO groups.

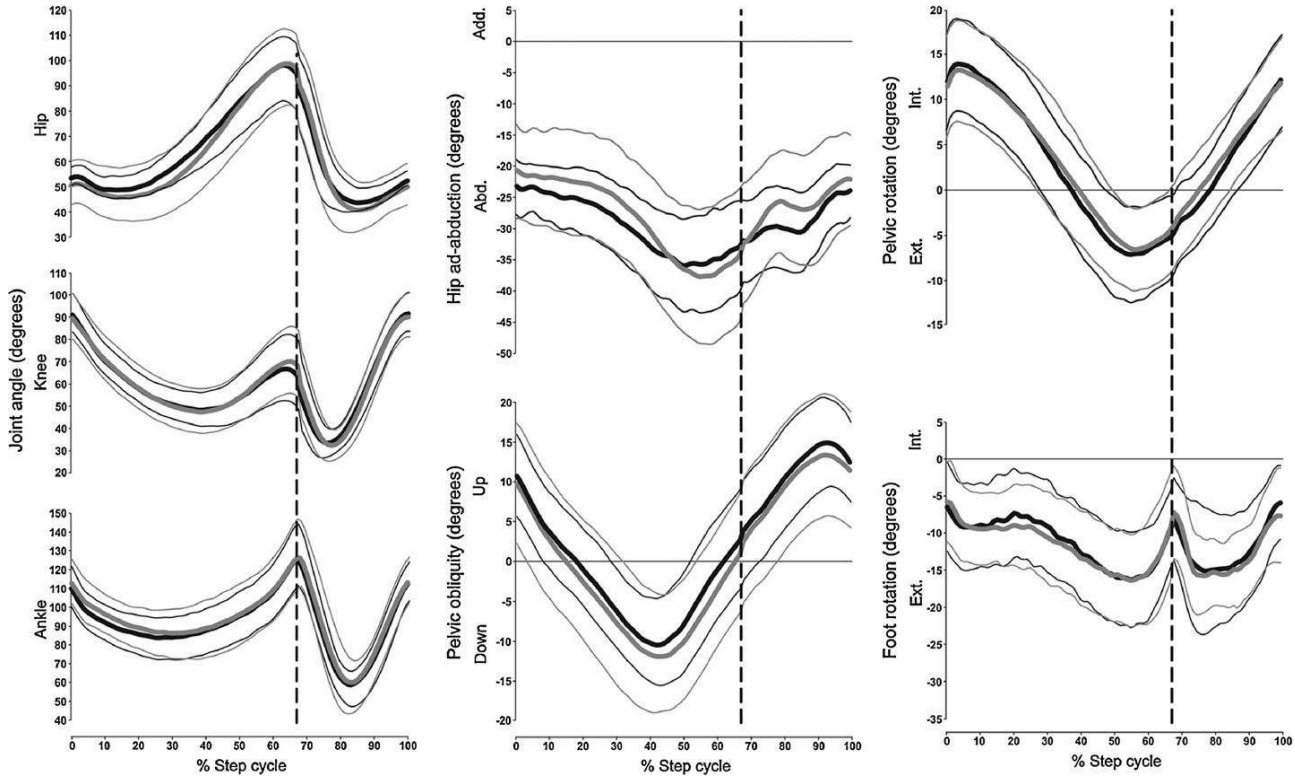


Fig. 4. Mean values for the joint kinematics in the sagittal plane (first column), coronal plane (second column), and transverse plane (third column) for Rolipram (black trace) vs. DMSO (grey trace) groups, before SCI. Standard deviation is plotted on either side of the mean. Stance and swing phases were normalized. The stance duration was set at 67% of the step cycle duration. The vertical dashed line corresponds to the stance–swing transition (n = 15 in each group).

Next, we studied the transverse plane for pelvic rotation, and foot rotation for Rolipram (black trace) and DMSO (grey trace) groups (Fig. 5, third column). In this plane, both healthy and spinal injured animals showed identical patterns of pelvic motion. At IC, through weight acceptance and mid-stance, the ipsilateral hemipelvis was internally rotated followed by progressive exter-nal rotation, reaching a maximum near TO. The hemipelvis then rotated internally until the end of the swing phase. We also assessed changes in the transverse plane kinematics throughout the anal-ysis of foot progression. During normal gait, the foot is rotated slightly external to the direction of progression. In contrast, both experimental groups showed increased external rotation during the entire step cycle following injury. However, analysis of the mean waveform for the Rolipram group shows a lesser external foot rotation. This decreased external foot rotation was significantly different during the stance phase at IC (P = 0.027). The difference in pelvic rotation at this same instant of the step cycle between the two groups was closed to statistical significance (P = 0.057).

3.4. Stereology

Results comparing stereological estimates of lesion volume, lesion length and spared white matter are shown in Fig. 6. Statistical analysis showed that numerical difference in aver-age lesion volume between

DMSO-treated (1745 ± 507_3 × 106) and rolipram-treated (1995 ± 712_3 × 106) groups was not significant (P > 0.05). Similarly, average lesion length was not significantly (P > 0.05) different between DMSO-treated group (6091_ ± 1666_) and rolipram-treated (4939_ ± 1260_) group. In contrast, rolipram-treated group displayed significantly (P < 0.05)
greater area of spared white matter at the lesion epicentre (461,313 ± 56,390_^{2}) then control group (301,168 ± 181,333_^{2}).

4. Discussion

Despite major progress in pharmacological, surgical and reha-bilitative approaches, SCI still induces devastating damage leading to irreversible loss of voluntary motor control and sensory func-tion [37]. While there is a wide range of insults that can impart SCI clinically, the vast majority of spinal injuries are characterized by severe spinal cord contusion rather than transection, even when there are massive vertebral injuries. The animal model of contus-ion injury we employed mimics the typical injury mechanism in humans [38]. In both rodent and human, contusion of the spinal cord induces a similar activation of secondary injury processes [39]. The Infinite Horizon spinal cord injury impactor uses force rather than tissue displacement as a user-defined variable to minimize potential errors that may arise from tissue movement during the surgical procedure [28]. In our study, the injury was delivered with a force of 200-kdyn. This moderate injury was chosen to allow repetitive plantar stepping on the treadmill during the recovery process [40].

An important goal of this investigation was to objectively and quantitatively analyse the effects of rolipram on gait following SCI in rats. Kinematic gait analysis allows for a more accurate assess-ment of gait deviations than visual gait assessment and is a widely accepted research tool [21]. For this study, locomotion was assessed using 3D kinematic movement analysis equipment. Additionally, we applied traditional methods such as BBB scoring and the beam walk. Employing a variety of tests to assess behavioural recovery

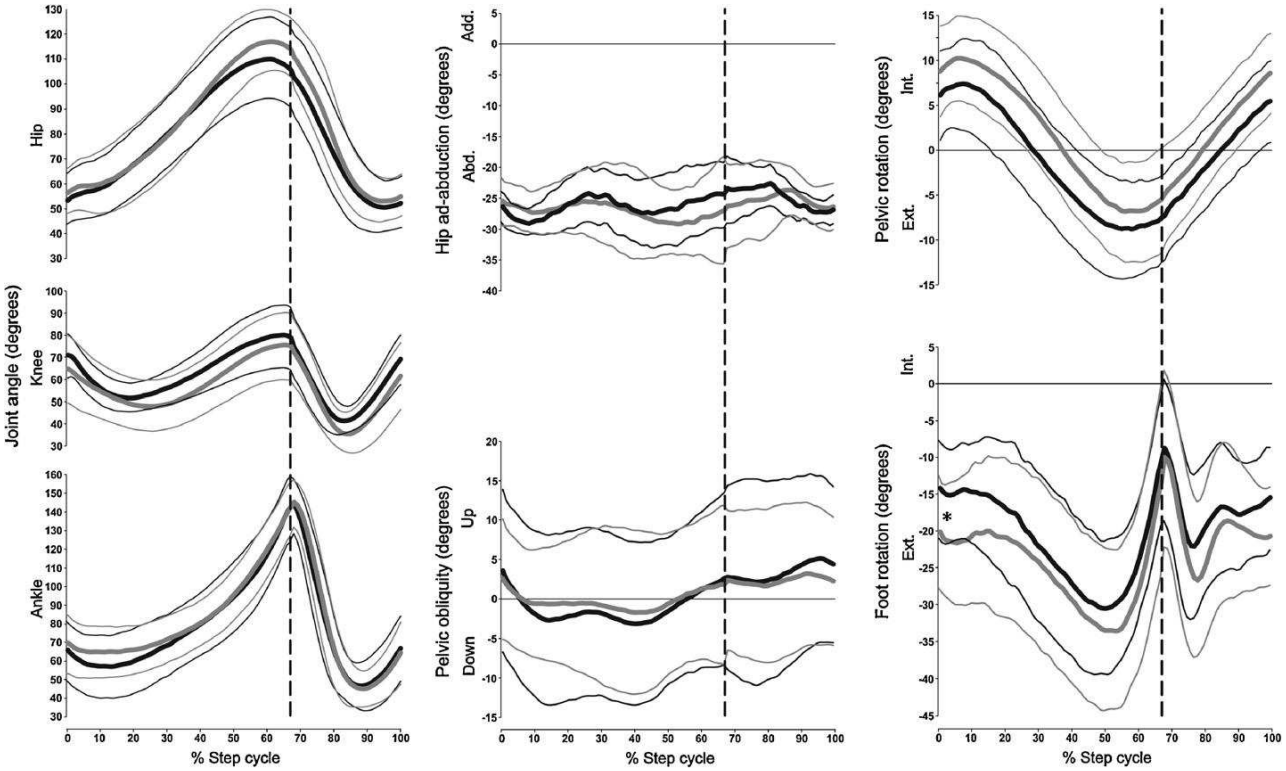


Fig. 5. Mean values for the joint kinematics in the sagittal plane (first column), coronal plane (second column), and transverse plane (third column) for Rolipram (black trace) vs. DMSO (grey trace) groups at 8 weeks post-contusion. Standard deviation is plotted on either side of the mean. Stance and swing phases were normalized. The stance duration was set at 67% of the step cycle duration. The vertical dashed line corresponds to the stance–swing transition (n = 8 in each group). * P < 0.05.

provides a better means to monitor progressive, postinjury changes in function [18,41–43].

The functional results of this study suggest that continuous administration of rolipram, given alone for 2 weeks following acute spinal cord contusion, has positive effects on neurological recovery after 8 weeks compared to controls. Open-field locomotor test-ing using the BBB scale showed that rolipram-treated rats had the most significantly improved hindlimb function throughout the entire post-injury testing period. Eight weeks following contusion, these animals displayed consistent coordinated plantar stepping and consistent forelimb–hindlimb coordination. Beam walk was used to assess deficits in descending fine motor control after spinal contusion. Three weeks post injury rolipram treated animals had significantly higher beam walk scores then DMSO treated rats.

The results of our 3D kinematic data after SCI showed that the general shapes of joint angular motion were very similar for Rolipram and DMSO groups. However, we observed statistically significant differences for foot progression along the transverse plane. In line with results obtained using traditional tests, we found that rolipram administration improved foot progression and induced a decreased external rotation during the entire step cycle. This may be a reflection of better lateral stability and body balance following spinal lesion in rolipram-treated animals [4].

Histological analysis supplements the results of behavioural tests by providing a quantitative evaluation of spinal cord damage and the potential tissue-protective effect of the applied therapy [44]. Stereological evaluation of the extent of lesion and spared white matter revealed a significantly better preservation of white matter in rolipram-treated animals than in controls. No significant differences were detected with respect to lesion volume and length. Our results corroborate data from previous studies showing that

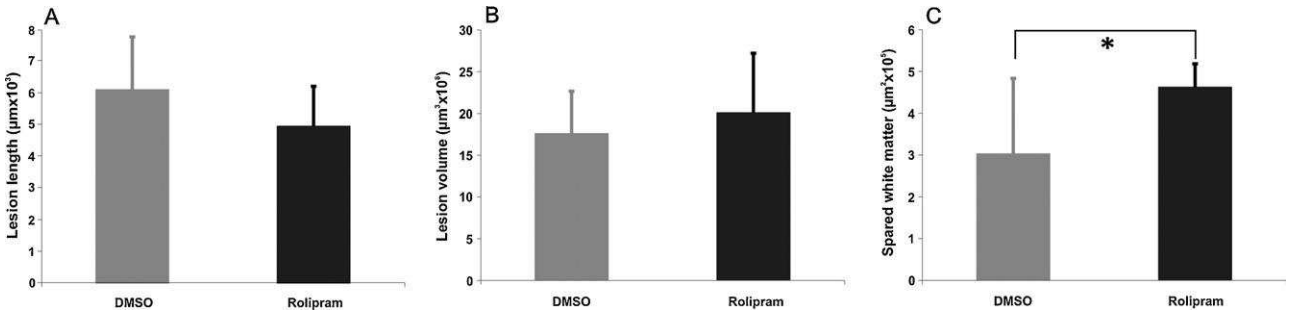


Fig. 6. Comparison of stereological estimates between Rolipram (black) vs. DMSO (grey) groups regarding the lesion length (A), the lesion volume (B) and the spared white matter (C) (n = 8 in each group). * $P < 0.05$.

functional outcome correlates most with the degree of sparing of white matter [32–35].

In agreement with our results, a number of experimental stud-ies have shown that rolipram treatment has a positive effect on acute SCI. The therapeutic potential of rolipram-induced cAMP elevation and PDE4 inhibition on SCI was recently assessed in several in vitro and in vivo studies involving different models of SCI. Rolipram administration to adult rats after C3/C4 spinal cord hemisection combined with embryonic spinal cord tissue transplantation improved growth of serotonergic fibres into the transplant compared with vehicle-treated animals. Consistent with these results, rolipram-treated animals also had greater functional recovery and improved forelimb control suggesting the establish-ment of synaptic connections from regenerating axons [11].

The neuroprotective effects of rolipram following SCI were also verified by Pearse and colleagues in a study involving moderate thoracic (T8) contusion injury in adult rats [12]. Results showed a significant sparing of oligodendrocyte-myelinated axons in all groups receiving rolipram with or without Schwann cell trans-plantation. Whitaker and colleagues have shown an increase in oligodendrocytes survival in the ventrolateral funiculus (VFL) fol-lowing the first 24 h after contusive cervical SCI in adult rats [45]. Increased oligodendrocyte survival in the adult rat VLF follow-ing contusive cervical (C5/C6) SCI and rolipram treatment was also demonstrated by Beaumont and colleagues. Rolipram-treated animals displayed improved axonal conductivity in the VLF in long-tract electrophysiological assessments and displayed fewer hindlimb footfall errors during grid-walking test. In contrast, BBB locomotor rating scores of treated and

non-treated animals at week 5 post-SCI were similar [14].

The combination of olfactory ensheathing cell transplants with systemic administration of rolipram resulted in improved locomo-tor performance in rats receiving a cervical (C4/C5) dorsolateral funiculus crush lesion [46]. Rolipram was also effective in the acti-vation of the crossed phrenic pathway and phrenic nerve recovery in unilateral cervical (C2) spinal cord injured rats [47]. When com-bined with thalidomide, rolipram produced a synergic effect on reduction of secondary injury following thoracic (T10) contusion lesion. The combinatorial treatment resulted in increased white matter sparing at the lesion epicentre and in improvement in BBB locomotor scoring [16].

The combined treatment of rolipram with clodronate, a first-generation bisphosphonate drug that induces selective apoptotic cell death of monocytes and phagocytic macrophages, resulted in significant amounts of axonal sparing following contusive thoracic (T8) SCI. These findings were associated with significant locomotor improvement as determined by BBB scoring [15].

Elevating cAMP levels with rolipram was previously shown to promote axonal growth [10], to be anti-inflammatory [48], and to induce plasticity [49]. Importantly, rolipram attenuates oligodendrocyte apoptosis in SCI model [45]. The oligodendrocyte-protective effect of rolipram could be a result of direct elevation of cAMP levels within oligodendrocytes that express PDE4s. In addi-tion, rolipram decreases the levels of pro-inflammatory cytokine TNF- [12], which is one of the major factors that determines oligo-dendrocyte secondary cell death after CNS injuries [50,51].

While the beneficial effects of combinatorial treatments incor-porating rolipram have been demonstrated, prospects for rolipram as a monotherapy after SCI remain controversial. Alongside studies demonstrating behavioural improvement and protection of tissues after applying rolipram as a single treatment after SCI in rats, there are studies that have not detected improvements in functional recovery [17,52]. The differing outcomes from rolipram treatment may result from differences in injury models (overhemisection vs. moderate contusion), time course of treatment and rolipram dose. Our results and reported data provide evidence that treating rats with rolipram after contusive SCI leads to a better behavioural recovery. It would follow that the effects of rolipram alone would be more prominent in less severe injury models where there exists a better chance to protect neuronal and glial cells in the vicinity of the injury site and to prevent injury-induced decline of function in originally unaffected axonal tracts [53].

A wide range of pharmacological agents have been studied extensively in acute SCI. To date, no therapeutic strategies have proven effective enough to provide a compelling consensus for uncontested translation in human clinical trials [54–56]. Here, we report that rolipram treatment enhanced functional recovery and increased spared white matter after contusive SCI. To date, among all agents tested on SCI, rolipram shows the greatest potential for modulating axonal regeneration and functional improvement. Rolipram is also superior to other agents as it readily crosses the blood–brain barrier, making it suitable for subcutaneous admin-istration. This decreases the risk of damaging axons that were spared initially by the trauma since the surgery is not needed to deliver treatment at the injury site [11]. Our results demonstrate that rolipram, delivered alone, improved locomotion and reduced secondary tissue degeneration after SCI. Thus, given its efficacy and the fact that it is a readily-available, approved drug, human clinical trials for rolipram in the treatment of SCI are warranted.

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