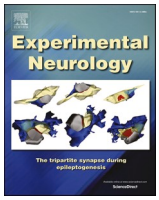
Experimental Neurology 382 (2024) 114993

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr



Research paper

Neuroprotective agents ineffective in mitigating autonomic dysreflexia following experimental spinal cord injury

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ARTICLE INFO

Keywords:
Spinal cord injury
Autonomic dysreflexia
Neuroprotection
Valproic acid
Glyburide
Fluoxetine
Indomethacin

ABSTRACT

Background and objectives: Loss of supraspinal cardiovascular control and secondary damage following spinal cord injury (SCI) lead to cardiovascular dysfunction, where autonomic dysreflexia (AD), triggered by stimuli below the injury, can cause uncontrolled blood pressure (BP) surges, posing severe health risks such as stroke and seizures. While anti-inflammatory neuroprotective agents have been studied for motor recovery, their impact on cardiovascular function remains under investigated. The objective was to assess the efficacy of four clinically approved neuroprotective agents in promoting cardiovascular recovery following SCI.

Methods: Male Wistar rats received contusion at the third thoracic spinal segment (T3). Fluoxetine, Glyburide, Valproic acid, and Indomethacin were first administered at 1 h or 6 h post-SCI, and every 12 h for two weeks thereafter. Four weeks following SCI, hemodynamics were measured at rest and during colorectal distension. Locomotor function was assessed prior to SCI and weekly for four weeks after SCI, using the Basso-Beattie-Bresnahan (BBB) locomotor scale. Quantitative comparisons of lesion area were performed.

Results: Contrary to the published literature, Indomethacin and Valproic acid resulted in high morbidity and mortality rates 60 % and 40 % respectively) within 2–3 days of administration. Fluoxetine, and Glyburide were well-tolerated. There were no differences in change in systolic BP with colorectal distension compared to control i.e., all experimental groups experienced severe episodes of AD [$F(6, 67) = 0.94$, $p = 0.47$]. There was no significant difference in BBB scores in any experimental group compared to control [$F(18, 252) = 0.3$, $p = 0.99$]. No between-group differences were observed in tissue sparing at the lesion epicentre [$F(6, 422) = 6.98$, $p = 0.29$].

Discussion: Despite promising beneficial effect reported in previous studies, none of the drugs demonstrated improvement in cardiovascular or motor function. Indomethacin and Valproic acid exhibited unexpected high mortality at doses deemed safe in the literature. This emphasizes the necessity for reproducibility studies in pre-clinical research and underscores the importance of publishing null findings to guide future investigations.

1. Introduction

While paralysis is perceived as a major consequence of SCI, autonomic dysregulation is a leading cause of mortality and morbidity and includes cardiovascular, urinogenital, gastrointestinal, and sexual dysfunction, which often present concurrently (Krassioukov, 2009; Phillips and Krassioukov, 2015). Among these, autonomic dysreflexia (AD) ranks as a critical cardiovascular concern (Anderson, 2004). Often triggered by innocuous stimuli, such as bowel and bladder management, AD results in a cascade of physiological responses that can pose substantial health risks, such as hemorrhage, stroke and even death (Cragg et al., 2013; Garshick et al., 2005; Krassioukov and Claydon, 2006;

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https://doi.org/10.1016/j.expneurol.2024.114993
Received 30 August 2024; Received in revised form 7 October 2024; Accepted 8 October 2024
Available online 10 October 2024

Teasell et al., 2000; Wu et al., 2012).

The aftermath of traumatic SCI presents a multifaceted challenge, initiating a complex cascade of events minutes within injury, including direct mechanical trauma to neurons and glia, vascular injuries resulting in hemorrhage and ischemia, and subsequent metabolic and biochemical changes (Ahuja et al., 2017; Alizadeh et al., 2019; Oyinbo, 2011; Tran et al., 2018). Secondary damage begins with hemorrhage and cell death, with inflammation causing spinal cord swelling and mechanical compression (Ahuja et al., 2017; Alizadeh et al., 2019; Oyinbo, 2011; Tran et al., 2018), eventually resulting in the development of a significant lesion site within the injured spinal cord, disrupting both intraspinal and supraspinal pathways (Ahuja et al., 2017; Alizadeh et al., 2019; Oyinbo, 2011; Tran et al., 2018).

The prominent causes for AD are the loss of supraspinal medullary input on sympathetic pre-ganglionic neurons, controlling spinal sympathetic outflow to postganglionic neurons and in turn to blood vessels (Fossey et al., 2022; Krassioukov et al., 2009; Krassioukov et al., 2002; Squair et al., 2015). Concurrent aberrant sprouting of small-diameter afferent fibers at the spinal level elicits an exaggerated response to incoming nociceptive input, which combined with the loss of supraspinal control, leads to severe unchecked BP spikes (Cameron et al., 2006; Krenz and Weaver, 1998). The extent of preserved descending vasomotor pathways has been shown to be directly related to the extent of cardiovascular dysfunction (Furlan et al., 2003). Therefore, preservation of descending spinal sympathetic fibers using neuroprotective agents may preserve BP control, and reduce the severity of AD.

Extensive research has been dedicated to investigating early interventions to minimize secondary damage and preserving sufficient intraspinal and supraspinal pathways crucial for sustaining functionality post-injury (Ditor et al., 2006, 2007; Fleming et al., 2008; Gris et al., 2004, 2005; Marsh and Flemming, 2011; Rabchevsky et al., 2012; Webb et al., 2006). Current experimental approaches have centered on anti-inflammatory agents, albeit primarily in preclinical settings (Kwon et al., 2011). These interventions, ranging from immune-modulating agents to cytokine inhibitors, have demonstrated limited clinical application due to their experimental nature and lack of regulatory approval for clinical use (Ditor et al., 2006, 2007; Fleming et al., 2008; Gris et al., 2004, 2005; Marsh and Flemming, 2011; Rabchevsky et al., 2012; Webb et al., 2006). Nonetheless, they have exhibited promising outcomes with respect to neurological recovery in rodent models, alluding to the prospect of preserving cardiovascular function following SCI.

Several drugs, originally FDA approved for conditions unrelated to SCI, have shown neuroprotective effects and have been successful in promoting functional locomotor recovery in experimental SCI studies. Among these, fluoxetine, a selective serotonin reuptake inhibitor used primarily as an antidepressant, has shown potential in maintaining the blood-spinal cord barrier and enhancing locomotor function following SCI (Bianchi et al., 1995; Cristante et al., 2013; Jin et al., 2021; Lee et al., 2015, 2016; Lee et al., 2012b; Lim et al., 2009; Ma et al., 2021; Scali et al., 2013). Glyburide, a sulfonylurea receptor 1 blocker primarily used in diabetes management, has emerged as a candidate for mitigating the effects of secondary hemorrhage through its potential to stabilize blood vessels and reduce edema (Popovich et al., 2012; Redondo-Castro et al., 2013; Simard et al., 2007, 2010, 2012). Similarly, valproic acid, a histone deacetylase inhibitor employed in epilepsy treatment, holds promise in reducing the expression of inflammatory mediators and thwarting apoptotic cell death (Abdanipour et al., 2012; Lee et al., 2014; Lee et al., 2012a; Lv et al., 2011; Yu et al., 2012). Indomethacin, a non-steroidal anti-inflammatory drug targeting prostaglandin production, also offers anti-inflammatory effect post-SCI, further expanding the repertoire of potential interventions (Guth et al., 1994; Nygård et al., 1994; Pantović et al., 2005; Sharma et al., 1993; Simpson et al., 1991; Winkler et al., 1993).

In the context of preserving cardiovascular function, the potential for these FDA approved drugs to promote locomotor recovery is particularly encouraging, as fewer preserved fibers of the autonomic pathways may be needed compared to motor function (Squair et al., 2015). Indeed, recent work from our laboratory demonstrates the neuroprotective effects of minocycline, a clinically available antibiotic possessing anti-inflammatory properties (Squair et al., 2018). Building on this work, we sought to expand our investigation to test the efficacy of four FDA-approved drugs, fluoxetine, glyburide, valproic acid, and indomethacin, in promoting neuroprotection and cardiovascular recovery after experimental SCI. Two timepoints were selected for the first dose: one hour following injury where the hemorrhage is relatively confined and the lesion volume similar to the initial mechanical trauma (Ek et al., 2010), and six hours post injury as the more clinically relevant timepoint, that would still target earlier stages of inflammation and ischemia, with the hypothesis that an earlier intervention is likely more beneficial for minimizing secondary damage.

2. Methods

2.1. Standard protocol approvals, registrations, and patient consents

All procedures were performed in compliance with the Canadian Council on Animal Committee guidelines. All protocols were approved by the Animal Care Committee at the University of British Columbia (Protocol numbers A18–0183 and A22–0137).

2.2. Study design

Adult male Wistar rats (~300 g, Envigo; USA) received a severe T3 contusion injury (Squair et al., 2017). Fluoxetine, glyburide, valproic acid, and indomethacin were selected (Fig. 1). Animals ($n = 110$) were randomly assigned to one of 5 experimental groups. Owing to mortality rates attributed to drug side effects and the severity of surgeries, final sample sizes used for analysis were (total $n = 73$), Vehicle ($n = 14$), fluoxetine (1 h, $n = 9$; 6 h, n = 9), glyburide (1 h, $n = 10$; 6 h, n = 9), valproic acid (1 h, n = 11; 6 h, n = 11). Although discontinued and not used for final analysis, indomethacin sample sizes were: 1 h, n = 10; 6 h, $n = 10$. BBB open-field locomotor assessment was performed at pre-injury state and weekly following SCI, continuing until 4 weeks post SCI. Telemeter implant and cardiovascular assessments occurred four weeks following injury, concluding with euthanasia and spinal cord tissue harvesting.

2.3. Spinal cord injury

Three days prior to surgery, animals were administered prophylactic enrofloxacin (Baytril™ 10 mg/kg, s.c., Associated Veterinary Purchasing (AVP), Elanco, Canada. On the day of surgery enrofloxacin, meloxicam (Metacam 5 mg/kg, s.c. AVP, Boehringer Ingelheim, Canada, buprenorphine (Bupaq; 0.02 mg/kg; s.c., CDMV) bupivacaine (5 mg/kg; s.c., AVP), and Ringer's solution (5 ml s.c., Baxter corporation, Canada) were administered pre-operatively. Rats were anesthetized with isoflurane (initially in an induction chamber at 2 l min^{-1} oxygen and a maintenance dose of approximately 1.5–2 l min^{-1} oxygen using a Bain Circuit). A dorsal midline incision was made in the superficial muscle overlying the C8-T3 vertebrae, followed by a laminectomy of the T3 vertebra. The animal was then transferred to the Infinite Horizons impactor stage, where the T2 and T4 spinous processes were securely clamped with modified Allis forceps and impactor tip (2.5 mm) aligned to be midline. The impact was set to be 400kdyn, with a 5 s dwell time. This severity of injury results significant disruption of the sympathetic cardiovascular control and severe autonomic dysreflexia as established previously (Squair et al., 2017). To ensure that the impact parameters were similar between groups, we examined the IH impactor output. The biomechanical output parameters (force [kdyn], displacement [mm] and velocity [mm/s]) after T3 contusion were consistent across all animals. Following impact, the muscle and skin were closed using 4–0 Monocryl (Ethicon, USA) and 4–0 Prolene (Ethicon, USA) sutures,

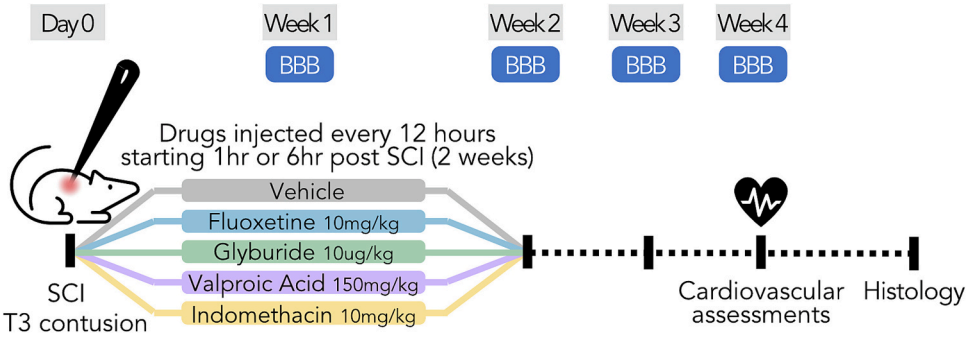

Fig. 1. Study Timeline. Animals were administered one of four neuroprotective drugs or a control vehicle for a total duration of two weeks starting 1- or 6- h(s) post injury. Four weeks after SCI, cardiovascular recovery was assessed. Concurrently, BBB assessment was conducted once per week to track locomotor recovery, starting 1 week following SCI. Finally, animals were euthanized to complete histological analyses. Continuous monitoring for potential adverse effects was conducted throughout the entire study duration.

respectively. Animals were then transferred to a temperature-controlled environment (Animal Intensive Care Unit, Los Angeles, CA) for 90 min for the effects of isoflurane to dissipate. Post-operative care included enrofloxacin (10 mg/kg, s.c.), buprenorphine (0.02 mg/kg, s.c.) meloxicam (Metacam 5 mg/kg, s.c) and Ringer's solution every 12 h for 3 days post-operatively. Manual bladder expression was performed three times daily post-injury until animals regained spontaneous bladder emptying.

2.4. Drug administration

Fluoxetine hydrochloride (Millipore Sigma), glyburide (Millipore Sigma), valproic acid sodium salt; (Millipore Sigma), and indomethacin (Millipore Sigma) stock solutions were prepared fresh daily and stored at 4 °C, in accordance with the manufacturers' instructions. Fluoxetine was dissolved in 0.9 % saline (Baxter corporation) and injected intraperitoneally (IP) at 10 mg/kg (Bianchi et al., 1995; Lim et al., 2009). Glyburide was dissolved in DMSO (Millipore Sigma) and injected at 10μg/kg IP (Simard et al., 2007, 2012). Valproic acid was dissolved in 0.9 % saline and initially injected at 300 mg/kg IP (Abdanipour et al., 2012; Lv et al., 2011), which was reduced to 150 mg/kg following high mortality rates. Lastly, indomethacin was dissolved in ethanol and saline and injected at 10 mg/kg. Vehicle injection consisted of an IP administration of 0.3 ml saline. Drugs or vehicle were first administered at 1 or 6 h after injury. Subsequently, animals received their respective drugs every 12 h for two weeks.

2.5. Telemeter implant

Four weeks after SCI, animals were implanted with telemeter device-TRM54P (Kaha Pressure Telemeter; KAHA Science, AD Instruments USA.) as described earlier (Sachdeva et al., 2021). Briefly, rats were anesthetized with isoflurane, and a skin incision was made on the ventral abdomen and inner thigh on the right side to expose the femoral artery. The vessel was secured distally with a permanent silk knot and temporary blocked proximally by a loose suture. Next, a small opening was formed in the femoral artery using a modified 25-gauge needle. The pressure transducer tip of the telemeter was inserted and secured within the femoral artery using silk ligatures. The body of the telemeter was placed subcutaneously and secured using suture tabs. Finally, the skin was sutured with 4–0 suture and the rat was given a minimum of 1 h recovery time prior to commencing physiological experiments.

2.6. Experimental autonomic dysreflexia and severity assessment

Following telemeter implantation, un-anesthetised animals in home cages were transferred to a Kaha SmartPad (TR181; AD Instruments USA), where resting beat-by-beat BP and HR were recorded during the daytime over a 10-min period prior to the assessment of autonomic dysreflexia and were converted from analog to digital (PowerLab/16S ML 795; AD Instruments, USA, LabChart 8; AD Instruments, USA). A Foley balloon-tipped catheter (AA6110 Coloplast, Canada) was used to perform colorectal distension (CRD), a stimulus that mimics clinical manifestation of colon impaction and induces severe AD (Maiorov et al., 1997). Following baseline hemodynamic assessment, the balloon-tipped catheter was inserted 2 cm into the rectum, and the measurements of BP and HR were collected, prior to balloon inflation (1 min). The balloon was inflated with 2 ml of air over 10s and maintained for 60s. The difference between the baseline before inflation and average BP and HR during CRD was calculated and averaged across two trials per rat to obtain AD severity.

2.7. Motor recovery assessment

Post-injury hind-limb function was assessed using the established Basso-Beattie-Bresnahan (BBB) open-field test (Basso et al., 1995). Rats were habituated to a 1.2 × 1.5 m clear, enclosed box prior to injury and pre-injury assessments were performed. Following SCI, two experimenters blinded to the animal groups individually scored the rats weekly for four minutes per session, up to 4 weeks post injury. Individual scores were discussed and averaged when disparity in scoring occurred. For each rat and timepoint, scores were averaged across both limbs.

2.8. Histology and lesion site analysis

Following physiological assessments, animals were euthanized and transcardially perfused with 250 ml of 0.1 M phosphate buffer (pH 7.4), followed by 400 ml of 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Spinal cords were harvested and stored overnight in 4 % paraformaldehyde at 4 °C before transfer into 30 % sucrose in 0.1 M phosphate buffer at 4 °C until analysis. Lesion epicenters were manually identified, and a tissue block corresponding to ±5 mm from the lesion epicentre was blocked in cryomatrix compound (VWR, Canada), for a total size of 1 cm. The rostral and caudal regions of interest (ROI) were selected to ensure the entire lesion cavity was captured and to avoid missing any damaged areas. Spinal cords were then sectioned in the transverse plane at 20 μm. Slides were stained with eriochrome cyanine (EC) for identification of spared white matter. Subsequently, slides were imaged and digitized for analysis in Aperio ImageScope (Leica Bio-Systems, version 12.4). Every tenth slide was used for analysis, corresponding to a ~ 200 μm distance between data points. Two regions of interest were manually traced. First, the total spinal cord cross section, consisting of the peripheral edges of the spinal cord. Second, the lesion site, identified as a region lacking regular cellular architecture, and EC staining. Tissue sparing was quantified by subtracting the lesion area

from the total area of the spinal cord. Finally, the percentage of tissue sparing was calculated for final analysis. To obtain average tissue sparing across rats in each experimental group, lesion epicenters were aligned before statistical analysis.

2.9. Statistical analysis and results reporting

All data were imported into Excel; final analysis was performed with GraphPad Prism (Version 10.0.0, Boston, Massachusetts, USA). Ordinary one-way ANOVA was used to detect differences between experimental groups for resting hemodynamics and severity of AD, and percentage of tissue sparing. A repeated measures two-way ANOVA was used to examine effects of drug and time post SCI (matched values) on BBB score. This approach was selected over regression, as we compared 4 time points. All post-hoc analysis was performed using Tukey's tests for multiple comparison. Data are reported as mean ± SD with statistical significance at $p < 0.05$. For ANOVA tests, we include η_p^2 (partial eta squared) values for effect size and accompanying 90 % confidence intervals (CI).

3. Results

3.1. Morbidity and mortality

Indomethacin and valproic acid resulted in severe morbidity and mortality within days of beginning administration. Within four days post SCI and beginning administration, Indomethacin resulted in >60 % mortality. The remaining rats in the Indomethacin group were euthanized in consultation with the veterinarian due to clinical signs indicative of a humane endpoint as per CCAC guidelines. This resulted in a discontinuation of the Indomethacin group of rats. Similarly, valproic acid resulted in a > 40 % mortality within four days after SCI and beginning administration. However, after reducing the drug dose by half to 150 mg/kg, rats recovered within 24–48 h. Selected pathological testing (Diagnostic Laboratory, Animal Care Services, University of British Columbia) revealed severe ulcerative gastritis and cystitis in the valproic acid and indomethacin treated group. The valproic acid group of rodents was continued at the lower dose, and sample size bolstered to sufficiently assess neuroprotective effects. Fluoxetine and glyburide were well-tolerated and continued as originally planned.

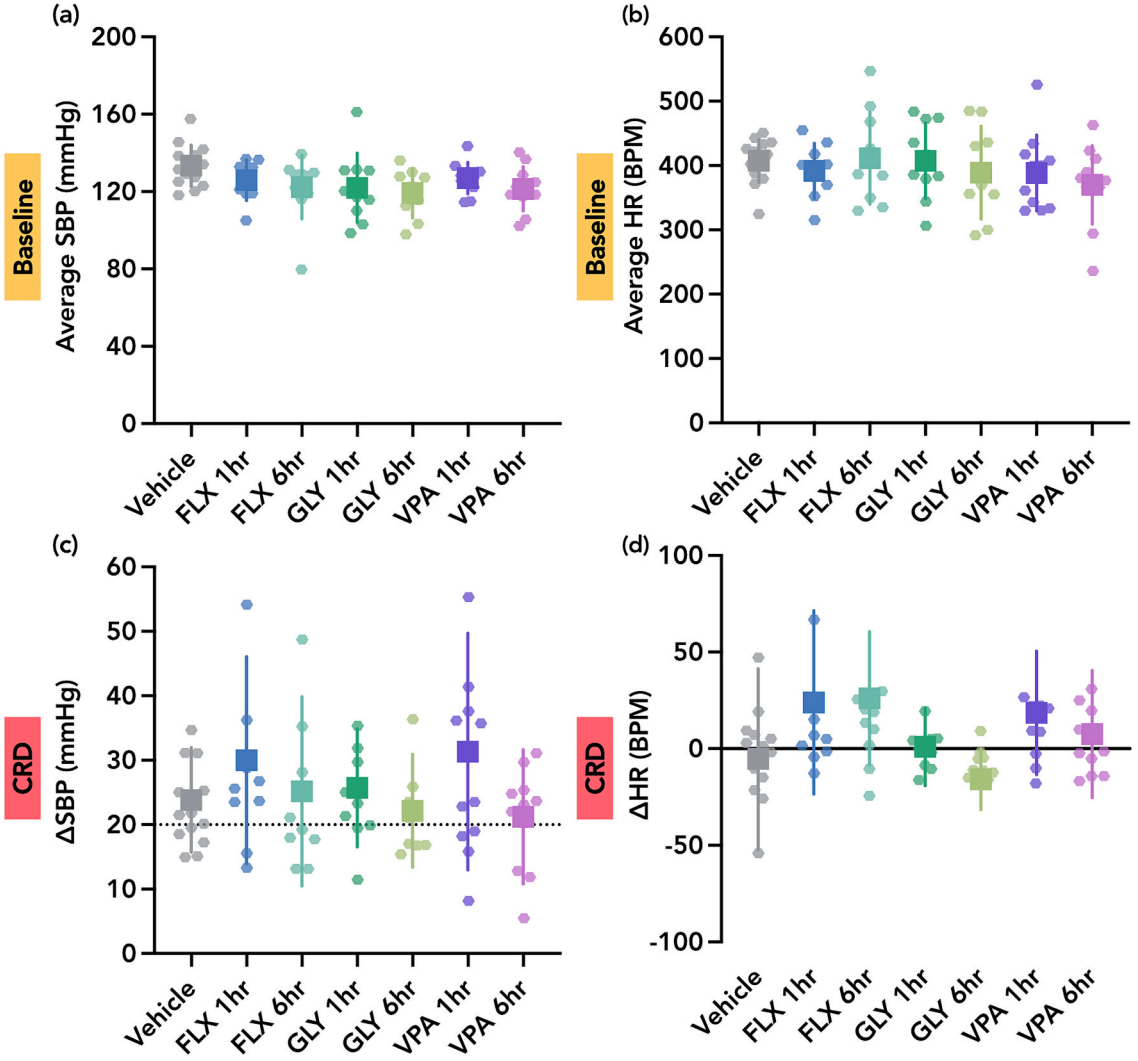

Fig. 2. Neuroprotective agents do not affect baseline hemodynamics (a, b) nor CRD-dependent average change in SBP (in mmHg) and HR (in BPM) compared with baseline in rats (b, c). Boxes represent mean values across all rats per experimental group; error bars represent SD. Vehicle $n = 14$; Fluoxetine (FLX) 1 h $n = 9$, 6 h n = 9; Glyburide (GLY) 1 h $n = 10$, 6 h n = 9; Valproic acid (VPA) 1 h $n = 11$, 6 h $n = 11$. (a) Average SBP (over 1 min) in mmHg for rodents prior to balloon insertion. (b) Average HR (over 1 min) in BPM for rodents prior to balloon insertion. (c) Change in SBP (ΔSBP) during CRD. Dashed line at 20 mmHg ΔSBP represents the clinical definition of AD. On average, ΔSBP was above this line for all groups, meaning all groups met the criteria for AD. (d) Change in HR (ΔHR) during CRD.

3.2. Cardiovascular function: baseline hemodynamics

We examined the impact of the drugs on resting hemodynamics (BP, HR). None of the drugs (fluoxetine, glyburide, valproic acid) showed any changes in BP (Fig. 2a) nor HR (Fig. 2b). There were no statistical differences between experimental groups (Table 1a) in baseline hemodynamics (SBP: [$F(6, 67) = 1.65$, $p = 0.14$, $\eta_p^2 = 0.13$, 90 % CI = [0 0.19]], DBP: [$F(6, 67) = 1.83$, $p = 0.11$, $\eta_p^2 = 0.14$, 90 % 90 % CI = [0 0.20]], MAP: [$F(6, 67) = 1.82$, $p = 0.11$, η_p^2 0.14, 90 % CI = [0 0.20]], HR: [$F(6, 67) = 0.7$, $p = 0.64$, $\eta_p^2 = 0.06$, 90 % CI = [0 0.09]]).

3.3. Cardiovascular function: autonomic dysreflexia

Next, we investigated the extent of cardiovascular dysfunction by quantifying the severity of AD during CRD. CRD resulted in AD regardless of experimental group (Fig. 2c, Fig. 3abc, Table 1b). Given the clinical definition of AD, which is a change in SBP of 20mmhg or more, none of the neuroprotective drugs ameliorated AD (Fig. 2c). Furthermore, there was no statistical difference between experimental groups in ΔSBP values [$F(6, 67) = 0.94$, $p = 0.47$, $\eta_p^2 = 0.07$, 90 % CI = [0 0.12]], indicating that the neuroprotective drugs have no impact on the severity of AD (Table 1). Similarly, there were no effects of experimental group on ΔDBP [$F(6, 67) = 1.67$, $p = 0.14$, $\eta_p^2 = 0.13$, 90 % CI = {0 0.19}], and ΔMAP [$F(6, 67) = 1.31$, $p = 0.27$, $\eta_p^2 = 0.1$, 90 % CI = {0 0.16}] (Table 1). No notable changes in ΔHR as result of neuroprotective drug were observed $F(6, 67) = 2.0$, $p = 0.42$, $\eta_p^2 = 0.16$, 90 % CI = {0 0.22}] (Fig. 2d, 3abc, Table 1).

3.4. Locomotor function

Previous studies examining fluoxetine, glyburide and valproic acid reported improvements in motor function following drug administration (Cristante et al., 2013; Lee et al., 2014, 2016; Lee et al., 2012a; Simard et al., 2007, 2012). Therefore, in addition to autonomic function, we also assessed BBB scores over four weeks following SCI. T3 contusion resulted in severe motor function deficit – BBB scores were < 1 (0.8) within the first week following SCI, meaning rodents had minimal movement in their hindlimbs. The two-way ANOVA revealed that there was no statistically significant interaction between neuroprotective drug and time post SCI [$F(18, 189) = 0.84$, $p = 0.66$, $\eta_p^2 = 0.07$] on BBB scores (Fig. 4). Simple main effects analysis showed no effect of neuroprotective drug on BBB score [$F(6, 63) = 0.67$, $p = 0.67$, $\eta_p^2 = 0.06$, 90 % CI = {0 0.09}], time post SCI however, showed a statistically significant effect [$F(2.346, 147.8) = 16.37$, $p < 0.0001$, $\eta_p^2 = 0.21$, 90 % CI = {0.11 0.29}], which was not entirely unexpected. (Squair et al., 2018) Post hoc tests were conducted to explore pairwise differences between groups. The analysis revealed no significant differences at the 0.05 significance level for nearly all pairwise comparisons. Within the vehicle and fluoxetine 6 h groups there was an effect of time on BBB score between 1- and 4-weeks post SCI (Vehicle mean difference = 2.13, 95 % CI = {0.21 4.04} $p = 0.03$; Fluoxetine 6 h mean difference = 2.05 95 % CI = {0.48 3.61}, $p = 0.012$). Over time, BBB scores increased for Vehicle and Fluoxetine 1-h groups. Additionally, there was an effect between 3- and 4-weeks post SCI in the glyburide 1 h group [mean difference = −1.85, 95 % CI = {−3.65–0.05}, $p = 0.04$], indicating that the BBB score dropped in the last week. Despite significance for some pairwise comparisons, the ANOVA indicates that no neuroprotective drug had an impact on functional motor recovery of rodents following a severe T3 contusion. Notably, on average, all rodents' BBB scores remained below 3 throughout the study duration, indicating that rodents had, at most, extensive movement of two joints – two of hip, knee and/or ankle (Basso et al., 1995). According to the scoring, this corresponds to an early phase of recovery, further highlighting the severity of injury (Basso et al., 1995).

Table 1
(a) Baseline hemodynamics at rest. Neuroprotective agents do not affect baseline hemodynamics (SBP, DBP, MAP in mmHg and HR in BPM) (b) Average change in hemodynamics following CRD across experimental groups and the result of statistical analysis. Neuroprotective agents do not affect severity of ΔSBP, ΔDBP, ΔMAP (in mmHg), and ΔHR (in BPM).

	Experimental Group						
	Vehicle	FLX 1 h	FLX 6 h	GLY 1 h	GLY 6 h	VPA 1 h	VPA 6 h
(a) Baseline hemodynamics							
SBP	133 ± 11	126 ± 11	122 ± 16	128 ± 18	119 ± 13	122 ± 18	127 ± 8
DBP	92 ± 10	84 ± 8	81 ± 15	81 ± 17	78 ± 8	81 ± 17	85 ± 7
MAP	106 ± 10	98 ± 9	95 ± 15	94 ± 17	92 ± 9	94 ± 17	99 ± 7
HR	407 ± 34	392 ± 43	411 ± 71	407 ± 59	389 ± 72	407 ± 59	388 ± 59
(b) Average change in hemodynamics following CRD							
ΔSBP	24 ± 8	30 ± 16	31 ± 18	26 ± 9	25 ± 15	22 ± 9	21 ± 10
ΔDBP	17 ± 5	22 ± 9	21 ± 12	17 ± 5	17 ± 9	16 ± 5	13 ± 7
ΔMAP	19 ± 7	25 ± 11	24 ± 14	20 ± 6	20 ± 11	18 ± 6	15 ± 8
ΔHR	−5 ± 47	24 ± 47	26 ± 35	1 ± 20	−16 ± 16	18 ± 32	8 ± 33

3.5. Tissue sparing

To characterize the neuroprotective effects of the selected drugs at the spinal cord level, we quantified the percentage of spared tissue surrounding the epicentre. In line with previous studies, T3 contusion SCI resulted in substantial lesion size at the injury epicentre. Here we observe spared tissue percentage of 14.36 ± 20.01 % at the epicentre for the vehicle group (Figs. 5, 6). Extending from the lesion center rostrally and caudally, the vehicle group indicates a rise in tissue density and a decline in lesion size (Fig. 5). This trend remains for the groups receiving neuroprotective drugs (Fig. 5). Specifically, we observed tissue sparing for: fluoxetine: 1 h = 13.07 ± 17.77 %, 6 h = 7.09 ± 4.87 %; glyburide: 1 h = 12.41 ± 8.84 %, 6 h = 11.27 ± 11.18 %; valproic acid: 1 h = 13.53 ± 16.67 %, 6 h = 3.87 ± 4.67 %. There was no effect of neuroprotective drugs on percentage of spared tissue [$F(6, 422) = 6.98$, $p = 0.29$], indicating no observable neuroprotective effects of drugs at the injury site. (Fig. 6).

4. Discussion

Contrary to our initial hypotheses and the results of previous studies, rats treated with the selected anti-inflammatory agents did not exhibit significant improvements in motor and cardiovascular function compared to the control group. One significant discovery was the unexpected toxicity of indomethacin and valproic acid at doses previously published to be safe in the SCI literature. There are multiple potential reasons for the results seen in this study, such as difference in injury models, route or timing of drug administration, or to some extent publication bias towards reporting positive outcomes.

4.1. Model selection

The disparity between present results and previous studies warrants a comparison between the previously employed injury models and the present study. Most previous studies examining these neuroprotective drugs utilized a lower thoracic to upper lumbar, mild to moderate SCI (Abdanipour et al., 2012; Cristante et al., 2013; Guth et al., 1994; Lee et al., 2014, 2016; Lee et al., 2012a; Lv et al., 2011; Ma et al., 2021; Redondo-Castro et al., 2013; Sharma et al., 1993; Winkler et al., 1993;

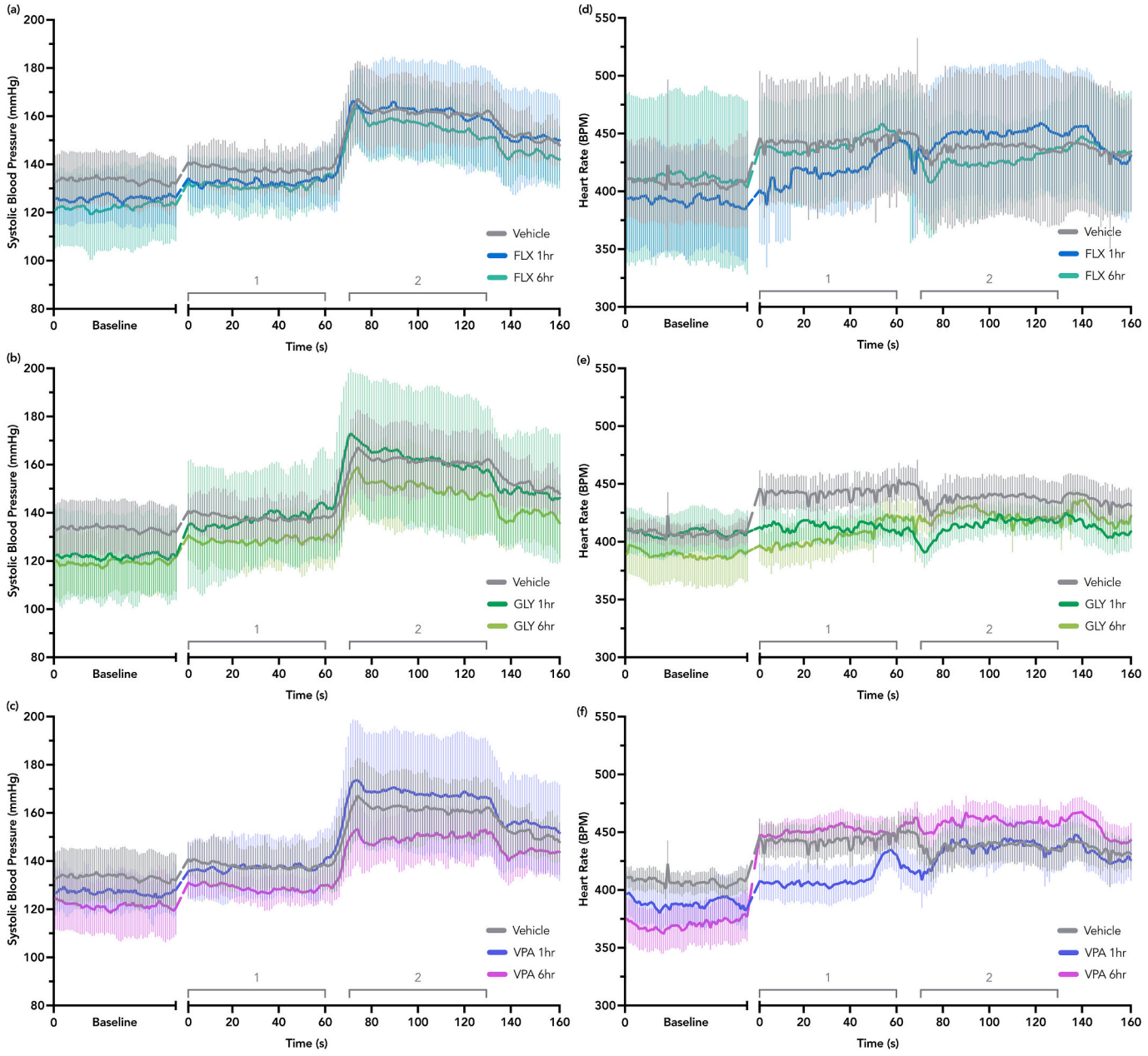

Fig. 3. SBP (in mmHg) and HR (in BPM) averaged across all animals. Average SBP and HR is plotted for three time intervals (baseline, 1 and 2) with discretization of $\Delta t = 1$ s. Bars represent SD. [Baseline] 60 s of SBP or HR averages prior to catheter insertion.[1] 60 s of SBP or HR averages after catheter insertion into rectum. [2] 60 s of SBP or HR averages after balloon inflation. (a, d) traces for vehicle (n = 14), fluoxetine (FLX) 1 h (n = 9) and 6 h (n = 9). (b, e) traces for vehicle, glyburide (GLY) 1 h (n = 10) and 6 h (n = 9). (c, f) traces for vehicle, valproic acid (VPA) 1 h (n = 11) and 6 h (n = 11).

Yu et al., 2012). In contrast, we employed a severe contusion injury model at T3, which as demonstrated here and in previous studies (Squair et al., 2017, 2018), results in significant cardiovascular dysfunction. This degree of severity is necessary to induce reliable and consistent AD. Furthermore, higher-level injuries are associated with greater metabolic impairment and disease risk, including lower glucose tolerance, increased insulin resistance, and impaired lipid profiles (Smith and Yarar-Fisher, 2016). Gut dysbiosis is also exacerbated in rats with T4 crush injuries compared to T10 (Du et al., 2021). It is possible that the higher and more severe model of SCI in the present study contributed to impaired tolerance of the drugs.

4.2. Relating tissue sparing and functional recovery

Previous investigations have established a direct connection between the severity of SCI and the preservation of connections with sympathetic pre-ganglionic neurons originating from the rostro-ventro-lateral medulla (RVLM), which is a major control center for cardiovascular function (Maiorov et al., 1998). Concurrently, post-mortem examinations of individuals with SCI have demonstrated a relationship between the extent of preserved descending vasomotor pathways below the injury site and the degree of cardiovascular dysfunction (Furlan et al., 2003). Although there is no definitive staining of descending fibers from the RVLM in our analysis, the low tissue sparing presumably contributes to the development of AD as shown previously (Squair et al., 2017, 2018). Consequently, given the neuroprotective drugs' inability to enhance tissue preservation and provide a neuroprotective effect, there was no observed improvement in cardiovascular recovery. Similarly, motor function did not improve, as the tissue loss not only affected descending fibers concerning cardiovascular function, but also fibers connecting to

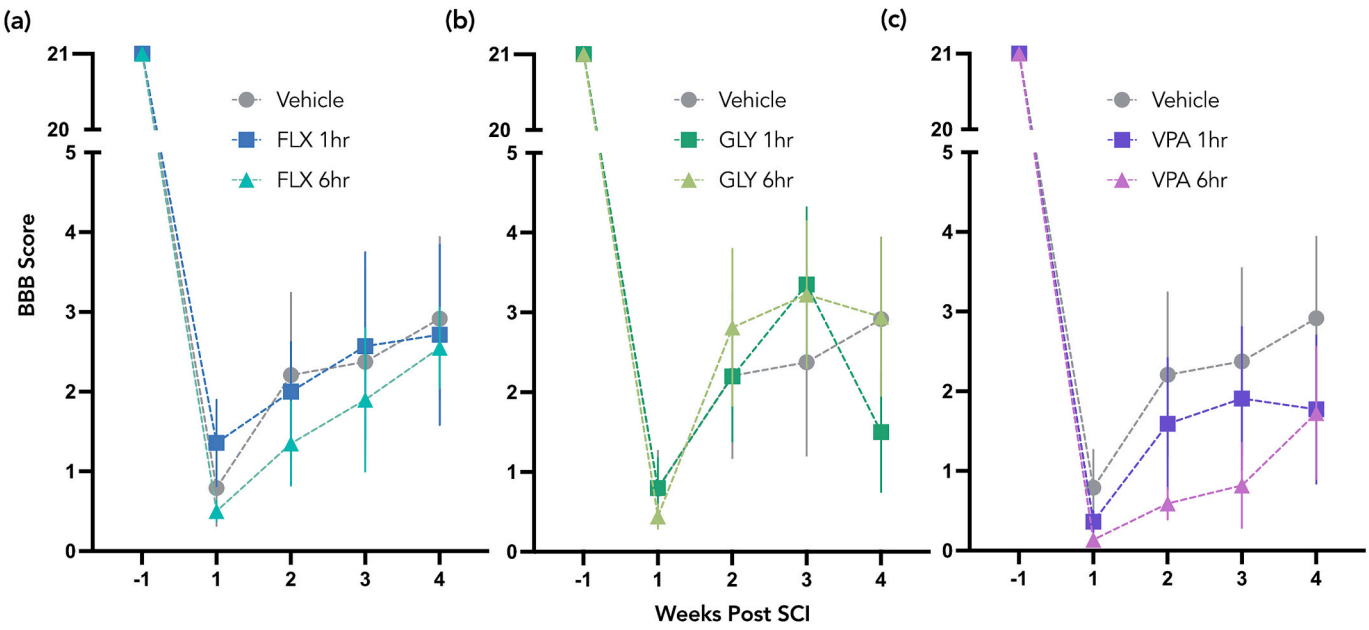

Fig. 4. BBB scores averaged across all animals grouped by weeks post SCI and neuroprotective drug. -1 weeks post SCI time point denotes average BBB before SCI. Points represent the average BBB score across all animals per given group, and bars represent SD. (a) BBB scores for vehicle ($n = 12$), fluoxetine (FLX) 1 h ($n = 7$) and 6 h (n = 10). (b) BBB scores for vehicle, glyburide (GLY) 1 h (n = 10) and 6 h (n = 9). (c) BBB scores for vehicle, valproic acid (VPA) 1 h (n = 11) and 6 h (n = 11).

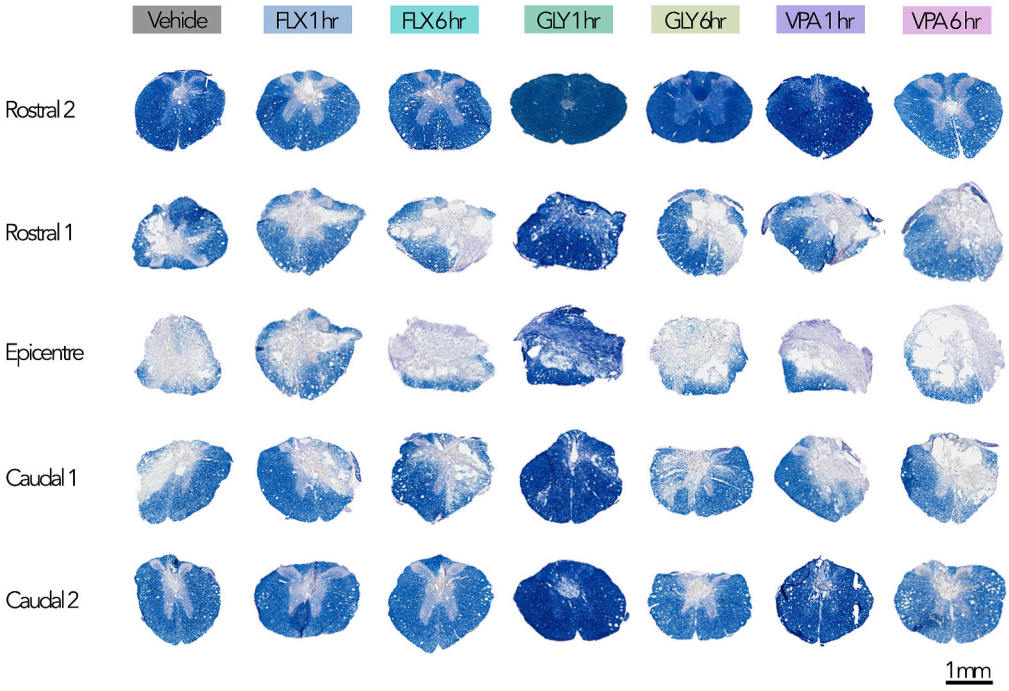

Fig. 5. Representative spinal cord cross sections for every experimental group (where FLX is fluoxetine, GLY is glyburide and VPA is valproic acid) stained with EC. The region from the epicentre to rostral 2 and caudal 2 is 4 mm in each direction, therefore each representative region is approximately 2 mm apart. Visibly reduced color intensity combined with a lack of normal architecture and white cavitations is more dominant in the epicentre compared to surrounding representative sections. Representative images show similar lesion areas at the epicentre between experimental groups, with similar increase in white and gray matter representation away from the injury epicentre caudally and rostrally.

other supraspinal centers (in the brainstem and midbrain) responsible for locomotor function (Laliberte et al., 2019; Zavvarian et al., 2020).

4.3. Fluoxetine

Fluoxetine has been previously administered at a dosage of 10 mg/kg, exhibiting favorable tolerability (Moyses et al., 2008) at this concentration, and demonstrating neuroprotective potential (Bianchi et al., 1995). For instance, it has exhibited neuroprotection against neuronal death in a rat cerebral ischemia model involving middle cerebral artery occlusion, even when administered as late as 9 h after the ischemia-reperfusion event (Lim et al., 2009).

In the context of the current study, the absence of discernible Fluoxetine effects may be attributed to several factors. Firstly, the choice

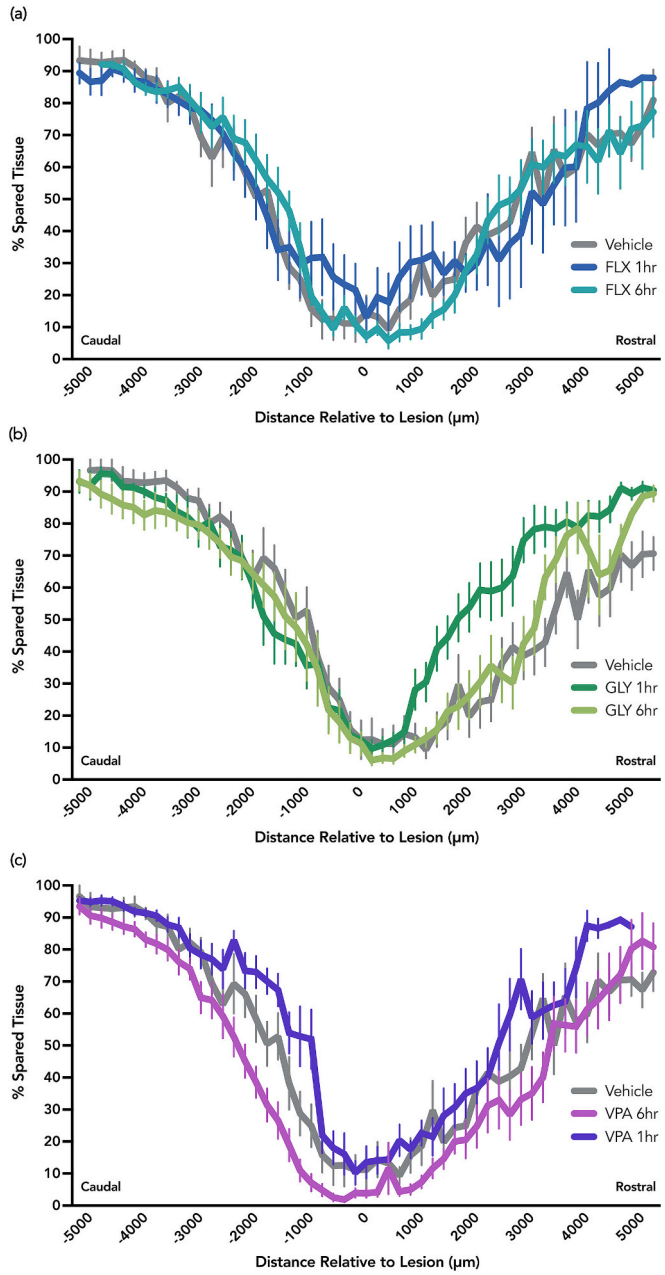

Fig. 6. Quantification of spared tissue area (consisting of regular cellular architecture, and EC staining) as a percentage of total area. Distance across lesion is relative to the lesion epicentre, denoted 0. (a) vehicle (n = 14), and fluoxetine (FLX) 1 h (n = 9) and 6 h (n = 9), with tissue sparing at 14.36 ± 20.01 % for vehicle, 13.07 ± 17.77 % for fluoxetine 1 h, and 7.09 ± 4.87 % for fluoxetine 6 h at the epicentre. (b) vehicle, and glyburide (GLY) 1 h (n = 10) and 6 h (n = 9), with tissue sparing at 12.41 ± 8.84 % for glyburide 1 h, and 11.27 ± 11.18 % for glyburide 6 h at the epicentre (c) vehicle, and valproic acid (VPA) 1 h (n = 11) and 6 h (n = 11) with tissue sparing at 13.53 ± 16.67 % for valproic acid 1 h, and 3.87 ± 4.67 % for valproic acid 6 h at the epicentre.

of the injury model; two studies investigating Fluoxetine's impact employed a C4 bilateral dorsal funiculi crush injury model, in which Fluoxetine-treated rats displayed recovery to pre-injury performance levels over an eight-week period (Jin et al., 2021) and reported the sprouting of intact corticospinal fibers (Scali et al., 2013). In contrast, other studies utilized T9 injury models, although also demonstrating improved BBB scores (Cristante et al., 2013; Lee et al., 2016), with one reporting a reduction in lesion volume (Lee et al., 2016). Interestingly, one study (Ma et al., 2021) assessed the recovery of bladder function in moderate and severe T10 mouse contusion models. While motor recovery was noted, Fluoxetine treatment did not have any effects on micturition function, suggesting a possible severance of descending serotonergic axons. Secondly, variations in the drug administration schedule should be considered. Although most of the aforementioned studies followed a similar administration schedule, with some administering their first dose one week after injury (compared to one hour in the current study) (Cristante et al., 2013; Jin et al., 2021), one study initiated Fluoxetine treatment 21 days prior to injury (Scali et al., 2013), citing prior research indicating that at least 21 days of antidepressant treatment are necessary to induce beneficial effects in the central nervous system (Berton and Nestler, 2006). While not definitive, it is possible that starting the Fluoxetine treatment before injury may have presented better recovery.

4.4. Glyburide

The efficacy of Glyburide as a neuroprotective agent is linked to the specific injury model utilized, which most likely accounts for the discordance between our findings and prior literature. This brings into question the clinical translation of the therapy where no two injuries are identical (Popovich et al., 2012). In one study, adult female Long-Evans rats underwent hemicervical spinal cord contusion at the C4-C5 level, with immediate subcutaneous administration of glyburide at a dosage of 200 ng/h (Simard et al., 2007). Glyburide-treated animals exhibited significantly reduced hemorrhage, smaller lesions at the 7-day post-SCI mark in comparison to controls, and improved motor functions, exploratory behavior, limb dexterity, paw placement, including vertical exploratory behavior. Looking at the lower cervical-upper thoracic level, glyburide's effects were examined in two rat models: (Simard et al., 2012) one with unilateral primary hemorrhage (UPH) and the other with bilateral primary hemorrhage (BPH). While Glyburide consistently showed benefits in the UPH model, its positive effect in the BPH model was less pronounced, with reduced neurological improvement and lesion volume reduction. Utilizing a more clinically relevant injury model (Redondo-Castro et al., 2013), a mild T8-T9 contusion, showed a significant improvement in BBB scores by day 21 post SCI. However, there was no significant preservation of spinal cord tissue, leaving open questions about whether this outcome can be attributed to the injury severity, especially compared to the current study, where the BBB score for the vehicle group at 3 weeks was 2.4, versus the 13 points reached in the saline group in the study by Redondo-Castro et al. (Redondo-Castro et al., 2013).

4.5. Valproic acid

Most studies regarding the neuroprotective effects of valproic acid appear to be concentrated around the T9 spinal cord level; encompassing diverse injury modalities such as contusion (Lee et al., 2014; Lv et al., 2011), weight drop (Lee et al., 2012a), and clip compression (Yu et al., 2012), apart from one (Abdanipour et al., 2012) utilizing a T12-L1 weight drop model. Interestingly despite the variation in injury models, the findings consistently demonstrated the efficacy of valproic acid. VPA significantly increased the hindlimb locomotor function, most often in BBB scores, with most reporting a decrease in lesion volume. Valproic acid was typically administered at dosages ranging from 100 to 400 mg/kg, via IP injection once or twice daily, not exceeding 600 mg/kg per day, over a period of 5 days to 2 weeks (Abdanipour et al., 2012; Lee et al., 2014; Lv et al., 2011; Yu et al., 2012). The revised dose we used, Valproic Acid at 150 mg/kg, has been shown to be effective in a traumatic brain injury model, where a single dose attenuated neurological impairment and reduced brain lesion size (Wakam et al., 2021). Remarkably, the reviewed studies did not report potential side effects or mortality rates. In fact, two studies (Lee et al., 2014; Lee et al., 2012a) stated that no significant side effects resulting from valproic acid administration, such as changes in body weight or an increase in

mortality, were observed. Although initially utilizing a dosage of 300 mg/kg IP, within the reported safe range, the severity and level of injury in the current study may be an explanation for the high mortality rates. Valproic acid has been demonstrated to influence lipid metabolism (Poolchanuan et al., 2020; Silva et al., 2008), while valproate has been shown to alter gut microbiota composition (Cussotto et al., 2019a; Cussotto et al., 2019b). Lipid peroxidation has been associated with ulcerative gastritis (Giamarellos-Bourboulis et al., 2003). Given the observed pathology in rodents administered 300 mg/kg of valproic acid, there is a possibility of a compounding or interaction events, leading to high mortality.

4.6. Indomethacin

There are few studies that directly observe effects of Indomethacin on locomotor function. One study utilized a T8 compression injury, and demonstrated that Indomethacin administered at 1 mg/kg, alone, is ineffective for driving locomotor recovery (Guth et al., 1994). Another study of rabbits utilized an L2 contusion, and administered indomethacin 0.1, 0.3, 1.0 or 3.0 mg/kg per day of treatment (Pantović et al., 2005). Motor activity was improved in all groups receiving indomethacin in comparison to the relevant vehicle-treated group in a dose-related manner. The dose of 10 mg/kg IP was previously used without reported mortalities, however, the survival timeline of five hours post injury, does not compare to the survival timeline in the current study (Sharma et al., 1993; Winkler et al., 1993). Concerning the issue of drug toxicity however, injury in the intestine has been reported, including microvascular injury and mucosal ulceration, following one oral administration of 15 mg/kg (Nygård et al., 1994). Similarly, we observed gastric ulceration in rodents receiving indomethacin at 10 mg/kg IP. However, given the relatively short survival time of the rodents, it's possible that ulcerations had not yet formed in previously mentioned studies utilizing the same dosage (Sharma et al., 1993; Winkler et al., 1993). Notably, the severity and level of the injury model employed in the current study, as well as the combined use of Meloxicam may also be compounding or interacting with the effects of indomethacin on the gut – while NSAIDs decrease inflammation, their side effects on the gut is well characterized, including gastrointestinal ulcers, bleeding or perforation (García Rodríguez, 1997).

5. Conclusion

Despite previous published studies reporting promising neuroprotective results in other models of rodent SCI, none of the drugs tested in this study resulted in improvement of either cardiovascular or motor function after severe SCI. Indomethacin and valproic acid were significantly toxic to rats at doses deemed safe in published relevant literature. This underlines the need for reproducibility studies in pre-clinical research and might indicate a potential lack of transparency, while emphasizing the importance of publishing null findings.

CRediT authorship contribution statement

Tamila Kalimullina: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Formal analysis, Data curation. **Rahul Sachdeva:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kiran Pawar:** Writing – review & editing, Project administration, Methodology, Data curation. **Steven Cao:** Methodology, Investigation, Data curation. **Arshdeep Marwaha:** Writing – review & editing, Methodology, Investigation, Data curation. **Jie Liu:** Writing – review & editing, Methodology, Data curation. **Ward Plunet:** Writing – review & editing, Methodology, Data curation. **Jordan Squair:** Writing – review & editing, Funding acquisition, Conceptualization. **Christopher R. West:** Writing – review & editing, Conceptualization. **Wolfram Tetzlaff:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Andrei V. Krassioukov:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Financial support for this study was provided by the Wings for Life Spinal Cord Research Foundation Research Grant. TK is supported by a Canada Graduate Scholarship through the CIHR and a WorkSafeBC research trainee award. RS is supported by the WFLSCRF and the US Department of Defense. AK is the endowed chair in the Division of Physical Medicine and Rehabilitation within the Faculty of Medicine at the University of British Columbia and is supported through grant funding provided by WFLSCRF, International Spinal Research Trust, Praxis, US Department of Defense, RHF, Onward, SpineX, Convatec, and Coloplast. We also thank Dr. Shelly McErlane for providing expertise regarding animal welfare, and Dr. Ian Welch for the histological assessment.

Data availability

Data will be made available on request.

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