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Research article

The triple monoamine re-uptake inhibitor DOV 216,303 promotes functional recovery after spinal cord contusion injury in mice

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ABSTRACT

Serotonin, noradrenaline and dopamine are important neuromodulators for locomotion in the spinal cord. Disruption of descending axons after spinal cord injury resulted in reduction of excitatory and neuromodulatory inputs to spinal neurons for locomotion. Receptor agonists or reuptake inhibitors for these neuromodulators have been shown to be beneficial in incomplete spinal cord injury. In this study, we tested a triple re-uptake inhibitor, DOV 216,303, for its ability to affect motor function recovery after spinal cord injury in mice. We impacted C57 mouse spinal cord at the T11 vertebral level and administered vehicle or DOV 216,303 at 10 mg/kg, b.i.d via intraperitoneal injections for 7 days. We monitored motor function with the Basso Mouse Scale for locomotion for 4 weeks. Spinal cords were harvested and histological examinations were performed to assess tissue sparing and lesion severity. Results showed that DOV 216,303-treated mice recovered significantly better than vehicle treated mice starting at 14 days post injury until the end of the survival period. Lesion size of the DOV 216,303 treated mice was also smaller compared to that of vehicle treated mice. This study suggests DOV 216,303 as a potential therapeutic after spinal cord injury warrants further investigation.

1. Introduction

Contusion injury is the most common type of spinal cord injury in humans [1]. Such injury not only interrupts the excitatory drive from major descending pathways, it also affects the descending fibres that modulate neuronal excitability. The brainstem-derived neuromodulatory inputs, mostly mediated via serotonin (5-HT) and noradrenaline (NA) actions on metabotropic receptors, affect the threshold and persistent inward current of motor neurons [2,3]. This in turn influences the ability of motor neurons to trigger an action potential and release of neurotransmitters at the motor end plate for muscle contraction. Without neuromodulation, motor neurons would not be able to sustain normal motor function, i.e. movement and posture, with excitatory ionotropic inputs alone [4–6].

The reduction of excitatory and neuromodulatory inputs immediately after injury places the spinal cord in a state of shock characterized by areflexia/hyporeflexia within 24 h [7]. Depending on the severity, the spinal cord will gradually recover some of its function due to compensatory mechanisms and plasticity [8]. Spinal motor neurons also regain their excitability by up-regulating constitutively active 5-HT_{2C} receptors to compensate for the loss of neuromodulatory inputs [9]. At the same time, supraspinal aminergic fibres regrow and rebuild connections with motor neurons, and serotonergic fibres in particular are known to regenerate through the lesion [10–12]. The importance of neuromodulation in locomotion prompted us to ask whether increasing the action of neuromodulators by inhibiting their re-uptake would improve functional recovery after traumatic spinal cord injury. Previous experiments showed that the selective serotonin reuptake inhibitor fluoxetine promotes functional recovery after T9 spinal contusion in mice due to its action on endothelial and glial cells, however its effects on the serotonergic system were not investigated [13,14]. A clinical trial studying serotonin modulation in spinal cord injured patients using the re-uptake inhibitor escitalopram is currently ongoing (NCT01788969). Experimental studies using a combination of buspirone, levodopa and carbidopa targeting serotonergic and dopaminergic pathways after spinal cord injury showed that mice receiving the triple-therapy had better movement than those with buspirone alone (i.e. serotonergic pathway modulation) [15].

We reasoned that a broader spectrum inhibitor might further improve recovery. DOV 216,303 ([(+/−)-1-(3,4-dichlorophenyl)-3-azabicycle [3.1.0] hexane hydrochloride]) is an anti-depressant with a high affinity for serotonin, noradrenaline and dopamine transporters

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(IC_{50} = 14, 20 and 78 nM respectively) [16]. A phase I clinical study has confirmed its safety and tolerability in healthy individuals [17]. Besides its effects on neuron excitability, we expected DOV 216,303 to exhibit pleiotropic effects which could be beneficial to spinal cord injury patients. First, because 5-HT and NA are involved in pain modulation [18,19], the 5-HT and NA re-uptake inhibitor duloxetine was shown to be beneficial for diabetic neuropathic pain and could therefore be beneficial in SCI patients [20,21]. Since DOV 216,303 exhibits similar potency [16], it is expected the drug can also alleviate pain. In addition, recent animal and human studies suggest pain is not just a symptom after spinal cord injury, but could also adversely influence spinal motor learning [22,23]. It was shown that pain induction by externally applied capsaicin cream modifies the motor strategies to carry out a performance in healthy human participants [24]. Second, although descending dopaminergic fibres do not have dopamine (DA) transporters [25], this may limit the utility of dopamine transporter reuptake inhibition in the spinal cord; however dopamine pathways in the brain are important for initiating movement [26,27]. Specifically, it has been shown that a group of dopaminergic cells in the posterior tuberculum in lamprey, homologous to the substantia nigra pars compacta/ventral tegmental area in mammals, sends projections to the mesencephalic locomotor region which controls locomotion. Taken together, we expected that a triple re-uptake inhibitor might work in concert in the brain and spinal cord to improve locomotion following SCI.

2. Material and methods

2.1. Animals

All animal experiments were approved by the Animal Care Committee at the University of Calgary. Female C57BL/6 mice from Charles River at 10–11 weeks of age were used. A total of 26 mice were used in the study.

2.2. Vehicle and drug preparation

DOV 216,303 was purchased from Sigma Aldrich (Catalog Number: D6446) and was dissolved in 5% dimethyl sulfoxide (DMSO) in sterile normal saline. Vehicle consisted of 5% DMSO in sterile normal saline.

2.3. Surgery, post-operative care and drug delivery

Mice were deeply anaesthetized using 1.5% isoflurane with oxygen, the surgical area was then shaved and sterilized with isopropanol and betadine. Buprenorphine (0.05 mg/kg) and Baytril (5 mg/kg) were given before surgery. A midline incision was made to expose the vertebral column, and a laminectomy was then performed at the eleventh thoracic vertebra. The vertebrae above and below the exposed spinal cord were stabilized with a pair of clamping forceps. The cord was then placed under the impact tip of an Infinite Horizon spinal cord impactor (Precision Systems and Instrumentation, Lexington, KY). An impact force of 75 kdynes was used. After the impact, the displacement of the tip was noted and only those animals with displacement between 600 μm and 670 μm were retained. The incision was sutured in layers and the animal returned to its home cage for recovery on a heating pad. Mice were then randomized into vehicle or drug treated group. The first dose of vehicle or DOV 216,303 (10 mg/kg) was given by intraperitoneal injection 1 h after the impact, then repeated twice daily every 12 h for 7 days. This dosage was chosen based on that study that shows 20 mg/kg/day exerts antidepressant effects in rat depression model [28]. We divided our dosing to twice a day since DOV 216,303 has a relatively short half-life (~3–4 h) [16,29]. Post-operative care included buprenorphine and Baytril administration for 2 days after surgery. Manual voiding of bladders was performed twice daily until return of bladder function.

2.4. Behavourial assessment

Mice were screened for pre-existing abnormality and allowed to become accustomed to the testing environment before surgery. After surgery, hindlimb function was monitored according to the Basso Mouse Scale for locomotion (BMS) [30]. Briefly, the mouse was placed in a 2 m diameter circular plexiglass enclosure. It was allowed to move freely in the tank for 4 min and its locomotor performance was scored by two observers blinded to the treatment conditions. Scores for left and right sides were averaged for each animal.

2.5. Perfusion and tissue processing

Four weeks after surgery, animals were given a lethal dose of sodium pentobarbital and perfused intracardially with normal saline followed by ~50 ml of fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Spinal cords were harvested and post-fixed with fresh fixative overnight and subsequently placed in 30% phosphate buffered sucrose. After the samples had sunk, the cords were cut into 10-μm cross-sections on a cryostat. The sections were collected on coated slides and stored at −20 °C for later use.

2.6. Histology

Luxol fast blue (LFB) was used to stain myelin and neutral red (NR) was used as a counterstain. Briefly, frozen sections were washed in 0.01 M phosphate buffered saline (PBS) and were treated with chloroform/methanol in 2:1 (v/v) ratio for at least 1 h at room temperature. The sections were then washed with 95% ethanol and left in LFB solution (0.1% Solvent Blue 38 in 95% ethanol with 0.5% acetic acid) at 56 °C overnight. The sections were then rinsed with 95% ethanol and distilled water, followed by differentiation in 0.05% lithium carbonate and 70% ethanol. Sections were rinsed in distilled water before counterstaining with 1% neutral red for 5 min, excess stain was washed off with 70% ethanol. The sections were then dehydrated with ethanol series and cleared in xylene before mounting with Surgimount and coverslips.

2.7. Measurement of normal and pathological tissue

Stained sections were imaged with an Olympus Virtual Slide Scanner (Olympus VS110, Tokyo, Japan). Spinal cord white and grey matter sparing and lesion were measured with the Cavalieri method as previously described [31]. Briefly, a grid with equal point spacing was overlaid on top of the spinal cord image in Photoshop, the number of "+" that landed on designated regions of interest were counted. The spacing was adjusted so that an average of ~60 "+" would cover the entire normal appearing spinal cord.

2.8. Statistical analysis

Data are presented as mean ± SEM, two-way repeated-measure ANOVA followed by Bonferroni's post-hoc multi-comparison or Student's *t*-test, where appropriate, were used to determine the statistical significance of differences among the means. A value of $p < 0.05$ was considered significant.

3. Results

3.1. DOV 216,303 promoted functional recovery after SCI

We used female mice in our study due to their ease of care and handling. We operated on 26 animals and excluded 3 of them since their displacements were lower than specified. One animal suffered a ruptured bladder on day 3 and was euthanized. The remaining animals suffered no unexpected adverse events. BMS results showed that DOV 216,303-treated animals performed significantly better than vehicle