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14. ABSTRACT The on-going research is to test the central hypothesis that post-SCI administration of the catalytic oxidoreductant BuOE will inhibit neuropathic pain after SCI. The research in years 1-3 focused on the goals of aims 1-3, to test the hypothesis that post-SCI administration of BuOE decreases inflammation and ROS activation in a rat, mouse and porcine model of SCI. The main finding of our work in this year was to evaluate the dose-effect curve of post-SCI administration of BuOE on the outcomes listed above. Our data from on-going experiments indicate that the dose of 0.2mg/kg BuOE was most effective in reducing inflammation and ROS activation acutely post-SCI in rats and mice. On-going experiments will evaluate additional outcome measures and behavioral outcomes in the subsequent year.						
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1. INTRODUCTION:

Although the neurobiological mechanisms that underlie neuropathic pain are poorly understood, we hypothesize that a highly efficacious treatment for neuropathic pain after SCI would be a molecule that scavenges ROS, inhibits NF- κ B activation, is bioavailable to the CNS, and is well tolerated. We have extensive experience in the development of a class of therapeutic catalytic oxidoreductants which *both* dissipate ROS *and* inhibit NF- κ B activation. Thus, the objective of this research is to evaluate MnTnBuOE-2-PyP⁵⁺ (BuOE2) as a novel therapeutic to treat neuropathic pain after SCI as it offers the unique and simultaneous chemistry of direct redox-regulation of pro-inflammatory promoting signaling pathways and dissipation of ROS. The proposed research will test the central hypothesis that post-SCI administration of the catalytic oxidoreductant BuOE2 will inhibit neuropathic pain after SCI by assessing the effects of post-SCI administration of BuOE2 on ROS, NF- κ B signaling, inflammation, and pain-associated behaviors in a clinically relevant *rat* model of *cervical contusion* SCI and a *mouse* model of *ischemic* SCI (Aims 1 and 2). The experiments of efficacy in rodent models will then be followed up in a large animal model of SCI, the porcine contusion model (Aim 3).

2. KEY WORDS:

Neuropathic pain, spinal cord injury, reactive oxygen species, cervical contusion, ischemic spinal cord injury, oxidative stress, inflammation

3. ACCOMPLISHMENTS

Major Goals of the Project in Years 1-3:

The years 1-3 major goals/ deliverables were to:

- **Perform pharmacokinetic analysis of subcutaneous BuOE2 in both rats and mice**
We have successfully completed this goal and determined the PK for administration of BuOE2 in rats in mice. Parts of these data are included in the manuscript by Tovmasyan et al, submitted to Redox Biology. This task is complete but the manuscript was not accepted and is now in revision.
- **Determine the dose of BuOE2 that is most effective in reduction ROS, inhibiting NF- κ B signaling, and inflammation in the spinal cord acutely post-SCI**
We have completed approximately 100% of the animal experiments in support of this goal. Thus far, the main finding is that the 0.1mg/kg dose is seemingly the most effective in reducing ROS and inflammation after SCI in the rodent models. Please see the data included under the details per task section, below.
- **Determine the dose of BuOE2 that is most effective in reducing SCI-induced neuropathic pain-like behaviors in mice.**
We have completed 100% of the animal experiment in support of this goal. Please see the data include under the details per task section below. Although we did not find an effect of BuOE2 in reducing functional deficits following ischemic SCI, we did observe an effect on neuropathic pain-like behaviors. Specifically, the high weekly dose and the low daily dose paradigms reduced mechanical allodynia. We are working to put the reporting of these data into a final form that can be submitted for consideration as a manuscript.
- **Use the rat grimace scale to assess neuropathic pain like behaviors in rats with and without BuOe2E.**

We have developed and characterized a procedure for utilization of the rat grimace scale in assessment of both spontaneous and evoked neuropathic pain. Those findings are in a manuscript that was submitted to the Journal of Neurotrauma and is currently in revision related to an acceptance pending major revision decision (appendix 1).

- **Determine the dose of BuOE2 that is most effective in reducing SCI-induced neuropathic pain-like behaviors in rats**

We have completed 100% of the animal experiment in support of this goal and are currently finishing the extensive data analysis required. We did not find an effect. These data were largely negative and are presented here in the details per task section. We are in the process of putting these data into a final form which can be submitted for consideration for publication.

- **Determine the effect of BuOE2 administration on cytokine/chemokine synthesis in the rat or mouse spinal cord**

We have completed 100% of the animal experiment in support of this goal and are currently finishing the extensive data analysis required. These data have been completed; please see the associated task section below. We are in the process of putting the data in a final form for submission in a manuscript.

- **Determine the pharmacokinetics of BuOE2 in pigs at 48 hours after SCI.**

These data are complete and presented in associated task section, please see below.

- **Determine the dose of BuOE2 that is most effective in reducing ROS, inhibiting NF-kB, and reducing inflammation in the spinal cord acutely post-SCI.**

These data are in the final analysis stages. Please see the data to date as described in the task section below.

- **Disseminate data concerning above to the medical and scientific community by publication in peer-reviewed journal and presentation at national meetings.**

--As described above, the group submitted 1 manuscript. (appendix 1). This manuscript is in revision for re-submission.

--Also as described above, a manuscript is in final revision for resubmission to the Journal of Neurotrauma related to an acceptance pending major revision. (see appendix 2).

Accomplishments by the Specific Objectives of the SOW for Years 1 -3 (note from approved revised SOW):

Year 1:

Task 1: Obtain required regulatory approval for project, including IACUC, Occupational Health Approval, and ACURO from UAB (Floyd and Tse) and from Duke (Warner, Batinic-Haberle, Spasojevic, Sheng). Months 1-3.

This goal was achieved by IACUC ACURO approval completed by February 2014. Updated in March 2015 with new dosing scheme (see task 5, below for more details)

Task 2: Quantitatively assure purity of sufficient BuOE2 for use at both UAB and Duke for Year 1 *in vivo* studies. Order all necessary surgical supplies and biochemistry/ molecular reagents (ALL). Months 1-12.

This goal was achieved. Purity of the compound was ensured by February 2014 and the research teams obtained necessary supplies to conduct the experiments detailed below.

Task 3: Order and acclimate adult male rats for evaluation in acute post-SCI time points related to Aim 1 at UAB (Floyd). Order and acclimate adult male mice and rats for BuOE2 subcutaneous pharmacokinetic studies (Warner and Spasojevic). Order and acclimate adult male mice for evaluation in acute post-spinal cord ischemia time points related to Aim 2 at Duke (Warner and Sheng). Months 3-12.

This goal was achieved and the data obtained from these animals are detailed below with other tasks.

Task 4: Perform pharmacokinetic analysis at Duke (Batinic-Haberle and Spasojevic) in rats and mice treated with 0.1 mg/kg BuOE2 SQ. Rodents will be anesthetized and spinal cord, liver, and blood samples will be collected for measurement of BuOE2 at various time points post-injection (4 animals/species / time point). Months 3-12.

Mouse and Rat PK Experiment:

Mouse and rat plasma and tissue PK was investigated. Plasma, spinal cord, and liver concentrations were measured at 0, 15 min, 2 h, 6 h, 24 h, 3 days (72 h) and 7 days (168 h). Three animals per time point were administered 0.2 or 1 mg/kg of MnBuOE in saline subcutaneously (SC) as 100 μ L saline per 20 g mouse and 200 μ L saline solution per 200 g rat. At a given time-point, animals were anesthetized with isoflurane (5% incubation, 1.5% maintenance) chest open, ~0.5 mL blood withdrawn by 23G needle from left ventricle into heparinized polypropylene vial (10 μ L of 1000 U/mL), plasma separated by centrifugation at 1300 g for 5 min at room temperature and stored in labeled 2-mL screw-cap polypropylene container at -80°C . A 20G cannula was advanced through the left ventricle into aorta and animals perfused with either 30 mL (mouse) or 90 mL (rat) heparinized saline (1 mL of 1000 U/mL heparin per 1L saline). Tissues were collected in appropriate pre-chilled (dry ice) polypropylene containers and stored at -80°C .

Analytical ASSAY:

Plasma and tissue processing

Organs were cryo-pulverized in a Bessman tissue pulverizer (BioSpec Products, Bartlesville, OK) under liquid nitrogen and then homogenized in a rotary homogenizer (PTFE pestle and glass tube) with 2 volumes of deionized water. A 50 μ L aliquot of either plasma or tissue homogenate was transferred into a 0.5 mL polypropylene vial and after adding 2.5 mm Zr/silica bead, 100 μ L 1% HFBA in H₂O/acetonitrile 70/30 with 100 nM MnBuOE-d₈, and 400 μ L of isopropanol/chloroform (1+4), agitated in Fast-Prep apparatus (Q-biogene, Carlsbad, CA) at speed 6.5 for 20 s, and centrifuged 10 min at 16,000 g. After pipetting-out the aqueous layer, 200 μ L of organic was transferred to 12 x 75 mm polypropylene test-tube and evaporated to dryness under nitrogen at room temperature for 15 min. The dry residue was dissolved in a 20 μ L of mobile phase B (see below) and sonicated for 5 min, 80 μ L of mobile phase B was added followed by sonication for 5 min and centrifugation for 5 min at 4500 g at 4 C. Finally, the tube content was transferred to the HPLC autosampler polypropylene vial equipped with silicone/PTFE septum, followed by another cycle of centrifugation for 5 min at 4500 g (4 C), placed in autosampler 4 C, and analyzed by LC-MS/MS.

Liquid Chromatography Tandem-Mass Spectrometry (LC-MS/MS)

Quantitative analysis of MnBuOE in plasma and tissue samples was performed on a Shimadzu 20A series HPLC (LC) - Applied Biosystems MDS Sciex 5500 QTrap tandem mass spectrometer (MS/MS) at PK/PD Core Laboratory of Duke Cancer Institute (I. Spasojevic, Director). The use of heptafluorobutyric acid (HFBA) as an ion-pairing agent increases overall lipophilicity/volatility and greatly improves retention and ionization efficiency of the analytes, affording an abundance of $[\text{MnP}^{5+} + 2\text{HFBA}]^{3+}$ and $[\text{MnP}^{5+} + 3\text{HFBA}]^{2+}$ ions. Mobile phase: A = 9:1 water:acetonitrile (0.05% HFBA); B = acetonitrile (0.05% HFBA). Analytical column: 2 x Phenomenex AJ0-4287, C18, 4 x 3mm at room temperature. Elution gradient: 0-1 min 0-70%B, 1-2 min 70%B, 2-2.1 min 70-100%B, 2.1-2.6 min 100%B, 2.6-2.7 min 100-0%B. Run time: 4 min. Mass transitions

used for quantification: MnTnBuOE-2-PyP5+ at $m/z = 857.3/599$ and MnTnBuOE-2-PyP5+-d8 (internal standard) at $m/z = 862.2/603.9$.

Calibration samples in appropriate range were prepared by adding known amounts of serially diluted pure standards into corresponding blank plasma or tissue homogenates obtained from drug-free animals and were analyzed along with study samples within the same analytical set. Response was calculated as the ratio between the standard peak area and internal standard peak area. Quantification was performed using Analyst 1.6.2 software.

PK modeling and calculations:

The obtained concentration/time data were processed by non-compartmental and compartmental modeling within WinNonlin software (Pharsight Corp.).

RESULTS and DISCUSSION

MOUSE and RAT plasma and tissue PK:

After subcutaneous (SC) injection of MnBuOE to mouse and rat, no absorption peak is observed in mouse (C_{max} at 30 min, the earliest time of plasma collection) in contrast with C_{max} at 2 h and 6 h observed in rat at 0.2 mg/kg and 1 mg/kg, respectively (Figures 1 and 2; Tables 1 and 2). It should be noted that the 2 h (rat, 0.2 mg/kg) is only marginally higher than at C_{30min} and C_{max} at 6 h (rat, 1 mg/kg) only 2x higher than the level at C_{30min} . The wide range of C_{max} values may be due to different injection volume/animal weight ratio (5 μ L/g for mouse and 1 μ L/g for rat) which means that the subcutaneous space is presented with different concentration of MnBuOE which may affect absorption/re-absorption processes in hypodermis and thus the dynamics of drug penetration into the systemic circulation (REF: Subcutaneous Absorption of Biotherapeutics: Knowns and Unknowns, Wolfgang F. Richter and Björn Jacobsen, *Drug Metabolism and Disposition*, 2014, 42 (11) 1881-1889); higher drug concentration in rat experiment may cause more drug to be initially absorbed by the epidermis tissue which is subsequently re-absorbed into the systemic circulation. The absorption process is mixed with early process of plasma elimination. The half-life ($t_{1/2}$) of this process (“apparent plasma elimination”), which eliminates 90% of the drug from plasma, is measured from the first “slope” (exponential decay in log-lin plot appears linear) observed after C_{max} . This $t_{1/2}$ value varies from 20 min to 2.5 h (Tables 1 and 2) and corresponds to tissue distribution as it clearly coincides with liver (and other tissue) loading process profile. This process is mixed with absorption process (and presumably renal elimination; drug was observed in urine at early time-points) and cannot be resolved with the limited number of time-points in this study. Practically 99% or more of the drug is cleared within 24 hours.

The distribution in well-perfused tissues (e.g. liver) is very efficient and those tissues (liver, kidney, spleen) serve as a *depot* for maintenance/accumulation of MnBuOE in tissues that are difficult to penetrate (brain and spinal cord). This is how we explain the observed accumulation of MnBuOE in brain and spinal cord over 7 days after single bolus SC injection. Two processes are observed in brain and spinal cord PK. First process appears to follow plasma decay which may correspond to the “shallow” compartments, followed by the distribution into “deep” (cellular) compartments, a slow process which needs a persistent (no matter how small) gradient from plasma levels provided by the leakage from liver, kidney, and spleen. This raises questions where the drug is on cellular and sub-cellular level which can be very important for the mechanism of the protection from injury.

The complexity of the PK behavior can be also illustrated by the very variable response of drug exposure parameters (C_{max} , C_{last} , AUC) to the five-fold change in dose (Table 3).

M O U S E PK Parameter	0.2 mg/kg				1 mg/kg			
	plasma	spinal cord	brain	liver	plasma	spinal cord	brain	liver
Tmax [h]	0.5	0.5	0.5	72	0.5	0.5	0.5	168
Cmax [ng/mL]	219.87	5.51	4.01	2615	1034.55	22.88	14.76	7507
Clast (7 day) [ng mL-1]	0.12	5.13	3.14	2600	0.57	14.73	11.65	7507
AUC (0-7 days) [ng mL-1 h]	267	565	341	408081	1740	1567	1372	1138043
AUC (0-24 days) [ng mL-1 h]	194	57	33	48426	1399	148	128	122815
t1/2 (apparent plasma half-life) [h]	0.32	N/A	N/A	N/A	0.66	N/A	N/A	N/A
AUCinf(area 0-infinity) [ng mL-1 h]	267	N/A	N/A	N/A	1740	N/A	N/A	N/A
C1/F (clearance as dose/AUCINF) [L h-1 kg]	0.70	0.30	0.60	0	0.60	0.60	0.70	0
MRT (mean residence time; 0-7 days) [h]	7.08	101.63	103.90	89	5.55	105.29	101.16	91

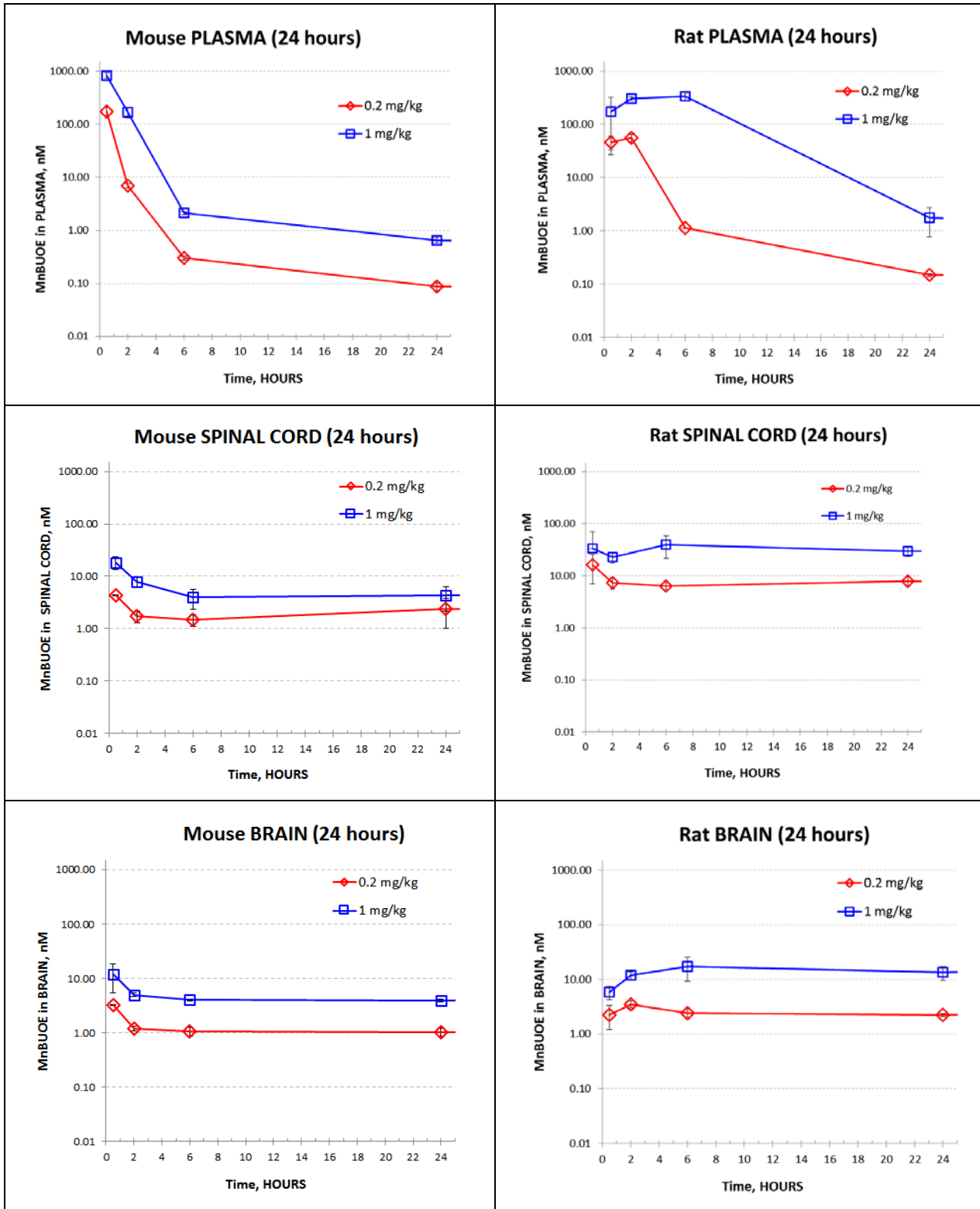
Table 1. Mouse plasma and tissue PK parameters obtained after non-compartmental analysis (WinNonlin software) of concentration-time data after administration of 0.2 and 1 mg/kg MnBuOE to mouse.

R A T PK Parameter	0.2 mg/kg				1 mg/kg			
	plasma	spinal cord	brain	liver	plasma	spinal cord	brain	liver
Tmax [h]	2	0.5	168	168	6	168	168	168
Cmax [ng/mL]	70.10	20.43	7.11	2888	418.84	54.09	48.49	7779
Clast (7 day) [ng mL-1]	0.25	8.81	7.11	2888	0.68	54.09	48.49	7779
AUC (0-7 days) [ng mL-1 h]	301	1547	838	409487	6064	7354	5323	1141167
AUC (0-24 days) [ng mL-1 h]	254	218	73	48861	1399	148	128	122815
t1/2 (apparent plasma half-life) [h]	0.71	N/A	N/A	N/A	2.38	N/A	N/A	N/A
AUCinf(area 0-infinity) [ng mL-1 h]	301	N/A	N/A	N/A	6066	N/A	N/A	N/A
C1/F (clearance as dose/AUCINF) [L h-1 kg]	0.70	0.10	0.20	0	0.20	0.10	0.10	0
MRT (mean residence time; 0-7 days) [h]	12.77	82.27	101.90	92	6.98	91.91	105.33	91

Table 2. Rat plasma and tissue PK parameters obtained after non-compartmental analysis (WinNonlin software) of concentration-time data after administration of 0.2 and 1 mg/kg MnBuOE.

PHARMACOKINETIC LINEARITY PK Param (1 mg/kg)/ PK Param 0.2 mg/kg)	MOUSE				RAT			
	plasma	spinal cord	brain	liver	plasma	spinal cord	brain	liver
Cmax	4.7	4.2	3.7	2.9	6.0	2.6	6.8	2.7
Clast	4.8	2.9	3.7	2.9	2.7	6.1	6.8	2.7
AUC(0-7d)	6.5	2.8	4.0	2.8	20.2	4.8	6.4	2.8
AUC (0-24 h)	7.2	2.6	3.8	2.5	5.5	0.7	1.8	2.5
t1/2 (apparent plasma half-life)	2.0				3.4			
AUCinf(area 0-infinity)	6.5				20.1			
C1/F (clearance as dose/AUCINF)	0.9	2.0	1.2	1.0	0.3	1.0	0.5	1.0
MRT (mean residence time; 0-7 days)	0.8	1.0	1.0	1.0	0.5	1.1	1.0	1.0

Table 3. Ratio of PK parameters given in Tables 1 and 2. Illustrates the pharmacokinetic nonlinearity for most parameters and thus the complexity of the PK behavior of MnBuOE after SC injection. Drug concentration and AUC are measures of exposure and depend on given dose (expected ratio = 5 for linear pharmacokinetics). Clearance, MRT, and t_{1/2} should not depend on dose (expected ratio=1).



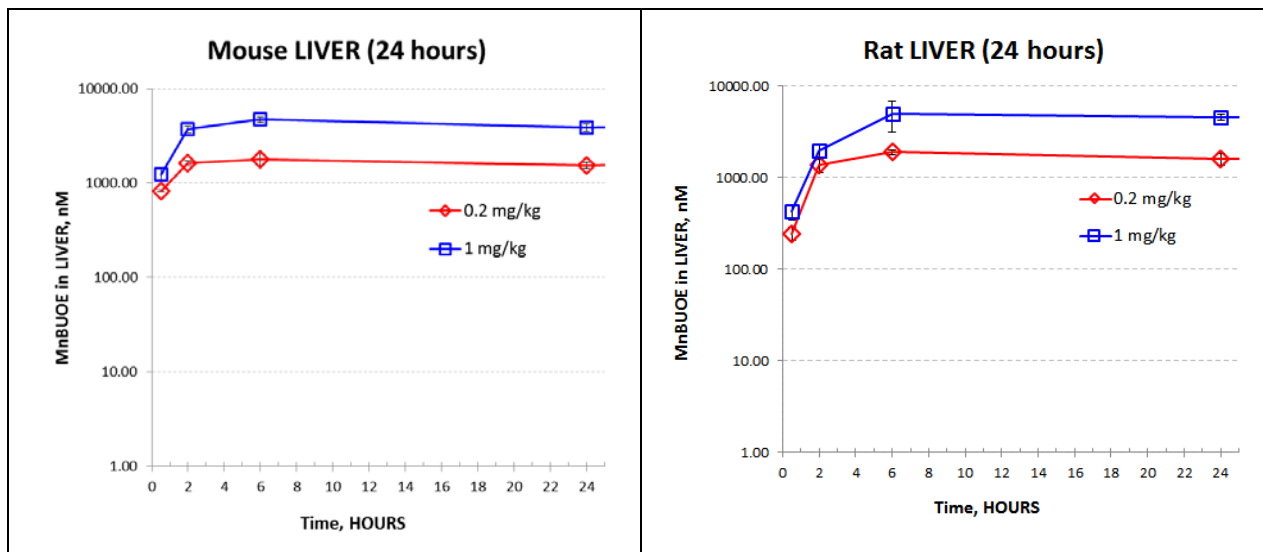
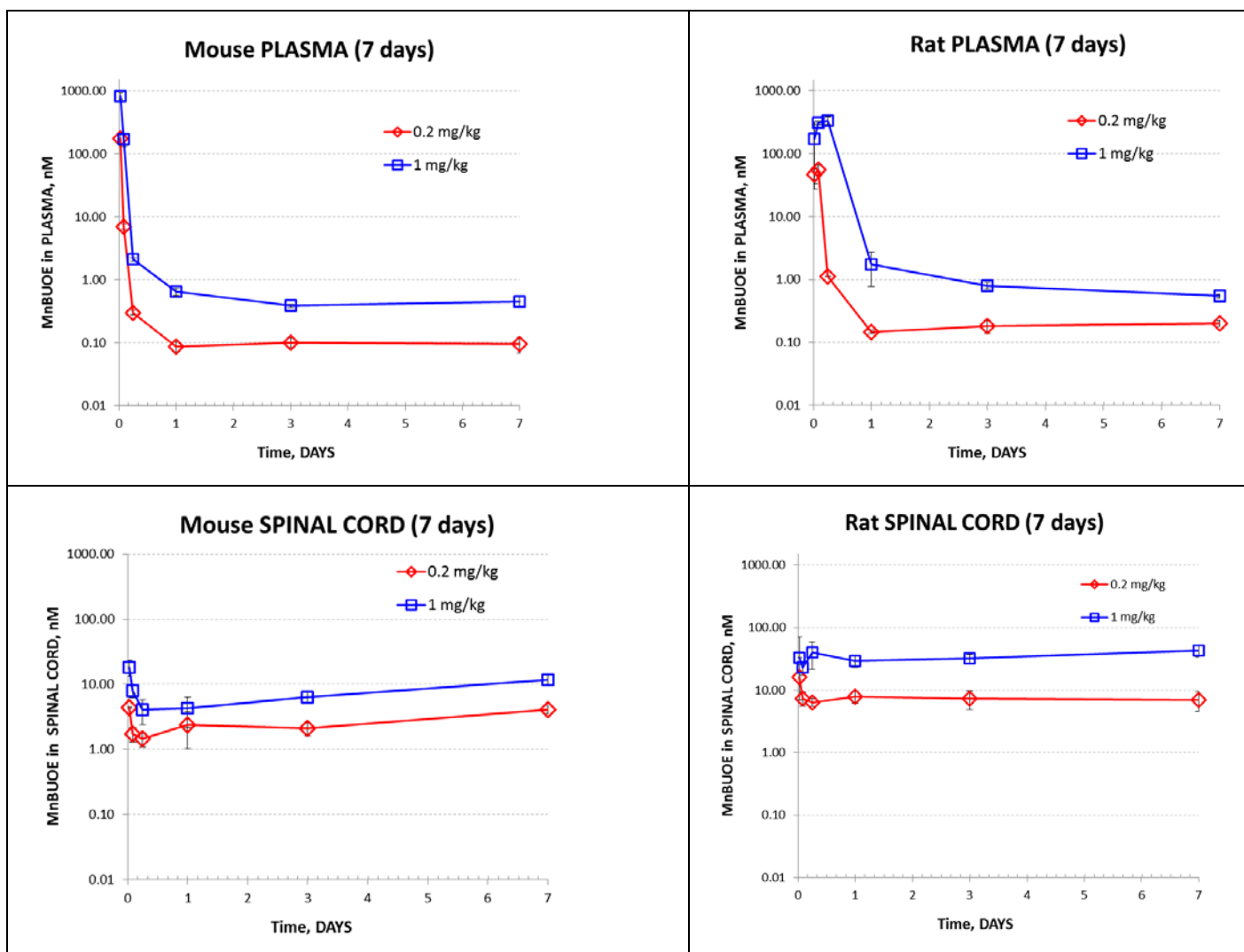


Figure 1. 24 hours PK. Mouse plasma and tissue PK. Log-linear concentration vs time data after 0.2 and 1 mg/kg SC injection of MnBuOE as 100 μ L saline per 20 g mouse (5 mL/kg).



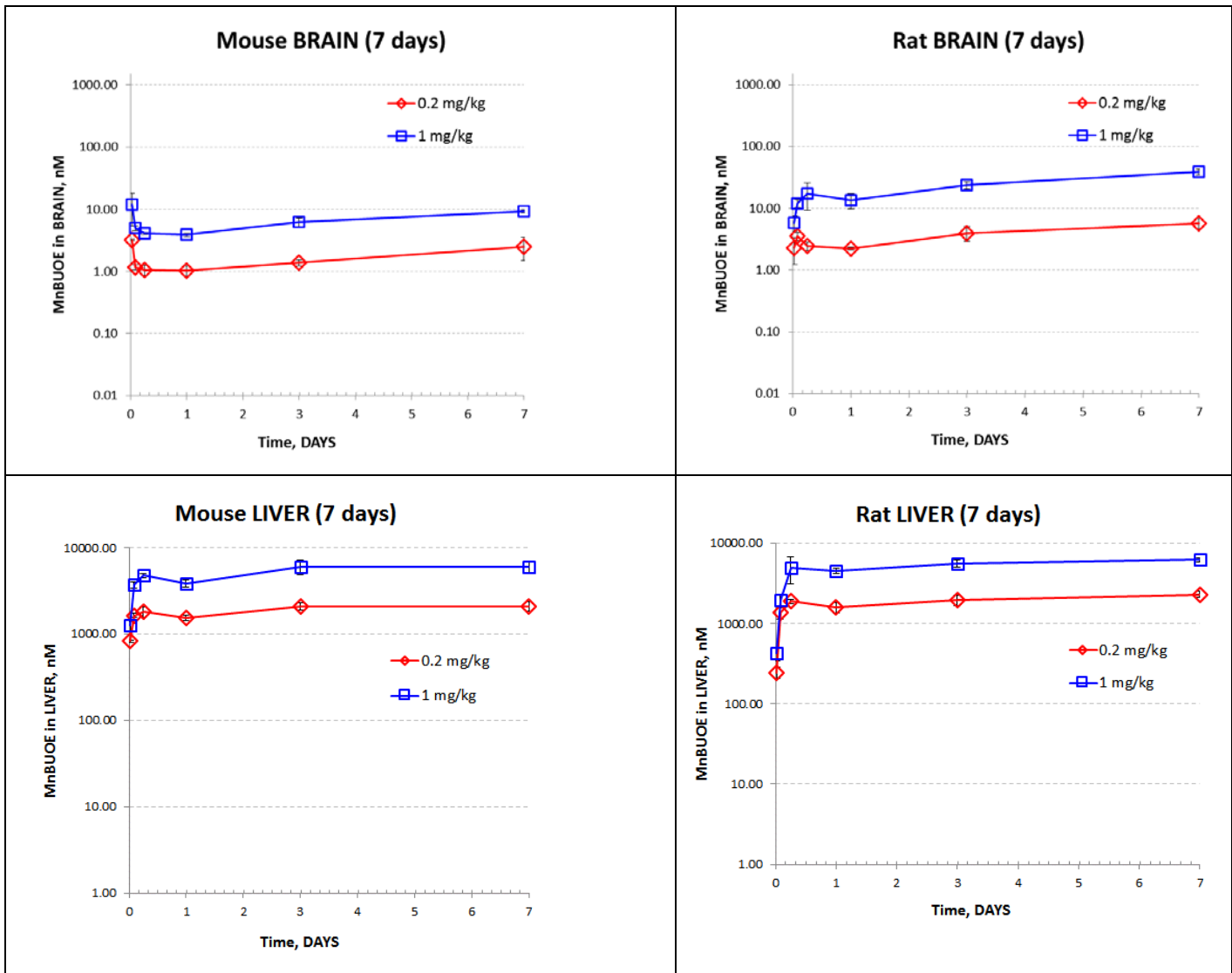


Figure 2: 7 days PK. Rat plasma and tissue PK. Log-linear concentration vs time data after 0.2 and 1 mg/kg SC injection of MnBuOE as 200 µL saline per 200 g rat (1 mL/kg).

Task 5: For evaluation of ROS, NF-κB, and immunological markers at 24, 72 hr or 7 d post-SCI, induce C5 hemiconfusion SCI in 225 male rats at UAB (Floyd) or spinal cord ischemia in 225 male mice at Duke (Warner and Sheng). Months 3-12.

- Sham (all procedures except SCI): n=15
- SCI + vehicle: n=15
- SCI + 0.03mg/kg/day BuOE2: n=15
- SCI + 0.1mg/kg/day BuOE2: n=15
- SCI + 0.3mg/kg/day BuOE2: n=15

UPDATED TO:

- Sham (all procedures except SCI): n=15
- SCI + vehicle: n=15
- SCI + 0.2mg/kg loading dose; 0.1mg/kg/day BuOE2 daily: n=15
- SCI + 1.0 mg/kg loading dose; 0.5mg/kg/day BuOE2 weekly: n=15
- SCI + 1.0 mg/kg loading dose; 0.5mg/kg/day BuOE2 daily: n=15

Due to the concerns with the lack of robust effects seen with the early dosing paradigm, we submitted a request to change the dosing scheme in March 2015. This request was approved and we therefore subsequently evaluated these higher doses. This change in the research plan caused modest delays in the progress, but we anticipate the completion of these associated tasks in 2016.

This task is completed in that 100% of the animal work is completed and data analysis on-going subsequent to the induction of SCI is on-going. As planned, experimental hemicontusion SCI surgery in the rat was performed at UAB and experimental ischemic SCI in the mice was being performed at Duke. There has been no change from the injury model as described in the original application. Briefly for induction of SCI in the rat, male Sprague-Dawley rats (250-275g) were anesthetized with inhaled isoflurane and body temperature was maintained at 37C. The mid-cervical vertebral column was exposed and a bilateral laminectomy was performed at vertebral level C5. A moderate (300kdyne) contusion injury was administered using the Infinite Horizons impactor. For induction of SCI in the mouse, male C57BL/6J mice were anesthetized with isoflurane and placed in the right lateral decubitus position. A left thoracotomy was performed by cauterizing the superficial layer of the intercostal muscle, then inserting the tip of a closed surgical scissor into the thoracic cavity and gently widening the intercostal space by opening the scissor. The thoracic aorta was visualized and a small aneurysm clip was placed on the aorta at the level of T8 to induce ischemic injury. The clip was removed after 10 min.

Task 6: At 24, 48 hours, or 7 days post-SCI, exsanguinate a subset of the rats at UAB (Floyd and Tse) or mice at Duke (Warner and Sheng) and rapidly remove and freeze the spinal cord tissue. Next, prepare tissue samples and lysates for subsequent biochemical and molecular analysis and send to Tse lab at UAB (Floyd, Warner, Sheng). Months 3-12.

This task has been 100% completed in that all spinal cords have been extracted, frozen and prepared for subsequent analysis.

Task 7: At 24, 48 hours, or 7 days post-SCI, exsanguinate a subset of the rats at UAB (Floyd and Tse) or mice at Duke (Warner and Sheng) and rapidly fix tissue with 4% paraformaldehyde. Remove the spinal cord, cryoprotect, and then block/ freeze the spinal cord segments for subsequent cryosectioning. Collect serial cryosections of the spinal cord for subsequent histochemical and immunohistochemical analysis at UAB and Duke (Floyd, Warner, Sheng). Months 3-12.

This task has been 100% completed in that all spinal cord have been extracted, fixed, and subsequently cryo-sectioned.

Task 8: At 24, 48 hours, or 7 days post-SCI, exsanguinate a subset of the rats (Floyd and Tse) or mice (Warner and Sheng) and freeze for subsequent purification of microglia cells and astrocytes from spinal cord tissue by discontinuous percoll gradients or with magnetic beads conjugated to microglia (CD11b)- or astrocyte (GLAST/ACSA-1)-specific antibodies. Conduct flow cytometry experiments from extracted rat or mouse microglia and astrocytes to detect ROS using redox-sensitive dyes and pro-inflammatory cytokines with fluorochrome-conjugated antibodies (Tse). Months 3-12.

All of the tissue extraction and purification steps have been completed. We are in the process of optimizing the flow cytometry experiments thus that portion of the task is on-going making this task 50% complete.

Task 9: Evaluate pro-inflammatory cytokine/chemokine synthesis in rat or mouse spinal cord protein lysates with a Luminex Beadlyte 21-plex multi-cytokine detection system (Tse). Months 3-12.

This task has been completed for the rat at the 24 hour time point, as detailed below. With regard to the mouse experimentation, all samples have been collected and prepared as lysates for the multi-cytokine detection system. We are currently optimizing the detection for the mouse tissue and then can complete the assay. Analysis for both species is on-going. This task is on-going, with an estimated completion of 80%. Thus far, we have preliminarily assessed the effect of BuOE2 administration on pro-inflammatory signals tumor necrosis factor alpha (TNF α) and interleukin 6 (IL-6) in the rodent spinal cord at 24 hours post-SCI using enzyme-linked immunosorbent assays (ELISA) for cytokine detection (R&D Systems, TNF alpha DuoSet #DY510; R&D systems IL-6 DuoSet #DY506). Experimental SCI and post-SCI administration of BuOE2 was induced in the rats and mice as described above. Spinal cord segments from the epicenter and one segment rostral (above the injury level, toward the head) and caudal (below the injury level, toward the tail) of the epicenter were also collected and assessed. Extracted spinal cord was snap frozen and then subsequently homogenized. All values are normalized to protein level and readings are within the standard curves.

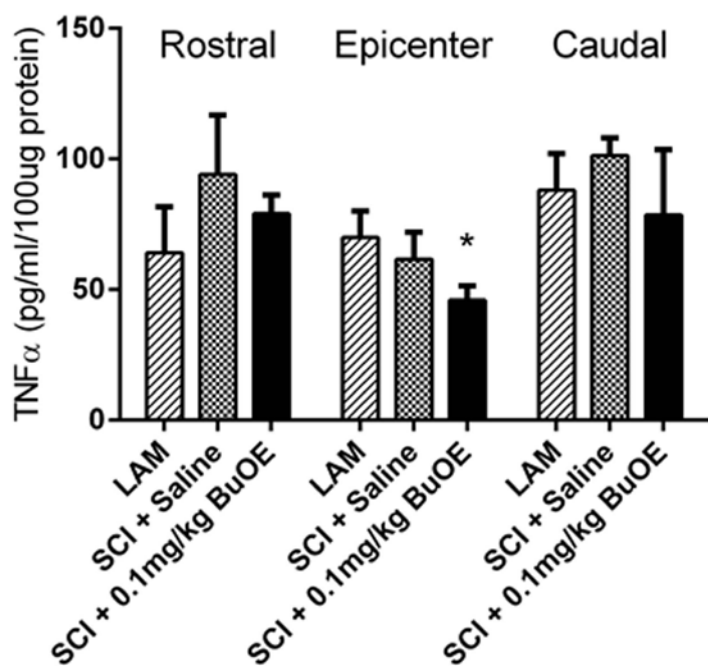


Figure 3: Effect of BuOE2 on expression of TNF α in the rat spinal cord at 24 hours post-SCI. In the rostral and caudal segments, SCI induced an elevation in TNF α as compared to the uninjured control (LAM) that was reduced in the groups administered BuOE2. The reduction in TNF α levels in the BuOE2 group as compared to the SCI + saline group reached statistical significance for tissue at the epicenter of the lesion (*).

Preliminary evaluation of the effect of SCI and post-SCI administration of BuOE2 on TNF α levels at 24 hours post-SCI in the rat is shown in figure 3. We found that SCI induced an increase in the levels of TNF α , particularly in the rostral and caudal segments and that post-SCI administration of BuOE2 reduced levels of TNF α as compared to the SCI+ saline group in tissue at the epicenter. We next evaluated the effect of SCI and post-SCI administration of BuOE2 on IL-6 levels at 24 hours post-SCI in the rat, figure 4. As with TNF α , we found that SCI caused an increase in the levels of IL-6 in the spinal cord (comparisons between LAM and SCI + saline groups). We also observed that levels of IL-6 were reduced in tissue from the group of animals that received BuOE2 post-SCI. With regard to the murine ischemic SCI, we did not find robust differences between groups for the levels of TNF α at 24 hours post-SCI (figure 5). However, when the levels of IL-6 were evaluated in the murine ischemic injury model (figure 6), we found that injury caused an increase IL-6 (comparisons between Sham (lam) and SCI + saline groups). Also, we observed that post-SCI administration of BuOE2 reduced the levels of IL-6, both at the 0.1mg/kg and the 0.3mg/kg group. Additional doses and time points are currently being evaluated to complete this data set and confer a more complete understanding of these effects.

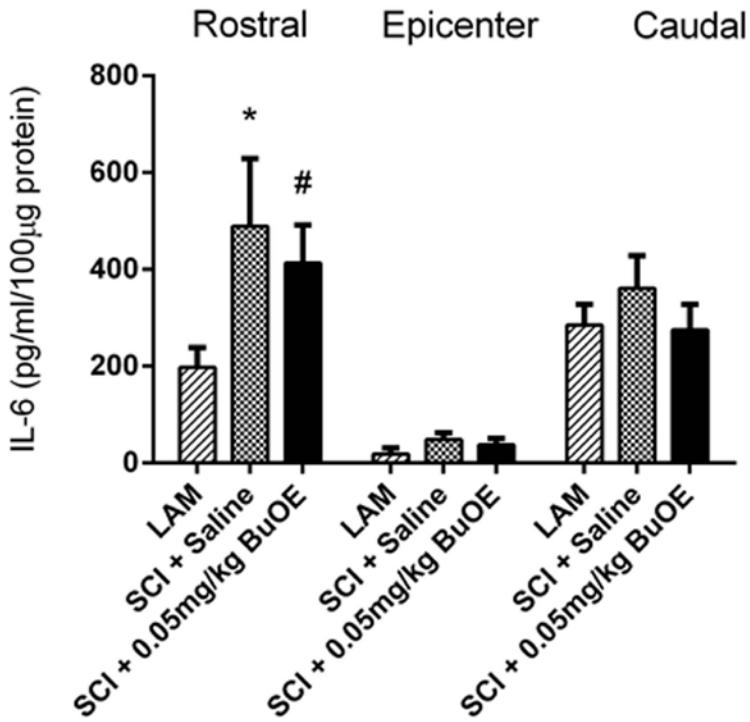


Figure 4: Effect of BuOE2 on expression of Il-6 in the rat spinal cord at 24 hours post-SCI. In the rostral and caudal segments, SCI induced an elevation in Il-6 as compared to the uninjured control (LAM) that reached statistical significant in the rostral segments (*). The levels of Il-6 were reduced in the groups administered BuOE2 as compared to the SCI + saline groups. This reduction reached statistical significance for tissue rostral to the epicenter of the lesion (#).

Mouse SCI - 24h after thoracic aorta clamping

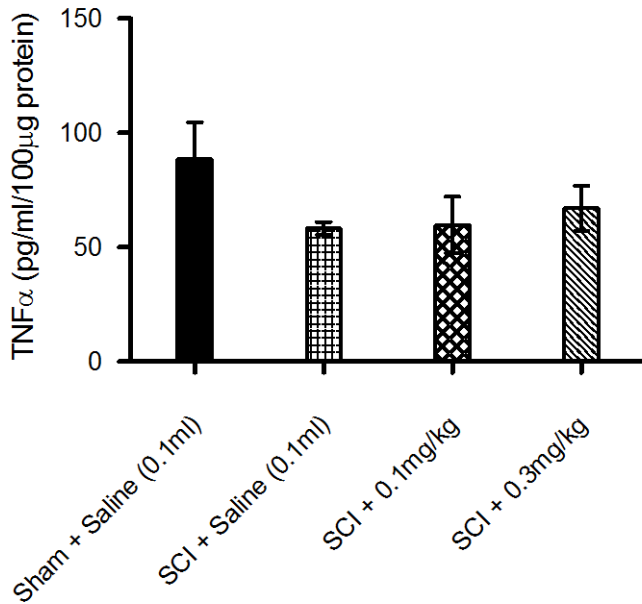


Figure 5: Effect of BuOE2 on expression of TNFα in the murine spinal cord at 24 hours post-SCI. No robust changes in TNFα levels were seen across treatment groups.

Mouse SCI - 24h after thoracic aorta clamping

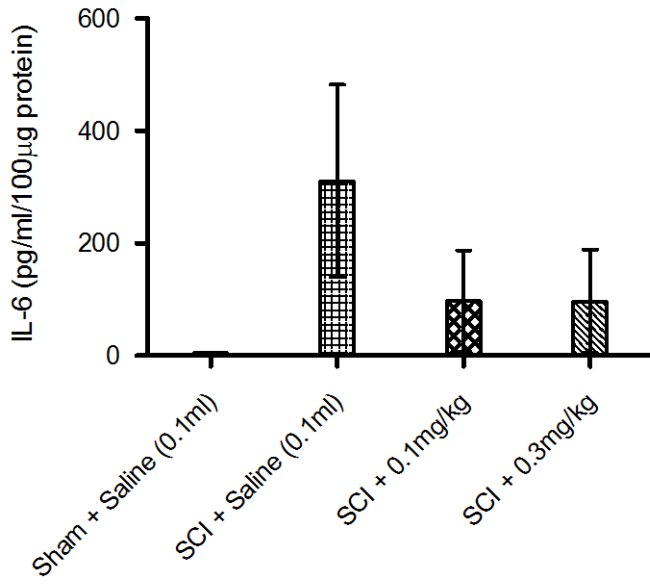


Figure 6: Effect of BuOE2 on expression of Il-6 in the murine spinal cord at 24 hours post-SCI. SCI induced an elevation in Il-6 as compared to the uninjured control (sham). The levels of Il-6 were reduced in the groups administered BuOE2 as compared to the SCI + saline groups.

We next used the multiplex chemokine system to assess the effect of SCI and BuOE2 treatment on several inflammation biomarkers in tissue extracted from the lesion epicenter and in segments rostral and caudal to the lesion epicenter. Note that these experiment groups received the higher doses from the revised dosing scheme. Data from the multiplex in tissue extracted at 24hours post-SCI is shown in figures 7- 28 with each figure legend describing the relevance of the biomarker assessed. We found the following changes to be of

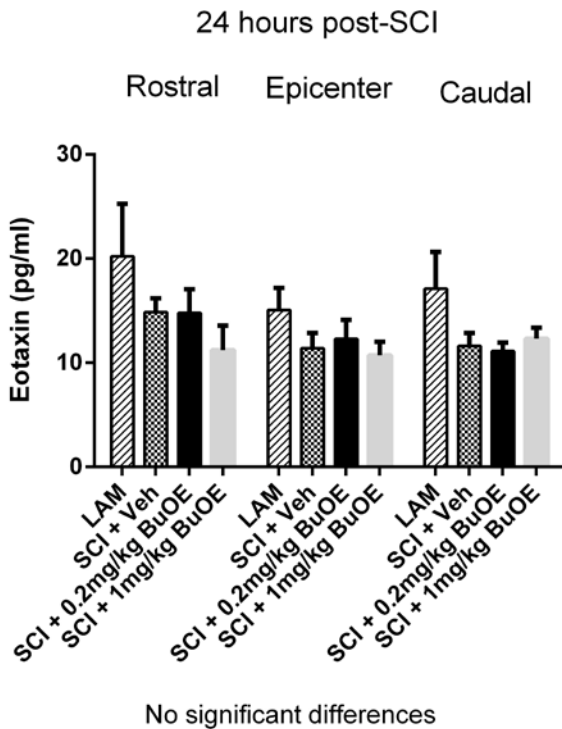


Figure 7: Effect of BuOE2 on expression of eotaxin in the rat spinal cord at 24 hours post-SCI. Eotaxins are in the chemokine subfamily of eosinophil chemotactic proteins that includes CCL11. No significant differences between groups were found.

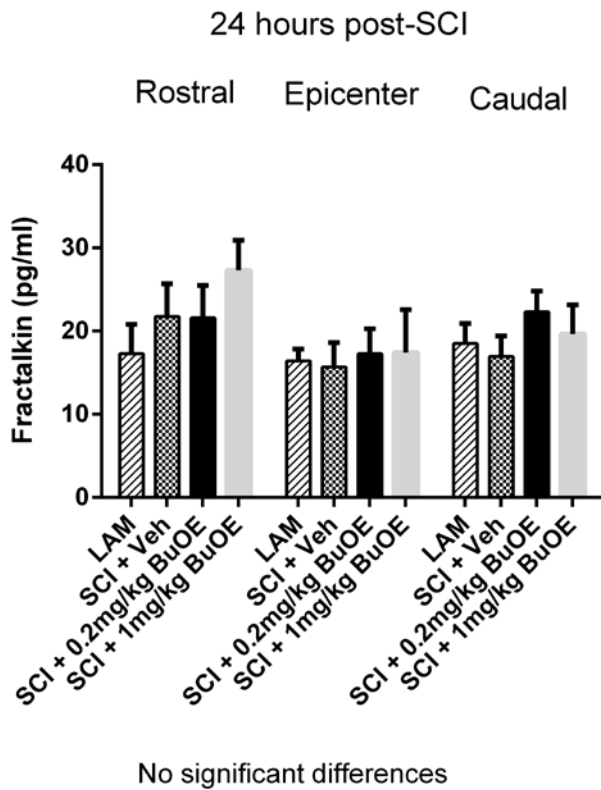


Figure 8: Effect of BuOE2 on expression of fractalkin in the rat spinal cord at 24 hours post-SCI. Fractalkine (CX3C) is a chemokine which is thought to chemo-attract T cells and monocytes. No significant differences between groups were found.

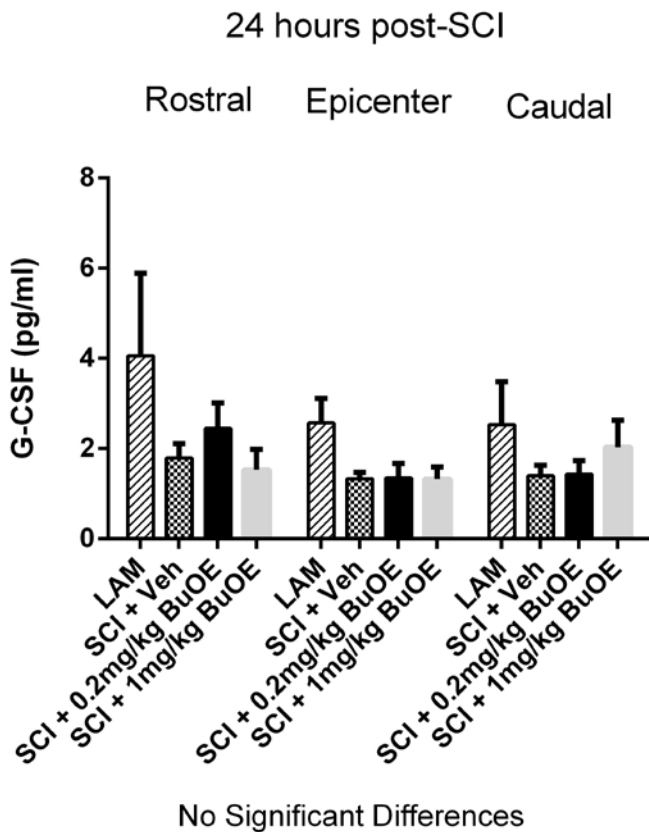


Figure 9: Effect of BuOE2 on expression of G-CSF in the rat spinal cord at 24 hours post-SCI. Granulocyte colony stimulating factor (G-CSF) is a glycoprotein that stimulates an immune response. No significant differences between groups were found.

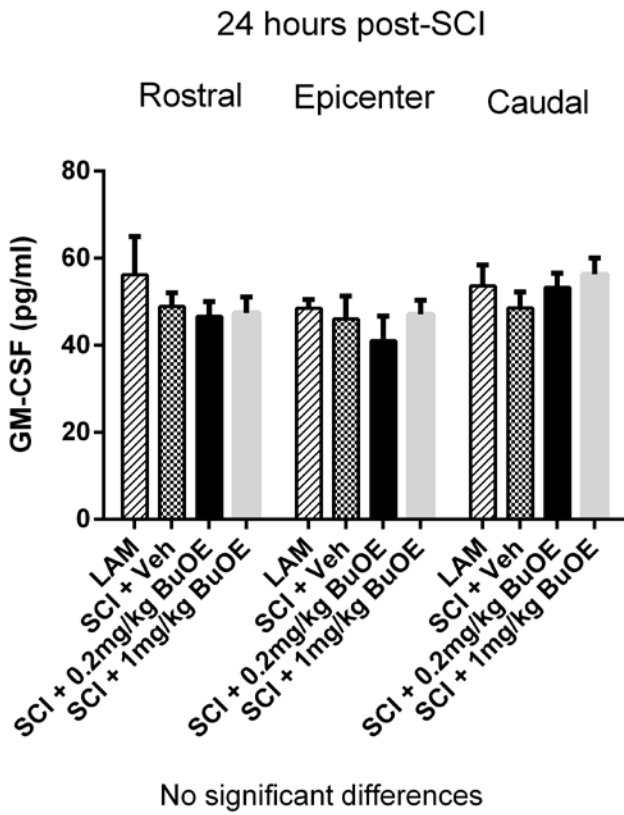


Figure 10: Effect of BuOE2 on expression of GM-CSF in the rat spinal cord at 24 hours post-SCI. Granulocyte macrophage colony stimulating factor (GM-CSF) is a glycoprotein that stimulates an immune response. No significant differences between groups were found.

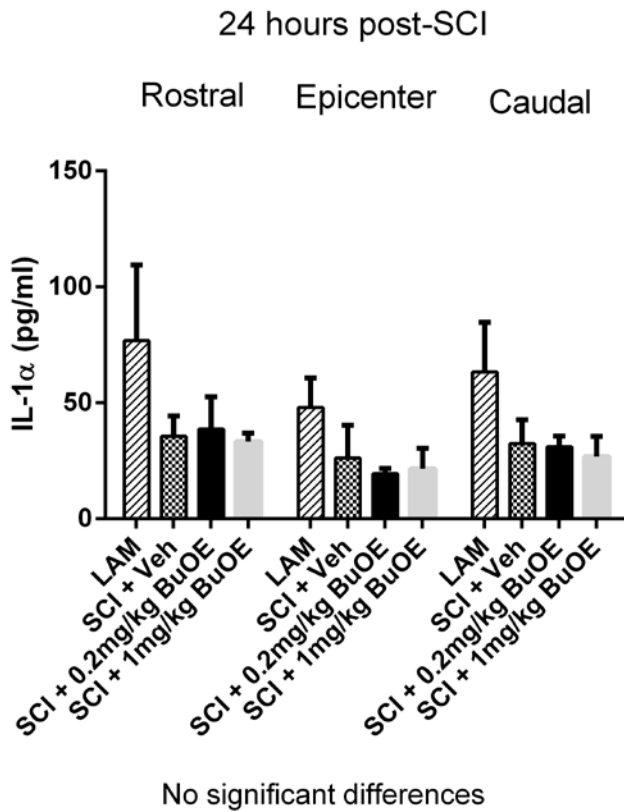
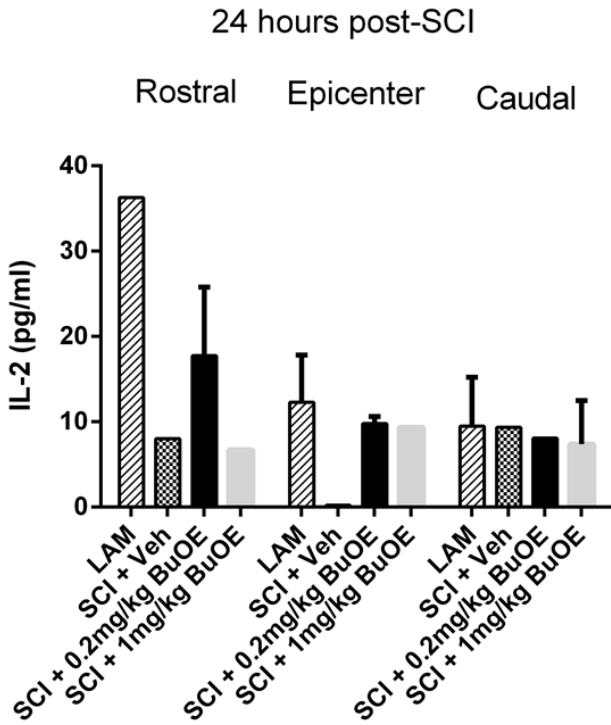
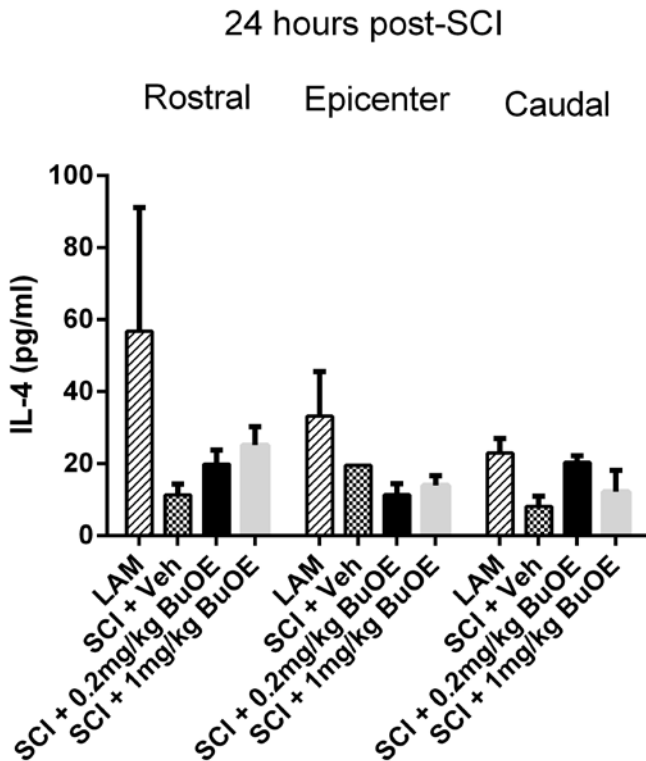


Figure 11: Effect of BuOE2 on expression of IL-1α in the rat spinal cord at 24 hours post-SCI. IL-1α is a protein of the interleukin 1 family that regulates the immune response. No significant differences between treatment groups were found, although a significant induction following SCI was observed.



No significant differences

Figure 12: Effect of BuOE2 on expression of IL-2 in the rat spinal cord at 24 hours post-SCI. IL-2 is a protein of the interleukin family that regulates the immune response. No significant differences between treatment groups were found, although a significant reduction following SCI was observed with a trend toward a drug effect for the 0.2 and 1.0 mg/kg dose at the rostral and epicenter segments.



No significant differences

Figure 13: Effect of BuOE2 on expression of IL-4 in the rat spinal cord at 24 hours post-SCI. IL-4 is a protein of the interleukin family that regulates the immune response. No significant differences between treatment groups were found, although a significant reduction following SCI was observed with a trend toward a drug effect for the 0.2 and 1.0 mg/kg dose at the rostral and caudal segments.

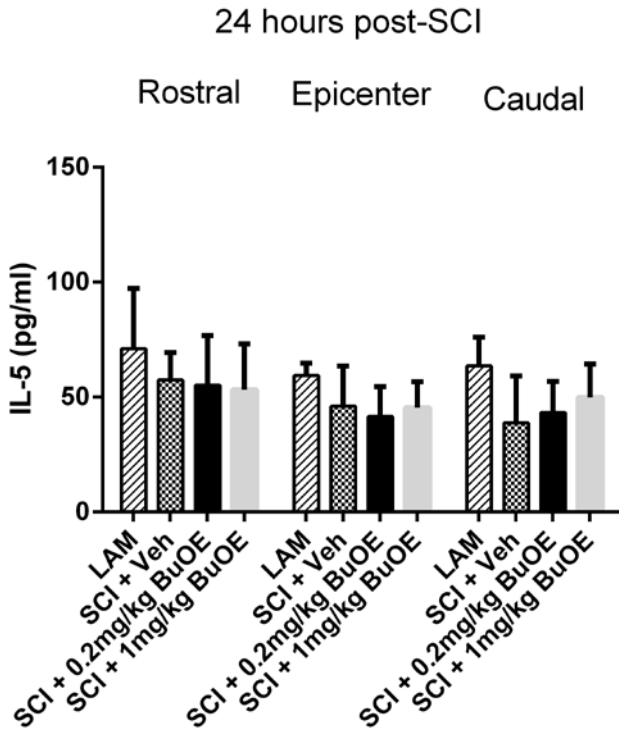


Figure 14: Effect of BuOE2 on expression of IL-5 in the rat spinal cord at 24 hours post-SCI. IL-5 is a protein of the interleukin family that regulates the immune response. No significant differences between treatment groups were found.

No significant differences

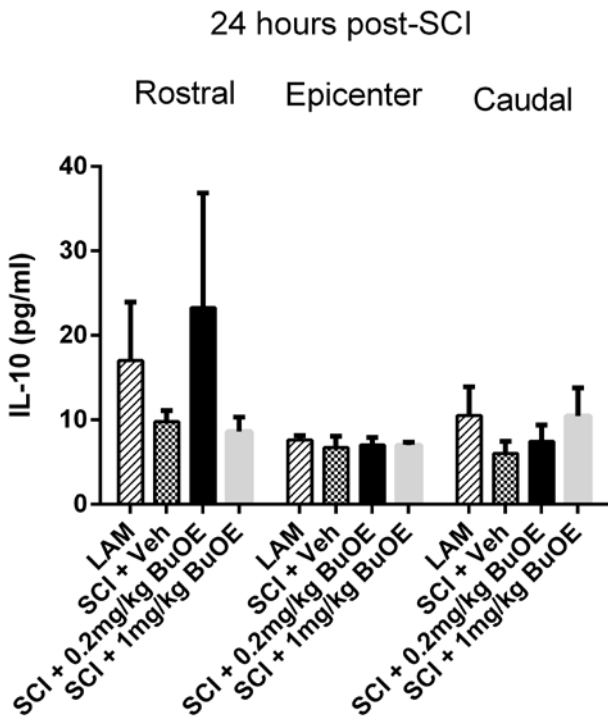


Figure 15: Effect of BuOE2 on expression of IL-10 in the rat spinal cord at 24 hours post-SCI. IL-10 is a protein of the interleukin family that regulates the immune response. In the rostral and caudal segments, a significant reduction in IL-10 was observed following SCI. Also, there was an trend toward effect of 0.2mg/kg BuOE2 in the rostral segment.

no significant differences

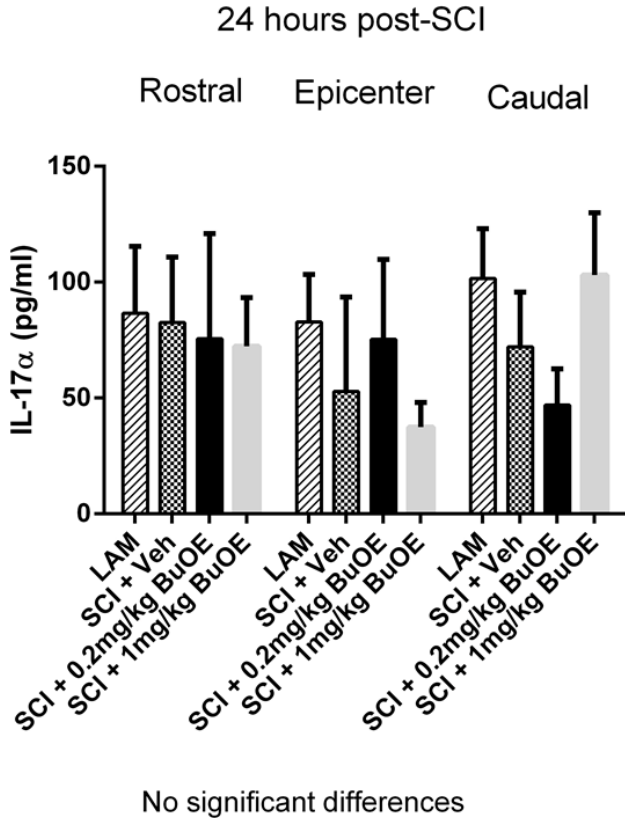


Figure 16: Effect of BuOE2 on expression of IL-17a in the rat spinal cord at 24 hours post-SCI. IL-17a is a protein of the interleukin family that regulates the immune response. No significant differences between treatment groups were found.

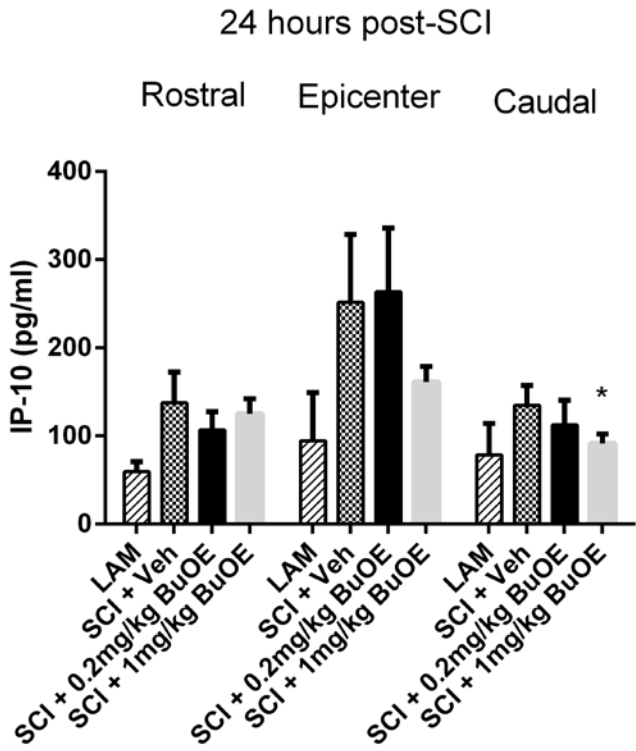


Figure 17: Effect of BuOE2 on expression of IP-10 in the rat spinal cord at 24 hours post-SCI. Interferon gamma-induced protein 10 is an inducible cytokine that activates the immune response. Trends were observed in relation to an upregulation following SCI with some effect of BuOE observed.

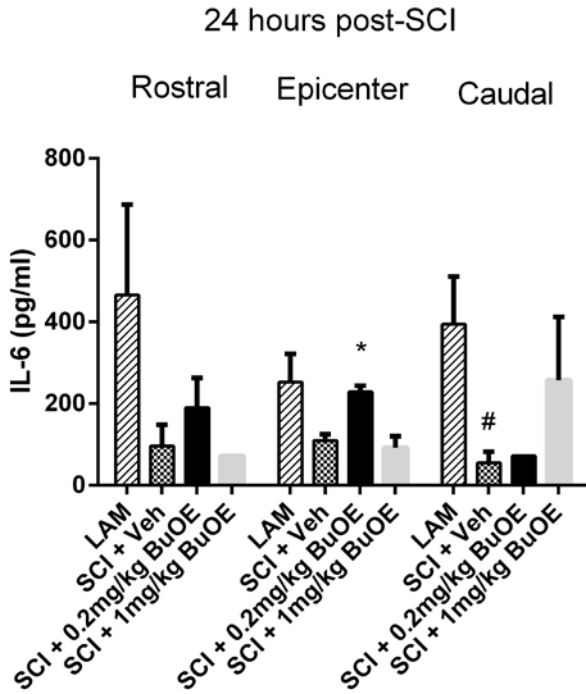


Figure 18: Effect of BuOE2 on expression of IL-6 in the rat spinal cord at 24 hours post-SCI. IL-6a is a protein of the interleukin family that regulates the immune response. A reduction following injury was observed as were some effects of BuOE treatment.

* p<0.01 SCI vs. SCI 0.2mg/kg BuOE

p=0.05 LAM vs.SCI

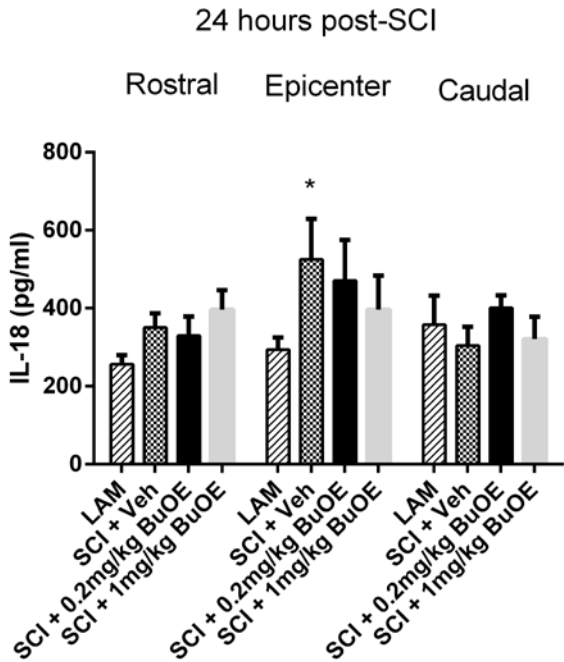
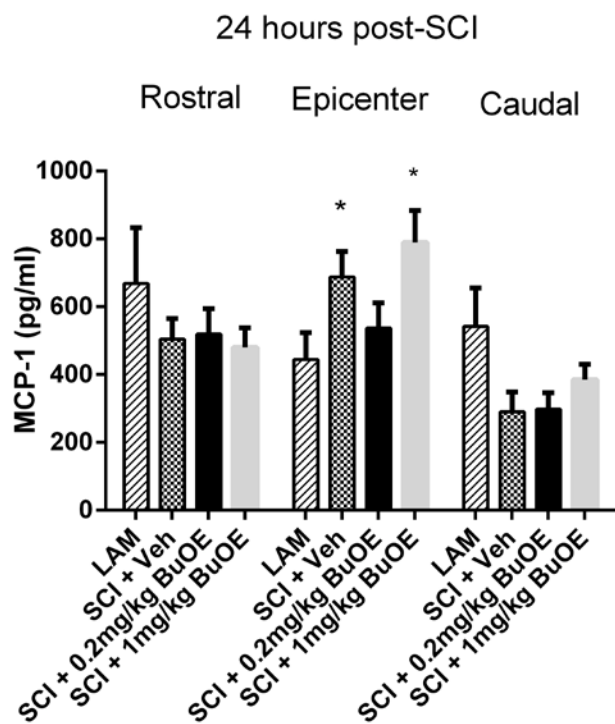


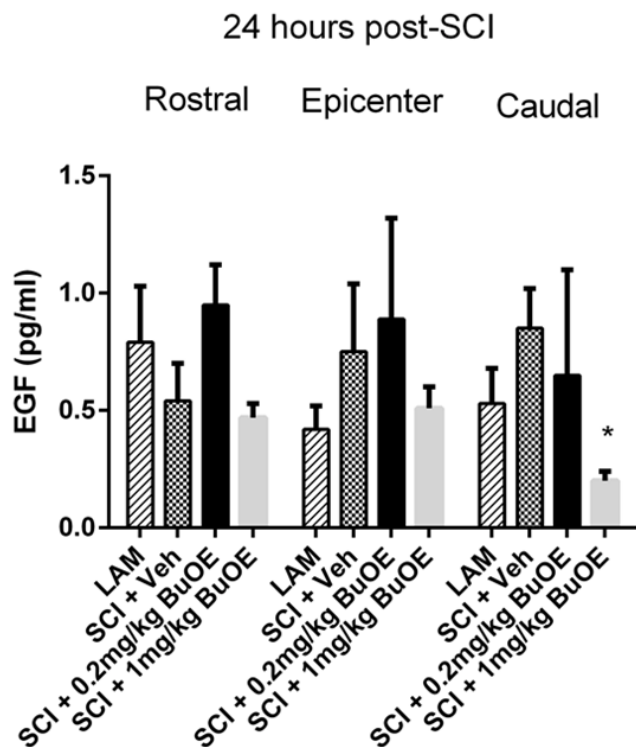
Figure 19: Effect of BuOE2 on expression of IL-18 in the rat spinal cord at 24 hours post-SCI. IL-18 is a protein of the interleukin family that regulates the immune response. A upregulation following injury was observed as were some effects of BuOE treatment.

*p<0.05 LAM vs. SCI



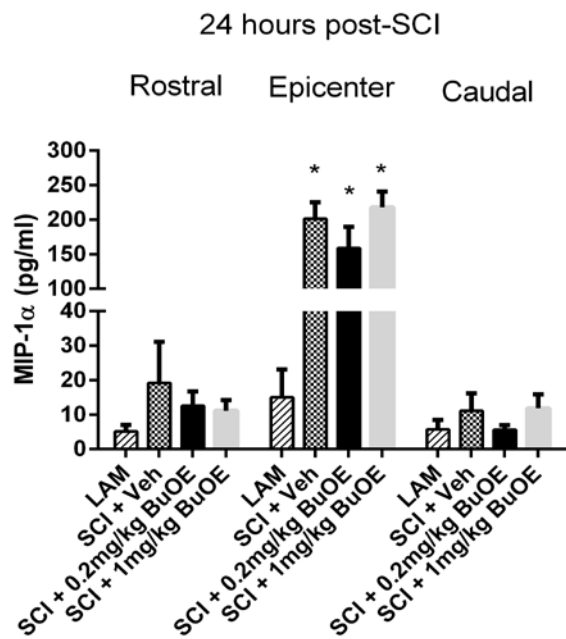
* $p < 0.05$ LAM vs. SCI + Veh

Figure 20: Effect of BuOE2 on expression of MCP-1 in the rat spinal cord at 24 hours post-SCI. Monocyte chemotactic protein 1 (MCP1) is an inducible cytokine that is pro-inflammatory. An upregulation following injury was observed as were some effects of BuOE treatment



* $p < 0.01$ SCI vs. SCI 1mg/kg BuOE

Figure 21: Effect of BuOE2 on expression of EGF in the rat spinal cord at 24 hours post-SCI. Epidermal growth factor (EGF) is a protein that supports cell proliferation. An upregulation following injury was observed in the epicenter and caudal segment, as were some effects of BuOE treatment

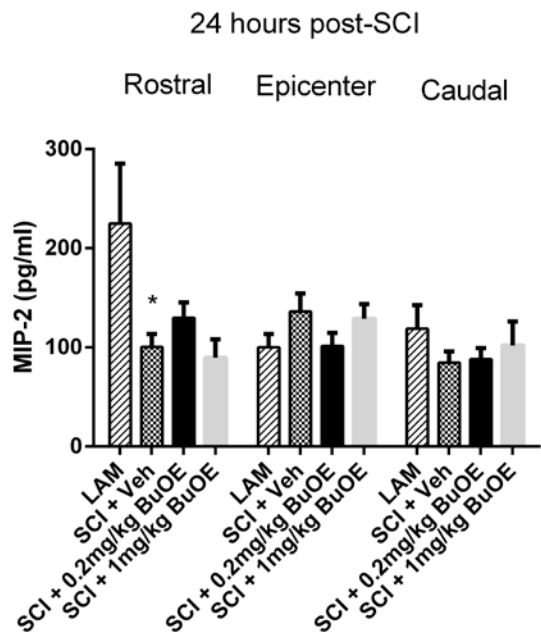


* $p < 0.01$ LAM vs SCI

Epicenter SCI groups are all significant compared to rostral and caudal

No drug effect

Figure 22: Effect of BuOE2 on expression of MIP-1a in the rat spinal cord at 24 hours post-SCI. Macrophage inflammatory protein 1A is a protein that recruits inflammatory cells. An upregulation following injury was observed in the epicenter and caudal segment.



* $p < 0.05$ LAM vs SCI

Figure 23: Effect of BuOE2 on expression of MIP-1a in the rat spinal cord at 24 hours post-SCI. Macrophage inflammatory protein 2 is a protein that recruits inflammatory cells. An downregulation following injury was observed in the rostral segment.

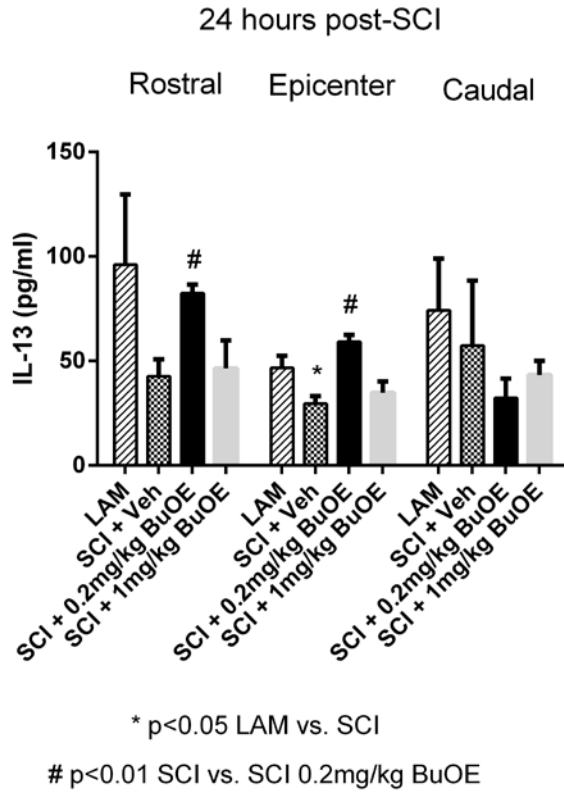


Figure 24: Effect of BuOE2 on expression of IL-13 in the rat spinal cord at 24 hours post-SCI. IL-13 is a protein of the interleukin family that regulates the immune response. A reduction following SCI was observed as well as effects of BuOE in the rostral and caudal segments.

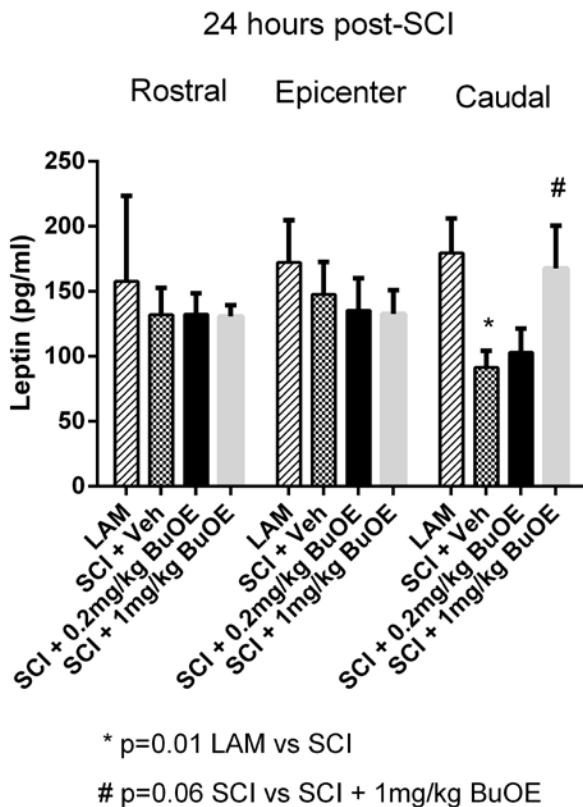


Figure 25: Effect of BuOE2 on expression of leptin in the rat spinal cord at 24 hours post-SCI. Leptin is a hormone which regulates energy homeostasis. A reduction following SCI was observed as well as effects of BuOE in the caudal segment.

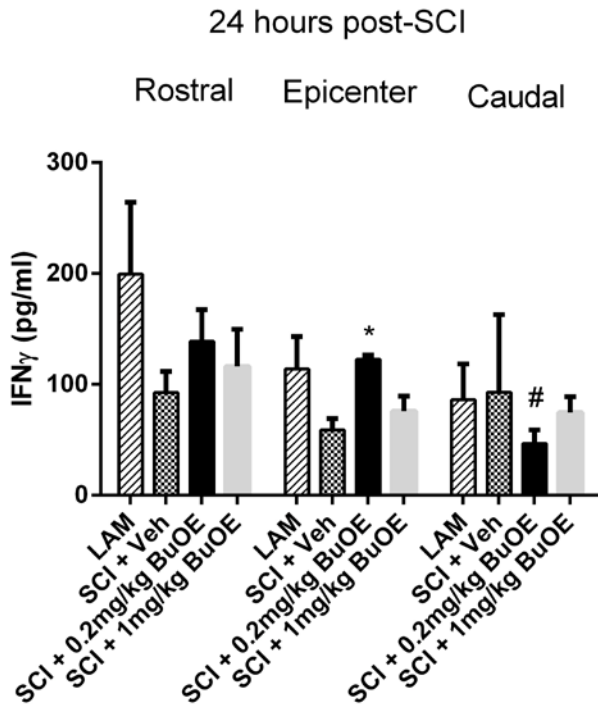


Figure 26: Effect of BuOE2 on expression of IFN γ in the rat spinal cord at 24 hours post-SCI. Interferon gamma (IFN γ) is a cytokine which regulates immune function. A reduction following SCI was observed as well as effect of BuOE in the caudal segment.

* $p < 0.01$ SCI vs SCI 0.2mg/kg BuOE

$p < 0.01$ 0.2mg/kg BuOE Epicenter vs. Caudal

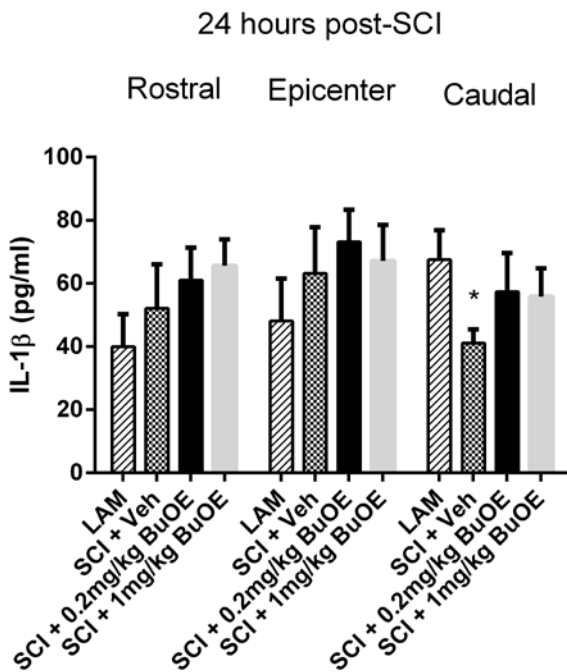


Figure 27: Effect of BuOE2 on expression of IL-1 β in the rat spinal cord at 24 hours post-SCI. IL-1 β is a protein of the interleukin family that regulates the immune response. A reduction following SCI was observed in the caudal segment, but no drug effects.

* $p = 0.02$ LAM vs. SCI

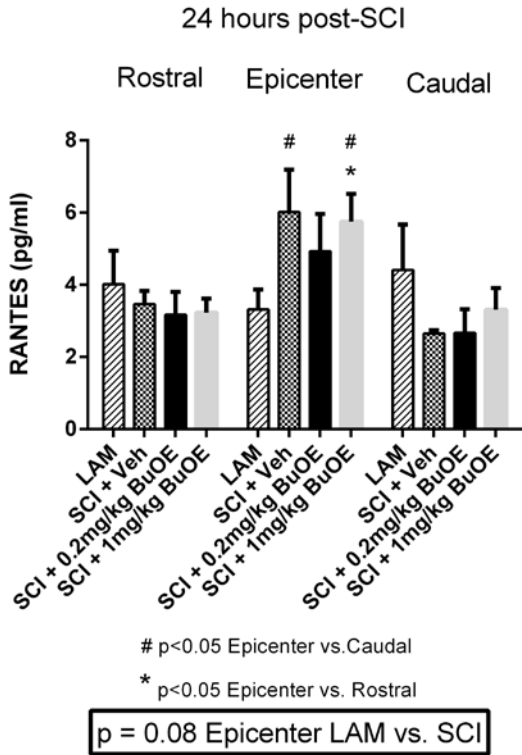


Figure 28: Effect of BuOE2 on expression of RANTES in the rat spinal cord at 24 hours post-SCI. Regulated on activation normal T cell expressed and secreted (RANTES) protein recruits immune cells to the injury site. An upregulation following SCI was observed in the epicenter, but no drug effects.

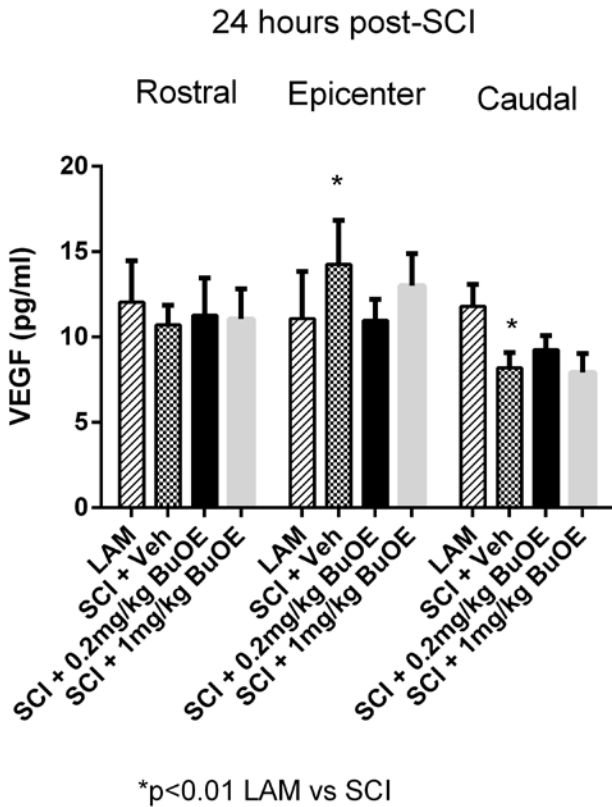


Figure 29: Effect of BuOE2 on expression of VEGF in the rat spinal cord at 24 hours post-SCI. Vascular endothelial growth factor (VEGF) stimulates a response to restore oxygen to a damaged system. An upregulation following SCI was observed in the epicenter and a downregulation in the caudal segment, but no drug effects.

particular interest: a) several protein in the interleukin family including IL-4, IL-6, IL-10, IL-13 as well as IFN- γ ; b) proteins which are chemoattractant including RANTES, MCP-1, MIP-1 α ; and c) proteins that regulate cellular energetics including Leptin and VEGF. We plan to continue these experiments by examining other acute time points as proposed.

Task 10: Conduct immunoblotting on extracts from rat and mouse spinal cord sections to evaluate activation of NF- κ B and post-translational modification of NF- κ B subunits (Tse). Months 3-12.

This task is on-going. Standardized protocols between the collaborative research team have been established and tissue samples have been effectively prepared according to these protocols. The tissue extraction completion parallels the task 5 completion, and is currently 100% complete.

We are continuing the evaluation of NF- κ B signaling post-SCI in the rat. For this experiment, rats were injured and administered BuOE2 after SCI as described above. Spinal cords were extracted at 24h post-SCI, flash frozen, and then homogenized. Tissue was also fractionated using the Active Motif Nuclear Extraction King (catalog #40010) to obtain nuclear fractions. Next, immunoblots were conducted to assess level of total NF- κ B (p65, Cell Signaling antibody #8242s) and activated NF- κ B (phosphorylated p65, Cell Signaling antibody #3033S). GAPDH (Protein Tech antibody # HRP-6004) was used as a loading control and all protein values as

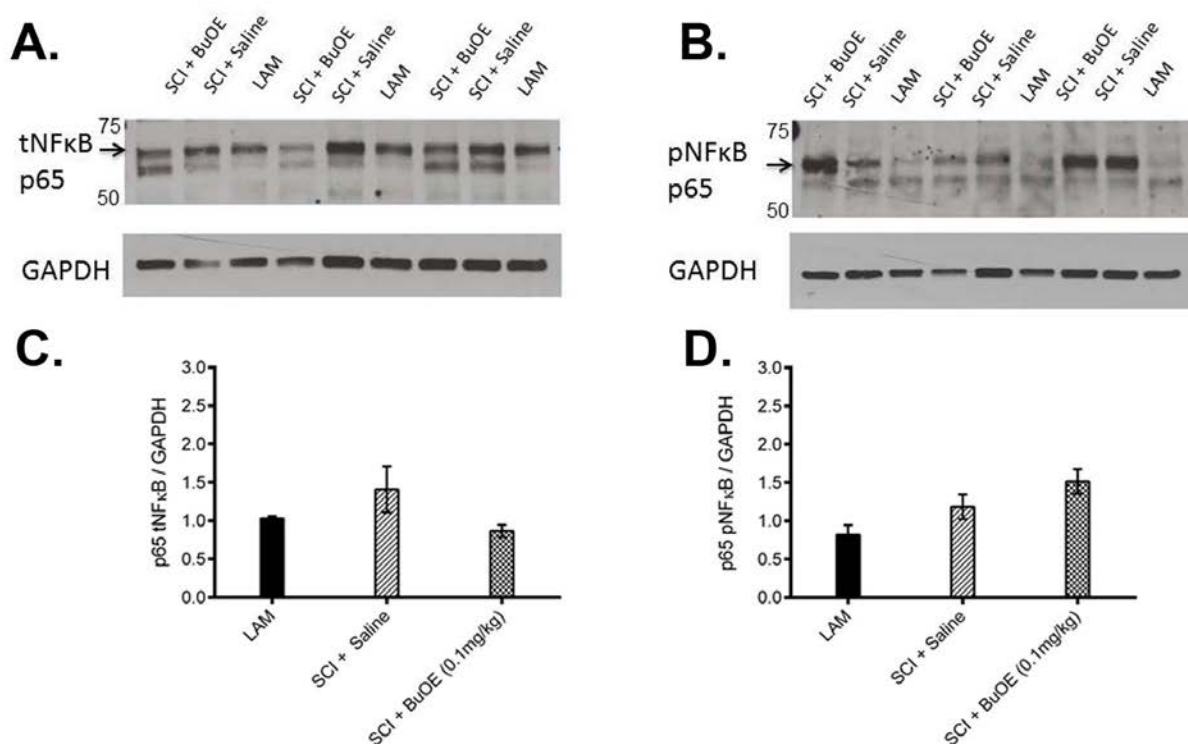


Figure 30: Effect of post-SCI administration of BuOE2 on NF- κ B signaling at 24 hours post-injury in the rats. All analyses were conducted at the epicenter of the spinal cord lesion. Panel A is a representative immunoblot probing for total NF- κ B (p65) from spinal cord tissue homogenates taken from 9 animals with the group of the animal listed above each lane. GAPDH was used as a loading control and the immunoblot corresponding to the lane above is pictured. Panel B is a representative immunoblot probing for phosphorylated (activated) NF- κ B (p65) from spinal cord tissue homogenates taken from 9 animals with the group of the animal listed above each lane. GAPDH was used as a loading control and the immunoblot corresponding to the lane above is pictured. Panel C is quantification using relative optical density of the total NF- κ B protein in the nuclear fraction (from A) protein normalized to GAPDH. Panel D is quantification using relative optical density of the phosphorylated NF- κ B protein in the nuclear fraction normalized to GAPDH.

assessed by relative optical density were normalized to GAPDH. Also, each immunoblot included tissue extracts from control (laminectomy only), SCI + vehicle (saline) and SCI + BuOE2 such that comparisons between experimental groups could be done between samples on the same immunoblot. As seen in figure 30, preliminary data indicate that post-SCI administration of BuOE2 induces a reduction in the level of total NF- κ B in the nuclear fraction. With regard to activated NF- κ B as detected by phosphorylation at the p65, no difference between injury groups were observed, suggesting that post-SCI BuOE2 may not affect activation of this subunit. Subsequent experiments will follow up on these preliminary findings in both rats and mice.

YEAR 2:

Task 1: Obtain required regulatory approval for project, including IACUC, Occupational Health Approval, ACURO from UAB (Floyd and Tse) and from Duke (Warner, Batinic-Haberle, Spasojevic, Sheng). Month 1.

This goal was achieved by IACUC ACURO approval annual renewal was completed by March 2015 with new dosing scheme (see task 5, below for more details)

Task 2: Quantitatively assure purity of sufficient BuOE2 for use at both UAB and Duke for Year 2 *in vivo* studies. Order all necessary surgical supplies and biochemistry/ molecular reagents (ALL). Months 1-12.

This task was completed.

Task 3: Order and acclimate adult male rats for evaluation in chronic post-SCI time points related to Aim 1 at UAB (Floyd). Order and acclimate adult male mice for evaluation in chronic post-SCI time points related to Aim 2 at Duke (Warner and Sheng). Months 2-12.

This task was completed.

Task 4: For evaluation of neuropathic pain and functional recovery as well as the assessment of ROS, NF- κ B, and immunological markers at 6 weeks post-SCI, induce C5 hemicondusion SCI in 100 male rats at UAB (Floyd) or conduct 10 min aortic occlusion in 100 male mice at Duke (Warner and Sheng). Months 2-12.

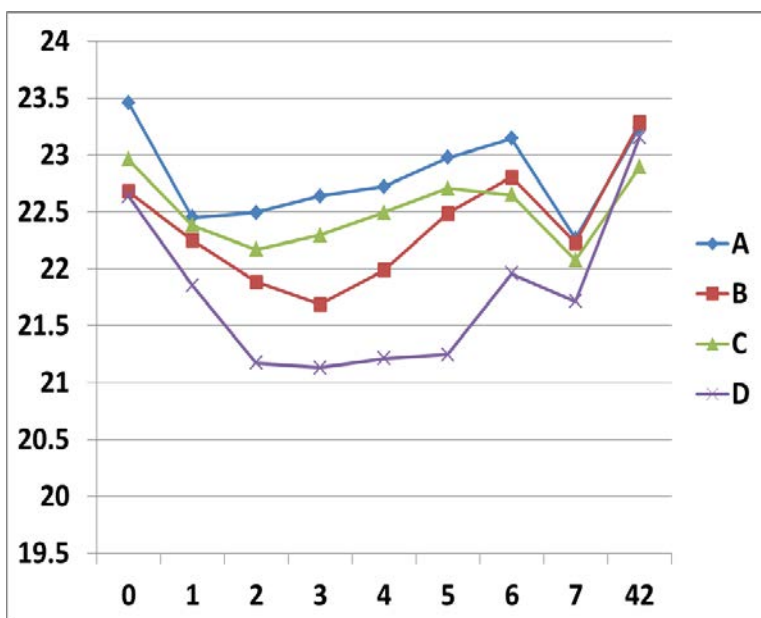
This task was completed and all rodent animal experimentation related to the chronic time point has been conducted. As described above, experimental hemicondusion SCI surgery in the rat was performed at UAB and experimental ischemic SCI in the mice was being performed at Duke. There has been no change from the injury model as described in the original application. Briefly for induction of SCI in the rat, male Sprague-Dawley rats (250-275g) were anesthetized with inhaled isoflurane and body temperature was maintained at 37C. The mid-cervical vertebral column was exposed and a bilateral laminectomy was performed at vertebral level C5. A moderate (300kdyne) contusion injury was be administered using the Infinite Horizons impactor. For induction of SCI in the mouse, male C57BL/6J mice were anesthetized with isoflurane and placed in the right lateral decubitus position. A left thoracotomy was performed by cauterizing the superficial layer of the intercostal muscle, then inserting the tip of a closed surgical scissor into the thoracic cavity and gently widening the intercostal space by opening the scissor. The thoracic aorta was visualized and a small aneurysm clip was placed on the aorta at the level of T8 to induce ischemic injury. The clip was removed after 10 min.

Task 5: Evaluate the effect of BuOE2 administration on neuropathic pain by assessing the daily incidence of dermatome-specific over-grooming, weekly assessment of tactile allodynia using Von Frey hairs, weekly assessment of thermal cold allodynia using the acetone test in conjunction with the rat grimace scale in rats at UAB (Floyd) and mice at Duke (Sheng). Months 2-12.

Mouse Results:

For this data set, the graphs are presented with the dosing schema coding (used for blinding of the experimenters) intact. The key for the code for the dosing scheme is:

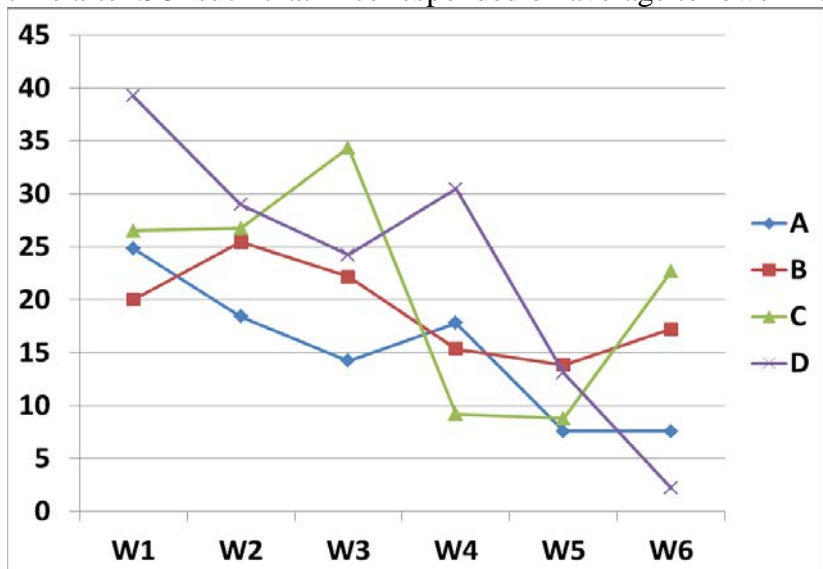
- A= SCI + 1.0 mg/kg loading dose; 0.5mg/kg/day BuOE2 daily: n=15
- B= SCI + 1.0 mg/kg loading dose; 0.5mg/kg/day BuOE2 weekly: n=15
- C= SCI + 0.2mg/kg loading dose; 0.1mg/kg/day BuOE2 daily: n=15
- D= SCI + vehicle: n=15



Body weight was evaluated as an indicator of the mouse health. Animals in the SCI + Vehicle group (D) exhibited greater reduction in body weight after the ischemic SCI than all other groups, which suggests some beneficial effects of BuOE2 on overall health.

Figure 31: Effect of drug treatment on body weight in mice following ischemic SCI. Mice in the SCI only group (D) exhibited greater decline in body weight than all the drug treated groups, which suggests that BuOE2 had a positive effect on health after SCI.

The main objective of this task was to evaluate the effect of BuOE2 treatment on neuropathic pain following ischemic SCI in the mouse. The first assessment was consideration of mechanical allodynia using paw withdrawal to von Frey filaments. In general, we observed that the sensitivity to the filaments increased over time after SCI such that mice responded on average to lower filaments which indicates an increased sensitivity



to tactile stimuli, or allodynia which developed by week 6 post-SCI (figure 31). When considering only the data at week 6 as compared to baseline, there was a trend for a reduction in sensitivity with the low dose daily dosing paradigm (group C) as well as the high dose weekly dosing paradigm (figure 32). These data suggest that dosing paradigms B and C conferred a reduction in neuropathic pain-like responses, specifically mechanical allodynia.

Figure 32: Effect of drug treatment on withdrawal threshold to von Frey filaments following ischemic SCI. SCI resulted in an increased sensitivity to mechanical stimuli (i.e. mechanical allodynia) in all groups.

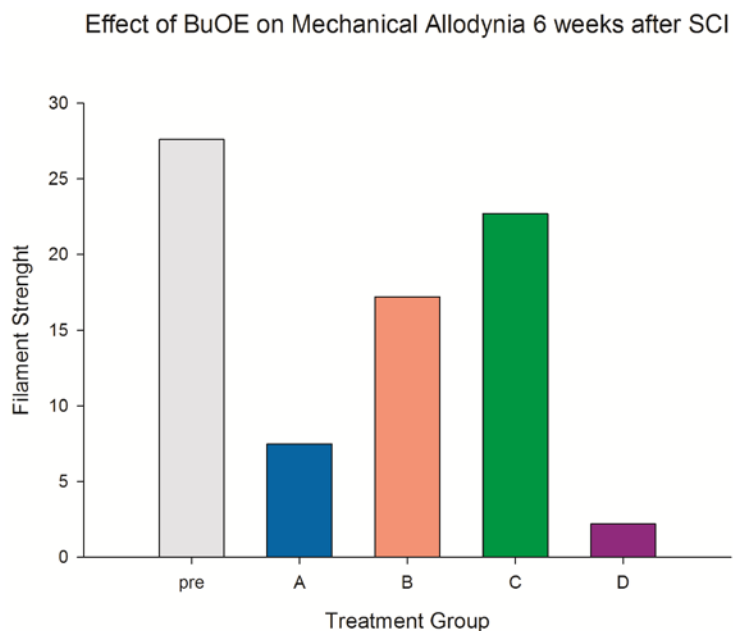


Figure 33: Effect of drug treatment on withdrawal threshold to von Frey filaments following ischemic SCI at week 6. SCI resulted in an increased sensitivity to mechanical stimuli (i.e. mechanical allodynia) in all groups as noted by the decrease in the filament strength compared to the pre-injury value. The most notable increase in sensitivity was in the SCI = vehicle group (group D). BuOE2 group B (high dose weekly) and group C (low dose daily) somewhat attenuated the effect of SCI.

Next, we evaluated the effect of BuOE2 on cold allodynia using the acetone test. We found that in general, mice became somewhat more sensitive to the acetone test after ischemic SCI, but that there was not a robust effect of BuOE2 on this sensitivity. Thus, these data suggest that post-SCI administration of BuOE2 does not affect cold allodynia.

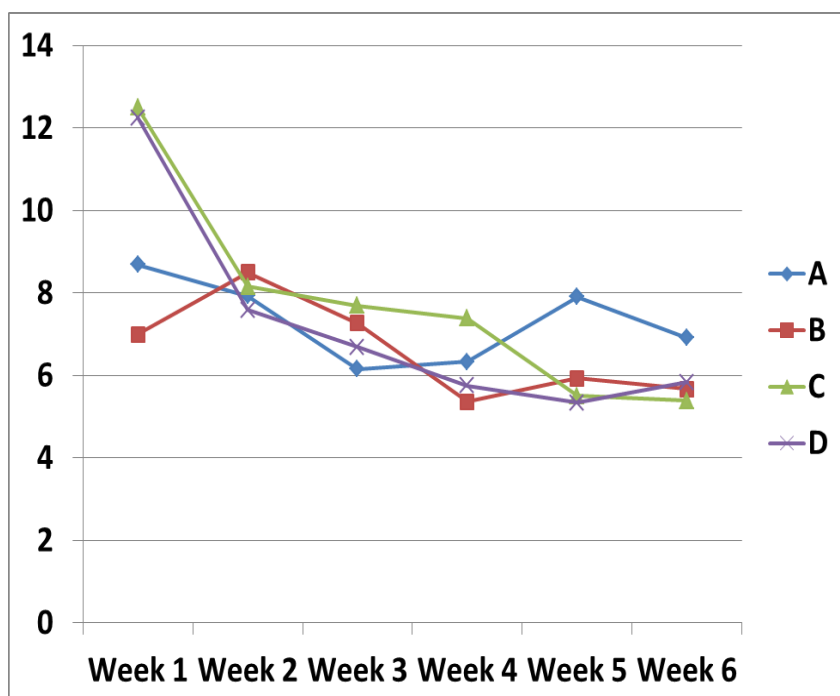


Figure 34: Effect of drug treatment on withdrawal threshold to acetone in mice. SCI resulted in a modest increased sensitivity to cold stimuli (i.e. cold allodynia). However, there was not a robust effect of BuOE2 treatment on responses.

Rat Results:

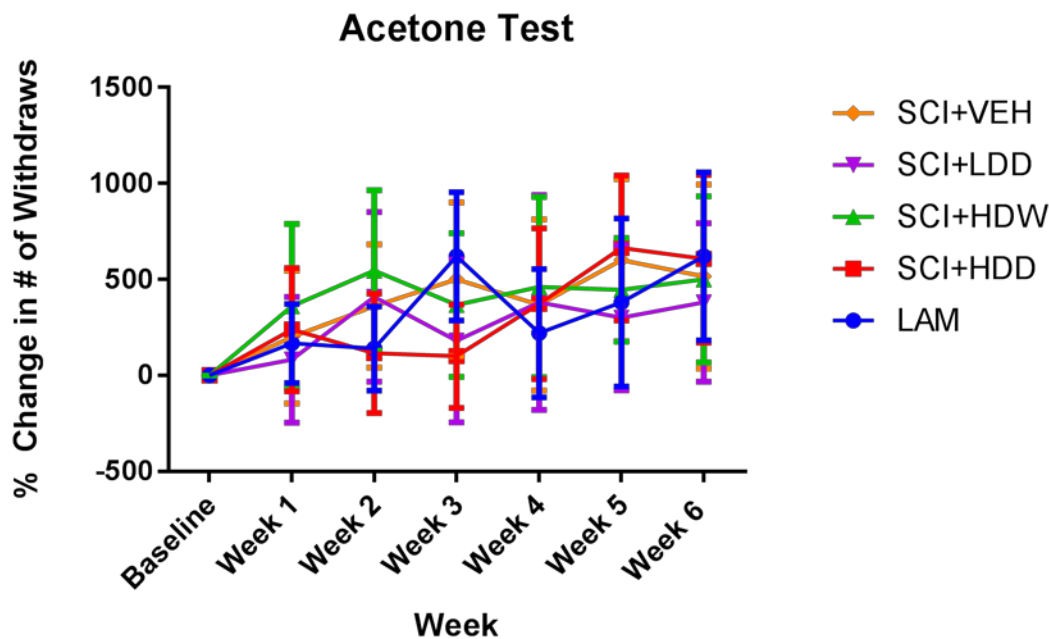


Figure 35: Effect of drug treatment on withdrawal threshold to acetone in rats. SCI resulted in a modest increased sensitivity to cold stimuli (i.e. cold allodynia). However, there was not a robust effect of BuOE2 treatment on responses.

As with the mice, the effect of BuOE2 on cold allodynia was evaluated in the rats following cervical hemiconfusion spinal cord injury. For this data set, the dosing paradigm is labeled as SCI-VEH, which is the group that received the cervical hemiconfusion SCI and vehicle drug treatment. LAM is the uninjured control laminectomy group. The nomenclature for the BuOE2-treated groups is as follows:
 SCI+LDD = SCI + 0.2mg/kg loading dose; 0.1mg/kg/day BuOE2 daily: n=15
 SCI + HDW = SCI + 1.0 mg/kg loading dose; 0.5mg/kg/day BuOE2 weekly: n=15
 SCI + HDD = SCI + 1.0 mg/kg loading dose; 0.5mg/kg/day BuOE2 daily: n=15

Prior to any surgical procedure (at baseline) we observed no reflexive withdrawals to the acetone stimulus. In the next 6 weeks following SCI, the rats were evaluated weekly on this test. In general, there was an increase in responsiveness to the acetone, indicating a hypersensitivity to cold. However, this hypersensitivity was seen in all groups and there was not a significant effect of drug treatment when the data from all animals is taken together. It is important to note that with this model, there is some heterogeneity in the development of neuropathic pain such that not all animals develop increases sensitivity. Thus, we propose to use the grimace scale scores to separate animals into neuropathic vs. non-neuropathic groups and conduct a subsequent analysis. This subsequent analysis is pending.

We also evaluate the effect of BuOE2 on mechanical allodynia following SCI using the von Frey test. When comparing responses after injury to the pre-injury baseline, we do not see a robust neuropathic pain-like

phenotype and also see a large degree of variability. As described above, these data include the scores of all animals. We hypothesize that the effects may be somewhat different when only the animals that exhibit neuropathic-pain like behaviors are considered. We propose to use the grimace scale to separate out the pain groups.

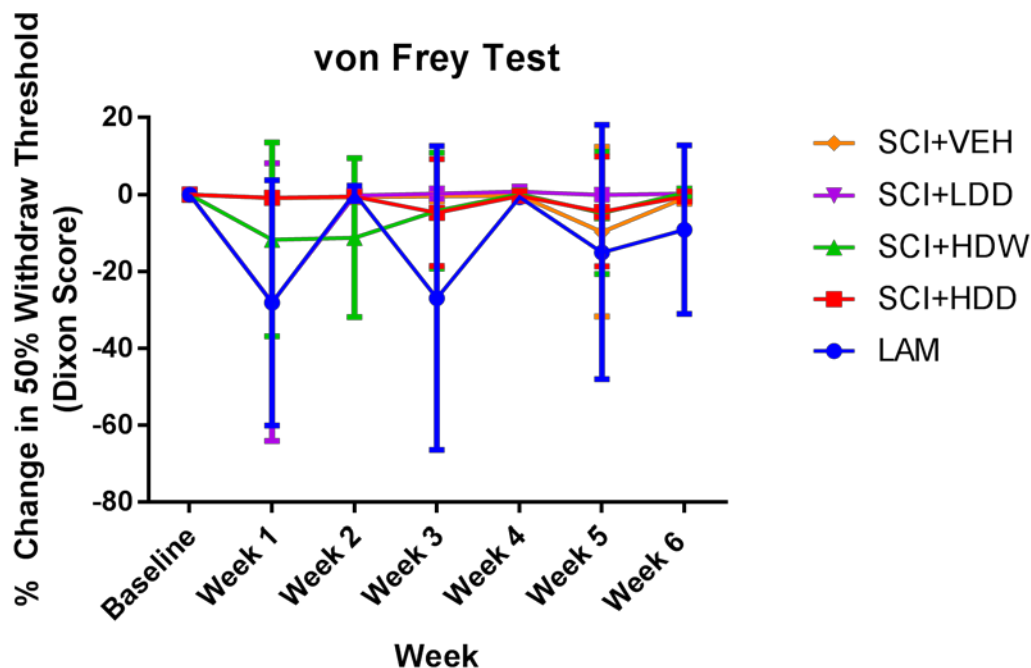
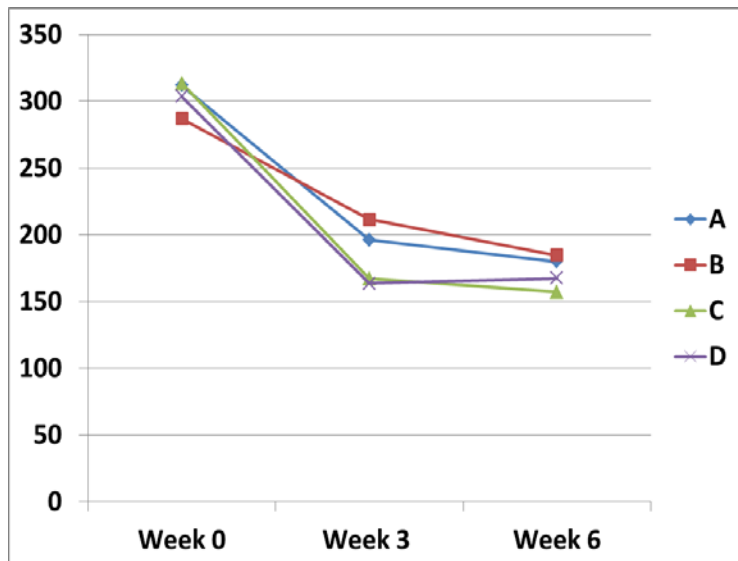


Figure 36: Effect of drug treatment on withdrawal threshold to von Frey hairs. SCI did not result in robust mechanical allodynia when all animals are considered. There was also not a robust effect of BuOE2 treatment on responses.

Task 6: Evaluate the effect of BuOE2 administration on functional recovery by weekly evaluation of forepaw use in skilled and unskilled use tests and locomotion tasks in rats at UAB (Floyd) and measurement of rotarod performance, and Basso mouse scale in mice at Duke (Sheng). Months 2-12.



We next assessed functional recovery via the

Figure 37: Effect of drug treatment on motor performance in mice following ischemic SCI. SCI resulted in impaired performance in the rotarod test with a decrease in the latency to stay on the rod (x-axis in seconds). However, no effect of BuOE2 was observed on performance assessed at 3 or 6 weeks after ischemic SCI.

performance of the mice on the rotarod test. Mice were evaluated prior to injury (week 0) and at weeks 3 and 6 after ischemic SCI. In general, we found that the SCI induced lasting impairment in this task. However, there was not a significant effect of drug treatment at any dose.

The next motor assessment that was conducted was the Basso Mouse Scale (BMS). This assessment was performed weekly following the ischemic SCI for 6 weeks. Please note that normal hind-limb locomotion on this scale is a 9. Our data indicate that mice were impaired in this locomotor assessment after ischemic SCI, but there was not an effect of drug treatment in that no drug group had significantly greater average scores than group D, the SCI + vehicle group.

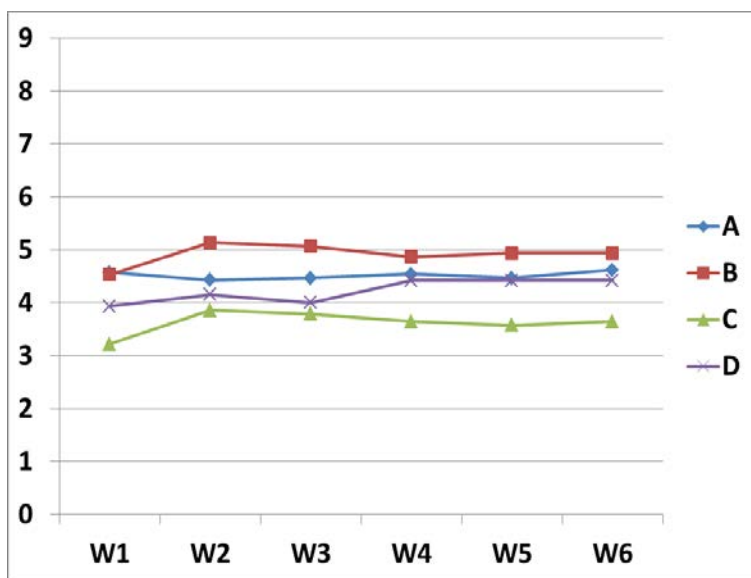


Figure 38: Effect of drug treatment on motor performance in mice following ischemic SCI. SCI resulted in impaired performance in the BMS test with a decrease in BMS score from the able-bodied score of 9 (y-axis in BMS score). However, no effect of BuOE2 was observed on performance assessed weekly throughout the 6 weeks after ischemic SCI.

Task 7: At 6 weeks post-SCI, exsanguinate a subset of the rats at UAB (Floyd) or mice at Duke (Sheng) and rapidly remove and freeze the spinal cord tissue. Transfer samples to Tse lab at UAB (Floyd and Sheng). Next, prepare tissue samples and lysates for subsequent biochemical and molecular analysis (Tse). (n=6/group). Months 2-12.

This task is complete. The animal work is 100% complete and all the tissues have been collected and transferred to UAB. Lysates have been prepared and are being examined.

Task 8: At 6 weeks post-SCI, exsanguinate a subset of the rats at UAB (Floyd) or mice at Duke (Sheng) and rapidly fix tissue with 4% paraformaldehyde. Remove the spinal cord, cryoprotect, and then block/ freeze the spinal cord segments for subsequent cryosectioning. Collect serial cryosections of the spinal cord for subsequent histochemical and immunohistochemical analysis at UAB and Duke(Floyd and Sheng). (n=6/group) Months 2-12.

This task is complete. The animal work is 100% complete and all the tissues have been collected, blocked and frozen.

Task 9: In a subset of the rats at UAB (Floyd) or mice at Duke(Sheng), extract the spinal cord for measurement of BuOE2 tissue concentration. Send samples to Spasojevic lab at Duke (Floyd and Sheng). Measure concentration of BuOE2 in spinal cord and blood from samples at Duke(Spasojevic). (n=6/group). Months 2-12.

The task has been complete, please see data presented above.

Task 10: Conduct flow cytometry experiments on spinal cord microglia and astrocyte cells from extracted rat or mouse tissue to detect ROS using redox-sensitive dyes and pro-inflammatory cytokines with fluorochrome-conjugated antibodies at UAB (Tse). Months 2-12.

We are continuing to optimize these procedures for the spinal cord tissue; thus, this task is on-going.

Task 11: Evaluate pro-inflammatory cytokine/chemokine synthesis in rat or mouse spinal cord protein lysates with a Luminex Beadlyte 21-plex multi-cytokine detection system at UAB (Tse). Months 2-12.

The animal work related to this task is completed and the tissue samples have been collected. The detection experiments are on-going and at 80% completion. We anticipate completion of all experimentation and of data analysis by June 2017.

Task 12: Conduct immunoblotting on extracts from rat or mouse spinal cord sections to evaluate activation of NF- κ B and post-translational modification of NF- κ B subunits at UAB (Tse). Months 2-12.

The animal work related to this task is completed and the tissue samples have been collected. The detection experiments are on-going and at 60% completion. We anticipate completion of all experimentation and of data analysis by June 2017.

YEAR 3

Task 1: Obtain required regulatory approval for project, including IACUC, Occupational Health Approval, and ACURO from UAB (Floyd and Tse) and from Duke (Warner, Batinic-Haberle, Spasojevic, Sheng). Month 1.

This task was completed.

Task 2: Quantitatively assure purity of sufficient BuOE2 for study of adult male Yucatan minipigs for evaluation in acute post-SCI time point related to Aim 3. Months 1-12.

This task was completed.

Task 3: Order and acclimate adult male Yucatan minipigs for evaluation in acute post-SCI time point related to Aim 3 at UAB (Floyd and Tse). Months 2-12.

This task was completed; see details along with tasks below.

Task 4: For evaluation of ROS, NF- κ B, immunological markers, and pharmacokinetics at 48 hours post-SCI, induce a moderate contusion SCI in 18 male Yucatan minipigs at UAB (Floyd, Warner, Sheng). Months 2-12.

This animal work on this task has been completed. We are currently evaluating tissue samples.

Task 5: At 48 hours post-SCI, exsanguinate pigs and rapidly remove and freeze the spinal cord tissue. Next, prepare tissue samples and lysates for subsequent biochemical and molecular analysis at UAB (Floyd, Tse, Sheng). Months 2-12.

This task has been completed.

Task 6: Send samples and portion of spinal cord tissue samples/lysate to Duke (Floyd and Tse) for pharmacokinetic analysis. Duke to receive spinal cord and blood samples from UAB and quantitatively measure tissue BuOE2 concentrations at Duke (Batinic-Haberle and Spasojevic). Months 2-12.

This task is completed and please see results below.

PORCINE plasma, serum, urine, CSF, and tissue levels:

The concentration of MnBuOE found 48 hrs after injury and 1 mg/kg intravenous (IV) injection, followed by 0.5 mg/kg at 24 hours, at the site of injury (epicenter) was 134 nM (168 ng/mL). The found at different rostral (R1, R2, R3) and caudal (C1, C2, C3) positions were gradually lower (Table 4) strongly suggesting that the tissue/vascular disruption contributed to the favorable MnBuOE accumulation. The rodent studies on spinal cord PK show very stable MnBuOE levels over 7 days after single SC injection so the levels found in our porcine contusion study at 48 h may be assumed to be present in tissue throughout the treatment. We do not have a spinal cord injury study to compare the levels found but the brain levels found in our study are comparable to those in brain in previous studies where MnBuOE or analogs were proven to be efficacious. (1) MnHex (analogue) given 0.075 mg IV on a first day followed by 2 x 0.225 mg/kg SC daily for 7 days, showed protective effect in brain ischemic stroke and subarachnoid hemorrhage mouse model [Ref 2, Sheng et al. 2011],

REF 2: Neuroprotective Efficacy from a Lipophilic Redox-Modulating Mn(III) N-Hexylpyridylporphyrin, MnTnHex-2-PyP: Rodent Models of Ischemic Stroke and Subarachnoid Hemorrhage, Huaxin Sheng, Ivan Spasojevic, Hubert M. Tse, Jin Yong Jung, Jun Hong, Zhiqian Zhang, Jon D. Piganelli, Ines Batinic-Haberle, and David S. Warner, *The Journal of Pharmacology and Experimental Therapeutics*, **2011**, Vol. 338, No. 3, p 906

(2) MnBuOE was radioprotective when given subcutaneously 1.6 mg/kg for 2 months and one week, and one month after last injection. The levels found in brain at the end of treatment were 31.30 nM. [Ref 3, Weitzel et al. 2014]

REF 3: Weitzel, DH, Tovmasyan, A., Ashcraft KA, Rajic, Z., Weitner, T., Liu, C., Li, W, Buckley, AF, Prasad, MR, Young, KH, Rodriguiz, RM, Wetsel, WC, Peters, KB, Spasojevic, I., Herndon II, JE, Batinic-Haberle, I, Dewhirst MW, Radioprotection of brain white matter by Mn(III) N-butoxyethylpyridylporphyrin-based superoxide dismutase mimic, MnTnBuOE-2-PyP5+, *Mol Cancer Ther*, **2015**, 14, 70-79 PMID:PMC4397941

(3) MnBuOE was radioprotective when given subcutaneously at a loading dose of 3 mg/kg twice weekly for 1 week) followed by a maintenance dose of 0.5 mg/kg twice weekly for 4 months. Brain levels found at the end of treatment were 12.73 nM [Ref 4, Weitzel et al. 2016]

REF 4: Weitzel DH, Ashcraft KA, Boico A, Birer SR, Roy Choudhury KR, Rodriguiz RM, Wetsel WC, Peters KB, Spasojevic I, Batinic-Haberle I, Dewhirst MW, Neurobehavioral radioprotection to standard brain cancer therapy regimens by Mn(III) n-Butoxyethylpyridylporphyrin-based redox modifier, *Environmental and Molecular Mutagenesis*, 2016, 2016 Jun;57(5):372-81

Fluid/Organ	MnBuoE, nM	std dev (n=3)
Plasma	3.7	0.7
Serum	3.5	0.9
Urine	1260.7	1089.4
Spinal Cord - Rostral 3	50.3	26.6
Spinal Cord - Rostral 2	52.6	36.0
Spinal Cord - Rostral 1	75.0	23.8
Spinal Cord - EPICENTER	133.7	10.0
Spinal Cord - Caudal 1	56.2	21.2
Spinal Cord - Caudal 2	40.0	9.6
Spinal Cord - Caudal 3	29.5	7.1
CSF	2.7	1.4
Brain	22.2	4.9
Kidney	11233.3	152.8
Liver	8583.3	182.3
Lung	672.3	46.2
Spleen	419.7	88.8
Heart	223.0	61.0

Table 4. MnBuOE levels in fluids and tissues at the time of sacrifice (48 hrs post injury).

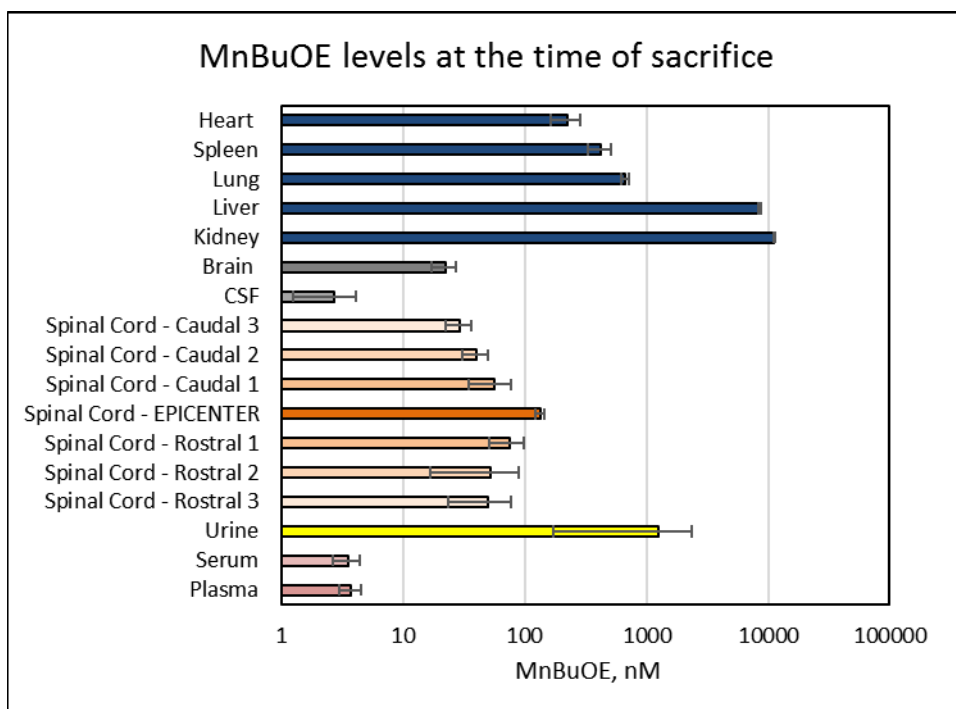


Figure 39. MnBuOE measured in fluids and tissues at the time of sacrifice (48 hrs post injury).

Task 7: At UAB, conduct flow cytometry experiments on spinal cord microglia and astrocyte cells from extracted pig tissue to detect ROS using redox-sensitive dyes and pro-inflammatory cytokines with fluorochrome-conjugated antibodies (Tse). Evaluate pro-inflammatory cytokine/chemokine synthesis in pig spinal cord protein lysates with a Luminex multi-cytokine detection system (Tse). Conduct immunoblotting on

extracts from pig spinal cord sections to evaluate activation of NF- κ B and post-translational modification of NF- κ B subunits (Tse). Months 2-12.

4. IMPACT

Development of the principal disciplines: The work thus far has had an impact in developing and extending our knowledge on the dose of BuOE2 that results in a steady level in the spinal cord in rodents. Secondly, these studies have begun to examine the effects of post-SCI administration of BuOE2 on inflammation and NF- κ B signaling in the spinal cord tissue after contusion and ischemic injury in rodent models.

Other disciplines: The knowledge concerning the dosing and tissue accumulation of BuOE2 will have an impact on other disciplines in which a therapeutic compound of this type could be used, including but not limited to traumatic brain injury, stroke, alleviation of chemotherapy-induced pain, and diabetic neuropathy.

Technology transfer: Nothing to report.

Society: Nothing to report.

5. CHANGES/ PROBLEMS:

Changes in approach:

1) Initially we proposed to evaluate lower doses of BuOE. However, our preliminary data indicated that these doses were too low which caused us to submit a change in the statement of work to evaluate higher doses. Additionally, our initial preliminary data suggested that either a weekly or daily dosing schedule would lead to differentially accumulated concentration of drug in the spinal cord with weekly achieving a steady state and daily causing an escalating dosing. As we are not sure which spinal cord drug concentration is the best for protection, we updated the statement of work to compare both strategies.

2) There is concern in the rodent literature that exclusive use of reflexive paw withdrawal data to indicate pain is not preferred. Thus, we added a subsequent analysis of facial grimace in rats. Our preliminary data indicate that grimace can be used to evaluate both spontaneous pain and evoked pain, see appendix 1. We have recorded the facial expression of rats during the acetone test and are evaluating these data as an additional pain marker.

Problems and delays with plan to resolve:

1) Due to the approved change in the dosing scheme, we are modestly behind in achieving our objectives in the rodent research. However, all the animal experimentation related to rodents has been completed and we are confident that we can complete the necessary data analysis by Oct 2017.

Changes with impact on expenditures: Nothing to report.

Changes in use and care of vertebrate animals: Nothing to report.

6. PRODUCTS

Submitted Publications in revision:

Comprehensive single and multiple, short and long-term pharmacokinetic studies of redox-active drug and SOD mimic, Mn(III) meso-tetrakis(N-butoxyethylpyridinium-2-yl)porphyrin, MnTnBuOE-2-PyP⁵⁺ Artak Tovmasyan,¹ Tin Weitner,¹ Huaxin Sheng,² Xinghe Chen,³ Kathleen Ashcraft,¹ Ping Fan,⁴ Dewhirst, M. W.,¹ David S. Warner,² Zeljko Vujaskovic,⁴ Ines Batinic-Haberle¹ and Ivan Spasojevic,^{6*} Departments of Radiation Oncology,¹ Anesthesiology,² Medicine,⁶ and Duke Cancer Institute,⁴ Duke University School of Medicine, Durham, NC 27710, Department of Neurosurgery, The First Hospital of Qinhuangdao City, Hebei, 066000 China,³ and Division of Translational Radiation Sciences, Department of Radiation Oncology, University of Maryland, Baltimore, Maryland⁵

Application of the rat grimace scale as a marker of supra-spinal pain sensation after cervical spinal cord injury.

Lonnie E. Schneider, Kathryn Henley, Omari Turner, Betty Pat, Tracy Niedzielko, and Candace Floyd. Department of Physical Medicine and Rehabilitation, University of Alabama at Birmingham, Birmingham AL

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

NAME	CANDACE FLOYD
PROJECT ROLE	PI
NEAREST PERSON MONTH WORKED	1.2
CONTRIBUTION	Project management, assessment of SCI in rodents
OTHER FUNDING SUPPORT	NIH, UAB, other DoD

NAME	HUBERT TSE, PH.D.
PROJECT ROLE	Co-PI
NEAREST PERSON MONTH WORKED	1.2
CONTRIBUTION	Assessment of cytokines and inflammation, data interpretation
OTHER FUNDING SUPPORT	NIH, UAB, American Diabetes Association

NAME	DAVID WARNER, M.D.
PROJECT ROLE	Co-PI
NEAREST PERSON MONTH WORKED	2.1
CONTRIBUTION	Subcontract project management and oversee the mouse SCI induction
OTHER FUNDING SUPPORT	DUKE

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PROJECT ROLE	CO-I
NEAREST PERSON MONTH WORKED	1.2
CONTRIBUTION	Conduct and coordinating the pharmacokinetic assessments
OTHER FUNDING SUPPORT	DUKE

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PROJECT ROLE	CO-I
NEAREST PERSON MONTH WORKED	0.9
CONTRIBUTION	assess the purity of BuOE2, assist with data interpretation and PK
OTHER FUNDING SUPPORT	DUKE

NAME	XUZXIN SHENG, M.D.
PROJECT ROLE	3.0

NEAREST PERSON MONTH WORKED	CO-I
CONTRIBUTION	Assessment of ischemic SCI in mice
FUNDING SUPPORT	DUKE

NAME	LONNIE SCHNEIDER
PROJECT ROLE	TECHNICAL (POST-DOC)
NEAREST PERSON MONTH WORKED	12
CONTRIBUTION	Conduct experiments related to rat SCI
FUNDING SUPPORT	NONE

NAME	GARY MASSEY
PROJECT ROLE	TECHNICAL
NEAREST PERSON MONTH WORKED	1.2
CONTRIBUTION	Assist with induction and evaluation of SCI in mice
FUNDING SUPPORT	DUKE

NAME	ARTAK TOVMASYAN
PROJECT ROLE	TECHNICAL (POST-DOC)
NEAREST PERSON MONTH WORKED	1.8
CONTRIBUTION	Assist with PK evaluations
FUNDING SUPPORT	DUKE

8. QUAD CHART

SEE NEXT PAGE

9. APPENDIX 1

SEE NEXT PAGES:
APPENDIX 1, SUBMITTED MANUSCRIPT

1 **Application of the Rat Grimace Scale as a Marker of Supra-spinal Pain Sensation**
2 **after Cervical Spinal Cord Injury**

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4 Tracy L. Niedzielko, and Candace L. Floyd, PhD*

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1 ¹These authors have contributed equally to the manuscript

2 Running title: Grimace as a marker of supra-spinal pain sensation after SCI

3

4 Table of Contents title: Application of the Rat Grimace Scale as a Marker of Supra-spinal Pain
5 Sensation after Cervical Spinal Cord Injury

6

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9

10 **Abstract**

11 Experimental models of neuropathic pain (NP) typically rely on withdrawal responses to assess
12 the presence and level of pain in laboratory animals. Reflexive withdrawal responses to a
13 stimulus are typically used to evaluate evoked pain and as such do not permit the assessment
14 of spontaneous NP or evaluation of the affective and emotional consequences of pain.

15 Additionally, withdrawal responses can be mediated entirely by spinal cord reflexes and not
16 thought to accurately represent supra-spinal pain sensation. This is especially true in models of
17 traumatic spinal cord injury (SCI) wherein spastic syndrome, a motor disorder characterized by
18 exaggeration of the stretch reflex secondary to hyper-excitability of the spinal reflex, can cause
19 paroxysmal withdrawals not associated with NP sensation. In order to establish consistent
20 methods for supra-spinal measures of pain it is important to compare these novel measures to
21 previous standard assessments such as reflexive withdrawal responses. Consequently, the
22 goal of this study was to utilize an assessment of supra-spinal pain sensation, the Rat Grimace
23 Scale (RGS), to measure both spontaneous and evoked NP in a model of SCI with a clinically
24 relevant cervical injury. Adult male rats received a hemi-contusion SCI at vertebral level cervical
25 level 5 (C5) followed by assessments of NP. Spontaneous pain was assessed using the RGS to
26 score facial action units prior to the application of a stimulus. Evoked pain from acetone induced
27 hyper-reflexia was assessed in conjunction with RGS scoring of action units after application of
28 the stimulus. Rodents exhibited significantly higher RGS scores at week 5 post-injury compared

1 to baseline and laminectomy controls prior to the application of the stimulus suggesting the
2 presence of spontaneous NP. Additionally, there was a significant increase in RGS scores after
3 the application of the acetone. These data suggest that in this model of SCI, the RGS can be
4 used to assess spontaneous NP and determine the presence of evoked supra-spinal pain
5 sensation after experimental SCI.

6 **Key words:** neuropathic pain, cold allodynia, pain sensation, ascending pain pathways

7

1 Introduction

2 Spinal cord injury (SCI) is a traumatic event affecting 238,000-332,000 people in the United
3 States with 12,000 new cases annually.¹ Chronic neuropathic pain (NP) can be a debilitating
4 consequence of SCI, affecting an average of 65% of patients.² Evoked NP occurs in response
5 to peripheral stimuli and can be categorized as hyperalgesia, in which sensitivity to noxious
6 stimuli is increased, or allodynia, in which innocuous stimuli become noxious. Spontaneous NP
7 occurs independently of external stimuli and is described by patients as an intermittent, burning
8 or stabbing sensation commonly rated as severe.^{2,3} The presence and severity of NP are
9 associated with greater impairments in physical, emotional, and social functioning⁴ and
10 alleviation of NP is one of the highest priorities for the patient.⁵

11 Numerous animal models of NP have been developed to elucidate mechanisms underlying
12 the development of NP and determine the analgesic potential of pharmacological interventions.
13 The most commonly used models involve compression of peripheral nerves using suture or
14 clamping to initiate a compression injury or axotomy of peripheral nerves innervating the rodent
15 hind paw, which result in both hyperalgesia and allodynia (see ⁶ for review). To emulate NP after
16 SCI several preclinical models have been developed, including the most prevalent spinal cord
17 contusion models.⁷⁻⁹ Previous studies have found significant mechanical allodynia,¹⁰⁻¹⁴ cold
18 allodynia,^{10, 14, 15} and thermal hyperalgesia^{11, 13, 16} after thoracic or cervical contusion injuries in
19 rodents. However, these assessments are unable to determine the presence, persistence
20 and/or intensity of spontaneous pain. As spontaneous pain is more prevalent among SCI
21 patients, increases over time, and is rated more severe and bothersome compared to evoked
22 pain,^{3, 17} there remains an on-going debate as to the importance of evaluating spontaneous pain
23 in preclinical models of NP. For example, Mogil and Crager reviewed studies published in the
24 journal *Pain* and found that only 10% (26 studies out of 259) included assessments of
25 spontaneous pain.¹⁸ Others have placed less emphasis on measures of spontaneous pain
26 sensation¹⁹ with regard to the clinical relevance of NP models. Evaluation of spontaneous pain

1 measures in the SCI literature is also rare and restricted to conditioned place preference or
2 avoidance tests²⁰ and over grooming.^{21, 22} As these paradigms require functional use of the
3 limbs, which is affected by SCI, and place preference and over grooming behavior can be
4 confounded by learning, memory and other affective states (e.g., anxiety).¹⁸

5 Injury-induced changes in pain sensation to evoked stimuli in SCI models are traditionally
6 assessed by determining changes in withdrawal responses when a stimulus is applied to the
7 paw or torso of the animal. An additional limitation to the classic reflexive withdrawal measures
8 as indicators of pain sensation that has received much discussion over the past decade is the
9 concept of hyper-reflexive versus hyper-sensitive states following SCI. This is due to the fact
10 that withdrawal responses can be mediated entirely by activation of spinal reflex pathways and
11 some research suggests that these measures are not valid indicators of pain sensation.^{18, 19}
12 Hyper-reflexia is demonstrated in thoracic contusion models of SCI, and is a common
13 secondary condition accompanying injury and spasms can be elicited by various stimuli.^{23, 24}
14 Indeed, after thoracic contusion injury Baastrup and colleagues found decreased thresholds for
15 withdrawal responses to von Frey and acetone (cold) stimuli, but did not observe a substantial
16 change in stimulus thresholds required to elicit spinal-brain stem reflexes such as guarding and
17 licking of the affected paw.²⁵ Similarly, S van Gorp and colleagues recently reported that
18 stimulus thresholds to elicit and escape response were lower than thresholds required to elicit a
19 withdrawal response, again suggesting divergence between withdrawal and supra-spinal
20 responses²⁶. Interestingly, van Gorp did not find a relationship between withdrawal responses
21 and spasticity. Other research indicates that there are increased brain stem and cerebral
22 responses from below level mechanical stimulus^{15, 27}. Taken together, these studies raise the
23 important point that the relationship between hind paw withdrawal responses and supra-spinal
24 responses requires further investigation. We hypothesize that expanding SCI models and
25 additional behavioral measures of supra-spinal responses should be characterized to further

1 elucidate the relationship between withdrawal responses and supra spinal responses in pre-
2 clinical models of SCI.

3 To that end, we selected the Rat Grimace Scale (RGS) to evaluate the presence of
4 spontaneous pain sensation and a supra-spinal component of evoked pain sensation in our rat
5 model of cervical (C5) hemi contusion SCI. Based off of successful use of facial coding to
6 interpret pain in young children and infants,^{28, 29} the grimace scale was first developed in mice
7 using a 0.9% acetic acid abdominal constriction pain model³⁰ and reliably quantified
8 spontaneous pain after laparotomy.³¹ The grimace scale has since been adapted to assess
9 post-procedural pain in rabbits,^{32, 33} horses,³⁴ and was translated to the rat by Sotocinal and
10 colleagues.³⁵ They demonstrated that in addition to being highly reliable in assessing
11 spontaneous pain after laparotomy and induced inflammation, RGS scores accurately
12 differentiated between rodents “in pain” and with “no pain.”³⁵ In addition to being a reliable
13 outcome measure in different species, Oliver and colleagues demonstrated that the RGS is a
14 also highly reliable with different models, raters and environments pertaining to pain.³⁶
15 Development, the RGS has been utilized to examine mechanisms underlying pain in various
16 models including laparotomy,³⁷ plantar incision,³⁸ induced inflammatory pain,³⁹ and experimental
17 tooth movement²⁰ as well as to test the efficacy of analgesics after laparotomy^{40, 41} and
18 implantation surgery.³⁶ Only one study has used the grimace scale in an SCI model; Wu and
19 colleagues used the Mouse Grimace Scale (MGS) to assess the effect of cell cycle inhibition
20 treatment on spontaneous pain after a T10 contusion.⁴² They demonstrate that the MGS can be
21 utilized to evaluate the effects of cell cycle inhibition on spontaneous supra-spinal pain. Here we
22 extend these findings to evaluate evoked and spontaneous supra spinal pain in the rat. The
23 RGS offers several advantages compared to other supra-spinal pain measurements such as it
24 evaluates above brain stem responses, requires no pre-training, is observational thus minimizes
25 affective influences in pain formation and doesn't require motor function, which is desirable after
26 SCI. Although the use of rats is widespread throughout the pre-clinical SCI literature, the

1 grimace scale has yet to be characterized in a rat model of SCI. Therefore, we hypothesized
2 that the RGS can be used to assess supra-spinal pain sensation occurring both spontaneously
3 and in response to a stimulus after cervical SCI.

4

1 **Materials and Methods**

2 *Animals*

3 Adult male Sprague-Dawley rats (275-300g) were obtained from Charles River Laboratories
4 (Hartford, CT, USA) and group-housed 2-3 per cage with access to standard rat chow and water
5 *ad libitum*. The room was maintained under a 12/12-hour light/dark cycle (6:00 am/6:00 pm) at a
6 temperature of 25°C. Animals were divided into two groups: (1) uninjured laminectomy control
7 (LAM, n=5) and (2) cervical spinal cord injury (SCI, n=10). All animals were allowed to acclimate
8 to the facility and investigators for five days after arrival, followed by one week of acclimation to
9 behavioral testing equipment. Baseline assessments were performed one week prior to injury.
10 Previous data from our lab using this model suggests that at week 5 animals exhibit NP using
11 reflexive responses to acetone and over grooming, thus for this initial study all assessments
12 were performed at week 5 after injury. All procedures were approved by the Institutional Animal
13 Care and Use Committee of the University of Alabama at Birmingham.

14 *Induction of C5-hemicontusion SCI*

15 The dominant paw of each animal was determined prior to surgery using the Paw
16 Placement test. Animals received a hemi-contusion at cervical level five (C5) on the side of the
17 spinal cord ipsilateral to the dominant paw. All injuries were done on the ipsilateral side to the
18 dominant paw to limit injury variability due to the possibility that stronger synaptic connections
19 may be present in the spinal cord on that side. Thus by maintaining injuries on the ipsilateral
20 side to the dominant paw promotes consistency of the hemi-contusion injury. The Infinite
21 Horizon SCI Impactor device (Precision Systems and Instrumentation, Lexington, KY, USA) was
22 used as previously described to induce a severe hemicontusion SCI.⁹ Briefly, each animal was
23 anesthetized with 4% isoflurane (Piramal Critical Care, Bethlehem, PA, USA) in oxygen for four
24 minutes and maintained on 2% isoflurane in oxygen for the duration of the procedure.
25 Normative body temperature was maintained at 37°C throughout surgery with the use of a rectal
26 thermometer and heating pad. The neck region was shaved, aseptically prepared for surgery,

1 and an incision was made from the C2 to T2 vertebrae. The muscles were retracted to reveal
2 the vertebral column and the underlying paravertebral muscles of C4-C6 were removed. The
3 lamina covering the C5 vertebrae was removed bilaterally and animal stabilized on the impactor
4 with clamps on the dorsal processes of C2 and T2. The impactor tip (0.8mm in diameter) was
5 aligned above the exposed dorsal aspect of the C5 spinal cord in order to cover the side
6 ipsilateral to the dominant paw. A hemi-contusion injury was delivered to all animals in the SCI
7 group by the impactor device at a force of 300 kdyn. Average tissue displacement was $1706.3 \pm$
8 $25.8 \mu\text{M}$. Animals in the laminectomy (LAM) control group received the full surgical procedure
9 through the C5 bilateral laminectomy but no hemi-contusion injury. Following impact (SCI) or
10 laminectomy, the musculature and skin was sutured in layers and animals remained in a heated
11 recovery cage until ambulant. Immediately post-surgery and twice a day (AM and PM) for five
12 days thereafter animals received subcutaneous injections of sterile saline (3 mL; Hospira Inc.,
13 Lake Forest, IL USA) and carprofen (5 mg/kg; Rimadyl, Pfizer, New York, NY, USA) as a fluid
14 replacement and analgesic. After surgery and once a day (AM) for five days thereafter the
15 injections included enrofloxacin (2.5 mg/kg; Baytril, Bayer HealthCare LLC, Shawnee Mission,
16 KS, USA) as a prophylactic antibiotic.

17 *Assessment of cold allodynia*

18 Cold allodynia was assessed by applying acetone to the hind paw, which has been shown to
19 elicit a significant increase in withdrawal responses after spinal cord contusion injuries in rats.¹⁰
20 ^{14, 15} We utilized methods previously described by Choi and colleagues⁴³ with slight adaptations.
21 Animals were placed in a clear Plexiglas box (20cm x 9cm x 10cm) on an elevated platform with
22 a wire mesh bottom to allow access to the plantar surface of the hind paws. After a five-minute
23 acclimation period, a single drop of acetone (Sigma-Aldrich, St. Louis, MO, USA) was produced
24 at the end of a 1 mL syringe. Avoiding contact of the syringe with the paw, the acetone drop was
25 applied to the plantar surface of the contralateral hind limb 1 cm posterior to the footpad of the
26 3rd and 4th phalanges. There is a short time delay as acetone evaporates before eliciting the

1 sensation of coolness;⁴³ therefore, a withdrawal response occurring within 20 seconds of
2 application was recorded along with the time of withdrawal. Acetone application was performed
3 using three trials for each animal with at least five minutes between each trial. The average time
4 in seconds to withdrawal of the three trials was used to determine withdrawal time. Importantly
5 for the comparison to the RGS, if the animal had a withdrawal response in any of the three
6 trials, the animal was placed in the “withdrawal” group for data analysis.

7 *Assessment of supra spinal pain*

8 Supra-spinal sensation of pain was assessed using the Rat Grimace Scale (RGS) methods
9 adapted from Sotocinal and colleagues.³⁵ Filming for the RGS was performed simultaneously
10 with the acetone test using a high-speed digital camera (Exilim EX-F0, Casio America Inc.,
11 Dover, NJ, USA). The nature of spontaneous pain is paroxysmal thus, to maximize the
12 possibility that the presence of pain was captured, the animal was filmed in high-definition at an
13 operating speed of 60 frames per second with auto focus and backlight correction to produce
14 images in which the animal’s face (including nose/cheek, whiskers, eyes, and ears) were clearly
15 visible. The animal’s face was filmed one minute prior to the application of acetone, and this
16 comprised the “before” acetone period and was used to assess spontaneous pain (i.e., prior to
17 the application of the stimulus). Filming continued as acetone was applied and for one minute
18 thereafter, which represented the “after” acetone period and assessment of evoked pain (Fig.
19 1). This was repeated three times during the testing session during each application of acetone.
20 The camera was held by the investigator for the entirety of filming in order to maintain focus on
21 the animal’s face as it changed position. To account for the fluctuation of spontaneous pain over
22 the course of the experiment we recorded animals for 2 minute intervals and analyzed images
23 every second to maximize the possibility that we could use the RGS to capture the presence of
24 pain. Preliminary studies in our lab (data not shown) demonstrated that the RGS was significant
25 for the determination of pain in LAM versus SCI groups by filming an animal for 20 seconds.
26 Thus we used this time interval to determine both spontaneous and evoked pain using RGS.

1 To prepare for scoring, one screen shot per second was extracted from the video recording
2 for the 20 seconds prior to and 20 seconds after the application of acetone by an investigator
3 naïve to the experimental group. The screen shots for all three tests were given a unique coding
4 number, combined into a PowerPoint slide presentation, and randomized using a PowerPoint
5 macro code. Two additional investigators naïve to the screen shot code scored the animal's
6 face on each PowerPoint slide (representing one second of the test) using the RGS. The scale
7 consists of four action units (AUs): orbital tightening, nose and cheek flattening, ear position,
8 and whisker position. Descriptions of each action unit during pain are provided in Table 1. Each
9 AU received a "0" if signs of pain were absent, "1" if there were moderate signs present, and "2"
10 if there were obvious signs of pain (Fig. 2). If the AU was not clearly visible, it was labelled as
11 "not assessable (N/A)" and excluded from further analysis. Following scoring the PowerPoint
12 slide deck was de-randomized and the scores for each AU were averaged for each slide. Slide
13 scores were then averaged across 3 raters, de-coded, and categorized into "before" acetone
14 and "after" acetone bins for analysis (Fig. 3) Inter-rater reliability analysis was performed
15 resulting in a score of 0.93. This resulted in two average RGS scores for each animal: one
16 represents the time period before and the other representing the time period after the stimulus.

17 *Assessments of Histology and Pathological Outcomes*

18 Animals were euthanized at week 5 post-SCI and perfused for histological analysis. Each
19 animal was anesthetized with 4% isoflurane (Piramal Critical Care, Bethlehem, PA, USA) for 4
20 minutes. Animals were then transcardially perfused with cold 0.1 M phosphate-buffered saline
21 (PB, pH 7.4) for 5 minutes, followed by Excell Plus (American Mastertech) for 20 minutes. The
22 epicenter was marked with tissue dye (Triangle Biomedical Sciences, Inc.), and the spinal cord
23 segments from C2 to T2 was removed. Spinal cord tissue was post-fixed in Excell Plus for 24
24 hours at 4°C, and cryoprotected with 10% sucrose/PB for 1 hour at 4°C, followed by 30%
25 sucrose for 48 hours. A 3mm section was blocked from the epicenter, and embedded into
26 Tissue Tek[®] optimum cutting temperature (OTC) medium and stored at -80°C until sectioning.

1 Serial 30µm transverse sections were sliced using a cryostat (Leica Microsystems) and
2 mounted onto 1% gelatin coated slides. To obtain neuronal counts, tissue sections were stained
3 for Nissl substance using cresyl violet acetate (Sigma Aldrich). Tissue sections were dehydrated
4 using increasing ethanol concentrations from 70% to 100% followed by clearing with xylene,
5 rehydrated in ethanol and stained with 0.1% cresyl violet acetate for 5 minutes. The tissue was
6 rinsed with double distilled water followed by 95% ethanol with acetic acid for 2 minutes,
7 dehydrated with alcohol, washed with xylene and finally cover slipped using Permount mounting
8 medium.

9 *Analysis of Lesion Volume*

10 *ADD SECTION AFTER KATIE GETS BACK*

11

12 *Statistical analyses*

13 All data were analyzed using GraphPad Prism 7 Statistical Software (San Diego, CA, USA)
14 with significance set at a p-value of $p \leq 0.05$. All data are presented as mean \pm standard error of
15 the mean (SEM). Pre- and post-injury differences in RGS scores were analyzed using a one-
16 way analysis of variance (ANOVA). Differences in RGS score between trials with and without a
17 withdraw response were analyzed using the Student's t-test.

18

19

20

1

2 **Results**

3 *RGS detects the presence of spontaneous NP sensation after SCI*

4 As not all patients that suffer from SCI exhibit spontaneous NP,² we hypothesized that only
5 a subset of animals with SCI may exhibit NP-like responses. To test this hypothesis, we used
6 the RGS to assess the presence supra-spinal indicators of spontaneous NP in the rat C5 hemi-
7 contusion model of SCI. As seen in Fig. 4A, we found a significant increase in average grimace
8 score at week 5 post injury (0.68 ± 0.10) compared to pre-surgery baseline (0.28 ± 0.06) and
9 week 5 laminectomy controls (LAM, 0.44 ± 0.06 ; $p \leq 0.05$). Based on Oliver et. al.³⁶(see
10 Discussion) we utilized this threshold score (rounded to 0.7) to categorize SCI animals into
11 “pain” and “no-pain” subsets.³⁶ As seen in figure 4B, no animals in the LAM group reached a
12 grimace score of 0.7 (dotted line) suggesting that none were exhibiting spontaneous pain. In
13 comparison, a subset of animals in the SCI group (50%) had a grimace score of 0.7 or above,
14 suggesting that these animals were experiencing spontaneous pain (Fig. 4B and 4C). We
15 concluded from these data that the RGS can also be used to distinguish between animals
16 exhibiting spontaneous pain from those with no spontaneous pain after SCI and that only
17 animals in the SCI group exhibited spontaneous NP.

18

19 *RGS detects supra-spinal pain sensation related to cold allodynia after SCI.*

20 We next examined the hypothesis that the RGS could be used to indicate supra-spinal pain
21 sensation to evoked stimuli. Specifically, we tested this by assessing RGS in conjunction with
22 cold allodynia using the application of acetone to the contralateral hind paw as previously
23 described⁴³. We first evaluated the effect of acetone application on the average grimace score
24 in animals prior to surgical manipulation (baseline). As seen in Fig. 5A, we found no significant
25 differences in average grimace score when comparing in the 20 seconds prior to the application
26 of acetone (0.25 ± 0.06 pre-acetone) with the 20 seconds after the acetone application ($0.29 \pm$

1 0.04 post-acetone) in non-surgical animals. Next, we assessed the effect of acetone application
2 on average grimace score in the uninjured laminectomy group at week 5 after surgery. Similar
3 to the baseline data, we found no significant differences in the average grimace score in the 20
4 seconds prior to the application of acetone (0.43 ± 0.07 pre-acetone) as compared to the 20
5 seconds after the acetone application (0.75 ± 0.13 post-acetone; Fig. 5B). Notably, a non-
6 significant trend toward a higher average grimace score was observed in the LAM group
7 following acetone application. This heightened response may be due to introduction of a new
8 stimulus, and although it doesn't reach significance, future studies should include a control
9 substance such as water in a sub-group of animals. In contrast, when the average grimace
10 score prior to and following the application of acetone was compared in the animals that
11 received SCI at week 5 after injury, we observed a significant increase in RGS score following
12 the application of acetone (0.68 ± 0.10 pre-acetone, 0.88 ± 0.07 post-acetone; $p \leq 0.05$; Fig.
13 5C). As we did for the spontaneous pain, we also evaluated the effect of group on the
14 percentage of animals with average grimace scores below versus at/above the 0.7 threshold as
15 a separator into "pain" and "no pain" groups related to evoked pain. (Fig. 5D). We found that
16 90% of the animals in the SCI group exhibited an average grimace score of 0.7 or higher
17 following the acetone stimulus (Fig. 5E). Also we observed that the animals that exhibited
18 spontaneous supra-spinal pain prior to acetone did not have a further increase in magnitude of
19 grimace score after acetone application, suggesting that the spontaneous and evoked pain
20 responses were not additive. Interestingly, while 90% of SCI animals responded to acetone,
21 only 50% of SCI animals had an RGS score above 0.7 suggesting that a most animals had
22 hyper-reflexia while only a subset exhibited spontaneous pain. Taken together, these data
23 indicate that the grimace score can be used as an indicator of supra-spinal sensation of evoked
24 allodynia and that animals in the SCI group exhibited evoked NP to a cold stimulus.

25

26 *The RGS parallels the reflexive withdrawal response in animals with SCI*

1 An ongoing debate in the study of pain in rodent models is the reliability of reflexive paw
2 withdrawal response to an evoked stimulus as an indicator of pain.^{18, 19} As we found that the
3 RGS can be used to indicate spontaneous pain, we also found that the RGS increases following
4 an evoked stimulus. An important aspect of the relationship between spontaneous pain and the
5 reflexive withdrawal response is that although mechanistically different⁴⁴, supra-spinal pain
6 indicators and reflex responses are not always mutually exclusive on a behavior level,
7 depending on injury magnitude and SCI model. Based on the literature in SCI contusion models
8 we hypothesized that, in the C5 hemi-contusion model the RGS would not be parallel to the
9 reflexive withdrawal response. To test this hypothesis, we examined the relationship between
10 grimace score and withdrawal in response to acetone in the SCI group (i.e. evoked responses).
11 First we analyzed all the acetone trials of every animal in the SCI group. In contrast to our
12 hypothesis, we found that the average grimace score after acetone was significantly higher in
13 the trials in which there was a paw withdrawal response as compared to trials without a
14 withdrawal response (0.69 ± 0.10 without withdrawal; 1.12 ± 0.08 with withdrawal, $p \leq 0.05$; Fig.
15 6A). Next, we evaluated the individual animals' average grimace score in instances with and
16 without a paw withdrawal to acetone (Fig. 6B). We found that the one animal that did not exhibit
17 paw withdrawal had a corresponding average grimace score below 0.7. Contrastingly, all nine
18 animals that exhibited paw withdrawals exhibited an average grimace scores greater than 0.7
19 (Fig. 6B).

20 We next considered only the animals in the SCI group that exhibited spontaneous NP (at or
21 above threshold of 0.7). In animals with spontaneous pain, we observed no statistical
22 differences between the average grimace score prior to or after the acetone stimuli (Fig. 7A),
23 which suggests that the pain sensation from the evoked stimulus is not additive to the
24 spontaneous pain. The subset of animals with spontaneous NP exhibited a paw withdrawal
25 response to acetone in 100% of the trials (Fig. 7B). Lastly, we evaluated the subset of animals

1 that did not exhibit spontaneous NP (below threshold of 0.7) to determine the relationship
2 between average grimace score and paw withdrawal. We found that in animals not exhibiting
3 spontaneous NP, 80% of the animals in which a paw withdrawal occurred corresponded to an
4 average grimace score at or above the 0.7 threshold (Fig. 7C). Taken together, these data
5 suggest that there is a strong correspondence between paw withdrawal and the average
6 grimace score with higher scores associated with withdrawal.

7 *No changes in percent lesion area or dorsal horn neuron counts between pain and no-pain*
8 *animals*

9 In order to determine if pathological features were different in animals that had pain, we
10 assessed lesion volume and neuronal cell counts in the dorsal horn of animals with SCI. The
11 average force across SCI groups was (FORCE) and the average displacement of the spinal
12 cord after impact was (IMPACT). We found no significant difference between change in percent
13 lesion area between “pain” and “no-pain” groups. (12.59 ± 4.95 and 8.95 ± 1.30 respectively). In
14 addition, we found no significant differences in dorsal horn neuron counts between “pain” and
15 “no pain groups”. (60.76 ± 37.12 and 898 ± 807 respectively).

1 Discussion

2 Here we found that the RGS can be used to determine the presence of spontaneous NP
3 sensation in a rat cervical hemi-contusion model of SCI. In addition, we found that the RGS can
4 also detect evoked pain from cold stimuli. The RGS was unable to detect a heightened pain
5 response from an evoked stimulus in the cases when the animal exhibited spontaneous pain
6 prior to the stimulus, which suggests that the super-spinal sensation of spontaneous and
7 evoked is not additive with regard to intensity. We also found a strong correspondence between
8 a high grimace score and a withdrawal response to a cold stimulus suggesting that the
9 presence of both high grimace score and withdrawal is a likely a robust indicator of pain.

10 *Spontaneous Supra-Spinal Pain after SCI*

11 SCI results motor and sensory dysfunction, which is accompanied by chronic, debilitating
12 NP in up to 65% of patients.² NP in persons with SCI manifests as both spontaneous and
13 evoked pain. Persons with SCI indicate that spontaneous pain is more severe and more
14 disruptive of everyday life than evoked pain;^{3, 17} yet, the experimental emphasis in pre-clinical
15 models often does not include evaluation of spontaneous pain.¹⁸ This is in part due to the
16 difficulty of evaluating spontaneous pain in animal models. The RGS has been developed to
17 measure spontaneous supra-spinal pain sensation³⁵ in animal models of NP, particularly those
18 based on peripheral nerve injury. This technique is based on facial coding in non-verbal or infant
19 humans^{28, 29} and has been applied in rodents,^{30, 35} rabbits,^{32, 33} and horses.³⁴ Here we
20 determined that rats with a C5-hemicontusion exhibit spontaneous pain that can be measured
21 with the RGS. In addition at week 5 (35 days) post-SCI we can use the RGS to distinguish
22 “pain” versus “no pain” in SCI rats. Previously, the RGS has been utilized to examine
23 mechanisms underlying pain in various models including laparotomy,³⁷ plantar incision,³⁸
24 induced inflammatory pain,³⁹ and experimental tooth movement²⁰ as well as to test the efficacy
25 of analgesics after laparotomy^{40, 41} and implantation surgery.³⁶ Here we used the RGS to
26 determine animals that exhibit pain versus those that do not after C5 SCI using an RGS score

1 cutoff of 0.7, where below 0.7 is “no-pain” and 0.7 and above was “pain”. We chose this score
2 based on a thorough evaluation of the sensitivity and reliability of the RGS done by Oliver et. al.
3 ³⁶ That study demonstrated that the RGS is highly reliable with different models, raters and
4 environments pertaining to pain.³⁶ The reliability in that study was demonstrated by internal
5 consistency measures of inter- and intra-rater reliability of 0.85 and 0.83 respectfully. In our
6 study in intra-rater reliability was 0.93. To determine the RGS’ utility as a tool for the
7 determination of pain, that study analyzed the sensitivity and specificity of the RGS in
8 heterogeneous settings using pain initiated from telemetry unit implantation surgery. The
9 analgesic intervention score was found to be 0.67. As a way to validate their findings, Oliver et.
10 al. applied this score to data published by Sotocinal et. al.³⁵ in which it correctly identified time
11 points that had peaks in the RGS after Freund’s adjuvant, intraarticular kaolin/carrageenan and
12 laparotomy .^{35, 36} In addition, 0.67 correctly identified the morphine treated animals versus the
13 controls in Freund’s adjuvant treated rats. The data from Oliver et. al. suggests that the RGS is
14 a useful tool to determine the effects of a analgesics using a RGS of 0.68 as a threshold for pain
15 vs. no pain. Based on these findings, for our seminal study we used a threshold RGS of 0.7.
16 (See Methods) to identify rats that have pain and distinguish them from those who don’t. It is
17 possible that the sensitivity of the RGS may vary depending on the magnitude or intensity of
18 pain for example in the Oliver et. al. study the pain was derived from implantation surgery
19 whereas in our model it was NP from SCI. We were however, able to identify and differentiate
20 animal with spontaneous pain, future studies are needed to determine the optimal analgesic
21 intervention score for this model of SCI.

22

23 | *Evoked Supra-Spinal Pain after SCI*

24 We found that acetone application results in a grimace score of ≥ 0.7 in 90% animals with
25 SCI, which indicates that this contusion SCI model induces NP to a cold stimulus. The
26 experimental endpoint for evoked pain is the reflexive withdrawal response, however since

1 reflexive withdrawal is largely mediated at the level of the spinal cord, this behavior following
2 SCI is more likely to indicate hyper-reflexia than pain due to the lack of supra-spinal
3 processing.^{18, 19} For example, van Gorp and colleagues found that thresholds for below-level
4 hypersensitivity increased after SCI and withdrawal responses decreased over time (by 5 weeks
5 post-SCI) and concluded that withdrawal responses are mostly a measure of hyperreflexia after
6 SCI.²⁶ In contrast, we determined that in the instances when a withdrawal response was
7 present, the RGS was always above 0.7. Also we found that all animals that had a
8 spontaneous RGS above 0.7 also had a withdrawal response to the evoked acetone stimulus.
9 Thus, our data suggest that supra-spinal sensation of NP in our C5 hemi-contusion SCI model
10 is highly coincident with a paw withdrawal response. We also found that the RGS did not
11 increase further upon evoked stimulus if they already exhibited spontaneous pain. Possible
12 interpretations to these data are that the evoked stimulus has a component of pain that is lower
13 than the spontaneous pain present or possibly that the evoked pain signal is a non-contributing
14 factor to the RGS. Although our data from increased RGS after acetone suggests the former,
15 we cannot rule out the possibility that during spontaneous pain generation, the evoked signal is
16 inhibited or is a minimal contributor.

17

18 *Evoked Supra-Spinal Pain and Hyper-reflexia*

19 One novel contribution of this study is the evaluation of supra-spinal pain and its
20 relationship to evoked pain and reflexive withdrawal response in a cervical hemi-contusion SCI
21 model. Two decades of research has focused on evaluating reflexive responses as an indicator
22 of pain, however as Baastrap, S von Gorp, Vierck and others report, reflexive responses are
23 insufficient to measure the presence of pain from a nociceptive stimulus. Furthermore reflexive
24 responses cannot evaluate the presence of spontaneous pain. Likewise, in the field of SCI, it
25 understood that injury induces hyper-reflexia as evidenced by spinally mediated reflexive
26 responses that remain present in completely transected spinal cord leading to spastic

1 syndrome^{19, 45}. This hyper-reflexive state precludes the use of evoked reflexive responses to
2 measure pain after SCI, however whether there is a painful component of the withdrawal
3 response may depend numerous factors, including injury severity, affective contributions and
4 experimental design.

5 Recently some studies show significant *increases* in withdrawal responses after SCI,
6 indicating loss of sensation (i.e., hypoesthesia), which was further evidenced by decreased
7 somatosensory cortex activation in response to stimuli on functional magnetic resonance
8 imaging (fMRI).^{26, 46} In response to these findings, investigators began assessing supra-spinal
9 indicators of pain in addition to withdrawal responses.^{27, 47-49} These include outcomes such as a)
10 vocalization, b) biting, licking, or guarding of the affected paw, and c) escape behaviors.
11 Concern has been raised that while these responses are indeed mediated by above-level
12 signaling, the main components of these behaviors are facilitated by the brainstem such that
13 cortical processing is not required as evidenced by the presence of these outcomes in de-
14 cerebrate rodents in response to mechanical and thermal stimuli.^{25, 26, 50-52} Furthermore
15 Baastrup found no significant change with below level stimuli on brain stem and cerebral
16 responses using the escape/avoidance paradigm. They did however find that, not all, but a large
17 subset of animals exhibited a threshold decrease in both brainstem reflexes and cerebral
18 responses after SCI with at-level stimulus both mechanical and cold thermal. Importantly, other
19 studies of thoracic contusion SCI have found increased spinal and brain stem responses from
20 below level mechanical^{15, 27} and thermal (cold) stimulus. S von Gorp²⁶ compared the reflexive
21 response to the supra-spinal pain measure of escape attempts in order to measure below-level
22 sensory thresholds in a rat thoracic contusion model of SCI. That study revealed that thresholds
23 for eliciting an escape response were lower than those required to elicit a withdrawal response
24 from a below-level stimulus leading to the conclusion that below level-thresholds do not reflect
25 pain hypersensitivity. An important consideration from these studies is the possibility that
26 different types and magnitudes of SCI may yield different conclusions when evaluating reflexive

1 and supra-spinal responses. In our study of the C5 hemi-contusion SCI, we found increases in
2 spinal reflexive responses paralleled increases in the RGS, which suggests that there is
3 involvement of painful sensation in the withdrawal response in this model of SCI. This finding is
4 at odds with S von Gorp et. al. and Baastrap and reasons for this are likely owing to injury
5 model, C5 hemi-contusion vs. thoracic contusion as well as application of different supra-spinal
6 pain outcome measures. Our finding that in C5 cervical injury, reflexive withdrawal is coincident
7 with supra-spinal pain outcomes is important because it has been noted that below-level pain
8 after SCI in humans is highly likely to have a cerebral component^{25, 53}, thus it is critical to
9 compare supra-spinal measures to reflexive withdrawal responses in pre-clinical models. In
10 fact, in a recent review by Vierck and Yeziarski it was noted that in order to determine if pain is
11 involved in the spinal reflexive response from a given stimulus, it is necessary to compare those
12 responses to supra-spinal pain measures. Here we have concluded that in the C5 hemi-
13 contusion model of SCI, the spinal reflexive response to cold stimuli is present in 100% of the
14 animals when they have a painful grimace score. Although we do not know the mechanism, this
15 study highlights the difference in pain evaluation outcomes across different SCI models.
16 Advantages to the C5 hemi-contusion model of SCI include cervical injury that is more common
17 in human SCI and the current findings that pain may have a different characteristic than thoracic
18 contusion models that are thought to not be effective for studies of below level pain.^{25,}

19 ⁵³*Evaluation of supra-spinal pain using the RGS*

20 Several methods are utilized to evaluate supra-spinal pain after SCI.^{25, 26, 42, 44} Included in
21 these are operant escape tasks⁵⁴, place escape/avoidance²⁵, and the escape response test.²⁶
22 These assessments have made it possible to identify and characterize pain however an
23 important consideration mentioned above is the heterogeneity in SCI injury models and
24 experimental systems. Supra-spinal pain measures are well designed to limit bias from the
25 experimenter as well as be performed in an experimental setting where the influence of the
26 experimenter is limited on the behavior of the animal. Some of the tasks such as operant

1 escape, place escape avoidance require pre-training of the animal and involvement of motor
2 function that is often compromised in SCI animals. Others such as the escape response task
3 requires the experimenter to handle the animal potentially resulting in increase in affective and
4 stress induced exacerbation of painful states.^{55, 56} These characteristics suggest that supra-
5 spinal pain evaluations should be carefully selected and the most appropriate method
6 depending on SCI model, experimental design and research question. Wu. et. al. evaluated the
7 effect of cell cycle inhibitors on supra spinal pain using the grimace scale in mice (MGS). That
8 study highlights the ability of the MGS to evaluate pain by manipulating biological variables.
9 Although Wu et. al. measured evoked pain with mechanical and thermal stimuli the study did not
10 directly compare the results to the MGS. However there were effects of cell cycle inhibition that
11 increased withdrawal thresholds to Von Frey, heat and MGS suggesting that there is a painful
12 component of the evoked response in this model. Interestingly in our model of cold thermal
13 stimulus we also describe overlap in RGS and evoked withdrawal responses. To our knowledge
14 our study and the Wu et. al. study are the only two studies that have used the grimace scale in
15 rodents to determine supra-spinal pain outcomes after SCI and our study is the only one to
16 compare evoked withdrawal responses to RGS. Importantly from a SCI standpoint, our study
17 also varies from Wu et. al. in that there are anatomical and genetic differences between mouse
18 and rat spinal cord as well as a difference in rodent species in response to injury,⁵⁷ thus it is
19 essential to develop the RGS as a tool to evaluate pain after SCI in the rat.

20 Although our study supports the current thinking that reflexive withdrawal measures should
21 be compared to supra-spinal pain measures and caution should be used in interpretation of pain
22 data, we recognize a number of caveats. The first is that we did not measure pain over a long
23 period of time. Our previous data indicate that the animals demonstrate pain behaviors
24 (overgrooming and paw biting) by 5 weeks post-SCI however we do not know when this pain
25 started and how long it persists. In fact one reason to undertake the current study was to
26 characterize more valid measures of pain in this model of SCI. This limitation is especially

1 important when considering the use of the RGS in evaluating therapeutic strategies and further
2 experiments are required in this model to determine the therapeutic window for pain
3 development to optimize analgesic effects of pharmacotherapy. Highlighting the potential
4 temporal relationship between the supra spinal pain measured by the RGS and hyper-reflexia, a
5 recent publication⁵⁸ found that paw hypersensitivity (as determined by withdrawal response)
6 coincided with a higher RGS in models of nociceptive pain from inflammation and surgical
7 incision, that hypersensitivity persisted while the grimace score decreased over time. If there is
8 a time dependent separation between the withdrawal response and the RGS in our SCI model,
9 it is likely at a time point later than the 5 weeks after SCI examined in the current work. Another
10 possibility is that the temporal profile of supra-spinal pain sensation is likely to vary between
11 injury models as has been reported for other behavioral indexes of pain.⁵⁹ It is important to note
12 that during thoracic contusion models of SCI it is thought that animals should be followed for a
13 period of 49 days or longer in order to ascertain the presence of pain^{25, 53}. Future studies
14 following this study will temporally evaluate pain using the RGS as we have in week 5 post-SCI.
15 Another caveat of this study is we did not perform threshold testing. Again our preliminary data
16 indicated that this model is most sensitive to acetone using reflexive withdrawal response. Due
17 to recent findings in the pain field that reflex testing should be re-evaluated and cannot be used
18 as a standard of pain measurement, we evaluated our model of C5 hemi-contusion SCI using a
19 supra-spinal pain measurement and compared it to reflex withdrawal using acetone. As such it
20 was not possible to conduct threshold testing using acetone however we recognize the need to
21 follow our current findings using experimental paradigms that evaluate increasing levels of cold
22 stimulus to determine at what point pain becomes apparent using the RGS. The RGS has yet to
23 be demonstrated as a stand-alone measure of SCI induced supra-spinal pain, thus it should be
24 compared to other measures of supra-spinal pain measures to validate the relevancy of the
25 RGS and place it in the context of current research future studies should incorporate this
26 comparison. Future studies will also be extended threshold testing to mechanical (Von Frey)

1 and thermal (heat) methods of stimulation to better situate our findings in the context of the
2 greater literature.

3 | In addition not all animals from this model develop pain according to our evaluation;
4 | however this phenomenon can be seen in other models of spinal cord contusion such as the
5 | thoracic spinal stenosis model in which 59% (13/22) develop hyperalgesia following SCI^{44, 54},
6 | and Detloff et al, who reported that 40% of rats injured by hemi-contusion at the C5 level
7 | exhibited allodynia to evoked stimuli.⁴⁷ Consistent with this, we found the percent of animals
8 | experiencing spontaneous pain sensations from this study is 50%. These studies are similar to
9 | clinical data that indicate that the prevalence of neuropathic pain in 60-70% of the human SCI
10 | population.^{2, 60} Although it is advantageous for all animals in an injury model do develop pain, it
11 | can also be beneficial for some do develop pain and some that don't, especially when a method
12 | of evaluation exists to separate the two, such as the RGS as demonstrated in this study. This
13 | opens the door for further studies of comparison between "pain" and "no-pain animals" for
14 | determination of mechanisms of pain formation. [We also performed a standard assessment of](#)
15 | [lesion area and dorsal and ventral horn neuronal counts to determine if there are differences in](#)
16 | [injury parameters after SCI that may contribute to pain in SCI rats. While our assesments found](#)
17 | [.....WAITING FOR NEW DATA.....](#)Another important consideration is that affective
18 | changes such as fear and anxiety could contribute to the behavioral changes observed in the
19 | RGS. Our experimental design sought to minimize these possibilities through rigorous
20 | acclimation prior to each set of experiments (see methods). We do not think animals were
21 | fearful during experiments however this parameter was not formally evaluated. Other possible
22 | contributing factors to the RGS may also be numbness, paresthesia or dysesthesias however
23 | our experiments did not account for these possible effects. Future experiments are required to
24 | understand if there is a specific effect of these on behavior and the RGS after SCI.

25 | An interesting, yet unexpected result from our study is that 2 out of 5 of the animal in the
26 | uninjured laminectomy only group exhibited a grimace score of 0.7 or greater post acetone

1 suggesting that some animals in the laminectomy group are experiencing evoked neuropathic
2 pain in response to acetone. Although the laminectomy group is typically used in preclinical SCI
3 studies as a procedural control, this is an invasive procedure. It is recognized that surgery can
4 induce chronic neuropathic pain³⁷ and in the clinical arena, complications and postoperative
5 pain following laminectomy are a frequent concern^{61,62}. Indeed, laminectomy is often used in
6 rats to model failed back syndrome⁶³, a condition associated with NP. Overall the laminectomy
7 control is has not been a perfect control for SCI experiments especially when evaluating pain
8 using the reflexive response. The data obtained in this study supports the idea that when
9 evaluating supra spinal pain, caution should be used when a laminectomy is a control group.
10 Although we can only speculate as to the cause of the increase RGS in the 2 animals that
11 received only laminectomy, a number of reasons are thought to be attributable to laminectomy-
12 associated pathobiology such as low level inflammation, ischemia, or epidural fibrosis.
13 Intriguingly, our data suggest that the RGS could be used in future preclinical studies of post-
14 laminectomy pain or failed back syndrome.

15 Neuropathic pain is a serious consequence of SCI with a limited currently available
16 treatment options⁶⁴. Improved modeling of SCI-induced NP is likely to improve understanding of
17 the pathobiology and therapeutic targets. Importantly, as most patients with SCI have multiple
18 concomitant pain types, including both spontaneous and evoked pain, a preclinical model which
19 measure multiple pain types are valuable. The results from the current study are consistent with
20 the prevailing theory that reflexive withdrawal responses may be measuring hyperreflexia more
21 than actual pain, however we found that the grimace score increases in response to cold
22 allodynia suggesting there is a supra-spinal component evoked by acetone that is concurrent
23 with a withdrawal response. Moreover, we report that the withdrawal response was parallel with
24 a pain grimace score, and suggest that our model may vary from other spinal contusion models.
25 Future studies will use RGS in conjunction with already successful and established supra-spinal

- 1 pain methods in this C5 hemi-contusion model of SCI to evaluate the pathobiology of SCI-
- 2 induced NP as well as the effects of analgesics and therapeutic strategies.
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3 **Disclosure:** No competing financial interests exist.

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