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Research article

CB1 cannabinoid receptor agonist inhibits matrix metalloproteinase activity in spinal cord injury: A possible mechanism of improved recovery

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HIGHLIGHTS

- We examined the effect of a selective CB1R agonist, ACEA in mouse spinal cord injury model.
- ACEA significantly improved functional deficit and reduced the compression lesion volume.
- MMP-9 activity was increased in SCI and inhibited by ACEA.
- The effect of ACEA on MMP-9 activity was abolished by CB1R, but not CB2R antagonism.

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ABSTRACT

Increased matrix metalloproteinase (MMP) activity contributes to glial scar formation that inhibits the repair path after spinal cord injury (SCI). We examined whether treatment with *N*-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA), a selective synthetic cannabinoid receptor (CB1R) agonist, inhibits MMP and improves functional and histological recovery in a mouse spinal cord compression injury model. Injured mice randomly received either intraperitoneal ACEA (3 mg/kg/day) or vehicle for up to 3 weeks. Behavioral, histological and biochemical assays were performed. Rotarod assessment and the Basso Mouse Scale score showed an improved performance following ACEA treatment concomitant with a decrease in compression lesion volume. MMP-9 and MMP-2 activity was measured at 1, 7 and 14 days post-SCI. SCI markedly increased MMP-9, but had negligible effect on MMP-2 activity. ACEA-treatment decreased MMP-9 activity by 80%, 49%, and 56%, respectively ($P < 0.05$) and had a smaller effect on MMP-2 activity. The CB1R antagonist SR141716, but not the CB2R antagonist SR144528, blocked ACEA-mediated decrease in MMP-9 activity confirming the role of the CB1R in the process. Collectively these data demonstrate that post-injury CB1R agonism can improve SCI outcome and also indicate marked attenuation of MMP-9 proteolytic enzyme activity as a biochemical mechanism.

1. Introduction

Fibrous scar formation presents a major obstacle to axonal regeneration following spinal cord injury (SCI) [10]. Increased matrix metalloproteinase (MMP) activity following SCI has been implicated in glial scar formation and the subsequent rehabilitation process [22]. The significance of and factors regulating MMP activity after SCI remain under investigation [49].

The endocannabinoid system is composed of two G protein-coupled receptors (the CB1 and CB2 receptors), the endogenous ligands that bind to those receptors (arachidonoyl ethanolamide or anandamide and 2-arachidonoylglycerol (2-AG)), and the specific enzymatic machinery for their synthesis and degradation [14]. Exogenous and endogenous cannabinoids produce physiological

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effects through CB1R and CB2R [20,21,37]. Endocannabinoids are usually synthesized on demand and thereby can act as a homeostatic regulatory mechanism against a variety of excitotoxic insults [4]. It has been shown that SCI induces a local and transient spiking of anandamide at 1 day and 2-AG at 7 days post injury [18]. Another study demonstrated that endocannabinoid 2-AG levels increased as early as 4 h following SCI [6] and single injection of 2-AG 30 min post-SCI protects white matter from secondary damage [5].

Both endogenous and exogenous cannabinoids have been shown to decrease inflammation and pain, and to provide neuroprotection in various models of spinal cord and brain injury [3,25,33,44]. The CB1R agonists have been shown to attenuate MMP activity in some [29,38], but not all pathologic processes [39]. The effect of CB1R in the regulation of MMP activity following SCI is undefined.

We hypothesized that the endocannabinoid system acts as a homeostatic protective mechanism in SCI, mediated through modulation of MMP-9 activity. This predicts that post-SCI systemic treatment with an exogenous CB1R agonist will result in decreased compression lesion volume and neurologic deficit. To test this hypothesis, we designed experiments to determine (a) the effect of post-SCI treatment with *N*-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA), a synthetic CB1R agonist, on long-term functional and histologic recovery in an established mouse spinal cord compression injury model, and (b) if effects on outcome are associated with CB1R agonist effects on MMP-9 activity.

2. Materials and methods

The Institutional Animal Care and Use Committee of Duke University approved all aspects of the study design. Experiments were conducted in adherence to the NIH Guide for the Care and Use of Laboratory Animals on young adult male C57BL/6J mice weighing between 20 and 25 g (Jackson Laboratories, Bar Harbor, ME). Mice were housed under a normal day night cycle and fasted overnight from food before injury with free access to water.

2.1. Spinal cord compression injury

Mice were anesthetized with 5% isoflurane in 40% O_2 balanced with N_2. The trachea was intubated with a 20 gauge IV catheter. The lungs were mechanically ventilated and rectal temperature was controlled at $37 \pm 0.5\,^{\circ}C$. Isoflurane was then decreased to 1.8% inspired during the surgical procedure. Spinal cord compression injury was induced as described previously [41,42]. Briefly, a 1.5 mm long silicone tube was transversely placed on the dorsal surface of thoracic spinal cord through the intervertebral gap created between the T10–T11 spinous processes, the wound was closed, and the mice were awakened. A suture, pre-placed through the tube was used to extract the tube after 60 min compression (for details see Supplement). This transient compression results in moderate functional deficit and histologic damage [42].

2.2. Cannabinoid drug preparation and treatment assignment

The CB1R agonist ACEA [*N*-(2-chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide], CB1R antagonist SR141716 [5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-1-piperidinyl-1H-pyrazole-3-carboxamide] and CB2R antagonist SR144528 [5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-*N*-[(1S, 2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide] were purchased from Cayman Chemical (Ann Arbor, MI), prepared fresh immediately before use by dissolving in 1:1:18 ethanol:alkamuls:saline [27,46]. This solvent was also used in vehicle-treated mice.

To assess functional and histologic outcome, injured mice were assigned by computerized randomization to one of two groups and received intraperitoneal injections of either vehicle or ACEA (3 mg/kg/day) for 21 days, beginning 1 h post-spinal cord compression.

2.3. Neurological function assessment

Vestibulomotor function was measured using a rodent rotarod (ENV-577m, Med Associate Inc., Georgia Vermont) as previously reported [41,42]. Latency to fall was measured one day prior to, and 1, 3, 7, 14, and 21 days after injury. After 21 days, the severity of SCI-induced paralysis was evaluated using the 9-point Basso Mouse Scale for locomotion based on hindlimb movement [8]. Scores were assigned for both hindlimbs separately and then averaged for statistical analysis. A separate cohort of SCI mice, treated with ACEA or vehicle, were assessed for open field locomotor behavior and anxiolysis (see Supplement for details).

2.4. Histology

At the completion of functional testing (21 days post-SCI), mice were perfused with saline and 10% formalin. A 1-cm long spinal cord segment spanning the injury site was harvested, cryopreserved, and serially sectioned (20 μm) through the rostral–caudal extent of the lesion. Every 16th section was collected (30–31 sections total), mounted on glass slides, and stained with hematoxylin and eosin. Using light microscopy (200×) and image analysis (MCID, Imaging Research Inc.), lesion margins were cursor-outlined. Compression lesion volumes were automatically calculated based on the lesion area measured in each section and the known interval between sections (see Supplement for details).

2.5. MMP zymography

After SCI, 32 mice were randomly assigned to receive either the CB1 receptor agonist ACEA (3 mg/kg/day) or vehicle as described above. At 24 h, 7 or 14 days post-injury, the mice were anesthetized and the spinal cords harvested ($n = 5$/time point/group). Five sham-operated mice were subjected to the same surgical procedures (without spinal cord compression) and the spinal cords were harvested 24 h post-operatively. The spinal cords were dissected to obtain tissue blocks spanning the compression zone, or corresponding anatomical tissue in sham-operated mice. To differentiate changes in MMP-9 and MMP-2 activities, we used zymography as described previously [30].

To further validate the specificity of CB1R in regulating post-SCI MMP activity, SCI injured mice were treated with CB1R agonist ACEA in the presence of CB1R specific antagonist SR141716 (SR1) or CB2R antagonist SR144528 (SR2). Mice were subjected to SCI ($n = 5$ per injury group, including vehicle, ACEA, SB1 + ACEA and SR2 + ACEA) or sham ($n = 3$ per sham group) surgery. Injured mice received either ACEA or vehicle treatment as above. SR1 or SR2 (3 mg/kg, i.p.) were given 5 min before ACEA treatment. Twenty-four hours later, the mice were euthanized and spinal cords were harvested for MMP-9 activity measurement as described above.

2.6. Statistical analysis

Individuals blinded to treatment group assignment collected all data. Parametric data (latency to fall, compression lesion volume and MMP density) are expressed as mean ± standard deviation and were analyzed using repeated measures of ANOVA (rotarod function with time as the repeat variable) or one-way ANOVA as appropriate. BMS scores were compared by the non-parametric Mann Whitney U statistic. Values are reported as

median ± interquartile range. A P value <0.05 was considered significant. Sham-operated mice were not included in the statistical analysis, but are presented to provide normal reference values.

3. Results

3.1. ACEA treatment improved post-SCI functional recovery

After recovery from anesthesia, all mice exhibited normal forelimb function. However, the hindlimbs were completely paralyzed during compression. This neurologic deficit severity persisted for several hours after decompression, after which progressive partial recovery of function was observed. One mouse in the vehicle group died. The remaining mice survived the full 21-day recovery interval. Latency to fall from the rotarod was less than 50 s at 24 h post-injury in all mice confirming SCI (Fig. 1A). Hindlimb function partially recovered during the first 2 weeks in both groups. Mice treated with ACEA (3 mg/kg/day) had improved rotarod function over the 21-day recovery interval versus the vehicle-treated group (P = 0.04).

In both groups, most mice were able to use the hindlimbs to support body weight by 3 weeks (BMS score > 5). Numerically, a greater number of mice were able to coordinate forelimb, hindlimb and tail function (BMS = 9) in the ACEA (5 of 12) versus vehicle (0 of 11) groups. The ACEA group had better BMS scores at 21 days compared to vehicle (Fig. 1B, ACEA = 8 ± 3, vehicle = 5 ± 6, P = 0.02). ACEA did not affect post-injury spontaneous activity or produce anxiolysis (see Supplement).

3.2. ACEA treatment attenuated compression lesion volume

Histological examination of the spinal cords revealed well-demarcated lesion extending from the dorsal column into the dorsal horn and gray matter near the central canal (Fig. 1C). In some cases, the ventral horn was also damaged. Compression lesion volume (Fig. 1D) was 0.34 ± 0.11 mm^3 in the vehicle group. ACEA decreased compression lesion volume by 29% (0.24 ± 0.11 mm^3, P = 0.04).

3.3. ACEA treatment inhibited post-SCI MMP-9 activity

MMP-9 activity was markedly increased at 24 h, 7 and 14 days post-SCI in vehicle mice. This was attenuated by ACEA (Fig. 2B). Post-SCI MMP-2 activity was changed from baseline. ACEA produced a small, but significant, decrease in MMP-2 activity at 7 and 14 days, but not at 24 h (Fig. 2C). Gelatinase activity measured at 24 h and 7 days post-SCI (see Supplement) is most consistent with the change in MMP-9 activity observed at same intervals.

3.4. Effect of CB1 and CB2R antagonist on ACEA-mediated MMP activity

CB1R and CB2R antagonists (SR1 and SR2, respectively) were used to determine (a) the specificity of ACEA for CB1R and (b) the role of CB2R in ACEA-mediated inhibition of MMP activity following SCI. SR2 did not block SCI-mediated increases in MMP-9 activity. The ACEA-mediated decrease in post-SCI MMP-9 activity

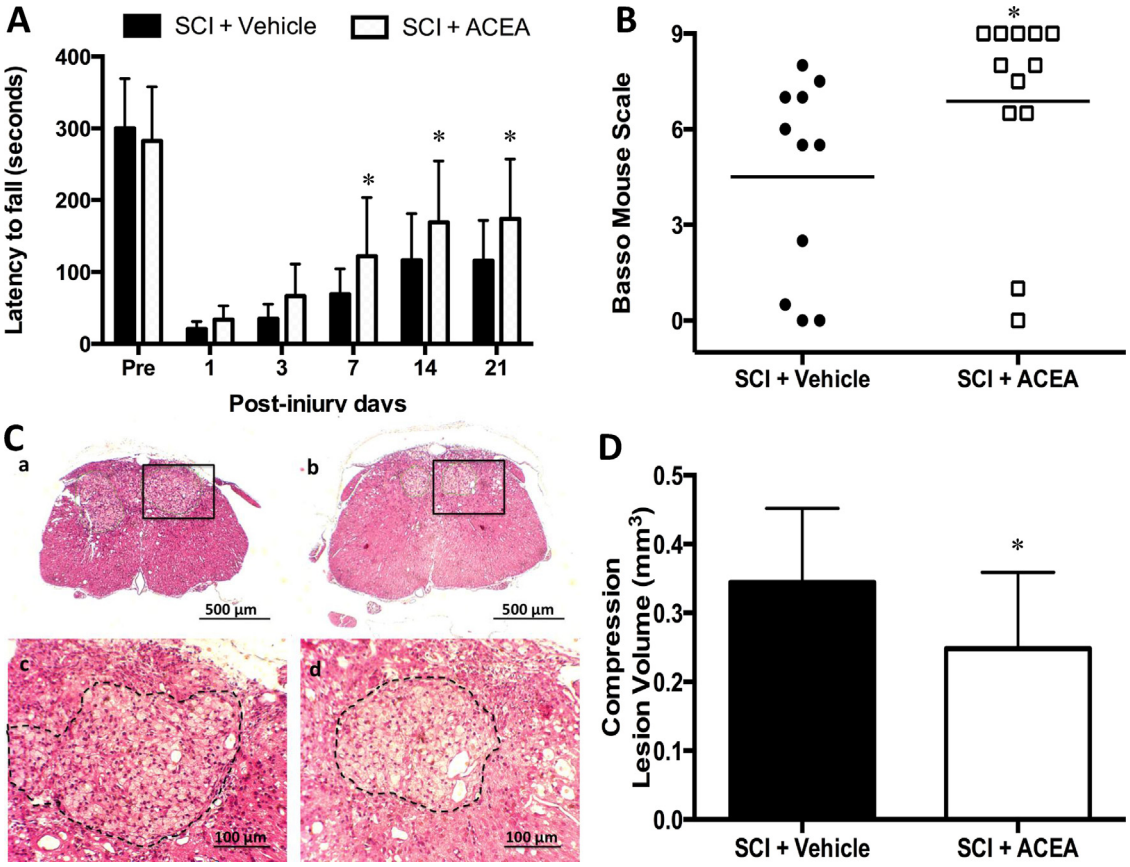

Fig. 1. Effect of ACEA on post-SCI functional and histological outcome. (A) Rotarod performance; (B) Basso Mouse Scale (BMS) scores; (C) representative images of lesion. a and b represent low magnification (50×) coronal thoracic (T11) spinal cord sections from the vehicle and ACEA groups, respectively. c and d depict high magnification (200×) images of cursor-outlined lesion margins within the rectangular regions of interest depicted in a and b. (D) Compression lesion volumes 21 days post-SCI (mean ± SD, ACEA n = 12 and vehicle n = 11). *P < 0.05 versus Vehicle.

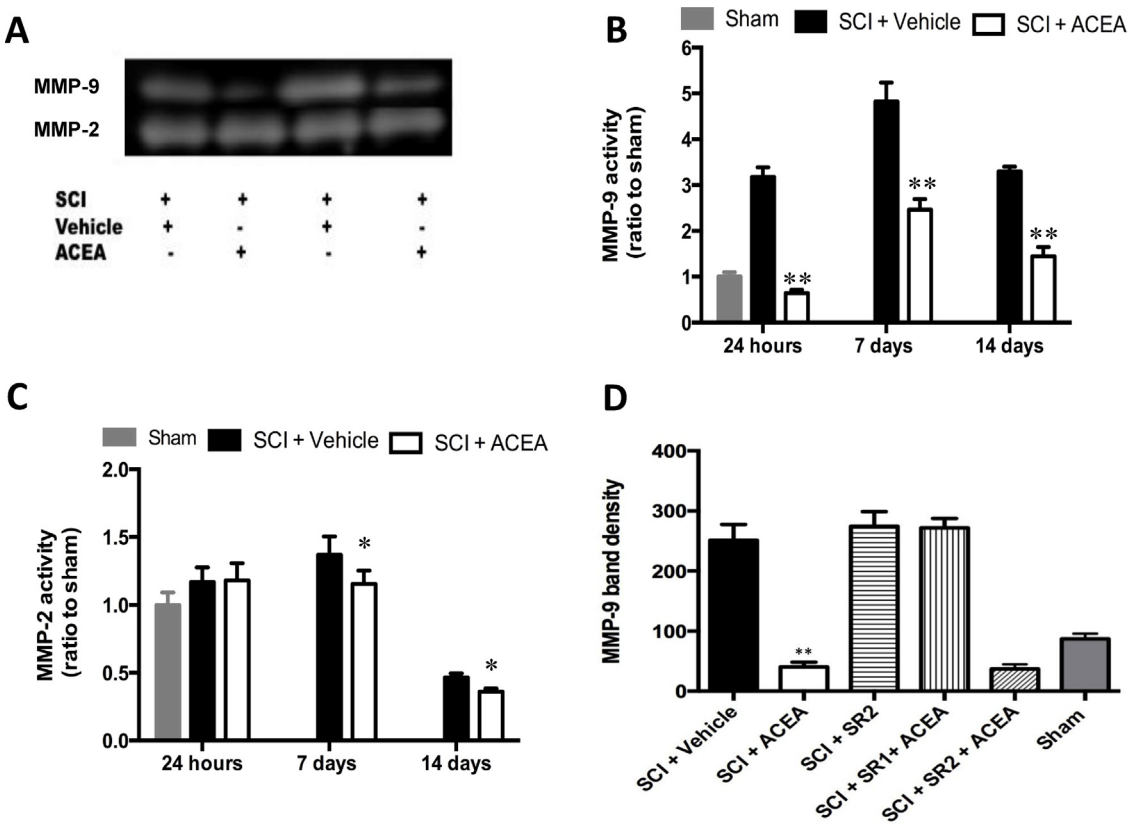

Fig. 2. ACEA-treatment differentially regulated post-SCI MMP-9 and MMP-2. (A) Representative zymograms of tissue samples at 24 h post-SCI. B anc C show MMP-9 and MMP-2 band densities at 24 h ($n=6$), 7 days ($n=4$) and 14 days ($n=6$) post-SCI, respectively. D shows effects of CB1R and CB2R antagonists on ACEA-mediated post-SCI MMP-9 decrease. SR1 blocked the effect of ACEA while SR2 did not. Data are expressed as mean ± SD. $^{*}P<0.05$ versus vehicle, $^{**}P<0.01$ versus SCI vehicle or SR1 + SCI ACEA.

was blocked by SR1 (Fig. 2D, $n=5$, $P<0.01$), but not by SR2 ($n=5$, $P=0.54$).

4. Discussion

Cannabinoids have been reported to decrease damage in neurodegenerative disorders through antioxidant, anti-inflammatory, and anti-excitotoxic mechanisms [13,24]. Synthetic cannabinoid compounds also have beneficial effects in experimental ischemic brain injury [7,9,17,28,31,48]. The current study found that treatment with ACEA, an exogenous CB1R agonist, improved both long-term functional and histologic outcome from a spinal cord compression insult in mice. SCI induced a sustained increase in MMP-9, but not MMP-2, activity. We further showed the specificity of CB1R in the process because ACEA mediated decrease in MMP-9 activity was blocked by SR1, but not SR2. Our findings of exogenous cannabinoid efficacy are consistent with investigations in other models of CNS injury, and further provide evidence that inhibition of injury-induced increases in MMP-9 activity has thepaeutic potential.

The CB1R is abundant in brain and spinal cord [20,40] and plays a significant role in neuroprotection [19] and neurogenesis [2,15,26]. In a mouse traumatic brain injury (TBI) model, intravenous treatment with the endocabinoid 2-AG improved functional recovery and prevented hippocampal neuronal death [36]. Beneficial effects of 2-AG have been associated with decreased NF-kB activity [34], cytokine expression, and blood–brain barrier dysfunction [35]. The therapeutic effect of 2-AG appeared to be mediated by CB1R because the CB1R antagonist SR141716 abolished beneficial effects of 2-AG, thus, segregating the CB1R (as opposed to CB2R) as the critical signal cascade for further focus on TBI therapeutics [34,36].

In a rat spinal cord contusion model, endocannabinoid concentrations and CB1R expression in neurons, oligodendrocytes and reactive astrocytes were increased [18]. In a different study, injury size was decreased in animals treated with 2-AG [5]. This response was blocked by the specific CB1R antagonist SR141716. In the current study, we found that the synthetic CB1R agonist ACEA also improved long-term post-injury motor function and decreased compression lesion volume. The CB2R antagonist did not block the effect of ACEA on SCI-induced MMP-9 activity (Fig. 2D). In contrast, the ACEA-induced suppression of MMP-9 activity was completed inhibited by co-treatment with a CB1R antagonist. Collectively, these results indicate that CB1R activation (either by a small molecule synthetic agonist or endogenous cannabinoid) has beneficial effects and thus, the CB1R is a potential therapeutic target for SCI treatment.

In the current study, we demonstrated the beneficial effects of CB1R agonist ACEA in inhibiting SCI-induced MMP activity and functional recovery. Other studies have highlighted the importance of the CB2R in the recovery process. Adhikary et al. [1] demonstrated that the CB2R agonist (O-1966) decreases inflammatory responses and promotes functional recovery following SCI. This was associated with decreased CXCL-9 and CXCL-11 levels and decreased expression of IL-23p19 and its receptor IL-23r. They also showed that O-1966 treatment inhibited toll-like receptor expression (TLR1, TLR4, TLR6, and TLR7). MMP activity was not measured. Collectively these studies and our results suggest that both the CB1R and CB2R agonism may provide beneficial effects in SCI.

MMPs are a family of extracellular zinc and calcium-dependent endopeptidases that degrade the extracellular matrix and other extracellular proteins [43]. They serve as physiological mediators regulating important processes in extracellular matrix remodeling, such as developmental morphogenesis and wound healing [47].

However, excessive proteolytic activities can be detrimental. Both animal and clinical investigations have shown that MMP levels and activity are increased several-fold after traumatic SCI [11,12,16,45]. MMP-9 is expressed in neurons, reactive atrocytes, infiltrating leukocytes [32]. MMP-9 activation is implicated in blood–spinal cord barrier (BCSC) disruption [32]. Enhanced MMP-9 activity decreases functional recovery following SCI by modulation of early vascular events [32]. MMP-9-null mice exhibit less BSCB disruption, attenuated neutrophil infiltration, and better locomotor recovery compared with wild-type mice [32]. The protection was attributed to attenuated MMP-9 expression. Interestingly, induction of MMPs in post-mortem SCI patients was not accompanied by an increase in endogenous tissue inhibitors of metalloproteinase. This indicates that uninhibited MMP activity may cause further damage in lesioned spinal cord [11]. Recently MMP-9 was found to facilitate glial scar formation in murine SCI [22]. Further, the size of glial scar was decreased in MMP-9 null mice. MMP-9 was ascribed to promote early migration of astrocytes to form an initially immature gliotic meshwork in the lesion, which later develops into a more complex glial scar. MMP-9 also enhances infiltration of inflammatory cells and increased cytokine production (e.g., interferon-γ and TGF-β), which are known to induce glial scar formation. Thus, MMP-9 inhibition, as detected in ACEA-treated mice in the current experiment, may have contributed to their improved long-term functional outcome.

ACEA treatment caused a sustained and marked inhibition of MMP-9 activity in our SCI model and improved long-term neurologic function. ACEA had no effect on MMP-2 at 24 h. ACEA produced a decrease in MMP-2 activity by 16% and 22% at 7 and 14 days post-SCI, respectively. Because MMP-2 is known to peak at 7–14 days post-SCI [23], the importance of this modest ACEA effect on MMP-2 is not clear. Hsu et al. [23] reported that MMP-2 null mice had impaired recovery after SCI, thus, sustained treatment with ACEA, to the extent that MMP-2 inhibition is adverse, plausibly could present an adverse effect on recovery, albeit this appears outweighed by its inhibition of MMP-9 activity.

Our study demonstrated that activation of CB1R using a synthetic agonist improved post-SCI functional recovery and decreased compression lesion volume. Outcome improvement may be mediated, at least in part, through inhibition of MMP-9 activity by CB1R agonism. Synthetic selective CB1R agonists with parenteral bioavailability are available. Further experiments with cannabinoids in SCI are required to define the therapeutic window, optimal treatment duration, and mechanisms associated with improved long-term recovery.

Conflict of interest statement

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2015.04.016.

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