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TITLE: Nociceptive (Pain) Input After Spinal Cord Injury (SCI) Enhances Secondary Injury:
Identifying Treatments that Can Be Translated to Clinical Practice

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14. ABSTRACT Using an animal model, we have shown that nociceptive (pain) input after spinal cord injury (SCI) amplifies secondary injury and undermines long-term recovery. These observations are important because SCI is often accompanied by additional tissue damage (polytrauma) that provides a source of nociceptive input. The consequences of pain (nociceptive) input on recovery after a lower thoracic contusion injury have been studied using an animal (rat) model. Prior work has shown that engaging nociceptive fibers 24 hr after injury increases tissue loss, undermines long-term recovery, and fosters the development of pain and spasticity. Recent studies have related these effects to pain induced hemorrhage at the site of injury and shown that this can be blocked by means of a local (lidocaine) or general (pentobarbital) anesthesia. Experiments will determine when nociceptive input affects tissue loss, the time period over which hemorrhage occurs, whether these effects are observed in both male and female animals, and whether chemically engaging pain (C) fibers with the irritant capsaicin has the same effect. Having demonstrated that capsaicin induces hemorrhage and impairs long-term recovery, we are assessing whether this effect can be prevented by means of a pharmacological transection or general anesthesia. To further evaluate the clinical relevance of these findings, we will test whether they are effective when initiated soon after injury or the initiation of painful stimulation.					
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1. INTRODUCTION

Using an animal model, we have shown that nociceptive (pain) input after spinal cord injury (SCI) amplifies secondary injury and undermines long-term recovery. These observations are important because SCI is often accompanied by additional tissue damage (polytrauma) that provides a source of nociceptive input. The consequences of pain (nociceptive) input on recovery after a lower thoracic contusion injury have been studied using an animal (rat) model. Prior work has shown that engaging nociceptive fibers 24 hr after injury increases tissue loss, undermines long-term recovery, and fosters the development of pain and spasticity. Recent studies suggest that nociceptive stimulation increases tissue loss because it induces a breakdown in the blood spinal cord barrier (BSCB) that allows blood to infiltrate spinal cord tissue. Because red blood cells are neurotoxic, this hemorrhage augments cell death (secondary injury). Importantly, we have shown that pretreatment with a local anesthetic (lidocaine), applied at the site of injury, blocks nociception-induced hemorrhage and the impairment in long-term recovery. Further work revealed that cutting communication with the brain, by means of a rostral transection, also blocks pain-induced hemorrhage. This suggests that the adverse effects of nociceptive input depend, in part, on surviving fibers and the brain—that brain-dependent processes can fuel tissue loss at the site of injury. Supporting this, preliminary data were presented to show that a pharmacological transection (induced by slowly infusing lidocaine rostral to injury) also has a protective effect. So too does placing the animals in a state analogous to a medically-induced coma, by administering the general anesthetic pentobarbital. The work outlined within the present proposal is designed to verify these observations and evaluate their generality. Experiments will determine when nociceptive input affects tissue loss, the time period over which hemorrhage occurs, whether these effects are observed in both male and female animals, and whether chemically engaging pain (C) fibers with the irritant capsaicin has the same effect. Additional studies are examining whether anesthesia given after painful stimulation or surgery attenuates hemorrhage. Parallel experiments are testing whether blocking communication with the brain (via rostral lidocaine) blocks pain-induced hemorrhage in awake animals.

2. KEYWORDS: spinal cord injury, contusion, recovery, polytrauma, pain, anesthesia, lidocaine, pentobarbital, anesthesia, coma

3. ACCOMPLISHMENTS

Major Goals

Task	Months	Status
IACUC/ACURO approval	2	Aug. 2018
Aim 1		
Major1: Detail the emergence of hemorrhage over time	3-6	Jan 2020
Major2: Detail when nociceptive stimulation affects tissue loss	7-10	Jan 2020
Major3: Does the effect of tonic pain depend on brain systems	11-13	
Sub1: Is hemorrhage blocked by spinal transection		July 2020
Sub2: Does afferent input initiate the breakdown of the BSCB		50%
Major4: Are similar effects observed in male and female animals	14-16	July 2019
Aim 2		
Major1: Is pentobarbital effective if given after stimulation?	17-19	Sept. 2019
Major2: Does anesthesia block the effect of capsaicin treatment?	20-22	Mar. 2020
Major3: Does anesthesia after surgery attenuate damage?	23-26	40%
Aim 3		
Major1: Does epidural lidocaine block the effect of capsaicin?	27-29	
Major2: Does rostral lidocaine block hemorrhage?	30-33	May 2020
Major3: Does epidural lidocaine after surgery reduce tissue loss?	34-36	

Summary of What has been Accomplished (Years 1 and 2)

Subtask 1: IACUC/ACURO approval

We had indicated that this would be accomplished within the first two months of the project. We completed the task in August of 2018, before the project was funded.

Aim 1: When does nociceptive input have an adverse effect?

Major Task 1: Emergence of hemorrhage over time

Prior work has shown that application of a noxious stimulus (intermittent electrical stimulation or the irritant capsaicin) a day after rats have received a contusion injury induces hemorrhage. Preliminary data, available at the time of grant submission, indicated that treatment with the irritant capsaicin produced hemorrhage when tissue was collected 3 hrs after treatment. The present experiment was designed to assess whether the capsaicin-induced hemorrhage is accompanied by an increase in blood pressure and the duration of these effects. Here, and in subsequent experiments, rats received a moderate contusion injury at T10. Hemorrhage was assessed using standard methods, which include the assessment of absorbance at the wavelength associated with hemoglobin (420 nm) and the Drabkin assay. In some cases, the data are reinforced with Western blotting.

Elucidation of a time-course requires a positive control, in this case a demonstration of the effect at 3 hrs after treatment. We coupled this to the assessment of hemorrhage at 24 hrs. Whether subsequent time points were needed was to be determined by the data from the 24 hr condition. Having found a positive effect, we assessed hemorrhage at a later time point, keeping the interval between conditions equivalent (with an 8 fold change [3, 8, 192 hrs]).

Below we present the data collected at 3 and 24 hrs after treatment in detail. When tissue collection was delayed for 8 days (192 hrs), no differences were observed. For this reason, just a brief description of those data are provided.

3 Hours After Capsaicin Treatment (details):

Locomotor scores were significantly lower after capsaicin injection. Locomotor scores did not differ between groups prior to treatment, $F(1, 14) = 0.071$, $p = 0.794$. Locomotor scores were obtained immediately, one, two, and three hours after injection. To examine whether capsaicin affected locomotor recovery over time, a repeated measures ANCOVA was used with injection as the between subjects variable, SCI locomotor scores as the covariate, and time as the repeated measure. Overall analysis revealed a main effect of injection, $F(1, 13) = 9.428$, $p = 0.009$ (Figure 3A). No other effects were statistically significant, all $F_s < 1.973$, $p > 0.134$. *Post hoc* analysis showed a reduction in locomotor scores in subjects receiving a capsaicin injection compared to vehicle controls.

Capsaicin injection had no effect on hypertension after SCI. Blood pressure measurements did not differ between groups prior to treatment, all $F_s < 1.020$, $p > 0.394$. Blood pressure measurements were obtained immediately, one, two, and three hours after injection to examine the effect of capsaicin on hypertension. A repeated measures ANCOVA with injection as the between subjects variable, SCI systolic blood pressure as a covariate, and time as a repeated measure found no significant effects, all $F_s < 0.617$, $p > 0.608$ (Figure 3B). Analysis of diastolic blood pressure and MAP showed similar null effects, all $F_s < 1.020$, $p > 0.394$ (data not shown).

Capsaicin treatment increased indices of hemorrhage at the SCI site within three hours after capsaicin injection. An ANOVA examining the magnitude of the peak in absorbance at 420 nm with injection as the between

subject variable found a significant main effect of injection, $F(1, 14) = 13.186$, $p = 0.003$ (Figure 3E). Subsequent analysis found that subjects treated with capsaicin showed an increase in hemorrhage. This effect was confirmed through Drabkin's analysis, $F(1, 14) = 12.651$, $p = 0.0032$ (Figure 3F). Additionally, the level of hemoglobin within the spinal cord tissue was analyzed using quantitative Western blotting. An ANOVA with injection as the between subjects variable found a significant difference between groups, $F(1, 14) = 5.070$, $p = 0.041$ (Figure 3G).

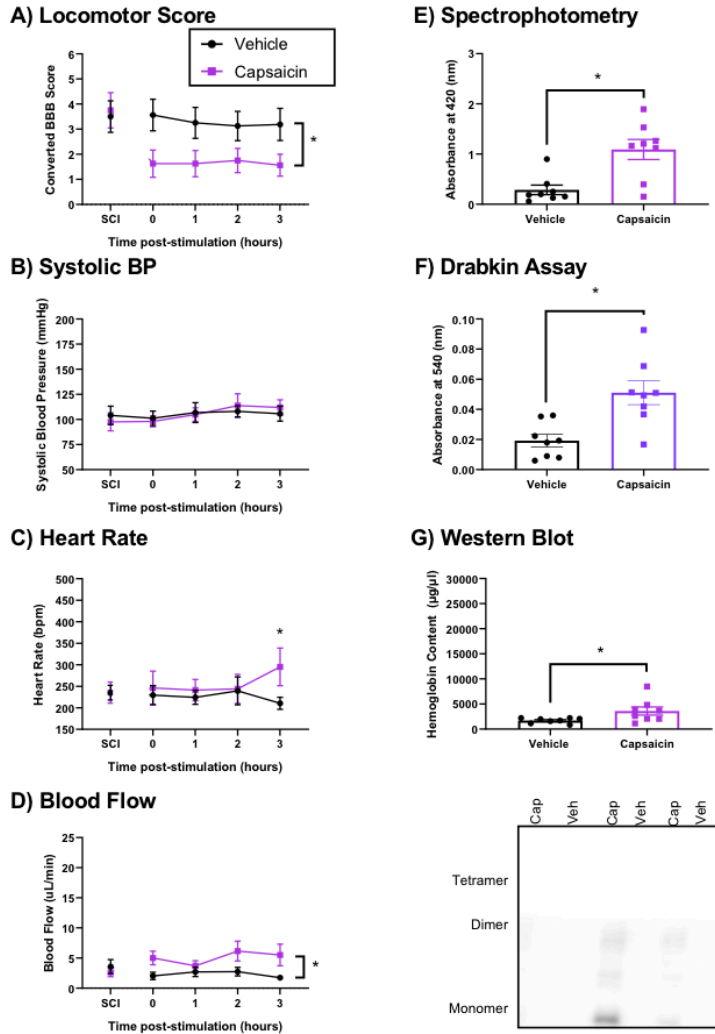


Figure 3. Locomotor performance over the three hours after capsaicin injection. BBB locomotor scores taken during the three hours post stimulation period decreased in subjects receiving capsaicin (A). Capsaicin treatment did not increase systolic blood pressure (B), but increased heart rate and blood flow (C and D). The absorbance at 420 nm taken at three hours was increased after capsaicin treatment (E). The amount of hemoglobin-α did not differ between groups (F). Representative western blot showing the three main bands associated with hemoglobin (G). Error bars represent SEM ($n = 8$).

24 Hours After Capsaicin Treatment (details):

Locomotor performance was reduced in capsaicin treated subjects. Locomotor scores did not differ between groups prior to treatment, $F(1, 14) = 0.069$, $p = 0.796$. Locomotor scores were obtained three, six, 12, and 24 hours after injection to examine whether a capsaicin injection would affect locomotor recovery. A repeated measures ANCOVA was used with injection as the between subjects variable, SCI locomotor score as the covariate, and time as the repeated measure. Overall analysis revealed a main effect of injection, $F(1, 13) = 12.839$, $p = 0.003$. No other effects were statistically significant, all $F_s < 0.327$, $p > 0.602$. *Post hoc* analysis revealed a significant decrease in locomotor performance in animals injected with capsaicin (Figure 4A).

A capsaicin injection had no effect on hypertension in the first 24 hours after injection. Blood pressure measurements did not differ between groups prior to treatment, all $F_s < 3.254$, $p > 0.093$. Blood pressure measurements were obtained at three, six, 12, and 24 hours after injection to examine the effect of injection on hypertension. A repeated measures ANCOVA with injection as the between subjects variable, SCI systolic

blood pressure as the covariate, and time as a repeated measure found no significant changes in systolic blood pressure, $F < 4.259$, $p > 0.060$. Although the main effect of injection was not significant, there is a notable increase in blood pressure for subjects given a capsaicin injection compared to vehicle controls, $F(1, 13) = 4.259$, $p = 0.060$ (Figure 4B). Neither diastolic blood pressure nor MAP showed any significant effects, $F < 2.914$, $p > 0.112$.

A capsaicin had no effect on heart rate in the first 24 hours after injection. Heart rate did not differ between groups prior to treatment, $F(1, 14) = 2.919$, $p = 0.110$. Heart rates were obtained at three, six, 12, and 24 hours after injection. A repeated measures ANCOVA with injection as the between subjects variable, SCI heart rate as the covariate, and time as a repeated measure, found no significant changes, all $F_s < 1.200$, $p > 0.323$ (Figure 4C). Preplanned analysis of the three-hour time point using an ANCOVA revealed no significant increase in heart rate, $F(1, 14) = 1.121$, $p = 0.309$.

A capsaicin had no effect on blood flow in the first 24 hours after injection. Blood flow did not differ between groups prior to treatment, $F(1, 14) = 1.121$, $p = 0.308$. Blood flow measurements were obtained at three, six, 12, and 24 hours after injection. A repeated measures ANCOVA with injection as the between subjects variable, SCI blood flow as a covariate, and time as a repeated measure found no significant changes, all $F_s < 2.698$, $p > 0.124$ (Figure 4D). Preplanned analysis of the three-hour time point using an ANCOVA revealed no significant increase in blood flow, $F(1, 14) = 3.353$, $p = 0.09$.

Capsaicin increased the amount of hemorrhage in the spinal cord 24 hours after injection. The magnitude of the peak in absorbance at 420 nm was analyzed using an ANOVA with injection as the between subjects variable. A significant main effect of injection was observed, $F(1, 14) = 11.515$, $p = 0.004$. Subsequent analysis found that subjects treated with capsaicin showed an increase absorbance at 420 nm (Figure 4E). Drabkin's reagent was used to confirm the increased concentration of hemoglobin in animals treated with capsaicin, $F(1, 14) = 87.341$, $p = 0.0001$ (Figure 4F). A quantitative Western blotting also found significantly different levels of hemoglobin- α within the spinal cord tissue, $F(1, 14) = 4.809$, $p = 0.046$ (Figure 4G).

We found that capsaicin treated animals exhibited greater absorbance at 420 nm, which is indicative of hemorrhage. An ANOVA yielded a significant main effect of injection was observed, $F(1, 14) = 11.515$, $p = 0.004$. Subsequent analysis found that subjects treated with capsaicin showed an increase absorbance at 420 nm (Figure 4E). Drabkin's reagent was used to confirm the increased concentration of hemoglobin in animals treated with capsaicin, $F(1, 14) = 87.341$, $p = 0.0001$ (Figure 4F). A quantitative Western blotting also found significantly different levels of hemoglobin- α within the spinal cord tissue, $F(1, 14) = 4.809$, $p = 0.046$.

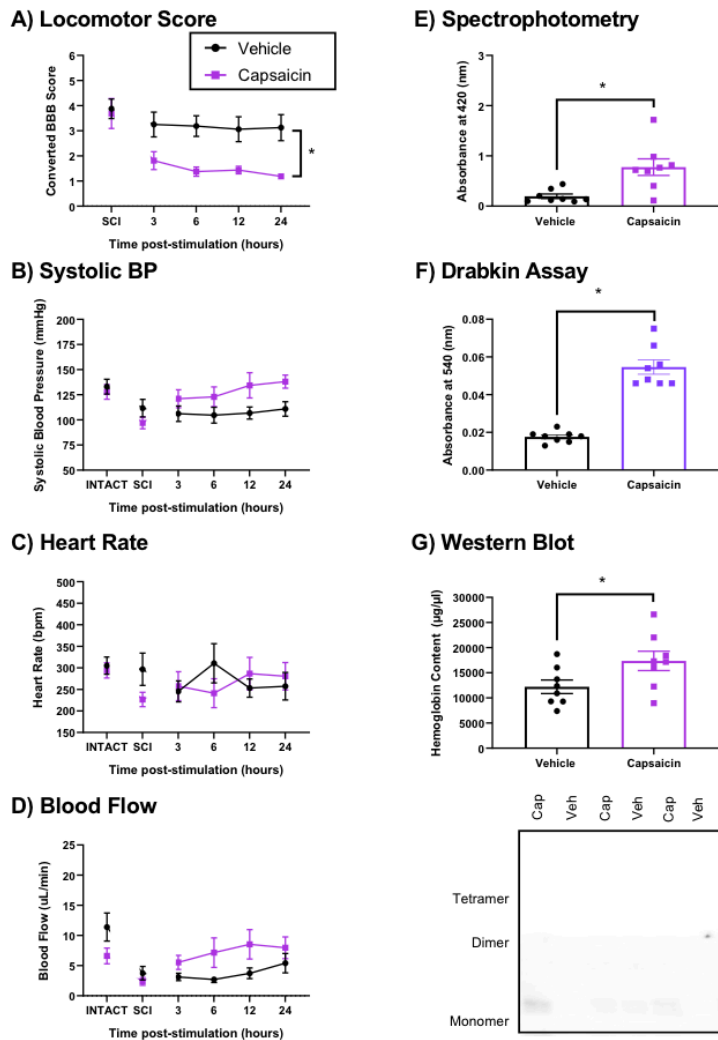
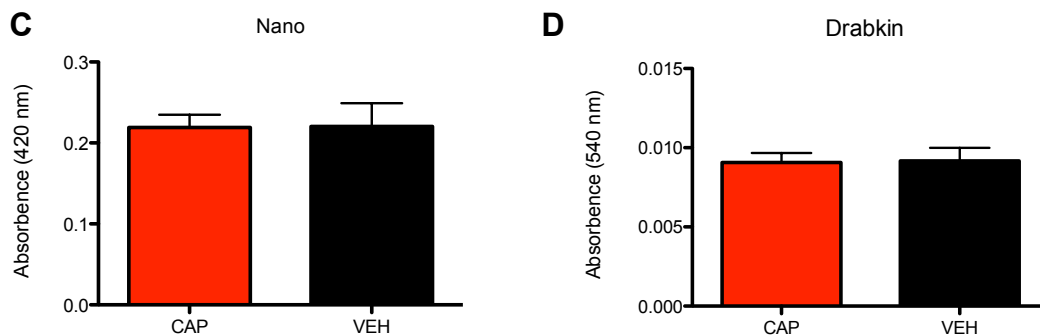


Figure 4. Hemorrhage 24 hours after capsaicin injection.

BBB locomotor scores were reduced in subjects treated with capsaicin (A). Capsaicin treatment did not increase blood pressure measurements (B-D). The absorbance at 420 nm taken at 24 hours was increased after capsaicin treatment (E). The amount of hemoglobin- α did not differ between groups (F). Representative western blot showing the three main bands associated with hemoglobin (G). Error bars represent SEM (n = 8).

192 Hours After Capsaicin Treatment:

Because biological processes typically decay in an exponential manner, we kept the ratio of time intervals constant across conditions (multiples of 8). Thus, our last time point examined hemorrhage at 192 hrs. At this time point, levels of hemorrhage in the spinal cord were not increased by capsaicin injection. Absorbance at 420 nm was assessed, and no significant differences between groups were found by an ANOVA, $F(1,14) = 0.002$, $p > 0.05$ (Figure 5C). Further evaluation with Drabkin's assay yielded similar results after an ANOVA was conducted, $F(1,14) = 0.020$, $p > 0.05$ (Figure 5D). Capsaicin also did not have a significant effect on blood pressure, $F(1,14) = 0.116$, $p = 0.739$.



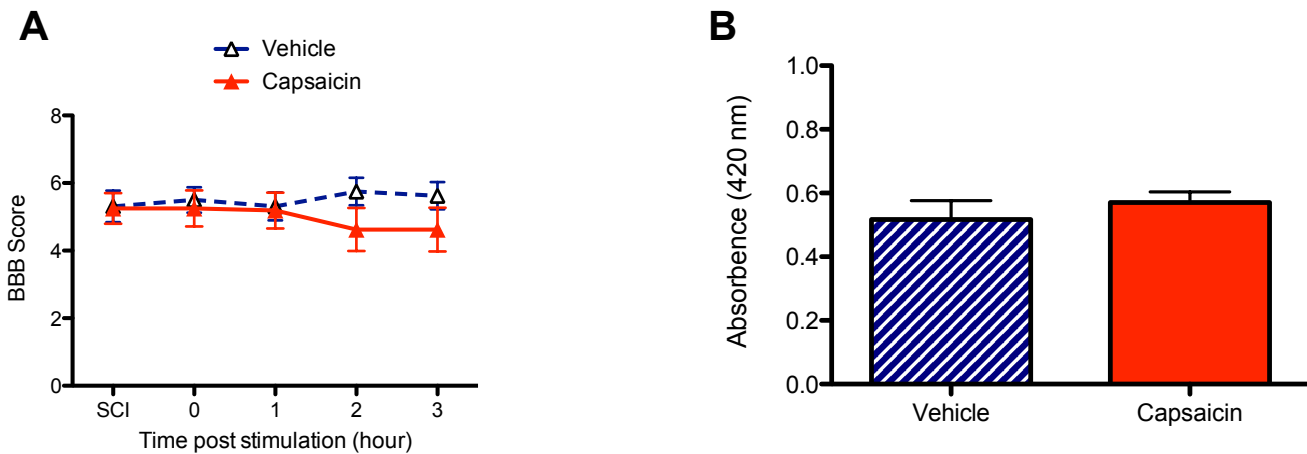
Major Task 2: Detail when nociceptive stimulation affects tissue loss

At the time of grant submission, we had collected preliminary data indicating that noxious stimulation (electrical stimulation or capsaicin) induces hemorrhage when applied 24 hrs after injury. The experiment outlined under this task was designed to detail when pain input has an adverse effect. Again, the exact time intervals selected were adjusted on basis of the initial results. Our results indicate that the effect of pain input wanes by 96 hrs and is most robust 6-24 hrs after injury. Interestingly, nociceptive stimulation just 90 min after injury had relatively little effect.

Capsaicin Treatment 90 min After Injury:

Treatment with capsaicin 90 minutes after injury did not significantly impair locomotor function. Before injection, BBB scores did not differ between groups, $F(1,14) = 0.008$, $p > 0.05$. Following injection, an ANCOVA yielded no significant differences between groups, $F(1,13) = 1.389$, $p > 0.05$ (Figure A).

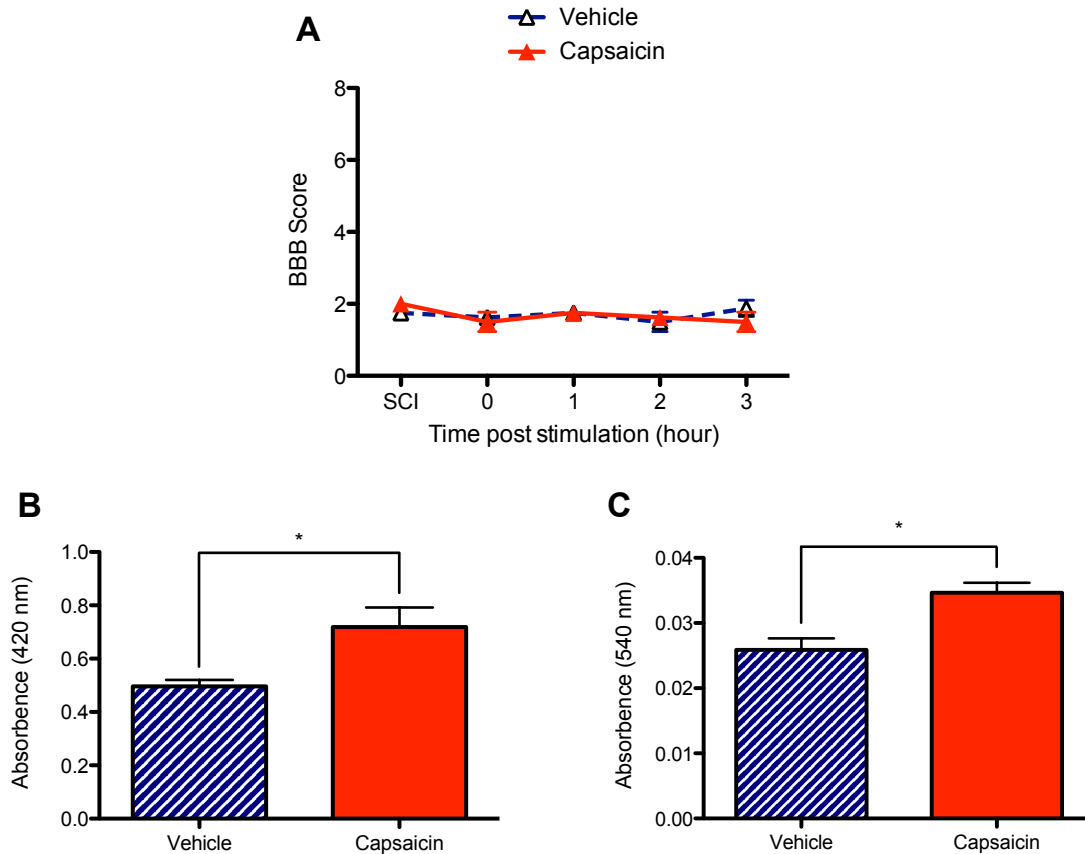
Capsaicin injection 90 minutes after SCI also did not impact hemorrhage at the injury site. Spectrophotometric analysis showed no differences between groups, $F(1,14) = 0.541$, $p > 0.05$ (Figure B).



Capsaicin Treatment 6 Hours After Injury:

When injected 6 hours after injury, capsaicin did not significantly impact locomotor function. Prior to treatment, locomotor scores did not differ between groups, $F(1,14) = 0.081$, $p < 0.7803$. An ANCOVA found no significant differences between groups after injection, $F(1,13) = 0.905$, $p > 0.05$ (Figure A).

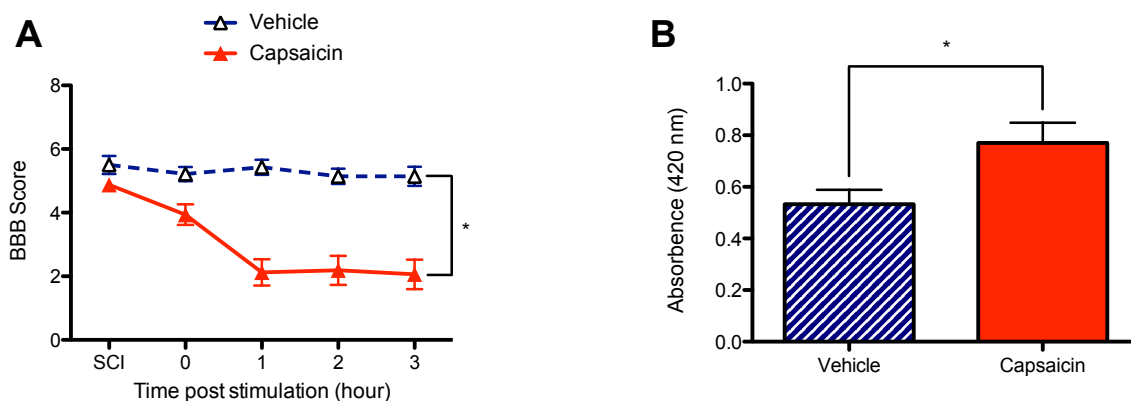
Hemorrhage at the injury site was significantly increased in animals administered capsaicin. An ANOVA found significantly greater absorbance in samples from animals treated with capsaicin compared to vehicle controls, $F(1,14) = 7.372$, $p = 0.0168$ (Figure B). Analysis with Drabkin's assay confirmed this effect, $F(1,14) = 12.401$, $p = 0.0034$ (Figure C).



Capsaicin Treatment 24 Hours After Injury:

In animals injected with capsaicin, locomotor function was significantly impaired relative to vehicle controls. Baseline locomotor scores did not differ between groups prior to treatment, $F(1,13) = 2.974$, $p = 0.108$. After treatment, an ANCOVA, using baseline scores as a covariate, found that animals treated with capsaicin had significantly lower BBB scores, $F(1, 12) = 34.807$, $p = 0.0001$ (Figure A).

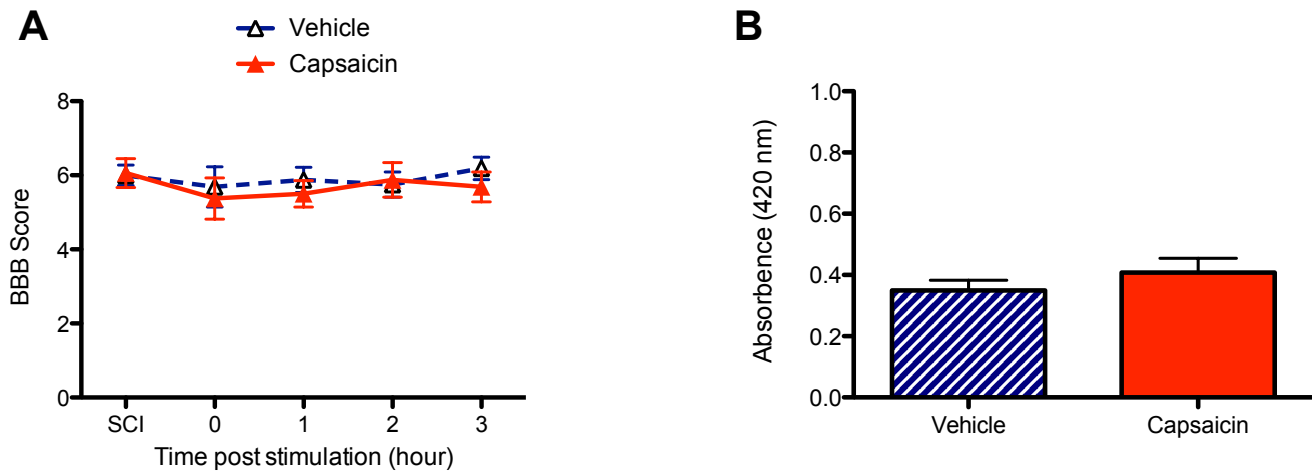
Hemorrhage at the injury site was increased in animals treated with capsaicin. After spectrophotometric analysis of protein samples from the injury site, an ANOVA found a significant difference between groups, $F(1,13) = 5.716$, $p < 0.05$ (Figure B).



Capsaicin Treatment 96 Hours After Injury:

Animals that received capsaicin treatment 96 hours after injury did not show any difference in locomotor function following injection. Before capsaicin injection, there were no differences in BBB score between groups, $F(1,14) = 0.015$, $p > 0.05$. After injection, an ANCOVA, using baseline scores as a covariate, found no effect of capsaicin on locomotor function, $F(1,13) = 0.500$, $p > 0.05$ (Figure A).

Additionally, capsaicin treatment did not impact hemorrhage at the injury site when delivered 96 hours after injury. An ANOVA found no effect of capsaicin after spectrophotometric analysis ($F(1,14) = 0.904$, $p > 0.05$) (Figure B).



Major Task 3: Does the effect of tonic pain (capsaicin) depend on brain systems

Prior work has shown that disrupting communication with the brain by means of a spinal transection blocks hemorrhage in animals exposed to noxious electrical stimulation. The present experiment was designed to test whether a similar effect is observed when animals are treated with capsaicin. Here, and in subsequent experiments, hemorrhage was assessed 3 hrs after noxious stimulation. We found that this effect was completely blocked by a complete transection at T2.

Detailed description:

Prior to transection surgery, systolic blood pressure ranged from 99.27 ± 7.09 to 101.88 ± 10.31 (mean $\pm$ SE) across groups. These differences were not statistically significant (all F s < 1.0 , $p > .05$). Contused animals that were treated with capsaicin showed no change in blood pressure and this was true independent of whether they had also received a spinal cord transection (Fig. 3). An ANCOVA confirmed that neither capsaicin nor spinal transection had a significant effect (all F s < 1.0 , $p > .05$).

Contused rats that had not undergone a spinal cord transection (Sham) exhibited greater absorbance at the wavelength associated with hemoglobin and this effect was blocked by spinal cord transection (Fig. 4A). An ANOVA analysis confirmed that the main effects of capsaicin ($F_{(1, 28)} = 5.40$, $p < .05$) and transection ($F_{(1, 28)} = 8.34$, $p < .01$) treatment, as well as their interaction ($F_{(1, 28)} = 5.65$, $p < .05$), were statistically significant. *Post hoc* comparisons confirmed that the sham group that received shock differed from the other three groups ($p > .05$).

A similar pattern of results was obtained on with the Drabkin assay. Again, capsaicin increased hemoglobin concentration at the site of injury in contused rats that were not transected (Sham). This effect was blocked by a spinal cord transection (Fig. 4B). An ANOVA confirmed that the main effects of capsaicin ($F_{(1, 28)} = 4.73, p < .05$) and transection ($F_{(1, 28)} = 9.42, p < .01$) treatment, as well as their interaction ($F_{(1, 28)} = 5.75, p < .05$), were statistically significant. *Post hoc* comparisons confirmed that the sham operated group that was treated with capsaicin differed from the other three ($p < .05$).

Western blotting confirmed that contused rats that received capsaicin had higher concentrations of hemoglobin at the site of injury relative to both the unshocked controls and transected rats that received shock (Fig. 4C). Because there was greater variability in behavioral performance after injury in this experiment, we analyzed the data using an analysis of covariance with baseline BBB score entered as a covariate. The main effects of transection and capsaicin treatment, and their interaction, were statistically significant (all $F_s > 4.33, p > .05$). *Post hoc* comparisons showed that the non-transected (Sham) group that received capsaicin differed from the other three ($p < .05$). No other group comparison was significant ($p > .05$).

T12 Contusion	18 Hours	Baseline BBB & BP	Sham T2 Transection	6 Hours	Vehicle Capsaicin	3 Hours BP T0-T3	Collect Tissue
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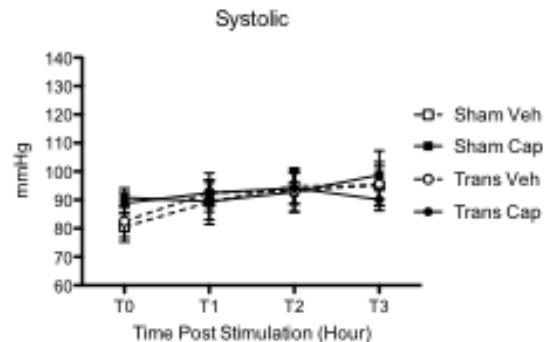


Fig. 3.. Application of capsaicin (Cap) to one hind paw did not induce a change in blood pressure ($n = 8$).

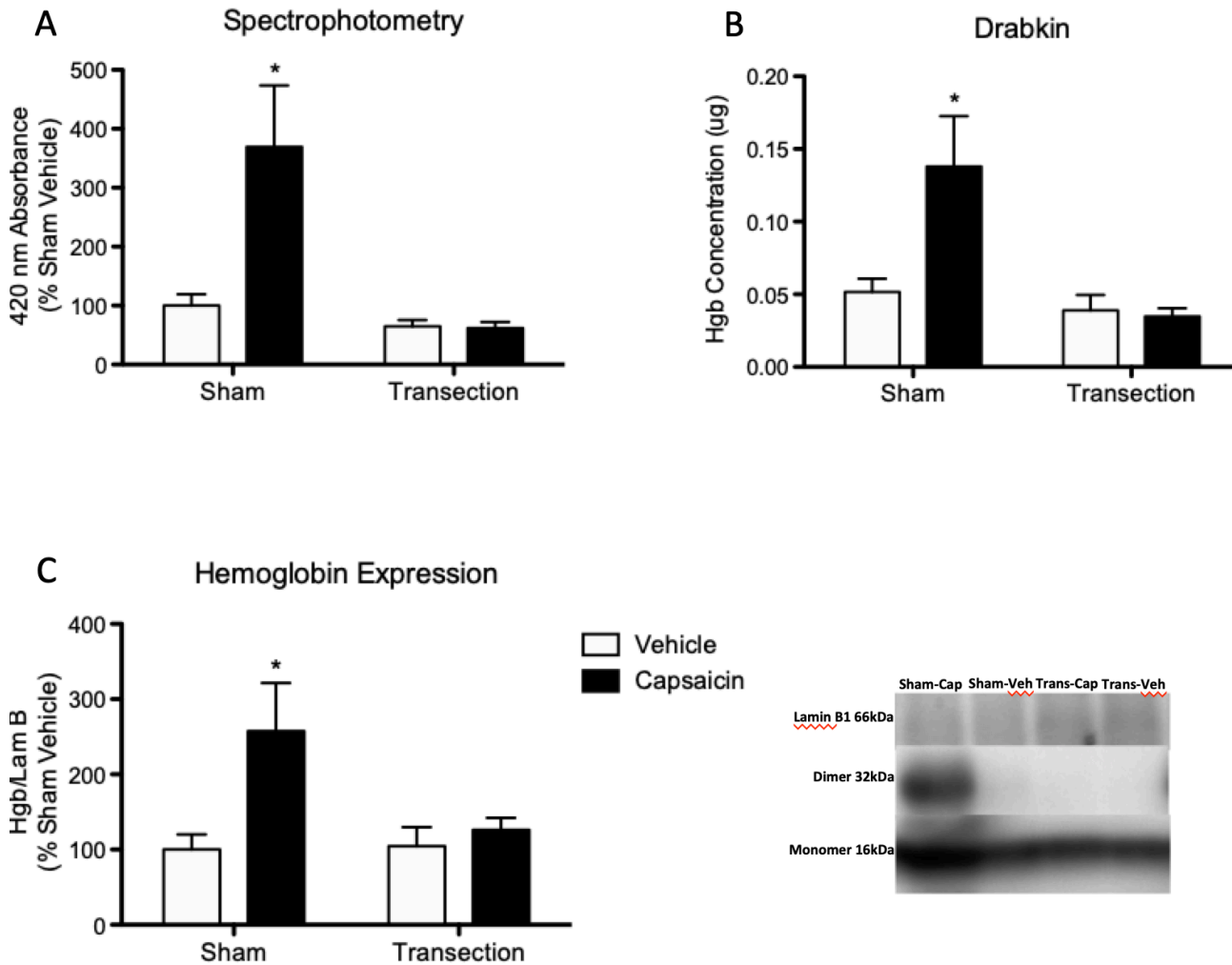


Fig. 4. Application of the irritant capsaicin to a hind paw increases hemorrhage after a lower thoracic contusion injury and this effect is blocked by a spinal transection. **(A)** Sham operated rats that were treated with capsaicin exhibited greater absorbance at 420 nm, the wavelength associated with hemoglobin. Transection surgery blocked this effect. **(B)** Drabkin assay and Western blot **(C)** showed similar results. Representative immunoblot is to the right of the figure. * Indicates statistical significance ($p < .05$, $n = 8$). Error bars represent the standard error of the mean (SEM).

Additional analyses are being performed to determine whether: 1) capsaicin treatment increases the expression of SUR1-TRPM4, which is indicative of progressive hemorrhagic necrosis; and 2) whether this effect is blocked by spinal transection. Preliminary analyses failed to find an effect of capsaicin treatment. We subsequently discovered that our immunoprecipitation procedure was adversely affected by a buffer (causing SUR1-TRPM4 to dissociate). We have addressed this issue and are repeating the assay.

Major Task 4: Are similar effects observed in male and female rats.

At the time of grant submission, all of our data had been collected using male rats. The present experiment was designed to determine whether treatment with capsaicin induces hemorrhage induces this effect in female animals.

Detailed description:

Experiment 1 observed the effect of intradermal capsaicin injection in female rats after SCI. Prior to treatment, baseline BBB scores did not differ between groups [$F(1,12) < 1.215$, $p > 0.05$], ranging from 3.79 (± 0.52) to 3.36 (± 0.28). Following treatment, locomotor function was impaired in animals that received capsaicin injection, $F(1,11) = 23.532$, $p = 0.0005$. This effect varied in a time-dependent manner, emerging after the first hour following injection and persisting until the animals were sacrificed, $F(3,33) = 11.289$, $p = 0.0001$ (Fig. 1A).

Nociceptive stimulation has also been shown to elevate blood pressure in male animals following spinal cord injury. Before injection of capsaicin in Experiment 1, MAP values did not differ between groups, $F(1,12) < 1.0$, $p > 0.05$. Following injection, an ANCOVA, using baseline MAP as a covariate, found no significant differences between groups [$F(1,11) < 1.0$, $p > 0.05$], though at two hours after injection, animals that received capsaicin showed significantly higher MAP than their controls, $F(3,33) = 3.136$, $p < 0.05$ (Fig. 1C).

Following the three hours of behavioral assessments in Experiments 1 and 2, animals were sacrificed and their spinal cord tissue collected for protein extraction and analysis. Past work has demonstrated increased amounts of hemorrhage at the injury site in animals treated with nociceptive stimuli. To determine the effects of capsaicin and shock on hemorrhage in female animals, the concentration of α -hemoglobin in samples was assessed with spectrophotometry and western blotting. Protein extracts from animals injected with capsaicin in Experiment 1 exhibited greater absorbance at 420 nm, the wavelength associated with hemoglobin, $F(1,12) = 6.77$, $p < 0.05$ (Fig. 2A). Immunoblotting was then used to confirm this effect. Levels of α -hemoglobin were significantly elevated in animals that received pain input, $F(1,12) = 10.873$, $p < 0.01$ (Fig. 2C).

Given that both forms of nociceptive input produced similar deficits acutely in female rats as shown in previous experiments in male rats, Experiments 3 and 4 examined the effects of capsaicin and shock on recovery over a 28-day period. It was hypothesized that the neuroprotective effect of estrogen might protect against the damage inflicted by secondary injury. As a result, any sex-dependent differences may only be apparent beyond the acute phase of injury, requiring observation of recovery over a longer period of time. Again, locomotor function was assessed using the BBB method. Animals were scored prior to noxious stimulation, each day for the week following injury, on day 10, then once a week thereafter until day 28. Prior to injection of capsaicin in Experiment 3, BBB scores did not differ between groups, $F(1,16) < 1.0$, $p > 0.05$, ranging from 2.222 (± 0.278) to 2.444 (± 0.242). An ANCOVA using baseline BBB scores as the covariate revealed that animals injected with capsaicin recovered less function over time, $F(1,15) = 5.764$, $p < 0.05$ (Fig. 3A). No other within subject factors were significant, all F s < 1.525 , $p > 0.05$.

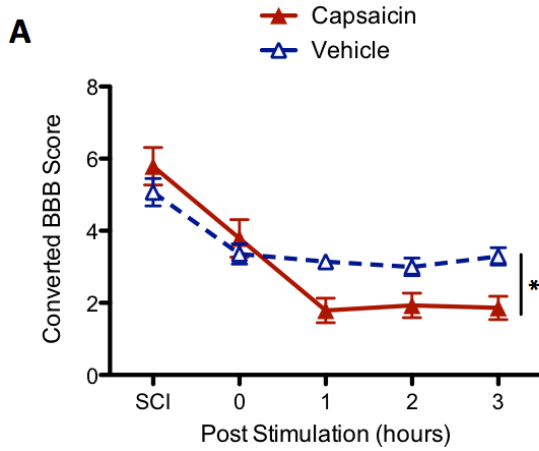


Figure 1. Acute effects of nociceptive input on locomotor function and blood pressure in female rats. (A) Animals that received intradermal injection of capsaicin to the hindpaw showed greater deficits in locomotor function after treatment. (C) MAP was only elevated for animals that received capsaicin at two hours after stimulation. * Indicates statistical significance ($p < 0.05$). Error bars represent standard error of the mean (Capsaicin $n = 7$, Vehicle $n = 7$, Shock $n = 8$, Unshock $n = 8$).

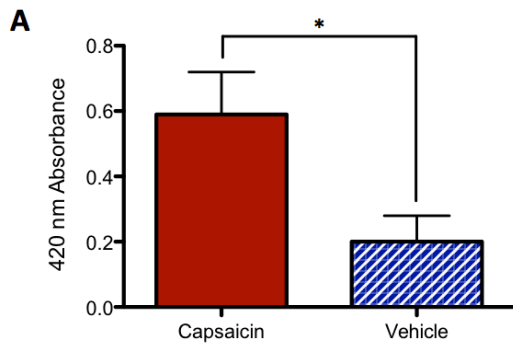
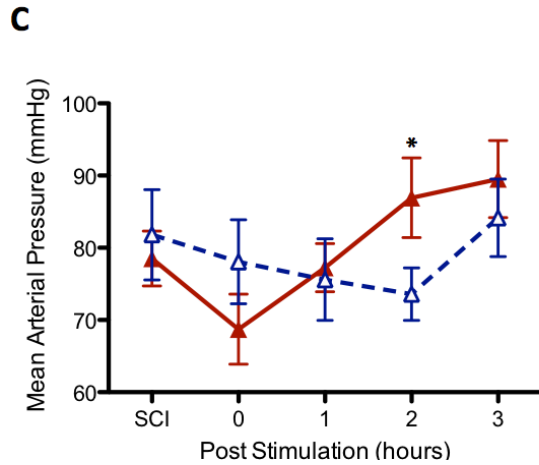
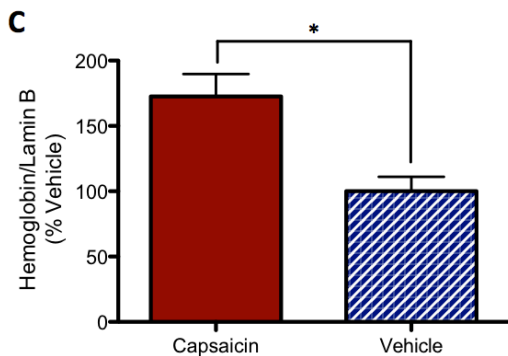


Figure 2. Acute effects of nociceptive input on hemorrhage after SCI in female rats. (A) Spectrophotometric analysis found greater absorbance of protein samples at 420 nm for animals that receive intradermal capsaicin injection. (C) Western blot analysis found greater hemoglobin content in capsaicin-treated animals. * Indicates statistical significance ($p < 0.05$). Error bars represent standard error of the mean (Capsaicin $n = 7$, Vehicle $n = 7$, Shock $n = 8$, Unshock $n = 8$).



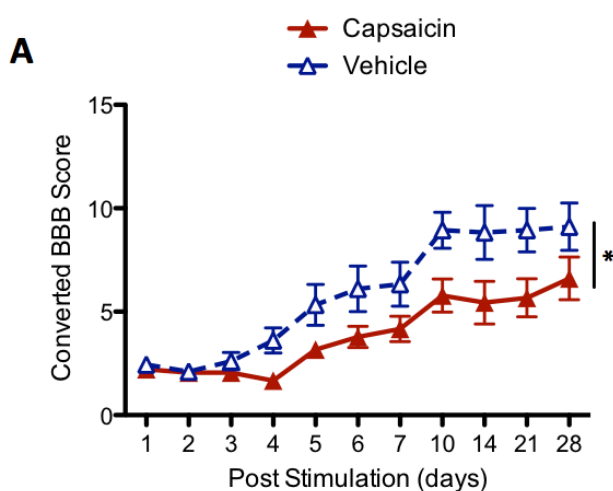


Figure 3. Effects of nociceptive stimulation on long-term recovery of locomotor function and weight. (A) Capsaicin-treated animals recovered less function over 28 days than their vehicle-treated controls. Error bars represent standard error of the mean (Capsaicin $n = 9$, Vehicle $n = 9$).

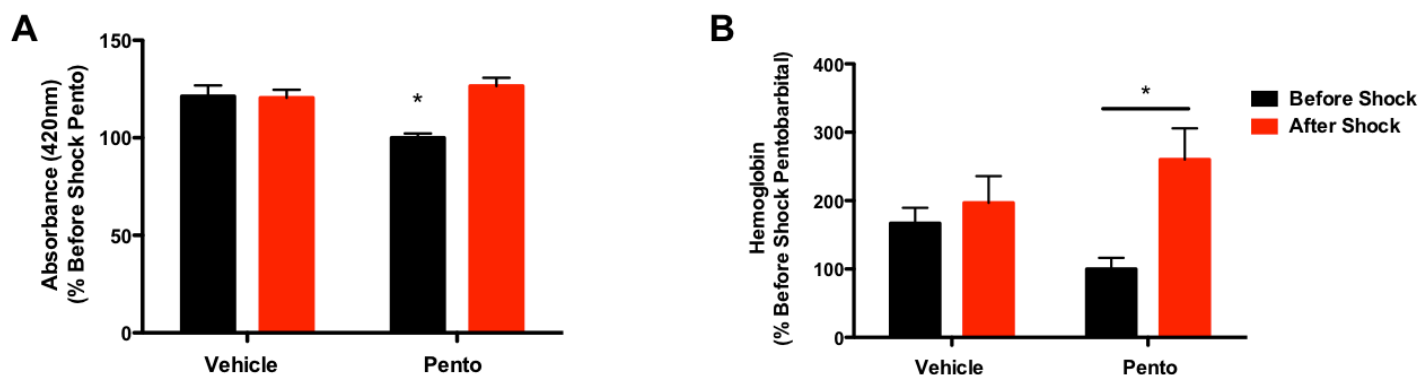
Specific Aim 2: Promoting recovery using general anesthesia

Major Task 1: Is anesthesia only effective if given prior to noxious stimulation

At the time of grant submission, we had shown that nociception induced hemorrhage and impaired long-term recovery can be blocked by pretreatment with the anesthetic pentobarbital. The current experiment examined whether inducing a state of anesthesia after noxious stimulation attenuates its adverse effect. Because the onset and offset of electrical stimulation is readily controlled, it was used here rather than capsaicin. We found that pentobarbital given before, but not after, nociceptive stimulation attenuates hemorrhage.

Detailed description:

Rats that received pentobarbital prior to shock treatment exhibited lower levels of hemoglobin at the site of injury. This protective effect was not observed when animals were anesthetized after shock treatment. An analysis of the absorbance at 420 nm confirmed that the effect of pentobarbital treatment depended upon when it was administered, $F(3,28) = 4.913$, $p < 0.05$. Likewise, an analysis of the western blotting data confirmed that anesthesia given before, but not after, reduced hemoglobin content, $F(3,28) = 4.047$, $p < 0.05$.



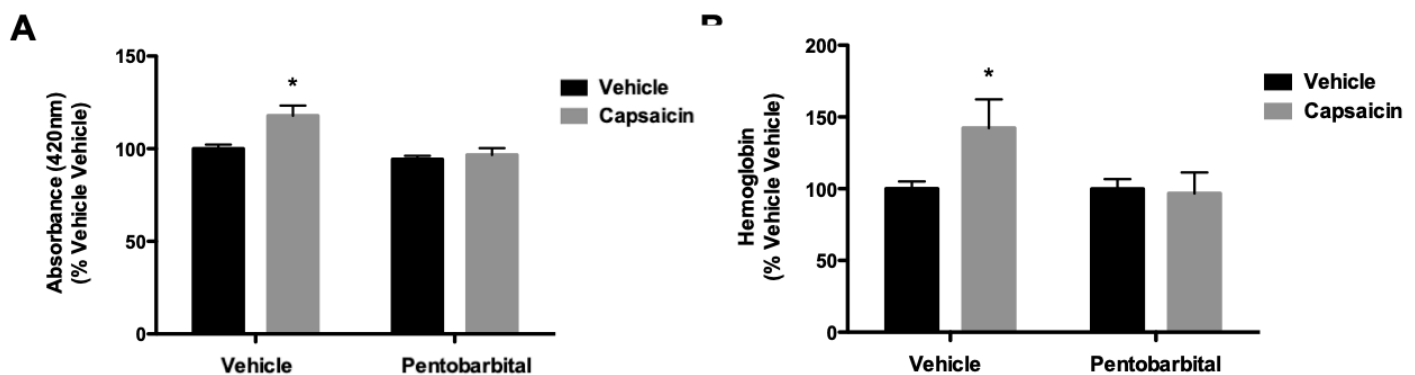
Hemorrhage was assessed via absorbance (A) and western blotting (B) in contused rats that had received noxious electrical stimulation. This effect was attenuated by pentobarbital given before, but not after shock.

Major Task 2: Does anesthesia block the effect of capsaicin treatment?

We have shown that anesthesia blocks the effect of noxious electrical stimulation. The present experiment was designed to test whether this treatment also attenuates capsaicin-induced hemorrhage. We found that it did.

Detailed description:

Protein extracted from injured spinal cord tissue of unanesthetized (vehicle treated) rats given hindpaw capsaicin exhibited significantly greater hemoglobin content. This effect was blocked by pretreatment with pentobarbital. A analysis of absorbance at 420 nm confirmed that the effect of capsaicin depended upon pentobarbital treatment, $F(3,32) = 9.374$, $p < 0.05$. Immunoblotting targeting hemoglobin yielded the same effect, $F(3,32) = 3.431$, $p < 0.05$.



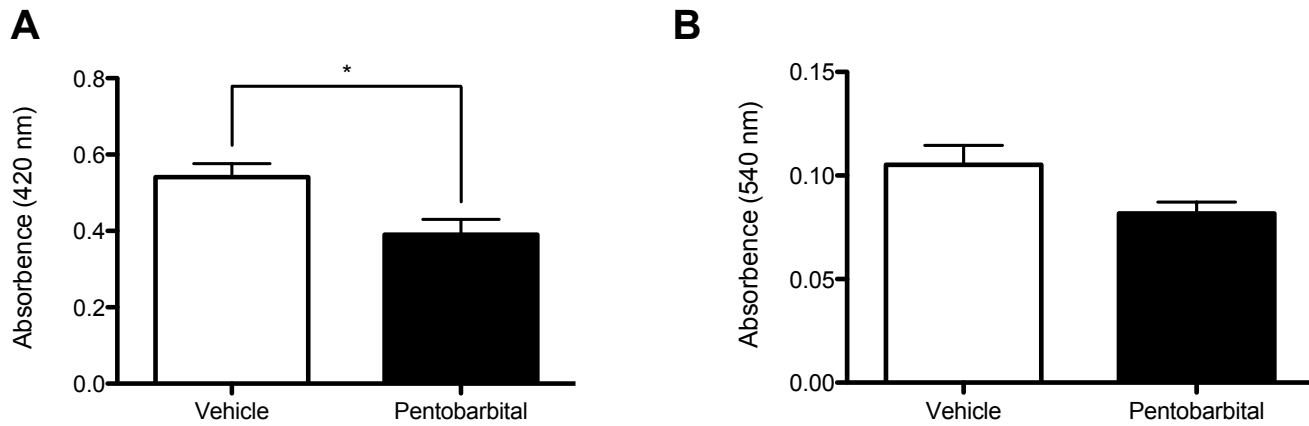
Hemorrhage was assessed via absorbance (A) and western blotting (B) in contused rats 3 hrs after capsaicin was applied. Capsaicin induced hemorrhage and this effect was blocked by pentobarbital anesthesia.

Major Task 3: Does anesthesia after surgery attenuate damage?

In all of our experiments, we have modeled polytrauma by providing nociceptive stimulation after injury. We have shown that inducing a coma-like state blocks pain-induced hemorrhage. We hypothesized that anesthesia could also counter the development of hemorrhage if initiated soon after injury. To test this, animals were maintained in an anesthetized state for a period of 12 hrs after surgery. We found that this treatment attenuated hemorrhage at the site of injury. Further work is now being conducted to evaluate whether a similar effect is observed when tissue is collected 24 hrs after injury. In addition, we will assess whether a shorter period of anesthesia has a protective effect.

Detailed description:

Spectrophotometric analysis of protein extracts found less hemoglobin at the injury site in animals maintained in an anesthetized state following surgery. An ANOVA found a significant difference in hemorrhage between vehicle-treated and anesthetized rats, $F(1,10) = 6.651$, $p = 0.0275$ (Figure A). This effect was further analyzed with Drabkin's assay, which found an effect of pentobarbital anesthesia that trended towards significance, $F(1,10) = 3.876$, $p = 0.0773$ (Figure B).



Specific Aim 3: Promoting recovery using local anesthesia

Major Task 3: Does rostral lidocaine block hemorrhage?

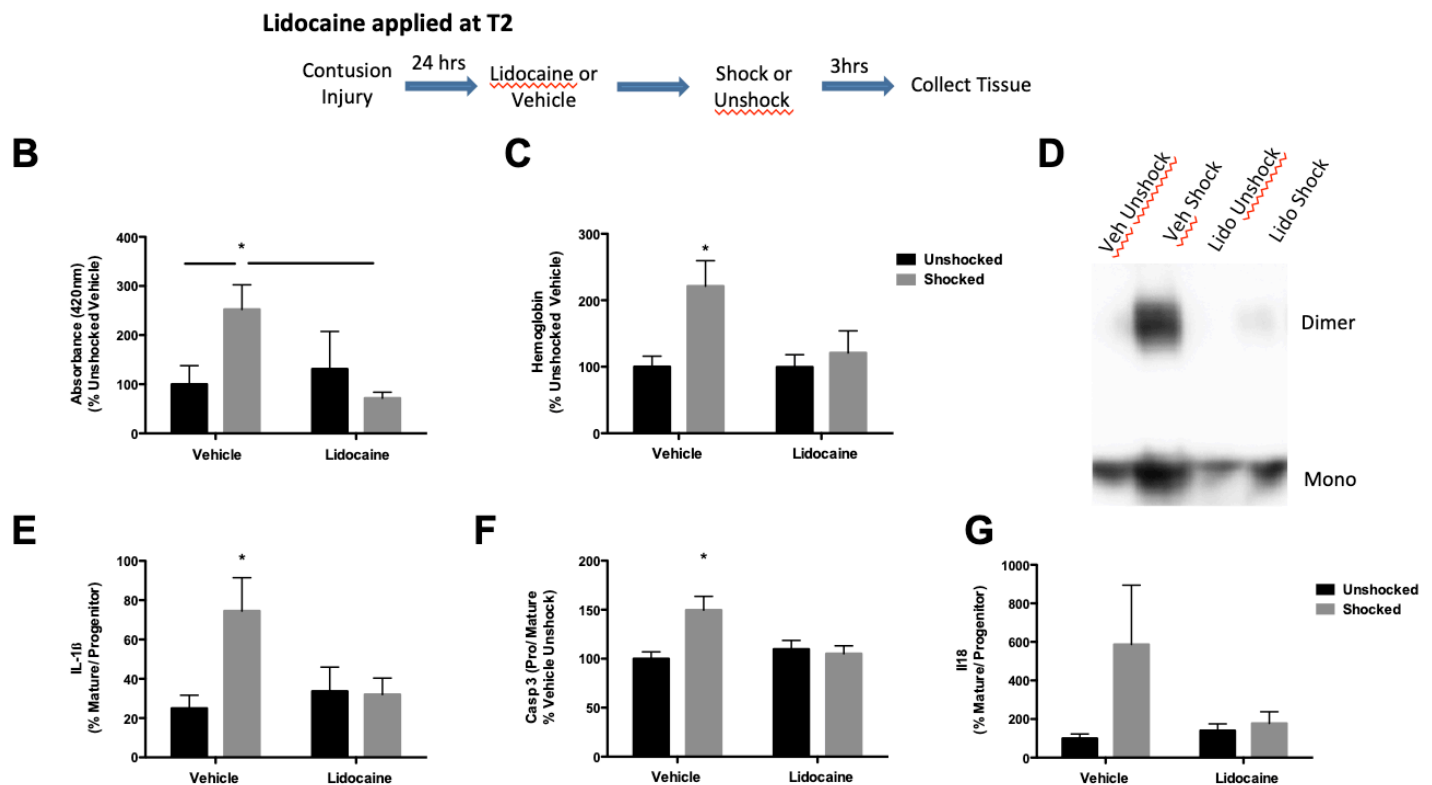
In animals that have received a lower thoracic (T11-12) contusion injury, nociceptive stimulation applied to the hind legs or tail increases the area of hemorrhage and impairs long-term recovery. Of course, a surgical transection does not represent a viable treatment option in the clinic. Given this, we suggested that a similar effect might be observed if lidocaine was slowly infused rostral to injury (at T2) and, at the time of submission, presented pilot data that suggested a protective effect. We have now completed this experiment (Aim 3, Major Task 3). As predicted, rostral lidocaine had a protective effect. Further, it also blocked the adverse effect pain input has on long-term recovery. These data were just published in the *Journal of Neurotrauma* (Davis et al., 2020).

Detailed description:

Rostral lidocaine was used to determine if blocking communication with the brain would attenuate nociception-induced hemorrhage. Hemorrhage was first analyzed by spectrophotometry at a wavelength associated with hemoglobin absorbance (420nm) and then confirmed by Western blotting targeting alpha-hemoglobin.

Spectrophotometry of protein samples revealed a protective effect of lidocaine against hemorrhage. Samples for vehicle treated rats given shock were tinted red, indicating a greater level of hemoglobin content. This effect was attenuated by lidocaine infusion (Figure 2A). An ANOVA confirmed that the effect of shock treatment depended upon whether lidocaine was administered [$F = 4.480$, $p < 0.05$, $\eta^2=0.103$]. No other terms were significant [both F s < 2.242 , $p > 0.05$]. *Post hoc* comparison revealed that vehicle shock animals differed from both lidocaine shock and lidocaine unshocked groups ($p < 0.05$).

Western blotting targeting hemoglobin-alpha proteins yielded a similar pattern of results. Animals treated with tail-shock had higher levels of hemoglobin at the site of injury. This effect was blocked by lidocaine infusion (Fig. 2B). An ANOVA revealed a main effect of shock treatment [$F = 6.368$, $p < 0.05$]. The main effect of drug administration and its interaction with shock approached significance [both F s > 3.123 , $p > 0.0857$]. *Post hoc* comparison of the group means showed that animals given a combination of vehicle infusion and shock treatment differed from all other groups ($p < 0.05$).



Overview of the results at the end of year 2:

We have worked to integrate the findings described above into four papers. Each of these papers has required additional experiments that we have funded from other sources (e.g., my professorship). One paper, describing the effect of rostral lidocaine on pain-induced hemorrhage, was just published (Davis et al., 2020). Two additional papers have been completed and should be submitted within the next 4 weeks. One shows that a rostral transection eliminates the nociception-induced rise in blood pressure and hemorrhage (Fauss et al.). A second shows that nociceptive stimulation induces hemorrhage in female rats (Baine et al.). We are also in the process of completing the data collection for a fourth paper, which shows that pentobarbital anesthesia has a protective effect (Davis et al.). We hope to submit that paper by the end of the year.

Our overall progress on the project has been excellent and we are on schedule to complete the proposed studies in year 3 of the project. Regarding Aim 1, Major Tasks 1, 2, and 4 have been completed. Major Task 3 had two components, the first of which (Sub1) is done. We hope to finish the last part of Major Task 3 in the next 2 months. For Aim 2, Major Tasks 1 and 2 are completed, and Major Task 3 has been initiated (and yielded some very interesting preliminary data, suggesting that initiating anesthesia soon after injury could have a protective effect). For Aim 3, we have already completed Major Task 2 and will be initiating the remaining tasks within the next few months.

While we are not in a position to translate these findings to humans, the work has begun to generate considerable interest, fueled in part by our frequent presentation of the material at national conferences. Indeed, the data are central to two keynote addresses at the spinal cord centers in Louisville and Vancouver. I also presented the data for Grand Rounds at Houston Methodist, a leading center in spinal cord injury. Other invited talks include ASIA and KSCHIRT. Data associated with this proposal have also been featured in two recent review articles (Grau, 2019; and Grau et al., 2020).

The pandemic began to affect research at Texas A&M University in March of this last year. We were granted “essential services” status for our Department of Defense research and were able to continue collecting data, albeit at a somewhat slower pace. In June, we were allowed to restart most aspects of our research, with safety measures in place to limit trainee contact. While limits on personnel, and a lack of hands (due to undergraduates not being allowed to participate in research), has slowed progress some, we have been able to keep this project on course. In part, this is because we entered the pandemic well ahead of schedule. Barring unforeseen problems this winter, we hope to move ahead with the remaining components of the grant as planned.

Training Opportunities

Though not explicitly designed to offer training opportunities, the project has yielded value in this domain. First, two graduate students (Jacob Davis and Travis Johnston) have been supported by the project. Both are completing their Ph.D., with a focus on research topics directly related to the aims of this grant. In both cases, the work has provided a learning opportunity, introducing the students to a host of behavioral, surgical, and cellular techniques. It has also provided a forum for discussing research impacting hemorrhage and recovery after spinal cord injury.

In addition, the proposal has bolstered undergraduate training in two ways. First, a number of students have elected to stay on after they graduate, prior to applying to medical school. These students work as technicians and have been instrumental to moving the project forward. Like the graduate students, they have gained expertise in a host of new domains, including surgery, behavioral testing, statistical analyses, experimental design, and cellular assays. They have also had an opportunity to prepare papers for publication. Two of these trainees (Melissa Henwood and Paris Bean) are now attending graduate school. A third (Megan Tarbet) has applied to MD/PhD programs and has three interviews this fall. Beyond this, the work helps to support independent study projects by undergraduates. Each semester, approximately 4-5 students gain individualized training through this mechanism.

Dissemination of Results

As noted above, we have completed data collection for 4 new papers, one of which was just published (Davis et al., 2020) and three of which are about to be submitted.

An article that reviews this work was published in *Experimental Neurology* (Grau et al., 2020; “Learning to promote recovery after spinal cord injury”). The project also helped to support a review article (Grau, 2019; “Achieving adaptive plasticity in the spinal cord”) that was published in the *Oxford Research Encyclopedia of Neuroscience*.

Finally, the work has been presented at three national conferences: the National Neurotrauma Society in Pittsburgh PA (July, 2019), the Society for Neuroscience in Chicago IL (October, 2019), and the International Symposium on Neural Regeneration in Monterey CA (January, 2020):

Baine, R.E., Strain, M.M., Henwood, M.K., Johnston, D.T., Davis, J.A., Grau, J.W. (2019). Pain input after spinal cord injury increases tissue loss and impairs long-term recovery in female rats. *Journal of Neurotrauma*. 36, A-72.

Fauss, G.N.K., Strain, M.M., Huang, Y.J., Reynolds, J.A., Davis, J.A., Henwood, M.K., West, C.R., & Grau, J.W. (2019). Role of blood pressure in pain-induced hemorrhage after spinal cord injury: Brain systems may drive an increase in blood pressure, but this effect was not sufficient to induce hemorrhage. Chicago, IL: Society for Neuroscience, 2019.

- Davis, J.A., Henwoow, M.K., Cox, C., Baine, R., & Grau, J.W. (2019). Pain-induced hemorrhage after spinal cord injury: Pentobarbital anesthesia and local application of lidocaine prevent hemorrhage when give before, but not after, noxious stimulation. Chicago, IL: Society for Neuroscience, 2019.
- Baine, R., Strain, M.M., Reynolds, J.A., Henwood, M.K., Lout, E., Haribhakti, P., West, C.R., & Grau, J.W. (2019). Role of blood pressure in pain-induced hemorrhage after spinal cord injury: Effect of shock and capsaicin treatment on blood pressure, behavioral performance, and hemorrhage over time. Chicago, IL: Society for Neuroscience, 2019.
- Fauss, G. N. K., Strain, M. M., Huang, Y. J., Reynolds, J. A., Davis, J. A., Henwood, M. K., Tarbet, M. M., Hudson, K. E., Bean, P. A., & Grau, J. W. (2020). Role of rostral neural systems on the development of pain-induced hemorrhage after spinal cord injury. (<https://neuralregen.org/wp-content/uploads/2020/01/ISNR-2020-Final.pdf>) International Symposium on Neural Regeneration. Pacific Grove, CA.
- Davis, J. A., Bopp, A. C., Henwood, M. K., Baine, R. E., & Grau, J. W. (2020). Pain-induced hemorrhage after spinal cord injury: Pentobarbital anesthesia and local application of lidocaine prevent hemorrhage when given before, but not after, noxious stimulation. (<https://neuralregen.org/wp-content/uploads/2020/01/ISNR-2020-Final.pdf>) International Symposium on Neural Regeneration. Pacific Grove, CA.

Plans for the Next Reporting Period

Our overall progress has been excellent. What has deviated slightly from our original plan is the order in which the experiments have been completed. The most technically challenging component of the grant [Sub2 of Major Task 3 (Aim 1)] has taken longer than expected. Filling in some of the time points for Major Tasks 1 and 2 of that Aim also took some additional effort. Progress on other components of the grant has been faster than we anticipated. For example, we are well into the last component (Major Task 3) of Aim 2, which is a few months ahead of schedule. Likewise, we have completed an experiment (Major Task 2 of Aim 3) that we had originally planned for year 3. Thus, on a balance, we remain on (or ahead) of schedule.

This next year we hope to complete Major Task 3 of Aim 2 by February of 2021. We will then initiate Major Tasks 1 and 3 of Aim 3, which we hope to have done by the summer. The remaining time would be used to finish up any loose ends (needed to complete papers for publication) and writing.

4. IMPACT

Impact on Principal Discipline

Our results are transforming how researchers and clinicians think about tissue loss after spinal cord injury in three ways. First, our work shows that the development is not a passive process, dictated solely by the magnitude of the initial physical injury. Instead, it is an active process that unfolds over the course of hours to days after injury, a process that is modulated by pain input.

Another way that our work is impacting the discipline is by showing that brain-dependent processes contribute to tissue loss after spinal cord injury. To our knowledge, this is the first evidence that the brain can have such an adverse effect. Indeed, prior work (including our own) suggested that brain systems act to quell neural excitation within the spinal cord, which should have a protective effect. Our work overturns this view and leads the field to think about tissue loss after spinal cord injury as a systemic process that is modulated by other on-going physiological processes.

Finally, our work is impacting the field by demonstrating two new procedures that could be translated to practice, to reduce tissue loss after injury. At present, the only method available to reduce neural excitation at the site of injury is hypothermia. This procedure is difficult to implement, especially in a battlefield situation, and hard to maintain over days-weeks after injury. Anesthesia, especially through the local application of lidocaine, offers an attractive alternative that could be readily administered by technicians soon after injury.

Impact on Other Disciplines

By moving the focus of research beyond the site of injury, to consider brain mechanisms and systemic processes, our work touches on a wide range of fields. What brain systems are critical? Are the effects of pain input mediated by lower-level processes within the brainstem or midbrain, or do higher-level structures (e.g., amygdala, cortical areas) contribute? Are these effects related to stress and activation of the pituitary adrenal axis?

Likewise, our work is relevant to work exploring the medical benefits of placing an individual in a coma-like state. Is complete anesthesia required or would a sedative be sufficient? Future studies will be exploring these issues.

Technology Transfer

The project does not involve components that would require technology transfer. The procedures used are regularly employed in a medical setting, albeit for different reasons. Implementation of the procedures would involve an “off-label” use and clinical verification of treatment effectiveness.

5. CHANGES/PROBLEMS

Changes in Approach

There have been no significant changes in our overall approach. As the research has progressed, it has become evident that the Drabkin assay provides an efficient means of quantifying hemorrhage extent and, for this reason, we have been using it more. For assays examining the expression of pro-inflammatory cytokines, we have increasingly focused upon interleukin 1 β and 18 because they are robustly expressed, provide an internal control (active vs. inactive form), and impact a number of important down-stream pathways. As the project continues, we will continue to refine our procedures.

Problems or Delays

As noted above, detailing the duration of hemorrhage and window of vulnerability has proved more challenging than we anticipated (Aim 1, Major Tasks 1 and 2). Nonetheless, we were able to complete these tasks this year. We also encountered some problems related to the procedures used to assay SUR1-TRPM4 (concerning the immunoprecipitation; Major Task 3, Subtask 2). We have now determined that this was related to a reagent used in our protein assays that was causing the complex to dissociate. That issue has been addressed and we are in the process of completing this subtask. Other aspects of the proposal (e.g., Aim 3) have progressed faster than anticipated.

Changes that Impact Expenditures

Issues related to the completion of Aim 1 has slightly inflated our animal budget. However, we anticipated some problems when we developed the proposal and, to date, it appears that this will provide sufficient cushion to complete the project.

Significant Changes in Vertebrate Animals

None

6. PRODUCTS

Journal Publications

Published

Grau, J. W., Baine, R. E., Bean, P. A., Davis, J. A., Fauss, G. N., Henwood, M. K., Hudson, K. E., Johnston, D. T., Tarbet, M. M., & Strain, M. M. (2020). Learning to promote recovery after spinal cord injury. *Experimental Neurology*, 330.

Davis, J. A., Bopp, A. C., Henwood, M. K., Baine, R. E., Cox, C. C., & Grau, J. W. (2020). Pharmacological transection of brain-spinal cord communication blocks pain-induced hemorrhage and locomotor deficits after spinal cord injury. *Journal of Neurotrauma*, 37, 1729-1739.

In Preparation (to be submitted by December, 2020)

Fauss, G. N. K., Strain, M. M., Huang, Y.-J., Reynolds, J. A., Davis, J. A., Henwood, M. K., West, C. R., & Grau, J. W. (in preparation). Spared fibers, but not hypertension, mediate development of pain-induced hemorrhage after spinal cord injury.

Baine, R. E., Strain, M. M., Henwood, M. K., Davis, J. A., Johnston, D. T., Reynolds, J. A., & Grau, J. W. (in preparation). Nociceptive input in females: Estrogen does not protect against the harmful effects of pain after spinal cord injury.

Davis, J. A., Bopp, A. C., Tarbet, M. M., Strain, M. M., & Grau, J. W. (in preparation). Pain-induced hemorrhage after spinal cord injury is blocked by pentobarbital anesthesia.

Other Publications

Grau, J. W. (forthcoming) Achieving adaptive plasticity in the spinal cord. *Oxford Research Encyclopedia of Neuroscience*, ed. Murray Sherman. New York and Oxford: Oxford University Press. doi:10.1093/acrefore/9780190264086.013.00243

Baine, R.E., Strain, M.M., Henwood, M.K., Johnston, D.T., Davis, J.A., Grau, J.W. (2019). Pain input after spinal cord injury increases tissue loss and impairs long-term recovery in female rats. *Journal of Neurotrauma*. 36, A-72.

Websites

Fauss, G.N.K., Strain, M.M., Huang, Y.J., Reynolds, J.A., Davis, J.A., Henwood, M.K., West, C.R., & Grau, J.W. (2019). Role of blood pressure in pain-induced hemorrhage after spinal cord injury: Brain systems may drive an increase in blood pressure, but this effect was not sufficient to induce hemorrhage. Chicago, IL: Society for Neuroscience, 2019.

<https://www.abstractsonline.com/pp8/#!/7883/presentation/52484>

Davis, J.A., Henwood, M.K., Cox, C., Baine, R., & Grau, J.W. (2019). Pain-induced hemorrhage after spinal cord injury: Pentobarbital anesthesia and local application of lidocaine prevent hemorrhage when give before, but not after, noxious stimulation. Chicago, IL: Society for Neuroscience, 2019.

<https://www.abstractsonline.com/pp8/#!/7883/presentation/52482>

Baine, R. E., Strain, M.M., Reynolds, J.A., Henwood, M.K., Lout, E., Haribhakti, P., West, C.R., & Grau, J.W. (2019). Role of blood pressure in pain-induced hemorrhage after spinal cord injury: Effect of shock and capsaicin treatment on blood pressure, behavioral performance, and hemorrhage over time. Chicago, IL: Society for Neuroscience, 2019.

<https://www.abstractsonline.com/pp8/#!/7883/presentation/52483>

Fauss, G. N. K., Strain, M. M., Huang, Y. J., Reynolds, J. A., Davis, J. A., Henwood, M. K., Tarbet, M. M., Hudson, K. E., Bean, P. A., & Grau, J. W. (2020). Role of rostral neural systems on the development of pain-induced hemorrhage after spinal cord injury. International Symposium on Neural Regeneration. Pacific Grove, CA.

<https://neuralregen.org/wp-content/uploads/2020/01/ISNR-2020-Final.pdf>

Davis, J. A., Bopp, A. C., Henwood, M. K., Baine, R. E., & Grau, J. W. (2020). Pain-induced hemorrhage after spinal cord injury: Pentobarbital anesthesia and local application of lidocaine prevent hemorrhage when given before, but not after, noxious stimulation. International Symposium on Neural Regeneration. Pacific Grove, CA.

<https://neuralregen.org/wp-content/uploads/2020/01/ISNR-2020-Final.pdf>

Technologies

None

Inventions

None

Other Products

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Individuals Who have Worked on the Project

Name:	James Grau
Project Role:	PI
Research Identifier (ORCID ID):	orcid.org/0000-0002-6191-0433
Person Month Worked:	1.1 cal. mos. yr. 1; 1.0 cal. mos. yr. 2
Contribution to Project:	Coordinates project, assists in designs/analyses, paper preparation
Funding Support:	National Institutes of Health

Name:	Jacob Davis
Project Role: PI	Graduate Student
Research Identifier (ORCID ID):	orcid.org/0000-0002-9293-9853
Person Month Worked:	5 cal. mos. yr. 1; 6 cal. mos. yr. 2
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Travis Johnston
Project Role: PI	Graduate Student
Research Identifier (ORCID ID):	orcid.org/0000-0003-3071-7996
Person Month Worked:	6 cal. mos. yr. 1; 5.75 cal. mos. yr. 2
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	Texas A&M University teaching assistantship

Name:	Rachel Baine
Project Role: PI	Graduate Research Assistant
Research Identifier (ORCID ID):	orcid.org/0000-0002-8576-6368
Person Month Worked:	1 cal. mo. yr. 1; 3.5 cal. mos. yr. 2
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Melissa Henwood
Project Role: PI	Technician
Research Identifier (ORCID ID):	orcid.org/0000-0003-2358-972X
Person Month Worked:	6 cal. mos. yr. 1
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Paris Bean
Project Role: PI	Technician
Research Identifier (ORCID ID):	orcid.org/0000-0002-1268-3588
Person Month Worked:	6 cal. mos. yr. 1; 9.5 cal. mos. yr. 2
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Megan Tarbet
Project Role: PI	Technician
Research Identifier (ORCID ID):	orcid.org/0000-0001-7802-9071
Person Month Worked:	6 cal. mos. yr. 1; 12 cal. mos. yr. 2
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Name:	Emerson Lout
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Project Role: PI	Technician
Research Identifier (ORCID ID):	0000-0001-8144-0486
Person Month Worked:	3 cal. mos. yr. 2
Contribution to Project:	Executes experiments, behavioral testing, hemorrhage assays
Funding Support:	National Institutes of Health

Has there been a Change in Other Support Since the Last Reporting Period?

No

What Other Organizations were Involved as Partners

None

8. SPECIAL REPORTING REQUIREMENTS

Collaborative Awards: None

Quad Chart: Attached

9. APPENDICES

We have attached two peer-reviewed papers that were published in the second year of this project.

Grau, J. W., Baine, R. E., Bean, P. A., Davis, J. A., Fauss, G. N., Henwood, M. K., Hudson, K. E., Johnston, D. T., Tarbet, M. M., & Strain, M. M. (2020). Learning to promote recovery after spinal cord injury. *Experimental Neurology*, 330.

Davis, J. A., Bopp, A. C., Henwood, M. K., Baine, R. E., Cox, C. C., & Grau, J. W. (2020). Pharmacological transection of brain-spinal cord communication blocks pain-induced hemorrhage and locomotor deficits after spinal cord injury. *Journal of Neurotrauma*, 37, 1729-1739.

Pharmacological Transection of Brain–Spinal Cord Communication Blocks Pain-Induced Hemorrhage and Locomotor Deficits after Spinal Cord Injury in Rats

Jacob A. Davis, Anne C. Bopp, Melissa K. Henwood, Rachel E. Baine, Carol C. Cox, and James W. Grau

Abstract

Spinal cord injury (SCI) is often accompanied by additional tissue damage (polytrauma), which engages pain (nociceptive) fibers. Prior research has shown that nociceptive input can increase cell death, expand the area of hemorrhage, and impair long-term recovery. The current study shows that these adverse effects can be blocked by the sodium channel blocker lidocaine applied rostral to a contusion injury. Rats received a lower thoracic (T12) contusion injury, and noxious electrical stimulation (shock) was applied to the tail 24 h later. Immediately before shock treatment, a pharmacological transection was performed by slowly infusing lidocaine at T2. Long-term locomotor recovery was assessed over the next 21 days. Noxious electrical stimulation impaired locomotor recovery, and this effect was blocked by rostral lidocaine. Next, the acute effect of lidocaine was assessed. Tissue was collected 3 h after noxious stimulation, and the extent of hemorrhage was evaluated by assessing hemoglobin content using Western blotting. Nociceptive stimulation increased the extent of hemorrhage. Lidocaine applied at T2 before, but not immediately after, stimulation blocked this effect. A similar pattern of results was observed when lidocaine was applied at the site of injury by means of a lumbar puncture. The results show that a pharmacological transection blocks nociception-induced hemorrhage and exacerbation of locomotor deficits.

Keywords: anesthesia; hemorrhage; lidocaine; pain; spinal cord injury

Introduction

TISSUE LOSS AFTER SPINAL CORD INJURY (SCI) is determined by both the acute primary injury and ongoing processes that expand the area of cell death (secondary injury). This expansion of cell death is characterized, in part, by the breakdown of the blood–spinal cord barrier (BSCB) and inflammation.^{1–3} SCIs are frequently encountered in contexts in which additional injuries (such as broken bones or lacerations) are present.^{4–6} These concurrent injuries (polytrauma) engage pain (nociceptive) fibers, inducing a form of maladaptive plasticity that can undermine long-term recovery.^{7,8} We have modeled this phenomenon by examining the effect of nociceptive stimulation on the development of secondary injury and long-term recovery in rats that have received a lower thoracic (T12) contusion injury. Just 6 min of intermittent electrical stimulation applied to the tail or the leg a day after injury, at an intensity that engages nociceptive fibers,⁹ impairs the recovery of locomotor function.^{7,8} Likewise, application of the irritant capsaicin, which engages unmyelinated nociceptive fibers that express the transient receptor potential vanilloid receptor subtype 1 (TRPV1) receptor after SCI impairs recovery.^{10,11} Further work has shown

that noxious stimulation soon after injury expands the area of tissue loss and fosters the development of chronic pain and spasticity.^{7,8,12}

The adverse effect of noxious stimulation has been related to the acute activation of pro-inflammatory cytokines (tumor necrosis factor [TNF], interleukin-1 β [IL-1 β], and interleukin 18 [IL-18]) and signals related to the initiation of cell death (e.g., caspase 1, 3, 8).^{12–14} More recently, we have discovered that noxious stimulation leads to a breakdown of the BSCB and the influx of blood into the neural tissue at the site of injury.^{15,16} Importantly, this effect was evident across multiple measures of hemorrhage, including absorbance at the wavelength associated with hemoglobin, Western blotting for α -hemoglobin, the Drabkin assay, and histological assessment of blood infiltration. Because blood contents are neurotoxic, this hemorrhage expands the area of secondary tissue loss, and ultimately undermines long-term recovery.^{15,17,18}

We had assumed that nociception-induced hemorrhage after SCI reflects an intraspinal process that is triggered by afferent pain signals. From this perspective, spared fibers and communication with the brain play no role. Indeed, given past work demonstrating that descending fibers can quell over-excitation,^{19–22} we posited that cutting communication with the brain would amplify

nociception-induced hemorrhage in contused rats. Contrary to our expectations, a complete transection of the spinal cord rostral (T2) to the initial contusion injury (T12), blocked nociception-induced hemorrhage and inflammation.^{23,24} The implication is that brain-dependent processes can fuel tissue loss after SCI.

Our research using a T2 transection suggests that the adverse effect of noxious stimulation on tissue sparing, hemorrhage, and long-term recovery depends in part on spared fibers and the engagement of rostral (brain) systems. The procedure is not, however, clinically applicable. To address this issue, we have adopted a common procedure (the local application of the anesthetic lidocaine) to perform a pharmacological transection. Lidocaine acts by blocking Na⁺ channels and is widely used to inhibit neural activity within the spinal cord during labor and after abdominal surgery.^{25–27} Here we show that infusing lidocaine at T2 prior to nociceptive stimulation blocks its adverse effect on long-term recovery. Lidocaine given prior to noxious electrical stimulation (shock) also attenuated hemorrhage, and the activation of pro-inflammatory cytokines at the site of injury. Lidocaine, whether given at T2 or by means of a lumbar puncture, was only effective if given prior to shock.

Methods

Subjects

Sprague–Dawley rats weighing from 300 to 420 g, ordered from Envigo (Houston, TX, USA) were used. Prior to surgery, rats were acclimated to handling and the behavioral apparatus (described subsequently). Rats were pair-housed in a 12 h light–dark cycle setting and allowed food and water *ad libitum*. All experiments were conducted in accordance with National Institutes of Health (NIH) standards for care and use of laboratory animals and guidelines set forth by Texas A&M University with full approval from its Animal Care and Use Committee. Every effort was made to minimize the use and suffering of laboratory animals in the study.

Surgery

All animals received a contusion injury at T12 vertebrae. A 5% isoflurane/oxygen mixture was used to anesthetize animals for surgery. This anesthetic was reduced to 2–3% during surgery. A 3 cm longitudinal incision was made through the skin along the vertebral column. After removing tissue to expose the spinous processes around the surgical site, a laminectomy was performed at T11–T12, exposing the spinal cord. Securing the rat to a MASCIS device²⁸ with clamps at opposite ends of the injury site, a 10 g weight was dropped onto the exposed spinal cord from a height of 12.5 mm. For experiments involving the application of rostral lidocaine, an 18.5 cm catheter was threaded 4 cm rostral to the injury site, placing the tip at T2. The surgical site was closed using Michel clips. Animals were injected intraperitoneally with 3 mL of saline and 100,000 units/kg of penicillin to compensate for fluid loss and to prevent infection, respectively, before being taken to recovery housing overnight.

Nociceptive stimulation

One day after injury, animals were secured in an opaque Plexiglas tube. Nociceptive fibers were engaged using intermittent electrical stimulation (1.5 mA, 100 ms). This type of stimulation engages pain (C, A δ) fibers without inducing tissue damage. In addition, both the onset and offset can be controlled, which allowed us to compare the effectiveness of lidocaine applied before versus after the period of nociceptive stimulation. A constant current AC shock source (660 V AC) was used to generate electrical stimulation, which was applied to the tail through a modified fuse clip

coated with electrode paste (Harvard Apparatus). Pulses of electrical stimulation (100 ms) were applied on a variable schedule (0.2–3.8 sec interstimulus interval). In Experiment 1, stimulation was applied for 30 min, as described in Grau and coworkers.⁸ Because subsequent studies revealed that a briefer period (6 min) of nociceptive stimulation can induce hemorrhage and impair recovery,^{8,29–31} this shock regimen was used in Experiments 2–4. Animals in the no-shock group were treated the same except that shock was omitted.

Sensory testing

Mechanical reactivity was assessed using von Frey filaments, which were applied to the mid-plantar surface of each hindpaw in a serial progression until a flexion response was elicited, as described in a previous study.¹⁹ Filament thickness/force data was transformed using the formula provided by the manufacturer, intensity = log₁₀ (10,000g).³² This allowed for a linear scale that more readily accommodates parametric analyses.

Reactivity to a noxious thermal stimulus was evaluated using a tail flick analgesia meter (IITC #33, IITC Life Science, Inc., Woodland Hills, CA, USA), as described by Hook and coworkers (2009). Briefly, the animal's tail was placed in a groove formed under a halogen lamp. Tail-flick latency was assessed three times at 2 min intervals prior to and after drug treatment. If a response did not occur within 8 sec, the thermal stimulus was terminated to prevent tissue damage.

Drug treatment

Lidocaine was administered at a concentration of 10%. Thirty microliters was applied rostral to injury through the catheter at a rate of 5 μ L per min prior to nociceptive stimulation. Treatment effectiveness was verified by monitoring vocalization to nociceptive stimulation. In the absence of lidocaine treatment, all rats exhibited a brain-dependent response (audible vocalizations) to shock. In every case, lidocaine applied prior to shock treatment blocked this behavioral response. In Experiment 1, the infusion was continued at a slow rate (0.5 μ L/min) for the 30 min of electrical stimulation. For experiments using a briefer (6 min) period of stimulation, pilot data revealed that the initial 30 μ L infusion remained effective and, for this reason, no additional lidocaine was given. Lidocaine was applied at the site of injury using a 25.4 mm, 25 gauge needle that was inserted caudal to the L5 vertebrae. Next, 30 μ L of lidocaine solution (10%) was slowly infused into the space between L5 and L6.

In a prior study, we examined fluid spread after our microinjection procedures by applying a comparable volume of India ink and measuring the extent of staining.³³ We found that the spread was limited to $\sim$ 1 cm along the rostral-caudal axis. The rostral infusion procedure used here yielded a similar pattern of staining, with negligible diffusion along the catheter path. Likewise, when India ink was applied by means of a lumbar puncture, staining was confined to the area of injury. The two treatment procedures also had distinct effects on reactivity to sensory stimulation applied to the tail or hindpaws. Lidocaine applied through a lumbar puncture reduced reactivity to von Frey stimuli applied to the hindpaws (133.2 [% change from baseline]) whereas rostral lidocaine had no effect (109.9%) ($F=4.889$, $p=0.0580$). Likewise, epidural lidocaine inhibited tail withdrawal from radiant heat (123.9% [% change from baseline]) whereas rostral lidocaine (79.8 %) had no effect ($F=9.996$, $p<0.05$).

Assessment of recovery

Locomotor performance was recorded while animals freely moved about an open field. A locomotor behavior score was obtained using the Basso, Beattie, and Bresnahan (BBB) scale¹⁸

during a 4-min observation period by two investigators. Investigators were tested for inter-rater reliability (all $r_s > 0.94$) and blinded to experimental treatment. BBB scores were converted as described previously.³⁴ This conversion improves the metric properties of the behavioral scale and helps assure that the data are amendable to parametric analyses.³⁴ Locomotor behavior was assessed once per day for the 1st week, every other day from the 2nd week, and every 3rd day from the 3rd week. At the end of the recovery period, coordination was assessed using the Beamwalk³⁵ and the Ladder beam tests.³⁶

Tissue collection

Rats were euthanized using a lethal dose of pentobarbital (100 mg/kg). One cm portion of the rat's spinal cord surrounding the injury site was flash frozen in liquid nitrogen. Protein was extracted using the QIAzol lysis reagent protocol. Tissue was processed as per the manufacturer's instructions. A Bradford assay (BioRad, Hercules, CA) was performed to evaluate the concentrations of protein in each sample. Given these concentrations, proteins were diluted in Laemmli sample buffer.

Spectrophotometry

Following protein extraction, a 1.5 μ L portion of protein from each subject's sample was examined through spectrophotometric analysis (Nanodrop, Thermo Scientific). This examined absorbance of light at wavelengths between 200 and 800 nm. Absorbance of light at 420 nm was used as a measure of hemoglobin content for each sample.³⁷

Immunoblotting

Following dilution of samples, gel electrophoresis was conducted using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on samples loaded into 12% pre-cast tris-HCL gels (BioRad, Hercules, CA). Before samples diluted in standard Laemmli buffer solution were loaded, they were heated in 96°C water for 10 min and centrifuged for a quick spin cycle (3–5 sec). After heating, proteins were inserted into Criterion cells. Ten μ L of each sample were loaded into each of the gel wells of for each sample. In addition, 5 μ L of biotinylated ladder (BioRad) was added to the well. Electrophoresis was conducted at 180 V for 1 h.

Proteins were transferred onto a polyvinylidene difluoride membrane (PVDF) (Millipore, Bedford, MA) as instructed by the manufacturer. Following assembly of the gel-cassette complex and its insertion into the transfer tank, the transfer unit was turned on and proteins were transferred for 1 h at 100 V.

After transfer was conducted, blots were blocked for 1 h in 5% blotting grade milk (BioRad, Hercules, CA) diluted in 1% Tris-buffered saline Tween-20 (TBST). Once the blocking phase was complete, blots were incubated overnight in primary antibodies at 4°C overnight on an orbital shaker. The antibodies used were for hemoglobin α chain proteins (1:1000; Abcam [Cambridge, MA] ab92492, RRID: AB10561594), II1B (1:1000; Novus Biologicals [Centennial, CO] NB600-633, RRID: AB10001060), II18 (1:1000; Invitrogen [Carlsbad, CA] PA5-79482, RRID: AB2746598), and Caspase 3 (1:2000; Novus Biologicals [Centennial, CO] NB100-56113, RRID: AB837848).

The next day, membranes were washed three times using TBST for 10 min each at room temperature. Then, membranes were treated with their respective horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (1:5000; Sigma-Aldrich [Saint Louis, MO] ab258649, RRID: AB228341). Following secondary antibody treatment, membranes were washed again three times using TBST for 10 min each at room temperature followed by a rinse with TBS.

Membranes were then developed using an electrochemiluminescence (ECL) substrate kit (Pierce, Rockford, IL) for 1 min.

Following development, membranes were imaged using Fluorchem HD2 (ProteinSimple, Santa Clara, CA).

Experimental designs

To assess whether rostral lidocaine blocks the effect of nociceptive stimulation on long-term recovery, rats received lower thoracic contusion injury. The next day, baseline locomotor performance was evaluated using the BBB scale. Animals were then randomly assigned to one of four experimental groups (vehicle shocked, vehicle unshocked, lidocaine shocked, lidocaine unshocked). Animals received 10% lidocaine or vehicle through the intrathecal catheter at a rate of 5 μ L/min. They were then moved to an opaque Plexiglas tube and restrained with their tails fixed with an electrode. Animals in shocked groups received 30 min of intermittent, uncontrollable electrical stimulation (shocked). Animals in the unshocked groups were treated the same except that shock was withheld. During this period, animals had a maintenance dose of their respective infusion (vehicle or lidocaine) at a rate of 0.5 μ L/min. Following nociceptive stimulation, or an equivalent period of restraint, locomotor performance and weight were monitored over the next 21 days. At the end of the recovery period, additional testing was conducted using the Beamwalk and Ladder beam tests. The complete experiment involved a 2 (lidocaine or vehicle) $\times$ 2 (shock or no shock) design with 10 animals per condition (Fig. 1A).

The impact of rostral lidocaine on acute hemorrhage was assessed 1 day after animals received a contusion injury. After locomotor performance was assessed, animals received 30 μ L of 10% lidocaine or vehicle infused intrathecally. They were then placed in the restraining tubes and given 6 min of electrical stimulation (shocked) or nothing (unshocked). Animals were euthanized 3 h later, and 1 cm of spinal cord tissue enveloping the site of contusion injury was collected. Tissue was processed for protein analysis as described. Spectral analysis was performed targeting a wavelength of light associated with hemoglobin (420 nm) and Western blotting was performed targeting α -hemoglobin. In addition, we assessed the expression of the inflammatory cytokines IL-1B and IL-18. The experiment involved a 2 (shocked or unshocked) $\times$ 2 (lidocaine or vehicle) factorial with 10 animals per condition (Fig. 2A).

A similar procedure was used to assess the effect of lidocaine administered after nociceptive stimulation. A day after a contusion injury, half the animals received 30 μ L of 10% lidocaine, or the vehicle, infused through the implanted catheter at a rate of 5 μ L/min. All animals were then placed in the restraining tubes and given nociceptive stimulation for 6 min. Immediately after, animals that had not received an infusion were given 30 μ L of 10% lidocaine, or the vehicle, infused at a rate of 5 μ L/min. Three hours after nociceptive stimulation, animals were euthanized, and 1 cm of spinal cord tissue enveloping the site of contusion injury was collected. Tissue was processed for hemoglobin content as described. The experiment involved a 2 (lidocaine or vehicle) $\times$ 2 (before or after) factorial design with 8 animals per condition (Fig. 3A).

The last experiment compared the effect of lidocaine given before, versus after, nociceptive stimulation using a lumbar puncture. One day after a contusion injury, half the animals received 30 μ L of 10% lidocaine, or the vehicle, given by means of a lumbar puncture. All animals were then placed in the restraining tubes and nociceptive stimulation was applied for 6 min. Immediately after, animals that had not received an infusion were given 30 μ L of 10% lidocaine, or the vehicle, by means of a lumbar puncture. Three hours after nociceptive stimulation, animals were euthanized, and 1 cm of spinal cord tissue enveloping the site of contusion injury was collected. Tissue was processed for hemoglobin content as described. The experiment involved a 2 (lidocaine or vehicle) $\times$ 2 (before or after) factorial design with 8 animals per condition (Fig. 4A).

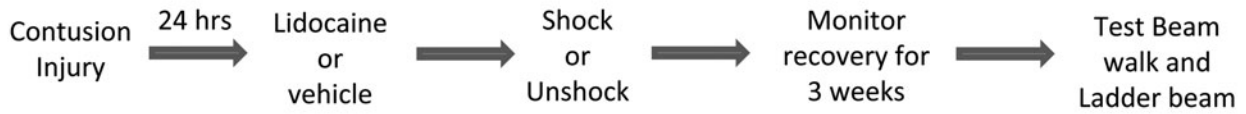
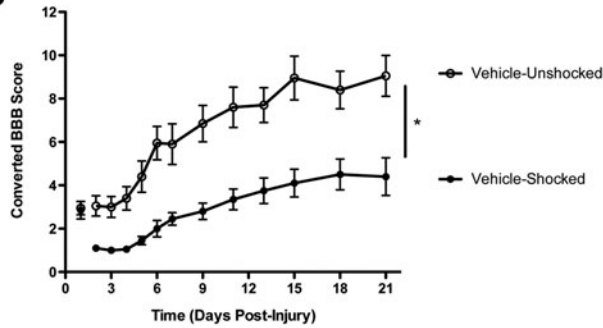
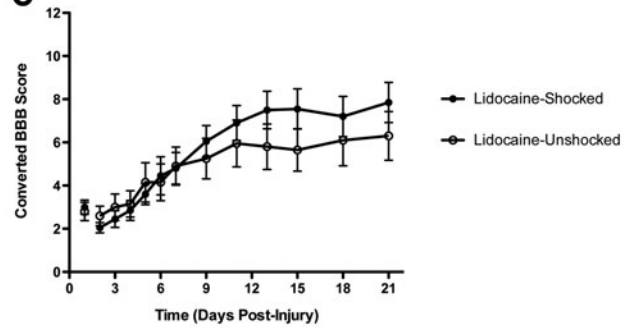
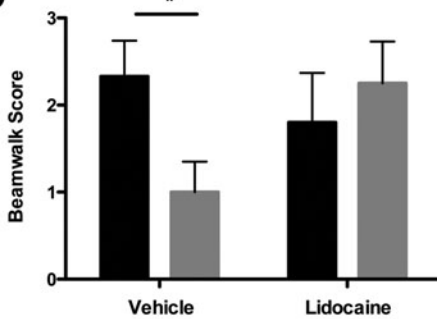
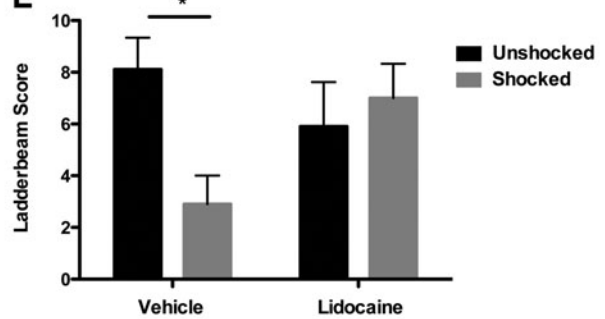
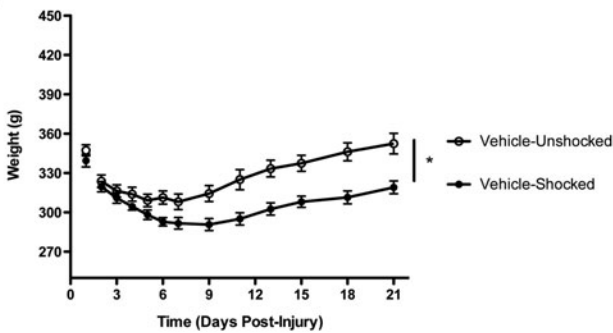
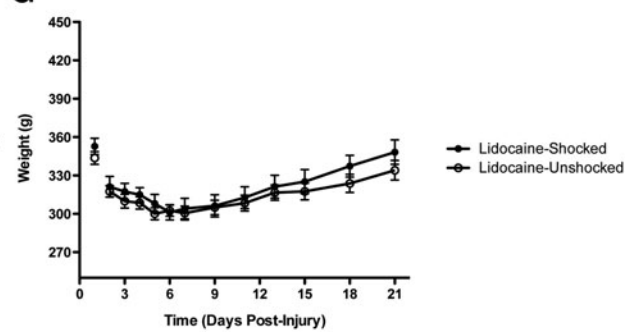
A**Lidocaine applied at T2****B****C****D****E****F****G**

FIG. 1. Rostral lidocaine attenuates the adverse effect that nociceptive stimulation (shock) has on long-term recovery. (A) Experimental design. (B) Unanesthetized (vehicle) animals that received shock exhibited poor locomotor recovery, assessed using the Basso, Beattie, and Bresnahan (BBB) scale. (C) Application of lidocaine at T2 prior to shock treatment attenuated its effect on locomotor recovery. Nociceptive stimulation impaired performance on the (D) Beamwalk and (E) ladder tasks; anesthesia blocks these pain-induced effects. (F) Vehicle-treated shocked animals showed diminished weight relative to unshocked controls. (G) Shock had no significant impact on weight in lidocaine-treated animals. *Indicates statistical significance ($p > 0.05$). Error bars represent standard error of the mean ($n = 10$).

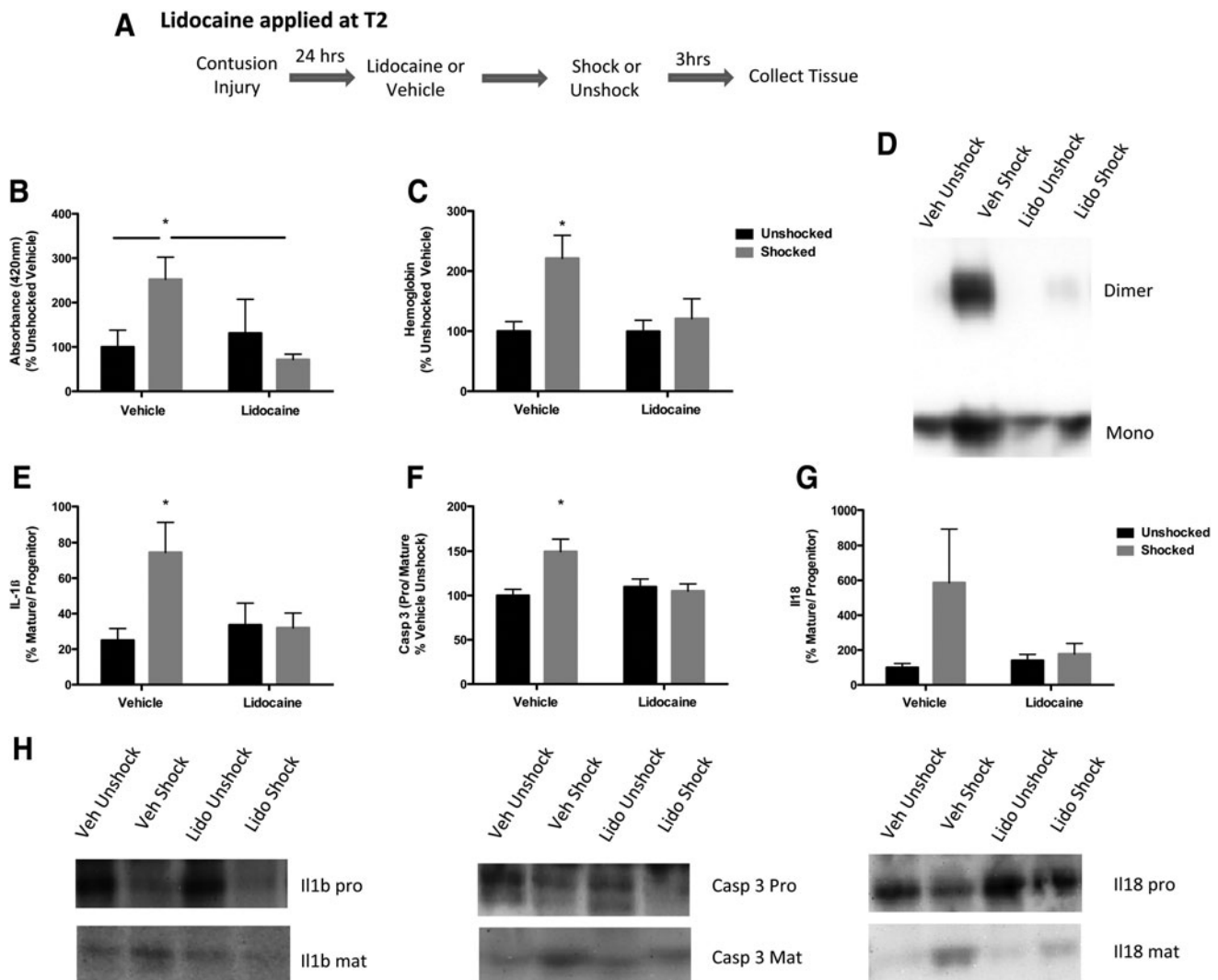


FIG. 2. Rostral lidocaine attenuates nociception-induced hemorrhage and the expression of pro-inflammatory cytokines. (A) Experimental design. (B) Protein samples from vehicle-treated animals given shock exhibited increased absorbance at 420 nm, the wavelength associated with hemoglobin. This effect was attenuated by rostral lidocaine. (C) Western blotting for α -hemoglobin confirmed that shock increased hemoglobin content in the vehicle, but not lidocaine, treated rats. (D) Representative blots. Western blotting for (E) Interleukin (IL)-1B, (F) Caspase 3, and (G) IL-18 showed increased protein expression in vehicle-shocked, but not lidocaine-shocked, rats. (H) Representative blots. *Indicates statistical significance ($p < 0.05$). Error bars represent standard error of the mean ($n = 10$).

Statistical analysis

Data were analyzed with analysis of variance (ANOVA). *Post-hoc* comparisons were performed using Duncan's New Multiple Range test. In all cases, a criterion of $p < 0.05$ was set as the threshold for statistical significance. For our primary measure, we also report the proportion of variance accounted for η^2 .

Results

Experiment 1: Rostral lidocaine blocks the effect of nociceptive stimulation on locomotor recovery

Prior work has shown that nociceptive stimulation undermines recovery after SCI.^{7,8} Here, we test whether interrupting communication with the brain by slowly infusing lidocaine rostral to the injury attenuates this effect.

Locomotor recovery was evaluated using the BBB scale. In vehicle-treated rats, exposure to shock impaired locomotor recovery (Fig. 1B). This shock-induced deficit was blocked by rostral lidocaine (Fig. 1C). An ANOVA confirmed that locomotor performance did not differ prior to shock treatment ($F < 1.0$, $p > 0.05$). An analysis of covariance (ANCOVA) performed on BBB scores from day 2 to day 21, using the day 1 score as a covariate, revealed a main effect of shock treatment ($F = 6.107$, $p < 0.005$; $\eta^2 = 0.549$) and a significant shock by drug treatment interaction ($F = 9.295$, $p < 0.05$). Locomotor performance generally improved over the recovery period ($F = 9.301$, $p < 0.0001$) and the extent of recovery depended upon both drug and shock treatment ($F = 5.253$, $p < 0.0001$). No other term approached statistical significance (all F s < 1.0 , $p > 0.05$). *Post-hoc* comparison of group means shows that saline shocked group differed from the other three ($p < 0.05$). No other group difference was significant ($p > 0.05$).

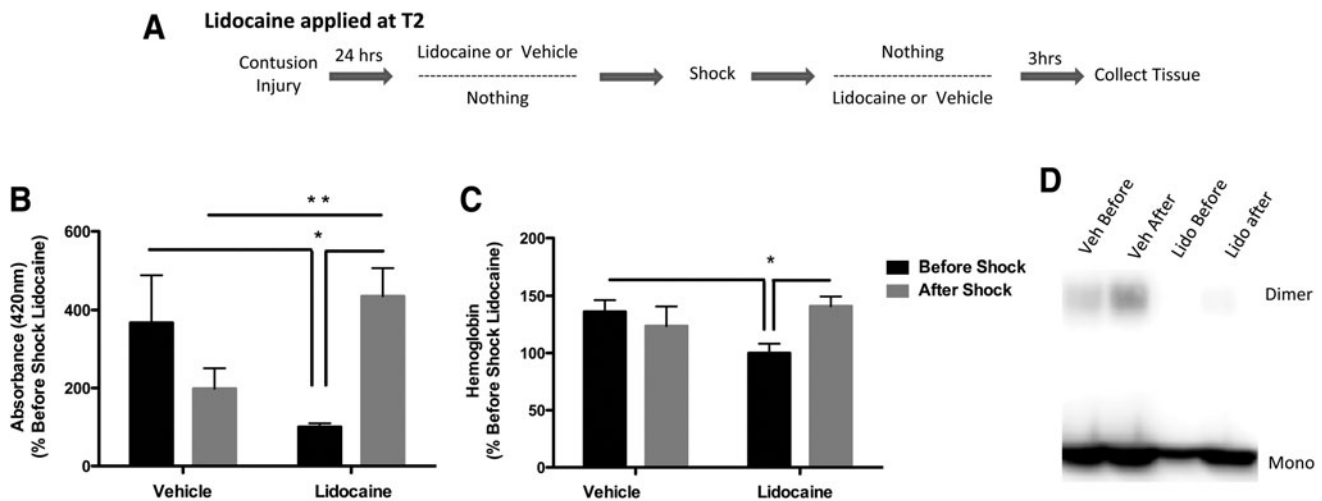


FIG. 3. Rostral lidocaine prior to, but not after, nociceptive stimulation attenuates hemorrhage. **(A)** Experimental design. **(B)** Rostral lidocaine reduced absorbance at the wavelength associated with hemoglobin (420 nm) when administered before, but not after, shock. **(C)** Hemoglobin measured by Western blotting revealed a similar pattern of results. **(D)** Representative bands for hemoglobin Western. *Indicates statistical significance ($p > 0.05$). Error bars represent standard error of the mean ($n = 8$).

To further evaluate locomotor performance across groups, animals were subject to Beamwalk and the Ladder beam tasks (Fig. 1D, E). Rats that had received the vehicle prior to shock treatment exhibited poor performance on both tasks relative to the vehicle-treated unshocked controls. Shock had no effect on performance in rats that received lidocaine. For the Beamwalk task, the interaction between drug administration and shock approached significance ($F = 3.375$, $p = 0.07$). No other term reached significance (both F s < 1.0 , $p > 0.05$). For the ladder task, the interaction was significant ($F = 4.71$, $p < 0.05$, $\eta^2 = 0.1117$). No other term reached significance ($F < 1.997$, $p < 0.05$). In both cases, *post-hoc* comparisons of the group means showed that saline shocked animals differed from saline unshocked animals ($p < 0.05$).

In addition to behavioral recovery, measurements of long-term health were also examined across time. Vehicle-treated animals that received shock displayed poor recovery of weight relative to the un-

shocked group (Fig. 1F). This effect was not observed in animals that received lidocaine prior to shock treatment (Fig. 1G). An ANOVA confirmed that the groups did not differ prior to treatment ($F = 1.083$, $p > 0.05$). After shock and drug treatment (days 2–21), both the main effect of shock, and its interaction with lidocaine treatment, were statistically significant (both F s > 2.860 , $p < 0.05$, $\eta^2 = 0.0271$). No other term reached significance (all F s > 2.765 , $p > 0.05$). *Post-hoc* comparisons of the group means showed that vehicle-treated shocked group differed from unshocked controls ($p < 0.05$).

Experiment 2: Rostral lidocaine blocks expression of shock-induced hemorrhage and inflammation

We have shown that uncontrollable nociceptive stimulation expands the area of hemorrhage at the site of injury, and that this effect is blocked by a rostral (T2) transection.^{29,30} The present

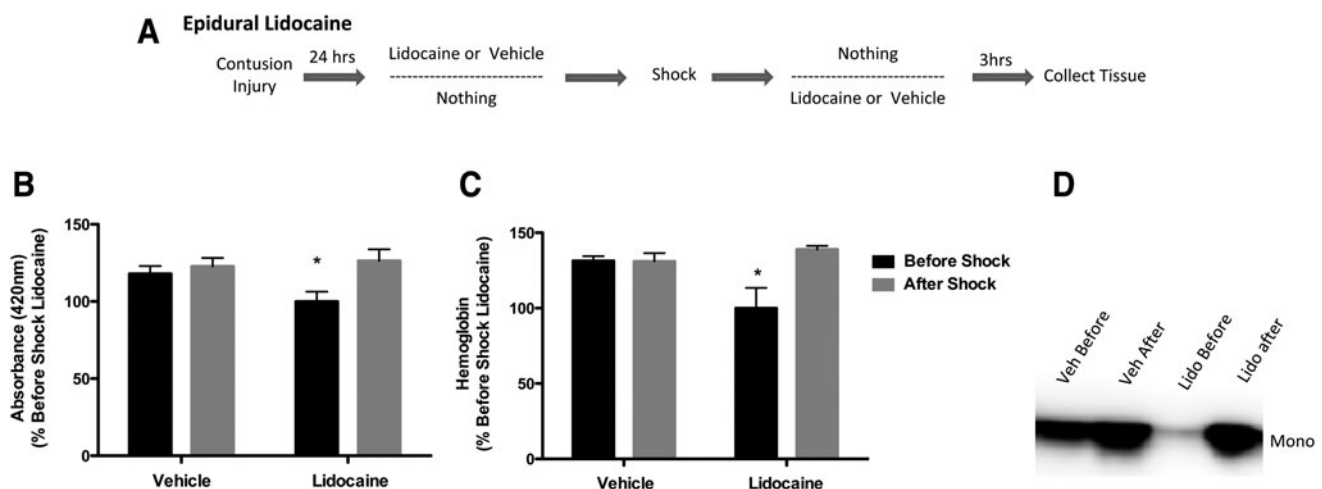


FIG. 4. Epidural lidocaine prior to, but not after, nociceptive stimulation attenuates hemorrhage. **(A)** Experimental design. **(B)** Absorbance at the wavelength associated with hemoglobin showed that lidocaine given before, but not after, shock attenuated hemorrhage. **(C)** Western blotting for α -hemoglobin revealed a similar pattern of results. **(D)** Representative bands for hemoglobin Western. *Indicates statistical significance ($p > 0.05$). Error bars represent standard error of the mean ($n = 8$).

experiment examines whether a pharmacological transection has the same effect. Hemorrhage was first analyzed by spectrophotometry at a wavelength associated with hemoglobin absorbance (420 nm),³⁷ and then confirmed by Western blotting targeting α -hemoglobin. In addition, we assessed the expression of IL-1 β , IL-18, and caspase 3.

Spectrophotometry of protein samples revealed a protective effect of lidocaine against hemorrhage. This effect was attenuated by lidocaine infusion (Fig. 2B). An ANOVA confirmed that the effect of shock treatment depended on whether lidocaine was administered ($F=4.480$, $p<0.05$, $\eta^2=0.103$). No other terms were significant (both $F_s<2.242$, $p>0.05$). *Post-hoc* comparisons revealed that vehicle-treated shocked animals differed from both the shocked and unshocked lidocaine-treated groups ($p<0.05$).

Western blotting targeting α -hemoglobin yielded a similar pattern of results. Animals treated with shock had higher levels of hemoglobin at the site of injury (Fig. 2C, D). Shock had little effect in animals that had received lidocaine. An ANOVA revealed a main effect of shock treatment ($F=6.368$, $p<0.05$). The main effect of drug administration and its interaction with shock approached significance (both $F_s>3.123$, $p>0.0857$). *Post-hoc* comparisons of the group means showed that animals given a combination of vehicle infusion and shock treatment differed from all other groups ($p<0.05$).

We have previously shown that nociceptive stimulation increases the expression of pro-inflammatory cytokines at the site of injury, and that this effect is blocked by epidural lidocaine.³⁸ Here, we examined the effect of rostral delivery of lidocaine on shock-induced expression of IL-1 β (2E), caspase 3 (2F), and IL-18 (2G) with Western blotting (2H). Our aim was to ascertain whether a pharmacological transection has the same effect as a surgical transection and for this purpose, we selected two upstream cell signals (IL-1 β and IL-18) and a common marker of cell death (caspase 3). Nociceptive stimulation increased the expression of IL-1 β , caspase 3, and IL-18, and these effects were blocked by rostral lidocaine. Independent ANOVAs revealed a main effect of shock and an interaction between drug administration and shock treatment (all $F_s<5.286$, $p<0.05$). For caspase 3, there was also a marginal effect of drug treatment *per se* ($F=3.106$, $p=0.0865$). No other comparison approached significance ($p>0.05$). *Post-hoc* comparisons of the group means showed that vehicle shocked animals differed from the other three groups ($p<0.05$).

Experiment 3: Rostral lidocaine does not block hemorrhage when delivered after shock

Lidocaine applied rostral to injury would disrupt both pain signaling to the brain and descending fibers that could fuel hemorrhage. The latter process could continue after the period of nociceptive stimulation. The present experiment examined this possibility by testing whether lidocaine applied immediately after the period of stimulation has a restorative effect.

As in Experiment 2, we first assessed hemoglobin content using spectrophotometry. Animals given shock and the vehicle exhibited greater absorbance at 420 nm, the wavelength associated with hemoglobin (Fig. 3B). Lidocaine blocked hemorrhage when given before, but not after, nociceptive stimulation. An ANOVA yielded a significant interaction between drug administration and timing of infusion (before vs. after) ($F=11.102$, $p<0.05$, $\eta^2=0.2752$). No other comparison reached statistical significance ($p>0.05$). *Post-hoc* comparisons showed that the lidocaine before shock group differed from the vehicle before and lidocaine after groups. In

addition, the vehicle before group differed from the lidocaine after shock group ($p<0.05$).

Western blotting was performed to corroborate observations from spectrophotometry. Hemoglobin content was reduced when lidocaine was given before, but not after, shock treatment (Fig. 3C, D). An ANOVA revealed an interaction between drug administration and timing of infusion ($F=7.055$, $p<0.05$, $\eta^2=0.1846$). *Post-hoc* comparisons of the group means confirmed that animals given lidocaine before shock treatment differed from the groups given vehicle before shock and given lidocaine after shock treatment ($p<0.05$). No other comparison reached statistical significance ($p>0.05$).

Experiment 4: Epidural lidocaine does not block hemorrhage when delivered after shock

Prior work has shown that lidocaine applied at the site of injury prior to nociceptive stimulation has a protective effect.³⁸ This treatment would be expected to block pain signaling to the brain and quiet neural activity at the site of injury. The latter effect could allow epidural lidocaine to have a protective effect when applied after noxious stimulation. The present experiment explores this possibility by comparing the effect of lidocaine applied using a lumbar puncture prior to, or after, shock treatment.

Analysis of the protein samples using spectrophotometry confirmed that epidural lidocaine attenuated hemoglobin absorbance when given before shock. However, lidocaine had no effect when delivered after shock stimulation (Fig. 4B). An ANOVA showed a main effect of timing of injection ($F=6.353$, $p<0.05$). *Post-hoc* comparison of the group means revealed that the lidocaine before shock group differed from the other groups ($p<0.05$). No other term was statistically significant ($p>0.05$).

A similar pattern of results was obtained using Western blotting for α -hemoglobin (Fig. 4C, D). Epidural lidocaine attenuated the shock-induced increase in hemoglobin when administered before, but not after, shock (Fig. 4C). An ANOVA revealed a main effect of time of injection and a drug by injection time interaction (both $F_s<6.902$, $p<0.05$). *Post-hoc* comparisons showed that the lidocaine before shock group differed from the other three groups ($p<0.05$). No other comparison was statically significant ($p>0.05$).

Discussion

Nociceptive stimulation caudal to a contusive SCI increases tissue loss and impairs long-term recovery.^{7,8,10,12,29} Prior work has tied these adverse effects to a pain-induced increase in the area of hemorrhage, and has shown that the local (epidural) application of lidocaine blocks these effects.³⁸ We assumed that epidural lidocaine has a protective effect because it counters nociception-induced neural/cellular activity at the site of injury. The present results show that lidocaine is equally effective when applied rostral to the site of injury. The findings suggest an alternative view of how lidocaine exerts a protective effect, and have implications for clinical application.

We chose to apply lidocaine at T2 based on prior work examining the effect of a spinal transection.²³ Our aim was to apply our experimental treatment as rostral as possible without inducing undesired side effects (e.g., fluid spread to the area of injury; impaired breathing). An assessment of fluid spread confirmed limited diffusion (see Methods). More importantly, we verified that lidocaine applied at T2, versus a lumbar puncture, had a distinct functional effect. As expected, lidocaine applied in either fashion blocked brain-dependent responses (e.g., vocalization) to nociceptive stimulation. However, spinally mediated responses to

nociceptive stimulation applied to the paw or tail were only affected by lidocaine given by means of a lumbar puncture. Because lidocaine applied in this manner should inhibit both neural activity in this region and afferent input, it is not surprising that lumbar application blocks pain-induced tissue loss and cytokine expression after SCI. What is surprising is that remote application of lidocaine, which has no apparent effect on these caudal circuits, has the same effect. The implication is that brain dependent processes fuel tissue loss after SCI.

We previously showed that lidocaine given by means of a lumbar puncture blocks the adverse effect that pain input has on recovery after a contusion injury. Lidocaine applied at T2 had the same protective effect. To explore whether this protective effect was related to an attenuation of hemorrhage, we collected tissue 3 h after the application of nociceptive stimulation. As previously reported, noxious electrical stimulation increased blood content at the site of injury in vehicle controls. We also observed increased expression of proinflammatory cytokines (IL-1 β and IL-18) and caspase 3, and these effects were blocked by rostral lidocaine.

Just 6 min of nociceptive stimulation was sufficient to induce hemorrhage at the site of injury. To evaluate whether this effect stems from the immediate effect of stimulation, or an ongoing process that continues after pain input has ended, we administered lidocaine after shock treatment. We found that lidocaine applied rostral to injury is only effective when given prior to stimulation. A similar pattern of results was obtained when lidocaine was administered at the site of injury by means of a lumbar puncture. These observations imply that pain input can rapidly, within minutes of application, initiate an irreversible process that expands the area of tissue loss.

Deleterious effects of nociception after SCI depend on brain systems

Our results suggest an alternative view for how lidocaine blocks the adverse effect that pain input has on tissue sparing after SCI. Previously, it was assumed that the drug was effective because it inhibited cellular activity at the site of injury.³⁸ Although such an effect may bring some benefit, the current results imply that this is not essential. Indeed, the most parsimonious explanation is that lidocaine has a protective effect because it disrupts communication with the brain.

Our results are consistent with prior studies demonstrating that a complete transection rostral to a lower thoracic contusion injury blocks nociception-induced hemorrhage.³⁹ Although studies using a complete transection provide evidence that brain–spinal cord communication plays a role, the surgery disrupts blood flow and can have secondary effects (e.g., spinal shock) that complicate interpretation. In addition, the procedure is not clinically relevant and cannot be used to assess the effect of treatment on long-term recovery. The present results provide converging evidence that the adverse effect of nociceptive stimulation depends on spared fibers using a clinically relevant treatment procedure. Although questions might be raised regarding the completeness of the pharmacological block (relative to a complete surgical transection), it is clear that the procedure is sufficient to block the adverse effect that stimulation has on tissue loss and long-term recovery.

We previously showed that nociceptive stimulation increases the expression of proinflammatory cytokines (e.g., IL-1 β , IL-18) and signals related to cell death (e.g., caspase 3) at the site of injury.¹² Although epidural administration of lidocaine has been shown to block these effects, this could reflect the direct inhibition of neu-

ral/cellular activity at the site of injury. The present results demonstrate that lidocaine applied rostral to injury has the same effect. So too does a T2 transection.²⁴ Disrupting communication with the brain also attenuates nociception-induced expression of TNF and caspase 1 at the site of injury.³⁹

Peripheral application of the irritant capsaicin also increases the area of hemorrhage in SCI rats.¹⁰ Cutting communication with the brain by means of a T2 transection blocks this effect.²⁴ Taken together, the results suggest that the adverse effect that noxious stimulation has on tissue sparing after SCI is not a direct (spinally mediated) consequence of the afferent input alone. Rather, it depends on spared fibers and the engagement of a brain-dependent process. Although the traditional view of secondary injury has focused on local processes, our data imply that a more molar approach is required, which considers the role of supraspinal and systemic processes.

Underlying mechanisms

To elucidate the stimulus conditions that impact tissue sparing after SCI, and to flesh out the clinical relevance of this line of work, we have explored the stimulus conditions that induce hemorrhage and impair long-term recovery. We found that just 72 sec of intermittent electrical stimulation induces hemorrhage and impairs long-term recovery.⁴⁰ Likewise, although the present study used relatively intense (1.5 mA) shocks, less intense stimulation (0.5) has a comparable effect.

Other studies have explored the cellular processes that contribute to the acute effect of nociceptive stimulation,¹⁰ relating the infiltration of blood to the phenomenon of progressive hemorrhagic necrosis (PHN).⁴¹ PHN is tied to the expression of sulfonyleurea receptor 1/transient receptor potential melastatin 4 (Sur1-Trpm4) channels in endothelial cells, which triggers necrotic cell death and capillary fragmentation. This breakdown in the capillary structure allows blood to infiltrate into the neural tissue at the site of injury, which expands the area of tissue loss (secondary injury). We have suggested that nociceptive stimulation has an adverse effect because it fuels PHN, upregulating the expression of Sur1-Trpm4 and the area of capillary fragmentation.¹⁰ The present results expand our understanding of this effect by showing that the expansion of PHN does not depend on local processes at the site of injury alone and that a brain dependent process provides an essential fuel.

The recognition that brain systems are involved raises a number of inter-related questions. First, there is the question of what spared fibers are essential, and how do they contribute to the expansion of tissue loss. What is clear is that ascending sensory fibers must play an essential role. Indeed, the degree to which the underlying brain systems are engaged is presumably proportional to the preservation of ascending sensory fibers; as damage increases, brain systems should be less activated by pain input, reducing the extent to which these processes drive PHN. As predicted, Strain and coworkers (2019) found that the magnitude of nociception-induced hemorrhage is inversely proportional to injury severity.

A second question concerns the role of the underlying brain processes. In all of the experiments reported to date, noxious stimulation was applied caudal to SCI. With a light to moderate injury, this should engage cellular processes at the site of injury and ascending sensory fibers. It is possible that the former effect plays no role, and that engaging brain-dependent systems with painful stimulation applied rostral to injury would be sufficient to fuel hemorrhage and tissue loss. To explore this possibility, we compared the effect of electrical stimulation applied below (to the

hindlimb) versus above (to the forelimb) a T12 contusion injury. Only the former induced hemorrhage and impaired long-term recovery.⁴² This implies that the afferent input must initiate a process at the site of injury that sets the stage for hemorrhage; that engaging brain processes is necessary, but not sufficient.

A final key issue concerns the brain-dependent processes that drive tissue loss after SCI in response to nociceptive input. An obvious possibility is that this is related to *perceived* pain. Such a view predicts that an analgesic, such as morphine, should attenuate nociception-induced hemorrhage. We evaluated this possibility using a systemic dose of morphine (20 mg/kg) that blocks behavioral reactivity to noxious electrical stimulation.³⁸ Morphine did not attenuate either nociception-induced hemorrhage or its adverse effect on long-term recovery.^{38,43} Indeed, local (intrathecal) application of morphine *per se* had an adverse effect on locomotor recovery.⁴³ These observations suggest that the expansion of hemorrhage after SCI is not related to psychological pain. The corollary to this is that nociceptive input has an adverse effect because it engages an alternative process, perhaps related to stress, anxiety, or fear. These processes could potentially foster hemorrhage by driving an increase in systolic blood pressure. This is consistent with prior studies, which have shown that a spike in blood pressure is associated with poor recovery after SCI.^{44,45} Supporting this hypothesis, recent work has shown that intermittent electrical stimulation induces a prolonged increase in systolic blood pressure and that this effect is blocked by spinal transection.^{42,46} This increase in blood pressure could drive hemorrhage by inducing perfusion in already weakened blood vessels at the site of SCI.⁴¹ From this perspective, nociceptive input may initiate processes at the site of injury (e.g., the upregulation of Sur1-Trpm4 on endothelial cells and capillary fragmentation) that weaken the BSCB. In this vulnerable state, a surge in blood pressure could expand the area of hemorrhage.

Generality and clinical implications

The current study has implications for both the processes that affect tissue loss after SCI and clinical treatment. Regarding the underlying mechanisms, it has long been recognized that processes triggered by physical injury of the spinal cord engage physiological changes (inflammation, a breakdown in the BSCB) that can increase the area of cell death, yielding an envelope of secondary injury that can double the volume of tissue loss.^{1,2,3,11,14,16} Recognizing that this is a tractable clinical target, researchers have worked to identify the factors that fuel secondary injury and treatments to counter it.^{23,24,38,42,46} In general, the focus has been on local processes that target the site of injury. Indeed, our work demonstrating that the adverse effect of nociceptive input can be blocked by the application of an anesthetic at, and caudal to, the site of injury was motivated by this line of thought.³⁸ What we had not recognized is that extrinsic processes played a critical role, engaged by spared nerve fibers. What we have discovered is that disrupting communication with the brain, by means of a surgical or pharmacological transection, attenuates pain-induced hemorrhage.^{23,24} These manipulations also reduce inflammation and indices of cell death (e.g., expression of caspase 3). The implication is that supraspinal (brain) systems fuel these processes, effects that may be secondary to the influx of blood (hemorrhage). This work refocuses attention on an old issue, how hemorrhage fuels cell loss after SCI.^{24,42}

Regarding treatment, the findings imply that the application of a local anesthetic, either at the site of injury or rostral to it, can reduce tissue damage after SCI. As discussed, a protective effect may be

especially evident when the injury is incomplete (with some spared fibers) and there is other tissue damage (polytrauma). A benefit to this approach is that blocking communication with the brain will inhibit pain signaling, which would reduce the need for opiate analgesics. Some caution is warranted, however, because lidocaine is not hemodynamically neutral and could, in and of itself, have an effect on tissue perfusion. Although this was not statistically significant, unshocked lidocaine treated rats appeared to exhibit some decrement in recovery relative to the vehicle controls in Experiment 1. Further work will be needed to assess the safety of this approach.

Our work implies that pain input can engage brain-dependent processes that fuel tissue loss. If this is true, treatments that generally inhibit brain function could block this process and reduce tissue after SCI. We have explored this possibility by giving contused rats a systemic injection of the anesthetic pentobarbital prior to nociceptive stimulation.⁴⁶ This produces a state akin to a medically induced coma. We found that pentobarbital anesthesia blocks the adverse effect that nociceptive stimulation has on long-term recovery. It also blocks hemorrhage in response to noxious electrical stimulation or application of the irritant capsaicin.

In another line of work, we have evaluated the generality of our effects by testing whether pain input expands the area of hemorrhage in rats that have received a traumatic brain injury (TBI). We have found that it does.⁴⁷ Further work is being done to determine whether this effect too is blocked anesthesia.

Our work is consistent with prior studies demonstrating that nociceptive input can sensitize pain pathways, alter BSCB permeability, and influence recovery after SCI.^{11,12,16,48} Here too, peripheral alterations could fuel cell loss through the sensitization of afferent neural activity and excitability within the dorsal root ganglia.⁴⁸ The findings are also consistent with the emerging view that SCI interacts with systemic processes. For example, SCI-induced gut dysbiosis can exacerbate the inflammatory response at the level of the spinal cord.^{49,50} In addition, nociceptive input could impact tissue loss by engaging the sympathetic nervous system, to drive a rise in blood pressure. This is consistent with prior work showing that a period of hypertension after SCI is associated with poor recovery in an animal model.⁴⁵ Further, we have shown that noxious electrical stimulation induces a rise in blood pressure and that this effect is blocked by both a T2 transection and pentobarbital anesthesia.⁴² The former observation has important mechanistic implications, because it implies that noxious stimulation is not directly engaging sympathetic drive through efferents that exit the spinal cord caudal to T2, as occurs in autonomic dysreflexia.^{51,52} Instead, it appears here that the efferent sympathetic drive is indirect, dependent on the activation of a brain-dependent process. Further work is being conducted to identify the brain systems involved.

Our work has shown that curtailing pain input to the brain can have a prophylactic effect that blocks noxious input's adverse effect on tissue loss after SCI. We have shown that a relatively brief period of noxious input is sufficient to trigger hemorrhage. What is unfortunate is that administration of lidocaine after a period of nociceptive stimulation had no beneficial effect. The implication is that, to be clinically useful, treatment would need to begin soon after injury, to prevent ongoing pain signals from fueling tissue loss over the hours to days that follow injury.

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Author Disclosure Statement

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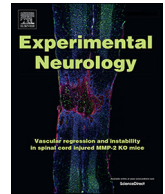
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Review Article

Learning to promote recovery after spinal cord injury

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ABSTRACT

The present review explores the concept of learning within the context of neurorehabilitation after spinal cord injury (SCI). The aim of physical therapy and neurorehabilitation is to bring about a lasting change in function—to encourage learning. Traditionally, it was assumed that the adult spinal cord is hardwired—immutable and incapable of learning. Research has shown that neurons within the lower (lumbosacral) spinal cord can support learning after communication with the brain has been disrupted by means of a thoracic transection. Noxious stimulation can sensitize nociceptive circuits within the spinal cord, engaging signal pathways analogous to those implicated in brain-dependent learning and memory. After a spinal contusion injury, pain input can fuel hemorrhage, increase the area of tissue loss (secondary injury), and undermine long-term recovery. Neurons within the spinal cord are sensitive to environmental relations. This learning has a metaplastic effect that counters neural over-excitation and promotes adaptive learning through an up-regulation of brain-derived neurotrophic factor (BDNF). Exposure to rhythmic stimulation, treadmill training, and cycling also enhances the expression of BDNF and counters the development of nociceptive sensitization. SCI appears to enable plastic potential within the spinal cord by down-regulating the Cl^- co-transporter KCC2, which reduces GABAergic inhibition. This enables learning, but also fuels over-excitation and nociceptive sensitization. Pairing epidural stimulation with activation of motor pathways also promotes recovery after SCI. Stimulating motoneurons in response to activity within the motor cortex, or a targeted muscle, has a similar effect. It is suggested that a neurofunctionalist approach can foster the discovery of processes that impact spinal function and how they may be harnessed to foster recovery after SCI.

1. Introduction

A growing body of research suggests that spinal cord systems are plastic rather than hardwired (Grau et al., 2006; Wolpaw, 2010; Grau, 2014). This has important implications for how we conduct our science and deliver treatment to those suffering from spinal cord injury (SCI). If sensory/motor programs within the spinal cord are hardwired, with no capacity to change, restoring function will hinge upon surviving fibers and rewiring the system through neural regeneration. And for the latter, a neural bridge would need to innervate the surviving tissue in a

manner that duplicates that which was lost. If the system is hardwired and immutable, we may be able to engage surviving circuits during physical therapy, but the training would have no lasting effect.

In this article, we review data that supports the alternative perspective that assumes spinal circuits and function can be modified through training and experience. Recent data suggest that SCI enables plasticity by fundamentally altering the neural context caudal to injury, removing a GABA-dependent brake on neural excitation and plasticity (Grau and Huang, 2018). This alteration presents both opportunities and challenges. On the positive side, plasticity allows

Abbreviations: 5HT, 5-hydroxytryptamine (serotonin); AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; BDNF, brain-derived neurotrophic factor; CPG, central pattern generator; CR, conditioned response; CS, conditioned stimulus; DLF, dorsal lateral funiculus; EMG, electromyography; FT, fixed time; GABA, γ -aminobutyric acid; GAD₆₇, glutamic acid decarboxase₆₇; H-reflex, Hoffman-reflex; IL-1 β , interleukin 1 beta; IL-18, interleukin 18; i.t., intrathecal; KCC2, K^+ - Cl^- cotransporter 2; L, lumbar; NKCC1, Na^+ - K^+ - Cl^- cotransporter 1; MEP, motor evoked potential; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; O, outcome; PLC γ , phospholipase C gamma; R, response; S, stimulus; SCI, spinal cord injury; S-R, stimulus-response; T, thoracic; TNF, tumor necrosis factor; TrkB-IgG, tropo- myosin-related kinase B-immunoglobulin G; TRPV1, transient receptor potential cation channel subfamily V member 1; UR, unconditioned response; US, unconditioned stimulus; VT, variable time

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neurorehabilitation and physical therapy (PT) to have a lasting effect (Edgerton et al., 2004; Harel and Carmel, 2016; Chen et al., 2018; Courtine and Sofroniew, 2019). At the same time, the enhancement in plastic potential places the system in a vulnerable state, wherein bouts of noxious stimulation can have unintended consequences, fueling an over-excitation that can drive pain and spasticity (Cramer et al., 2008; Boulenguez et al., 2010; Edgerton and Roy, 2010; Ferguson et al., 2012; Huang et al., 2016).

Elsewhere, we have reviewed how the study of spinal cord plasticity informs the field of learning (Grau, 2014), how pain (nociceptive) input impacts recovery (Grau et al., 2017), and how neurobiological mechanisms enable plasticity (Grau and Huang, 2018). Here, we explore the implications of this work for neurorehabilitation. As defined by Barrett, neurorehabilitation involves a broad range of treatments, to include: “neural regeneration, repair, and dynamic reorganization of functional neural systems, as well as increasing awareness of behavioral principles that may support best return to function and freedom, brought forward treatments based on experience-dependent learning, neurophysiologic stimulation, and a combination of these concepts” (Barrett et al., 2013). The present review focuses upon the role of learning. We discuss how the study of learning can help to elucidate the circumstances that encourage plasticity, its functional consequences, and inform our analysis of how training impacts spinal cord function.

2. The process of learning

2.1. Learning within the context of neurorehabilitation

Our central thesis is that concepts from the field of learning (Fig. 1) can help researchers and clinicians discover, characterize, and implement principles to optimize the effectiveness of physical therapy and neurorehabilitation. Treatments after SCI aim to create a lasting change, engaging processes that will promote behavioral and physiological function. The aim is to *retrain* the nervous system, stimulating a form of learning that restructures the surviving nervous system and promotes new neuronal connections.

At a functional level, learning allows the organism to encode environmental stimuli and relations, structure behavior, and predict future events. From this perspective, learning is engaged when the system is challenged by an environmental puzzle—an event or relation that was not anticipated. In this way, the process of learning can serve as a form of error correction, a system engaged when the internal model of the world deviates from reality (Rescorla, 1988).

There are a number of ways to characterize the processes that mediate learning. Traditionally, these have been wedded to environmental stimulation and the consequent behavioral relation: does it involve exposure to a single stimulus, the relation between two stimulus events (Pavlovian conditioning), or the relation between a response and an outcome (instrumental conditioning)? In each case, it is typically assumed that an event/relation can be encoded in multiple ways, with the process engaged shaped by evolution and the organism's experiential history (Timberlake and Lucas, 1989; Domjan, 2015). We have suggested that this approach provides a useful framework for characterizing the circumstances that engage a particular process and its function, providing a model to explore the underlying neurobiological mechanisms. To recognize these roots, we coined the term *neurofunctionalism* (Grau and Joynes, 2005a).

A neurofunctionalist approach begins by asking: Under what conditions is a particular process engaged, and how does it impact the organism? The answer to this question is framed in terms of observable events and responses, with the aim of abstracting core principles that describe how the system functions across a range of situations (Fig. 2). Researchers and clinicians seeking to promote recovery after SCI face the same problem of characterizing and defining the stimuli and forms of training that will improve function. In doing so, it is important to distinguish between two types of effects—those that are learned (that

have a lasting effect) and those that are unlearned (unconditioned). To conclude that learning is involved, the stimulation/training must have a lasting effect on function, establishing a kind of *memory*. We suggest that treatments of this kind will have the most value for promoting long-term recovery.

2.2. Defining learning

As Domjan (2015) has noted, learning and memory are like two sides of a coin, one cannot exist without the other. Learning refers to the process(es) used to encode new environmental stimuli and relations, whereas memory is seen as a mechanism that preserves the consequences of learning over time. Embedded within this definition is a key criterion for learning and an important methodological issue. To count as an instance of learning, an experience must have a lasting effect (Fig. 3). At a minimum, it must outlast the training conditions used to induce the behavioral modification, remaining evident when subjects are tested under *common conditions* (Rescorla, 1988; Grau, 2014).

Of course, learning impacts the CNS because it engages a form of plasticity. While it is recognized that non-neuronal cells impact CNS function (Perea et al., 2009; Vichaya et al., 2009), we will focus on neurally-mediated events. And just as it is assumed that a particular environmental puzzle can be solved in multiple ways, a neuro-functional approach assumes that a particular neurobiological process (e.g., NMDA receptor-mediated plasticity) may serve many masters (Grau and Joynes, 2001).

Learning theorists have sought principles that describe how a process works across a range of circumstances. For example, Pavlovian conditioning brings explanatory value by providing a set of rules that help us characterize and understand how an organism encodes stimulus-stimulus (S–S) relations. The same is true for optimizing treatment after SCI; we must discover the forms of stimulation and the training conditions that promote recovery, and these principles must be stated in clinically translatable terms.

2.3. Traditional view: Learning and memory are the province of the brain

Our model of CNS function determines the way that we characterize the influence of learning on recovery. The traditional view assumed that learning is the province of the brain (Patterson, 2001a). Of course, neural systems within the brain are adept at registering stimuli and behavioral relations and can integrate signals across time, space, and modalities. Further, it is evident that brain-dependent learning can impact recovery in multiple ways, bringing alterations in mood, motivation, and systemic processes that will influence long-term health and well being. A perceived lack of control (learned helplessness) can fuel depression, undermine motivation, and engage neurobiological processes that contribute to the maintenance of chronic pain (Maier and Seligman, 2016). Additionally, learning can influence how brain/cortical processes are mapped onto surviving fibers, by expanding the area of innervation and modifying when they are engaged (Knikou, 2012).

Brain-dependent learning can also influence how circuits within the spinal cord operate. For example, animals can be trained using an appetitive reinforcer (e.g., fruit juice) to exhibit a change in the Hoffman-reflex (H-reflex), an electrical analog of the spinal stretch reflex (Wolpaw and Tennissen, 2001). For some animals, fruit juice may be given whenever they exhibit a weaker force (down trained). Over days, the animals learn to maintain this behavioral response to obtain reward. Here, the brain registers the behavioral contingencies and maintains (through descending fibers) the consequent change in H-reflex vigor.

The traditional view of spinal cord function was embedded within a system of hierarchical control, wherein higher-level processes are charged with interpreting sensory signals and issuing motor commands. It was generally assumed that this required a faithful representation of

Allodynia: Pain due to a stimulus that does not normally provoke pain.

Analgesia: An absence of pain in response to stimulation that would normally elicit this state.

Antinociception: A process that counters neural activity in (nociceptive) pathways engaged by noxious stimulation.

Appetitive stimulus: A stimulus that engages approach and that may elicit a pleasant psychological state.

Associationism: The view that knowledge is acquired by linking (associating) discrete psychological elements (e.g., sensations, responses, and their compounds).

Associative learning: A psychological process that has been used to describe how organisms encode the relation between stimulus events and behavior (responses).

Aversive stimulus: A stimulus that engages withdrawal and may elicit an unpleasant psychological state.

Avoidance learning: An instrumental conditioning procedure wherein the performance of a response prevents the delivery of an aversive stimulus.

Backward conditioning: A form of Pavlovian conditioning wherein the conditioned stimulus occurs after the unconditioned stimulus.

Behavioral metaplasticity: A behavioral facilitation (positive transfer) or inhibition (negative transfer) of learning linked to a prior episode of learning.

Biological preparedness (aka belongingness): A process attributed to evolution that tunes the nervous system to foster learning about particular stimuli and relations, and how this learning impacts behavior.

Blocking: A disruption in relational learning that arises when a new cue is presented in compound with one that was previously trained.

Central sensitization: An increase in the responsiveness of nociceptive neurons within the central nervous system.

Conditioned response (CR): A behavioral response elicited by a cue (the conditioned stimulus) attributable to its being paired with a biologically significant event (the unconditioned stimulus).

Conditioned stimulus (CS): A cue in a Pavlovian paradigm that may acquire the capacity to generate a (conditioned) response due to its temporal relation with another event (e.g., the unconditioned stimulus).

Contiguity: The occurrence of two events close together in time and space.

Controllable stimulation: An instrumental learning paradigm wherein the onset and/or offset of an event is tied to a behavioral response.

Delayed conditioning: A form of Pavlovian conditioning in which the conditioned stimulus precedes the unconditioned stimulus and terminates upon its onset or offset.

Devaluation: A procedure used to reduce the biological significance of an event after training has occurred.

Differential conditioning: A form of Pavlovian conditioning in which the same subject experiences one conditioned stimulus (the CS+) paired with the unconditioned stimulus while another (the CS-) is presented in an explicitly unpaired manner.

Discriminative stimulus: A cue that controls the performance of an instrumental response because it signals the availability (or nonavailability) of the outcome.

Disinhibition: Recovery of a habituated response due to the presentation of an extraneous cue.

Escape learning: An instrumental conditioning procedure wherein the performance of a response terminates an aversive stimulus.

Explicitly unpaired control: A procedure wherein a subject repeatedly experiences two cues (e.g., the CS and US) in a manner that assures that they are never paired.

Extinction: The weakening of a previously trained behavioral response due to the withdrawal of the stimulus relation used to establish the behavior.

Fixed time (FT): A training paradigm in which an event is presented repeatedly over time and the interval between presentations remains constant.

Forward conditioning: A form of Pavlovian conditioning wherein the conditioned stimulus is presented before the unconditioned stimulus.

Functionalism: A view that focuses upon what a system is designed (through evolution) to accomplish (i.e., its function), recognizing that a particular capacity can be achieved in multiple ways.

Habituation: A reduction in the behavioral/psychological response to a stimulus due to stimulus exposure.

Hyperalgesia: Increased pain to a stimulus that normally elicits this state.

Hypoalgesia: Reduced pain to a stimulus that normally elicits this state.

Instrumental conditioning: A form of learning that depends upon the temporal relationship between a behavioral response (R) and an environmental outcome (aka reinforcer).

Interstimulus interval (ISI): The temporal gap between two events.

Latent inhibition: A disruption in Pavlovian conditioning attributed to pre-exposure to the conditioned stimulus alone before it is paired with an unconditioned stimulus.

Learned helplessness: A disruption in instrumental learning attributed to prior exposure to an uncontrollable outcome (e.g., variable intermittent electrical stimulation).

Learning: The process that mediates the encoding of environmental events and relations, establishing a memory that preserves the experience over time.

Memory: The retention of an acquired (learned) modification in behavioral/psychological function over time.

Negative transfer: An example of behavioral metaplasticity wherein a prior (distinct) training episode inhibits the capacity to learn.

Neurofunctionalism: An approach that assumes environmental events and behavioral relations can be encoded and executed by multiple processes and that detailing how these systems function will bring explanatory value and facilitate the identification of the underlying neurobiological mechanisms.

Neuropathic pain: Pain caused by damage or disease related to the body, often associated with enhanced pain to touch (allodynia).

Nociception: Neural activity in the pathways that encode noxious stimulation.

Nociceptive stimulus: An event that engages neural pathways designed to detect noxious stimuli.

Noxious stimulation: A stimulus that engages receptors (nociceptors) designed to detect events that induce tissue damage or have the potential to do so.

Non-relational learning (aka single stimulus learning): A form of learning in which exposure to a single cue (simple or complex) brings about a modification in its capacity to elicit a behavioral or psychological response.

Omission training: An instrumental conditioning procedure wherein the outcome is omitted whenever the organism performs the target response.

Operant learning: Skinner's term for examples of instrumental learning that appear less biologically constrained, where the organism can operate on its environment in a flexible manner and performance can be modified using a variety of outcomes.

Outcome (aka reinforcer): A biologically significant event that is contingent upon a behavioral response in an instrumental learning paradigm.

Overshadowing: A disruption in relational learning that arises when a cue is trained in compound with another stimulus that is more salient (noticeable).

Pain: An unpleasant experience tied to actual or potential tissue damage or described in these terms.

Pavlovian (classical) conditioning: A form of learning that depends upon the temporal relationship between two stimulus events.

Performance variable: A factor that affects the organism's propensity/capacity to execute a response (e.g., motivation) independent of whether it was learned or unlearned.

Positive transfer: An example of behavioral metaplasticity wherein a prior (distinct) training episode facilitates the capacity to learn.

Punishment: An instrumental conditioning procedure wherein the performance of the target response leads to the presentation of an aversive stimulus.

Reinforcer: A term used to describe the outcome that brings about a modification of the target response in instrumental and/or operant conditioning.

Reinforcement: A term used to describe the process that mediates the consequence of instrumental and operant learning, that brings about a change in the organism's propensity to perform a behavioral response.

Relational learning: A form of learning that depends upon the relationship between two events.

Respondent: A behavioral response that is reflexively elicited by a particular stimulus. A respondent can involve an innate stimulus-response (S-R) reflex or an acquired (conditioned) response.

Reward training: An instrumental conditioning procedure in which the performance of a response produces an appetitive stimulus.

Sensitization: An increase in the behavioral/psychological response to a stimulus due to stimulus exposure.

Second-order conditioning: Acquisition of a conditioned response to a cue that has paired with a conditioned stimulus that was previously paired with an unconditioned stimulus.

Sensory preconditioning: Acquisition of a conditioned response to a cue that has been paired with a conditioned stimulus that was subsequently paired with an unconditioned stimulus.

Shaping: An instrumental training procedure in which the organism is given an outcome (reinforcer) for performing successive approximations of the target response.

Spontaneous recovery: The recovery of a response after a period of rest.

Stimulus generalization: Responding to a cue that is different from the one used in training.

Stimulus salience: The degree to which a cue engages sensory processing, a characteristic that is often tied to intensity.

Temporal conditioning: A form of learning wherein an internal cue related to the passage of a fixed period of time serves as a stimulus.

Trace conditioning: A form of Pavlovian forward conditioning wherein a temporal gap separates the offset of the conditioned stimulus and the onset of the unconditioned stimulus.

Unconditioned stimulus (US): A biologically significant event that innately elicits a behavioral/psychological response and is capable of supporting Pavlovian conditioning.

Unconditioned response (UR): The response that is innately elicited by an unconditioned stimulus.

Uncontrollable stimulation: An instrumental training paradigm wherein an outcome occurs independent of the animal's behavior.

Variable time (VT): A training paradigm in which an event is presented repeatedly over time and the interval between presentations is varied.

Yoked control: A training paradigm in which the presentation of the stimuli is controlled by another (Master) subject.

Fig. 1. A glossary of relevant terms from the learning and pain literatures. Adapted from Domjan (2015), APA Dictionary of Psychology (<https://dictionary.apa.org>), IASP Terminology (<https://www.iasp-pain.org/Education/Content.aspx?ItemNumber=1698#Nociceptivestimulus>) and the Stanford Encyclopedia of Philosophy (<https://plato.stanford.edu/index.html>).

the world, relayed by afferent pathways in a mechanical manner. Likewise, higher-level processes would need to know that their behavioral orders were reliably executed. A circuit within the spinal cord might organize a response and be sensitive to local perturbances, but this mode of operation would remain constant over time, unchanged by experience. In the present review, we advance a more distributed view of learning that assumes neural function within the spinal cord is adjusted in response to environmental stimulation and relations.

2.4. Challenges to the traditional view

Three sets of observations led researchers to reject the view that the spinal cord is a mechanical system incapable of change. One challenge came from studies demonstrating that brain systems can induce a lasting modification in how spinal circuits operate. For example, Wolpaw and his colleagues used the paradigm described above to train animals to exhibit either an increase or decrease in the magnitude of the H-reflex (Wolpaw and Lee, 1989). After extended training, they eliminated brain-dependent regulation of the spinal H-reflex by transecting the spinal cord. Remarkably, the consequences of training were still evident after the spinal transection, implying that the brain-dependent learning had induced a lasting modification (a kind of memory) within the spinal cord. Another example of how the brain can alter spinal function is provided by the phenomenon of *spinal fixation* (Patterson,

2001b). Localized cerebellar lesions can induce a postural asymmetry wherein animals exhibit a stronger flexion response in one hind leg. If the spinal cord is transected soon after the cerebellar lesion, the postural asymmetry wanes. However, if the spinal cord is transected 1–3 h later, the behavioral modification persists. More recently, Vahdat et al. (2015) showed, using functional magnetic resonance imaging (fMRI), that learning to perform a complex motor sequence is accompanied by a change in the pattern of neural activity elicited within the cervical spinal cord.

A second observation that challenged the traditional view stemmed from studies by Rossignol, Edgerton, and their colleagues, who showed that treadmill training can re-establish a complex behavior (hind limb stepping) after communication with the brain is disrupted by means of a rostral transection (Grillner and Rossignol, 1978; Lovely et al., 1986; Rossignol, 2017). Subsequent studies have linked this rhythmic behavior to an oscillator [central pattern generator (CPG)] in the rostral lumbosacral spinal cord (Cazalets et al., 1995; Calancie et al., 1994; Magnuson et al., 1999). Interestingly, animals can also be trained to stand (Edgerton et al., 1997a). While it might be presumed that each form of training would enable the other, the opposite is observed—stand training interferes with learning to step on a treadmill, and treadmill training interferes with the ability to learn to stand.

A third challenge to the traditional view stemmed from studies examining whether spinal neurons can learn without input from the brain.

A. Modes of Description	B. Application	C. Example
Environmental Stimuli & Behavioral Responses	Training Experience	Exposure to a Response → Outcome relation
Principles/Model, Functional Process	Learning	Controllable stimulation enables learning, Behavioral control counters sensitization
Neurobiological Mechanisms	Neural Plasticity ┌ - Ionic Plasticity + └	Learning depends on the NMDA receptor, BDNF promotes adaptive plasticity

D. An Example in Neurorehabilitation

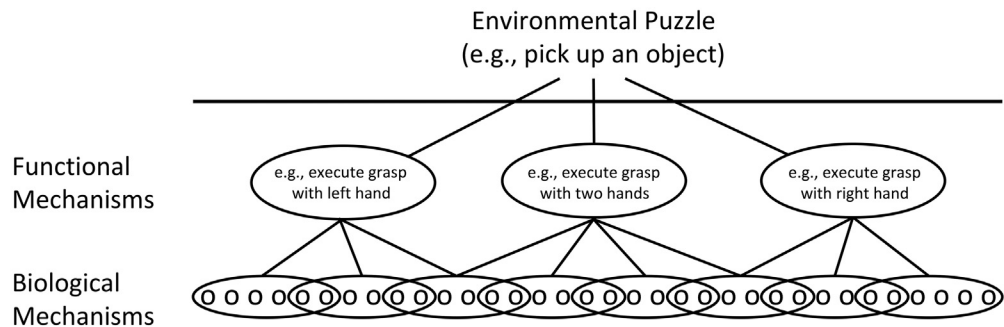


Fig. 2. A framework for learning. (A) A neurofunctionalist approach aims to discover regularities in how the CNS processes environmental events (stimuli) and transforms these into physiological and behavioral responses (Grau and Joynes, 2005a). It is assumed that this processing occurs within an evolved structure designed to perform a particular function. Principles are sought that describe regularities in how events are processed, organized within a model of the system. A thorough description of the rules and eliciting (efficient) conditions provides a framework to explore the underlying neurobiological mechanisms. (B) The present review focuses on how a training experience engenders the process of learning. It is suggested that the potential for change within the CNS (neural plasticity) is regulated by GABA (ionic plasticity) (Rivera et al., 2005). (C) As an example, the spinal cord is sensitive to response→outcome (instrumental) relations. Instrumental conditioning engages a process that fosters learning and counters the development of nociceptive sensitization. These processes have been related to NMDA receptor-mediated plasticity and the expression of the neurotrophin BDNF (Joynes et al., 2003; Huie et al., 2012b). (D) A neurofunctionalist approach to the loss of hand function after neural injury would characterize how the individual recovers the capacity to grasp an object, recognizing this can be accomplished by either hand in multiple ways and that these alternative solutions depend upon both unique and common biological processes.

To explore this issue, researchers routinely perform a complete SCI (e.g., a thoracic transection) and examine the capacity to learn in animals using stimuli applied to the hind limbs and tail. As reviewed below, this line of work has revealed that stimulus exposure (non-relational learning) can have a lasting effect on spinal function (Thompson and Spencer, 1966; Groves et al., 1969; Ji et al., 2003). Further work has shown that spinal systems are also sensitive to environmental and behavioral relations (Grau et al., 1998; Durkovic, 2001; Patterson, 2001a; Grau, 2014). The implication is that not only can the spinal cord store a memory, as shown by Wolpaw and his colleagues (Wolpaw, 2010), it can also learn. In the sections that follow, we review the evidence for spinally-mediated learning and the implications of this work for neurorehabilitation.

3. Non-relational learning

It is well established that stimuli that engage pain (nociceptive) fibers can elicit a withdrawal response, and this behavior can be elicited after communication with the brain has been disrupted by means of an upper thoracic (e.g., T2) transection (Sherrington, 1906). Under natural conditions, these defensive responses would be driven by noxious (stimulation that can potentially induce tissue damage) mechanical or

thermal stimuli. In the laboratory, nociceptive reflexes are often studied using electrical stimulation of the skin or afferent sensory nerves. An advantage of this approach is that key stimulus parameters (e.g., onset/offset and intensity) are easily controlled. In addition, electrical stimulation at moderate intensities can be repeatedly applied without inducing tissue damage. Classic studies by Groves and Thompson used this approach to characterize the development of spinally-mediated habituation and sensitization, laying the groundwork for their Dual Process Model (Groves and Thompson, 1970). In this model, *habituation* (a stimulus-induced decrement in response magnitude; Fig. 3) is mediated by an intraspinal decrease in synaptic efficacy within the stimulus-response (S-R) pathway that mediates the elicited behavior. *Sensitization* (a stimulus-induced increment in response magnitude) was attributed to a process that more generally enhances behavioral reactivity/neural excitability to augment responsiveness across multiple S-R pathways.

3.1. Nociceptive sensitization

Because relatively intense electrical stimulation that engages unmyelinated (C) pain (nociceptive) fibers is needed to induce sensitization, researchers reasoned that this phenomenon could impact pain

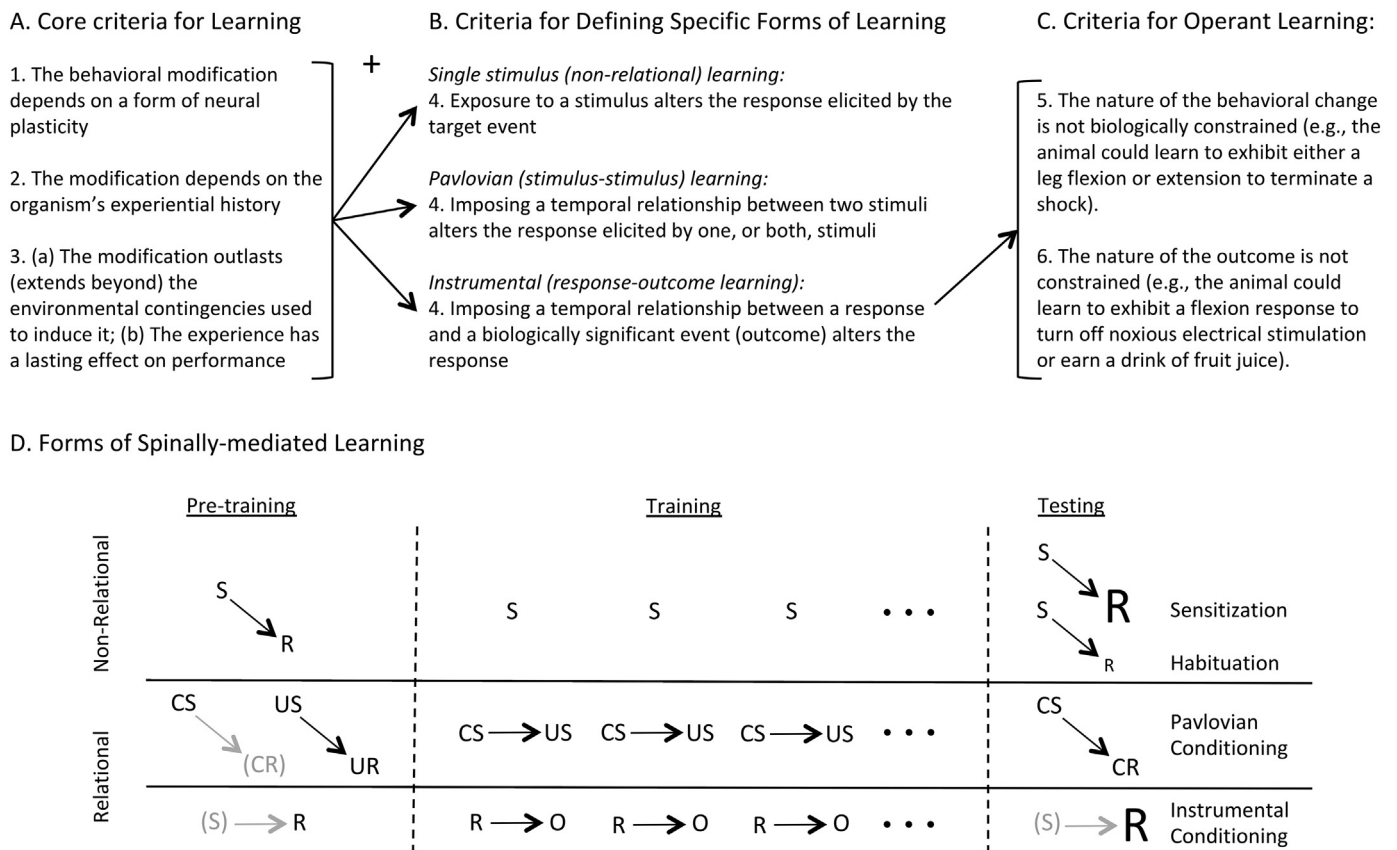


Fig. 3. Defining alternative forms of learning. (A) Common criteria for learning. (B) Specific forms of learning are defined by detailing the environmental event/relation that engages the process. (C) Operant learning involves a form of instrumental conditioning that appears more flexible, wherein the organism can perform the target response in multiple ways and the behavioral change can be established using a number of distinct outcomes. (D) Spinally-mediated learning typically depends upon biologically prepared behaviors that have some capacity to elicit the target response prior to training (Pre-training). In non-relational (single stimulus) learning, repeated exposure to a stimulus (S) brings about an increase (sensitization) or decrease (habituation) in the magnitude of the elicited response (R). Spinally-mediated examples of Pavlovian conditioning typically involve conditioned stimuli (CS) that have some capacity to elicit a behavioral response (light gray) prior to training. Pairing the CS with an unconditioned stimulus (US) strengthens the CS elicited conditioned response (CR), relative to a CS that was explicitly unpaired. In instrumental learning, a contingency is established between the performance of a response and an environmental outcome (O). For example, animals can learn to perform a flexion response (R) if given noxious electrical stimulation (the O) whenever the leg is extended. It is proposed that this training strengthens a pre-existing connection (light gray), enabling a cue indicative of leg position (S) to drive a stronger flexion response.

processing. Building upon this concept, work in the 1990s showed that peripheral application of the irritant capsaicin, which engages the C-fibers that express the transient receptor potential cation channel subfamily V member 1 (TRPV1) receptor (Willis, 2001), induces a lasting increase in neural excitability (nociceptive sensitization) in neurons within the dorsal central gray matter (Ji et al., 2003; Latremoliere and Woolf, 2009; Sandkuhler, 2009). At the cellular level, nociceptive sensitization engages cellular signals (e.g., c-Fos, ERK) linked to neural excitation (Gao et al., 2010). Importantly, the sensitization of nociceptive processing within the dorsal horn is accompanied by an enhancement in pain signaling to the brain and pain reactivity (Simone et al., 1989). Indeed, nociceptive sensitization can transform the psychological response elicited by a light touch, causing a normally innocuous stimulus to elicit pain (allodynia), a defining feature of neuropathic pain.

Subsequent research showed that treatments that induce nociceptive sensitization can have a lasting effect on neural function within the spinal cord, linking this memory-like effect to signal pathways implicated in learning and memory within the brain (Ji et al., 2003). For example, nociceptive sensitization depends upon processes that contribute to neural plasticity throughout the CNS: the release of glutamate, the induction of NMDA receptor (NMDAR) mediated plasticity, and an alteration in AMPA receptor signaling. Further, electrophysiological stimulation of C-fibers can induce a physiological

correlate of memory within the spinal cord, NMDAR-dependent long-term potentiation (Sandkuhler, 2009). The discovery that afferent signals can sensitize neural activity within the dorsal horn, and that this modification involves signal pathways analogous to those implicated in brain-dependent learning and memory, fundamentally altered our view of spinal cord function. From this point onward, the question became not whether spinal cord can learn, but how.

As the stimuli used to induce nociceptive sensitization lead to pain, and movement can interfere with electrophysiological recordings, work on this phenomenon was usually conducted in reduced preparations, using animals that were decerebrate or lightly anesthetized (Woolf, 1983; Sandkuhler and Liu, 1998). In our laboratory, the phenomenon has been studied in animals that have undergone a thoracic (T2) transection (Huang et al., 2016). We have shown that the application of capsaicin to one hind paw induces a lasting (3 h and longer) increase in reactivity to mechanical stimulation and that this effect is evident (albeit, typically less robust) on the untreated (contralateral) leg. These changes in behavioral reactivity are accompanied by an increased expression of cFos and ERK within the dorsal horn.

Interestingly, while capsaicin generates an increase in mechanical reactivity in both sham-operated and transected animals, activation of c-Fos and ERK within the dorsal horn is much greater in animals that have received a spinal cord transection (Castellanos et al., 2007; Huang et al., 2016; Grau and Huang, 2018). This suggests that descending

fibers normally quell neural excitation and the development of nociceptive sensitization in adult uninjured animals. Similarly, electrical stimulation of C-fibers induces a robust long-term potentiation in transected, but not intact, animals (Sandkuhler, 2000). Subsequent studies linked the brain-dependent inhibition of neural excitability within the dorsal horn to descending serotonergic fibers within the dorsal lateral funiculi (DLF) and the serotonin (5HT)-1A receptor (Gjerstad et al., 2001; Hains et al., 2001; Hains et al., 2003; Crown and Grau, 2005; Huang and Grau, 2018). These findings imply that, in the absence of SCI or pathology, nociceptive sensitization within the spinal cord may contribute little to the enhancement of pain. The corollary to this is that spinally-mediated nociceptive sensitization is especially relevant to the development of chronic pain after SCI.

3.2. Ionic plasticity

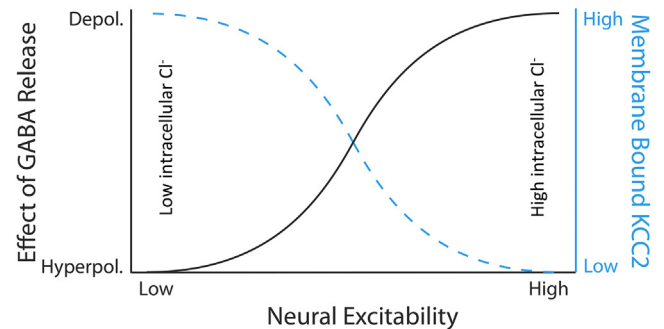
There is little doubt that neurons within the spinal cord, surgically transected from the brain, can support learning. At the same time, our conclusions need to be tempered by the observation that nociceptive sensitization appears far stronger after SCI, which suggests that removing input from the brain enables plasticity within the spinal cord. Recent work has linked this transformation to an alteration in GABA function (Grau and Huang, 2018).

Neural excitability within the spinal cord is regulated by interneurons and the release of GABA, which can engage the ionotropic GABA-A receptor, allowing the anion Cl^- to pass across the neural membrane (Medina et al., 2014). In adult uninjured animals, cotransporters maintain a low concentration of Cl^- within the cell (Kaila et al., 2014). Under these conditions, engaging the GABA-A receptor allows Cl^- to flow into the cell, which has a hyperpolarizing (inhibitory) effect that quells neural excitability and plasticity. Interestingly, these cotransporters mature at different rates, resulting in a higher intracellular concentration of Cl^- early in development (Ben-Ari, 2002; Rivera et al., 2005). The first to emerge is the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter 1 (NKCC1), which transports Cl^- into the cell. Because the co-transporter [K^+/Cl^- cotransporter 2 (KCC2)] that pumps Cl^- out of the cell develops later, the intracellular concentration of Cl^- is greater early in development (Kaila et al., 2014; Medina et al., 2014). Under these conditions, engaging the GABA-A receptor allows Cl^- to exit the cell, a depolarizing effect that excites, rather than inhibits, neural activity (Fig. 4). These changes are relevant to SCI because injury can impact the concentration of membrane-bound KCC2, inducing a down-regulation that recapitulates an earlier developmental state (Drew et al., 2004; Cramer et al., 2008; Boulenguez et al., 2010; Medina et al., 2014).

Recent studies have shown that a T2 transection leads to a reduction in membrane-bound KCC2 within the dorsal horn of the lumbosacral spinal cord within 24 h (Huang et al., 2016). This alteration is accompanied by an enhancement in cellular indices of neural excitability (Fig. 4A). SCI also transforms how brain-derived neurotrophic factor (BDNF) affects nociceptive sensitization and KCC2. In uninjured animals, BDNF promotes nociceptive sensitization (Coull et al., 2005a), whereas after SCI it has the opposite effect (Huie et al., 2012b; Huang et al., 2017). Research suggests that i.t. BDNF has these divergent effects because SCI transforms how it impacts KCC2 (Boulenguez et al., 2010; Huang et al., 2017). After SCI, BDNF up-regulates KCC2, which would act to re-establish GABAergic inhibition. In contrast, i.t. BDNF down-regulates KCC2 in uninjured animals, lessening GABAergic inhibition and promoting the development of nociceptive sensitization.

Parallel studies have linked the development of spasticity to a down-regulation of KCC2 in lumbar (L2-L4) motoneurons. To explore how this affects neural excitability, Boulenguez et al. (2010) assessed the effect of repeatedly eliciting the H-reflex. Past work has shown that this normally leads to a decline in the H-reflex (rate dependent depression), a habituation-like effect attributed to GABA-dependent inhibition (Kakinohana et al., 2006). If the down-regulation of KCC2 weakens

A. Effect of GABA release on neural excitability varies with KCC2



B. Relation between adaptive potential and neural excitability

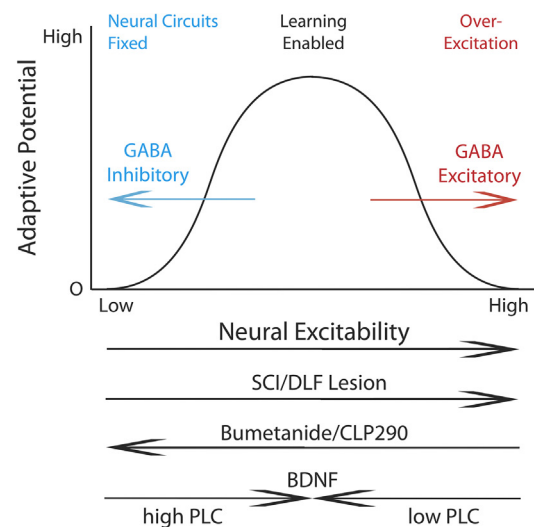


Fig. 4. Regulation of spinal cord plasticity by GABA (ionic plasticity). (A) The effect of GABA on neural excitability is inversely related to the level of membrane-bound KCC2. In the adult uninjured nervous system, membrane-bound KCC2 helps to maintain a low intracellular concentration of Cl^- . Under these conditions, engaging the GABA-A receptor allows Cl^- to enter the cell, which has a hyperpolarizing (inhibitory) effect. After SCI, membrane bound KCC2 declines caudal to injury, which allows the intracellular Cl^- concentration to increase. Now, engaging the GABA-A receptor allows Cl^- to exit the cell which has a depolarizing (excitatory) effect. As membrane bound KCC2 declines, neural excitability increases. (B) The hypothesized relation between neural excitability and the development of learning/maladaptive plasticity. In adult uninjured animals, neural plasticity is low due to GABA-dependent inhibition. SCI can remove this brake to allow spinal cord learning. In the absence of brain-dependent regulatory systems, noxious input can induce a state of over-excitation that saturates plasticity and disrupts adaptive learning. GABA-dependent over-excitation can be reduced by pretreatment with a KCC2 agonist (CLP290) or a NKCC1 antagonist (bumetanide) (Huang et al., 2016; Chen et al., 2018). BDNF has a homeostatic effect that fosters plasticity. Evidence suggests that this bidirectional effect is regulated by PLC γ (Rivera et al., 2004; Rivera et al., 2005).

GABAergic inhibition of motoneurons, it should block the development of rate dependent depression. In fact, SCI had this effect. Interestingly, here too BDNF appears to have divergent effects—in uninjured animals, it down-regulates KCC2 within the ventral horn and attenuates the emergence of rate dependent depression, whereas after SCI, BDNF has the opposite effect. These opposing effects on motor function and KCC2 have been related to a loss of corticospinal input (Shulga et al., 2008).

GABAergic regulation of spinal cord circuits also contributes to the adaptive rewiring of the spinal cord. This is nicely illustrated by a recent study by (Chen et al., 2018) who examined recovery after

contralateral hemisections that transected the spinal cord along the mid-line at T7 and T10. Combined, these two transections severed all ascending/descending fibers. Remarkably, animals exhibit some recovery in sensory/motor function over time, an effect that depends upon neural plasticity within the intervening central gray that provides a functional bridge. Using a drug screen, [Chen et al. \(2018\)](#) discovered that pretreatment with a KCC2 agonist (CLP290) improves functional recovery. Genetically over-expressing KCC2 had a similar effect. These KCC2-dependent effects were linked to GABAergic interneurons within the relay zone and the re-establishment of GABA-dependent inhibition. Supporting this, an index of neural excitability (c-Fos expression) revealed that contralateral hemisections induced a state of over-excitation within the dorsal horn of the relay zone. Re-establishing GABA-dependent inhibition with CLP290 attenuated c-Fos expression and enhanced functional recovery.

3.3. Nociceptive stimulation impairs recovery

We have shown that, after a complete SCI, noxious stimulation can induce a state of over-excitation and that this interferes with adaptive learning ([Ferguson et al., 2012](#); [Huang et al., 2016](#)). Such an effect could have clinical significance because many injuries are accompanied by other tissue damage (polytrauma) that would drive the activation of pain fibers. After a contusion injury, this nociceptive input could fuel processes (e.g., microglia activation, release of pro-inflammatory cytokines, over-excitation) that undermine adaptive potential, foster the development of chronic pain, and promote cell death ([Beattie and Bresnahan, 2000](#); [Grau et al., 2017](#)). To explore this possibility, rats were given a lower thoracic (T11–12) contusion injury and received noxious electrical stimulation the next day ([Grau et al., 2004](#)). We found that just 6 min of intermittent stimulation applied caudal to the injury impaired long-term locomotor recovery. Noxious stimulation also undermined the recovery of weight and bladder function, fostered the development of chronic pain and spasticity, and increased mortality. More recently, we have shown that application of the irritant capsaicin also impairs the recovery of locomotor function ([Turtle et al., 2018](#)). Collectively, these findings imply that nociceptive input induces a kind of *maladaptive plasticity* that undermines function ([Ferguson et al., 2012](#)).

The physical impact of a contusion injury induces a region of cell loss (primary injury) that impacts the surrounding cellular milieu, placing the surviving tissue in a vulnerable state for a period of days to weeks after injury ([Beattie et al., 2002](#)). In our rodent model, noxious stimulation impairs long-term recovery when given within 4 days of injury, but it does not have a lasting effect when applied two weeks after injury. Further work has shown that acute nociceptive stimulation, from shock or capsaicin, engages the expression of pro-inflammatory cytokines (IL-1 β , IL-18, TNF) at the site of injury and up-regulates signal pathways related to cell death (caspase 1, 3, and 8) ([Turtle et al., 2018](#)). Nociceptive input also fuels processes related to progressive hemorrhagic necrosis ([Simard et al., 2010](#); [Simard et al., 2013](#); [Turtle et al., 2019](#)), which expands the area of hemorrhage and secondary tissue loss. Recent studies have shown that this effect is observed in both male and female animals ([Baine et al., 2019](#)) and that pain input also increases the area of hemorrhage after traumatic brain injury ([Bean et al., 2019](#)).

While there is little evidence that pain input during the chronic phase impacts tissue loss, engaging nociceptive fibers by exerting a physical force to the lower limbs (e.g., by stretching) disrupts locomotor performance ([Caudle et al., 2011](#); [Caudle et al., 2015](#)). In addition, engaging nociceptive fibers with capsaicin can disrupt retention in a locomotor task in humans ([Bouffard et al., 2014](#)). Further work is needed to explore how pain input affects physiological systems during the chronic stage of injury. Of particular concern is the possibility that it could trigger unregulated sympathetic activation (autonomic dysreflexia) and foster processes (chronic pain, spasticity) related to neural

over-excitation.

3.4. Protecting the injured spinal cord

Pain input after SCI is problematic for multiple reasons. Our work has shown that soon after injury, it will induce a form of maladaptive plasticity within the spinal cord that expands the area of secondary injury and undermine long-term recovery ([Grau et al., 2004](#); [Grau et al., 2017](#)). Acutely, spared sensory projections to the brain can drive pain. Nociceptive activity can also sensitize peripheral inputs to the spinal cord and neural circuits within the dorsal horn, setting the stage for chronic pain. These observations suggest that attenuating pain soon after injury could improve patient wellbeing and recovery. To explore this possibility, Hook assessed whether systemic morphine attenuates the adverse effect of nociceptive stimulation in contused rats ([Hook et al., 2007](#)). Morphine was administered at a dose that blocked behavioral reactivity to noxious stimulation. The drug did not, however, prevent nociception-induced hemorrhage or the impairment in long-term recovery ([Hook et al., 2007](#); [Turtle et al., 2017](#)). Thus, while drug treatment brought acute relief from pain, it had no long-term benefit. Indeed, quite the opposite—treatment with morphine increased the area of tissue loss and, in combination with nociceptive stimulation, enhanced mortality.

Seeking a safe alternative, we explored the effect of administering the anesthetic lidocaine with an epidural injection immediately before nociceptive stimulation ([Turtle et al., 2017](#)). Like morphine, lidocaine blocked acute pain behavior to noxious stimulation applied caudal to injury. Unlike morphine, lidocaine attenuated the activation of pro-inflammatory cytokines and nociception-induced hemorrhage at the site of injury. Most importantly, lidocaine blocked the adverse effect of nociceptive stimulation on long-term recovery.

We have shown ([Crown and Grau, 2005](#)) that descending fibers can counter the development of nociceptive sensitization. Given this, we hypothesized that cutting these pathways rostral to a contusion injury would amplify nociception-induced hemorrhage and the activation of pro-inflammatory cytokines ([Reynolds et al., 2019](#)). Rats received a lower thoracic (T11–12) contusion injury. Eighteen hours later, the spinal cord was transected at T2 and nociceptive stimulation was applied 6 h later. The overall pattern of results was opposite to what we had expected—cutting communication with the brain blocked nociception-induced hemorrhage and the activation of pro-inflammatory cytokines. More recently, we showed that a pharmacological transection at T2, induced by slowly infusing lidocaine, also blocks nociception-induced hemorrhage and the impairment in long-term recovery ([Davis et al., 2018](#)). Likewise, inducing a state akin to a medically-induced coma with pentobarbital blocked the adverse effects of nociceptive stimulation ([Davis et al., 2017](#)).

These findings have a number of important implications. First, they suggest that treatment with a local or general anesthetic may provide a safe method for treating acute pain that has the added advantage of blocking nociception-induced tissue loss. Second, the results imply that preserved sensory fibers allow nociceptive signals to engage brain-dependent processes that drive tissue loss within the spinal cord. To our knowledge, this is the first evidence that the brain plays a role in regulating the development of secondary injury. On-going studies are exploring how the brain can impact processes that influence tissue loss at the site of SCI. One possibility is that this is fueled by spared descending fibers. In addition, brain systems may drive an increase in blood pressure that fuels hemorrhage ([Fauss et al., 2018](#); [Strain et al., 2019](#)). This is consistent with past work demonstrating that hypertension at the time of injury is associated with poor recovery ([Nielson et al., 2015](#)). Finally, additional work is needed to evaluate whether brain dependent processes adversely affect other tissue/physiological systems after SCI, disrupting the healing process in response to polytrauma, potentiating the stress response, and inducing a state of neural over-excitation that could fuel anxiety and chronic pain after injury.

3.5. Beneficial effects of stimulus exposure

The studies reviewed above demonstrate that exposure to a noxious stimulus can induce a form of maladaptive plasticity that can foster pain and impair behavioral function after SCI. Interestingly, less intense stimulation can have the opposite effect. Early evidence of this emerged from the pain literature, motivated by the idea that activity in large diameter sensory fibers has an inhibitory effect on small unmyelinated C-fibers (Melzack and Wall, 1965). Supporting this, tonic stimulation to the skin [transcutaneous electrical nerve stimulation (TENS)] or spinal cord (epidural stimulation) at an intensity and frequency that minimizes the activation of nociceptive fibers has an antinociceptive effect (Levins and Moritz, 2017).

Mild epidural stimulation can also elicit stepping in decerebrate cats and drive rhythmic locomotor behavior in rats when applied over the lumbar (L2) region thought to contain the CPG (Ichiyama et al., 2005). In humans, subthreshold epidural stimulation can enable volitional movements in individuals who are otherwise paralyzed (Harkema et al., 2011; Angeli et al., 2014). Likewise, stimulation applied to the cervical region has been shown to improve hand function (Lu et al., 2016). The prevailing view is that epidural stimulation promotes performance by engaging proprioceptive fibers that provide mono- and polysynaptic drive to motoneurons (Harkema et al., 2011; Formento et al., 2018).

Ideally, the beneficial effect of spinal cord stimulation would grow with experience and outlast the period of stimulation, providing evidence for learning. In general, however, epidural stimulation appears to act in an unconditioned (unlearned) manner, producing an effect that remains stable during stimulation and wanes soon after the stimulus is terminated (Rejc et al., 2017). As we will see below, encouraging a lasting effect may require fine-tuning these procedures, so that stimulation occurs in a conditional (e.g., response-contingent) manner (McPherson et al., 2015; Moraud et al., 2016).

4. Relational learning

It is well established that neurons within the spinal cord can organize some simple behaviors, and that these are affected by stimulus exposure. What has been more controversial is whether spinal cord circuits can support forms of learning that depends upon the relation between two events. Within the learning literature, researchers have traditionally distinguished between two kinds of relational learning: Pavlovian and instrumental conditioning (Rescorla, 1988; Domjan, 2015). Pavlovian conditioning (classical conditioning) concerns the consequence of establishing a relationship between two stimulus events. Instrumental conditioning examines whether introducing a behavioral contingency between the performance of a response (R) and an environmental outcome (O; aka reinforcer) impacts the likelihood or magnitude of the R. In the following sections, we briefly review evidence that spinal cord neurons are sensitive to stimulus-stimulus (S–S) and R–O relations, the processes involved, and the implications of this work for treatment after SCI.

4.1. Learning about stimulus relations (Pavlovian conditioning)

Learning about S–S relations is typically studied by pairing a cue [the conditioned stimulus (CS)] with a stimulus [the unconditioned stimulus (US)] that innately elicits an unconditioned (unlearned) response (UR). As a consequence of this training (Fig. 5B), the paired CS (the CS+) acquires the capacity to produce a stronger response [the conditioned response (CR)], relative to an unpaired CS (the CS-). To examine whether neurons within the spinal cord can support Pavlovian conditioning, researchers have used electrical stimulation of a hind paw or a nerve (e.g., the superficial peroneal) as the US (Fig. 6A), with the stimulation applied at an intensity that elicits an unconditioned flexion response (Durkovic, 2001). For the CS (S1), a weaker stimulus can be applied to the thigh or a distinct nerve (e.g., the saphenous) at an

intensity that elicits a weak response. After multiple CS-US pairings, the CS elicited a conditioned flexion response, relative to a group that had experienced these same events in an explicitly unpaired manner.

Further work showed: that a conditioned response (CR) develops when the CS is presented before (forward) the US but not after (backward); that presenting the CS alone after a CR has been acquired causes the behavioral response to wane (extinction); and that the learning involves a form of NMDAR-mediated plasticity (Durkovic and Prokovich, 1998; Durkovic, 2001). As observed with brain-dependent examples of Pavlovian conditioning, presenting a CS alone prior to training undermines learning (latent inhibition) (Illich et al., 1994). Presenting the target CS in conjunction with one that has been previously trained (blocking), or that is more salient (overshadowing), also undermines learning.

While Pavlovian conditioning is typically studied using an US that induces a behavioral response, this is not a requirement. Indeed, there is ample evidence that animals can learn a S1-S2 relation in situations where neither cue elicits an overt response (e.g., sensory pre-conditioning; (Domjan, 2015)). In addition, animals can learn that a cue predicts an internal state (e.g., illness).

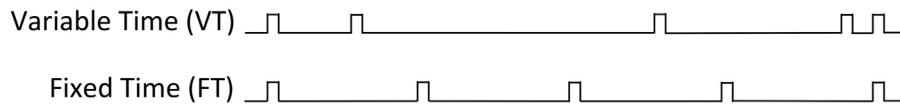
4.2. Why did studies of spinal conditioning elicit controversy?

These findings show that establishing a S–S relation can induce a lasting alteration in spinal function—that spinal neurons can support a form of Pavlovian conditioning. In some regards, this is not surprising because this form of learning has been demonstrated in a variety of simple systems, including the invertebrate *Aplysia* (Kandel and Schwartz, 1982). Given that it has been repeatedly shown that spinal cord neurons are sensitive to S–S relations, why did this claim elicit controversy? One reason stems from the nature of the paradigm used to establish the phenomenon, which requires that we eliminate communication with the brain. As we have seen, SCI transforms the caudal neural tissue, removing a GABA-dependent brake on neural excitation and plasticity. This modification may amplify spinal conditioning and explain why learning (using the same stimuli) typically appears brain-dependent in uninjured animals (Thompson, 2001).

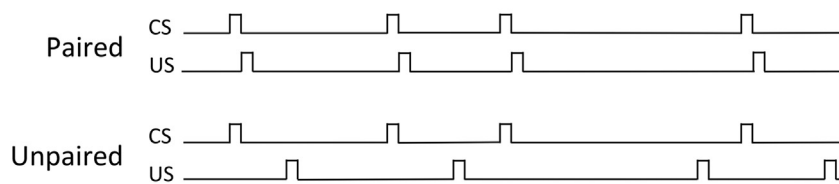
A second key issue concerns the limits of spinal conditioning. While it is clear that spinal systems are sensitive to S–S relations, and exhibit a range of Pavlovian phenomena, learning is limited in some important ways. Brain-dependent systems allow organisms to span gaps in time (trace conditioning), integrate stimuli across modalities, and update behavior given a change in US value (Domjan, 2015). These capacities have been linked to specialized structures within the brain, like the hippocampus (Rudy and O'Reilly, 1999; Rudy, 2009). Without these brain structures, learning appears more constrained and habit-like (Joynes and Grau, 1996). There is also a quantitative difference in both the rate of learning and the stimulus intensities needed to motivate spinal and brain-dependent learning. For example, both brain and spinal circuits can support a form of conditioned antinociception (Fanselow, 1986; Grau et al., 1990). However, the brain-dependent form of this phenomenon can be engaged with just a few CS-US pairings and a relatively weak US. Producing a lasting modification at the level of the spinal cord normally requires more training and higher levels of stimulation.

A final issue concerns a historical link to the concept of associative learning (Grau and Joynes, 2005a). Traditionalists saw Pavlovian conditioning as a way of studying the formation of *de novo* associations. Ideally, this would entail evidence that a neutral CS, that shows no evidence of producing a CR-like behavior prior to conditioning, gains the capacity to generate a new response through its association with the US. The link to *associative learning* has become so wedded to the concept of learning that the term is sometimes used as a synonym for Pavlovian conditioning. The erroneous implication is that, if a CR develops, it is due to an associative process; therefore, a behavioral change that fails the criteria for an associative process does not reflect

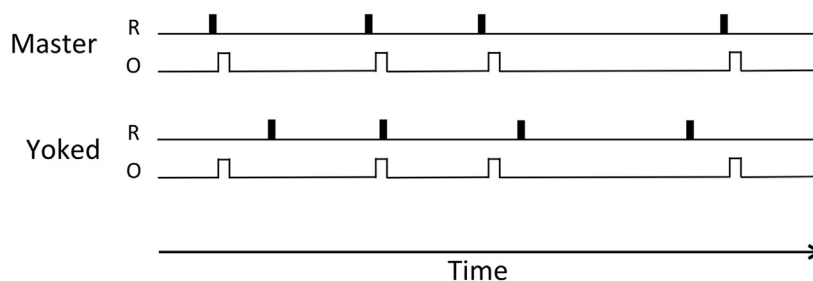
A. Intermittent Stimulation



B. Pavlovian Conditioning (CS → US)



C. Instrumental Conditioning (R → O)



an example of learning. Elsewhere, we have discussed why this historical reference should be retired (Grau and Joynes, 2005a; Grau and Joynes, 2005b). First, few, if any, examples of conditioning appear based on the development of a *de novo* link. Instead, learning is typically built upon biologically prepared connections, with most CS's exhibiting some potential to drive a CR-like behavior prior to conditioning. Secondly, a CS-elicited response can be strengthened through non-associative processes. For example, presenting a noxious stimulus can sensitize a CS-evoked response and the magnitude of this effect is amplified when the events are paired, a phenomenon known as pairing-specific enhanced sensitization (Hawkins et al., 1983; Walters and Byrne, 1983).

The problems with the traditional approach led us to suggest a new view (neurofunctionalism) that assumes environmental puzzles can be solved in multiple ways, at both a functional and neurobiological level (Grau and Joynes, 2005a). The aim here is to discover the mechanisms involved and the circumstances under which they are enlisted. A dogmatic focus on just one process can obscure the wealth of processes that contribute to learning and how these processes are inter-linked to solve a range of problems. To avoid these conceptual pitfalls, we reference the environmental demands that drive the learning rather than a singular (presumed) underlying process, characterizing both Pavlovian and instrumental conditioning as forms of *relational learning*.

4.3. Pavlovian conditioning in the context of neurorehabilitation

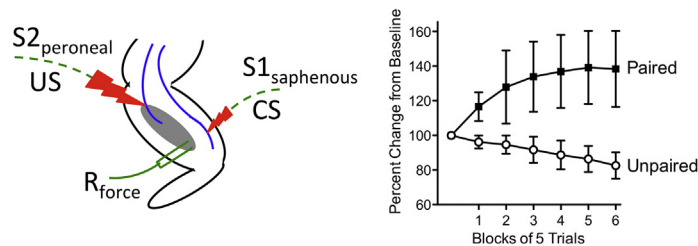
We saw earlier that tonic epidural stimulation can foster locomotor performance after SCI (Iwahara et al., 1992; Ichiyama et al., 2005; Harkema et al., 2011). In general, this appears to reflect an unconditioned response to stimulation that provides little benefit after the stimulus has terminated. Presenting the stimuli in a relational manner, by pairing epidural stimulation with activity in descending motor pathways, can produce a more robust and longer-lasting effect. For example, Mishra et al. (2017) engaged spinal circuits in rats by applying electrical stimulation over the cervical dorsal root entry zone at an intensity

Fig. 5. Alternative modes of stimulation. The lines illustrate the onset (upward dash) and offset (downward dash) of stimulation across time. (A) Intermittent stimulation can be applied in an irregular manner [variable time (VT)] or at regular intervals [fixed time (FT)]. (B) A Pavlovian relation is established by pairing a cue (the CS) with a second stimulus (the US). In most instances, optimal learning is observed when the CS occurs prior to US (forward conditioning). To demonstrate pairing matters, other animals may be exposed to CS and US in an explicitly unpaired manner. (C) In instrumental conditioning, a relationship is established between the performance of a response (R) and the presentation of an environmental outcome (O). Animals in this condition (Master) have behavioral control over the O. To demonstrate that the R → O relation matters, other animals can be experimentally coupled (Yoked) to animals in the Master condition. Yoked animals receive the O at the same time, independent of their behavior (R). In all cases, the observable events (S, R, and O) may correspond to external stimuli/responses or electrophysiological correlates. For example, a R can be inferred from electrical activity in a particular neuron or muscle. Likewise, a S can be applied using the techniques of *neuromodulation*, which involves the manipulation of neuronal activity using electrical, chemical, or electromagnetic (optogenetics) stimulation.

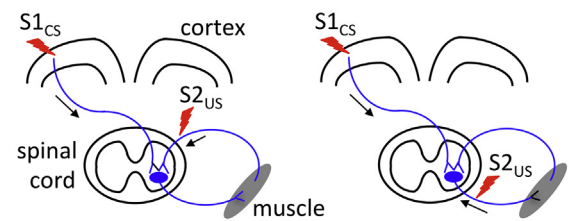
that was sub-threshold for eliciting a forelimb response. Descending fibers were engaged by electrically stimulating the cortex at a site that elicited a motor evoked potential (MEP) within the bicep (Fig. 6B). When cortical stimulation was applied 10 to 15 ms before epidural stimulation, a stronger MEP was observed. Given the estimated time for neural impulses to travel from the cortex to the cervical region (a little less than 10 ms), this temporal lag assured that the corticospinal input arrived just prior to the activity evoked by epidural stimulation. Engaging corticospinal neurons well before or after epidural stimulation eliminated the pairing-induced augmentation of the MEP. Most importantly, repeated pairing (150–300 trials) induced an effect that outlasted the period of stimulation by 30–40 min. At a neurobiological level, the findings were related to the development of spike-timing-dependent plasticity within the spinal cord (Dan and Poo, 2004). At a functional level, the findings imply that the training induced a kind of Pavlovian conditioning, with epidural stimulation serving as the US. In this paradigm, pairing cortical stimulation (the CS) with the US strengthens its capacity to engage a motor response (the CR).

Others (Urbán et al., 2017) have demonstrated a similar effect in humans with SCI. In this case, activity was evoked in a corticospinal pathway using transcranial magnetic stimulation (TMS) over the cortical region that innervates a leg. This stimulation was paired with antidromic potentials evoked in motoneurons elicited by electrical stimulation of the common peroneal nerve. Again, repeated pairing (200 trials) increased the MEP elicited by cortical stimulation and this effect outlasted the period of nerve stimulation by 30 min. The emergence of this effect depended upon the timing of the two events, with the strongest effect observed when the cortical stimulation (the CS) was timed so that spinal activity preceded the input from peroneal stimulation (the US) by 2 ms. Interestingly, the development of this effect appears to depend upon a form of NMDAR-mediated plasticity (Donges et al., 2018).

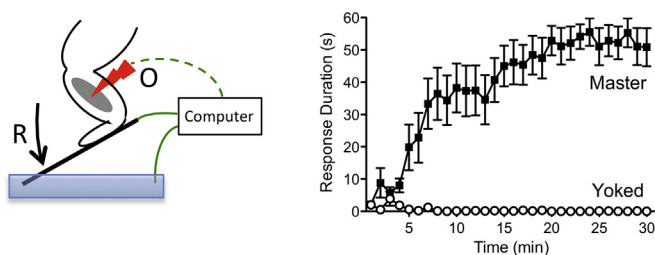
A. Pavlovian Conditioning (S1 → S2)



B. Open-loop Stimulation



C. Instrumental Conditioning (R → O)



D. Closed-loop Stimulation

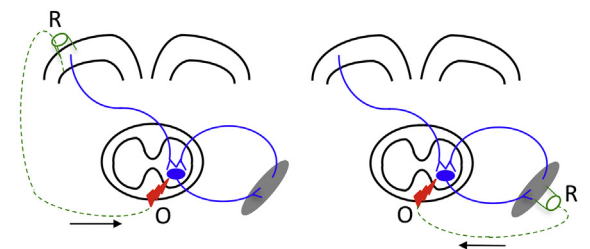


Fig. 6. Alternative ways of establishing a Pavlovian or instrumental relation. (A) Pavlovian conditioning can be established by pairing electrical stimulation of the saphenous nerve (S1) with stimulation of the peroneal (S2) nerve of one hind leg (Durkovic, 2001). Here, S1 corresponds to the CS and S2 serves as the US. S1 intensity is adjusted so that it elicits a weak behavioral response (R), inferred from a strain gauge attached to the tendon of the tibialis anterior muscle. S2 is adjusted to an intensity that engages nociceptive fibers. Pairing S1 with S2 generates an increase in the response elicited by S1, relative to animals that receive the stimuli in an unpaired manner (Durkovic, 2001). (B) Electrical stimulation of the motor cortex (S1) can engage surviving corticospinal fibers after SCI. Pairing S1 with epidural stimulation (S2) in a Pavlovian manner (open-loop stimulation) enhances motor performance (Harel and Carmel, 2016). Pairing S1 with antidromic activity (S2) elicited by stimulating an efferent motor pathway has a similar effect (Urbán et al., 2017). (C) Instrumental learning can be studied in spinally transected rats by instituting a relation between leg position (the R) and the application of shock (the O) to the tibialis anterior muscle (Grau et al., 2012). Leg position is monitored using a rod that is taped to the base of the rat's paw. Whenever the rod touches an underlying salt solution, electrical stimulation (shock) is applied to the tibialis anterior muscle. Over 30 min of training, animals that have behavioral control (Master) learn to maintain the hindlimb in a flexed position that reduces net shock exposure. Animals in the Yoked group, that receive shock independent of leg position, do not exhibit an increase in flexion duration. (D) Establishing an instrumental relation (closed-loop stimulation) between neural activity within the motor cortex (the R) and targeted stimulation of the spinal cord (the O) can foster motor performance after SCI. An instrumental relation can also be established by monitoring electrical stimulation within a muscle (the R). A small evoked response serves as a proxy for neural activity within a surviving corticospinal projection. Motor performance is strengthened by stimulating the motor neurons immediately after the R is detected (McPherson et al., 2015; Bonizzato et al., 2018; Nishimune and Shigemoto, 2018). Error bars indicate the standard error of the mean.

4.4. Learning about behavioral relations (instrumental conditioning)

In instrumental conditioning, a contingency is established between the performance of a response (R) and an environmental outcome (O). R-O relations are an integral component of physical therapy and can influence recovery in multiple ways. At the most general level, behavioral contingencies guide performance through brain-dependent processes, with effort maintained by a combination of appetitive reward (positive reinforcement) and pain reduction (negative reinforcement). As noted earlier, Wolpaw and his colleagues have shown that appetitive reinforcement can be used to shape the magnitude of a spinal (H) reflex (Wolpaw and Carp, 1990; Wolpaw, 2010; Thompson and Wolpaw, 2014). With extended training, this modification survives a spinal transection. The implication is that brain-dependent learning can, through descending fibers, induce a lasting alteration (a form of memory) within the spinal cord. The question we pose here is somewhat different: Can experience with a R-O relation alter spinal function without input from the brain? Can the spinal cord learn?

Again, to explore the potential for learning within the spinal cord, researchers have examined how an experience affects performance after communication with the brain has been eliminated by means of a rostral transection. For example, DeLeon and Edgerton (Edgerton et al., 1997b) examined whether transected animals that were trained to step with their hind legs on a treadmill could adjust locomotor performance

in response to an obstacle. The obstacle was placed so that one paw hit it during the swing phase. Over time, the cat learned to exhibit a stronger flexion that minimized contact with the obstacle. This behavioral modification persisted for several steps after the obstacle was removed, implying a kind of spinal learning wherein a response (increased flexion) was strengthened by exposure to a noxious outcome (contact with the bar).

While the observations of DeLeon and Edgerton were intriguing, the complexities of the treadmill paradigm made it difficult to assess the circumstances under which the learning occurred and the underlying neurobiological mechanisms. To address this issue, we focused on the key components, developing a learning paradigm in which rats received electrical stimulation (shock) to one hind leg [the outcome (O)] whenever the leg was in an extended position [the response (R)]. Leg position was monitored by taping a rod to the rat's paw (contact electrode). When the leg was extended, the tip of the rod touched an underlying salt solution, completing a circuit that was monitored by a computer (Fig. 6C). Rats received a thoracic (T2) transection and were trained for 30 min with response-contingent stimulation. Over the course of training, they exhibited a progressive increase in flexion duration that minimized net shock exposure (Grau et al., 1998). Further work has shown that this learning is blocked by pretreatment with an NMDAR antagonist (Joyne et al., 2004). More recently, we showed that learning is also blocked by i.t. application of a drug (bumetanide)

that should restore GABA-dependent inhibition after SCI, which again suggests that SCI enables spinal learning by down-regulating KCC2.

We recognized that exposure to shock, per se, might sensitize behavioral reactivity, independent of the R-O relation. To address this possibility, a second group was experimentally coupled (yoked) to animals that received (response-contingent) shock whenever its hind leg was extended (Master). Each yoked rat received shock at the same time as its master partner, independent of leg position (Fig. 5C). In behavioral terms, master animals receive *controllable* stimulation, whereas shock is applied to the yoked animals in an *uncontrollable* manner. During training, only master animals exhibited an increase in flexion duration (Grau et al., 1998), implying that the R-O relation matters.

There are a number of methodological issues here that deserve attention. The first concerns the dependent variable, an increase in flexion duration. Earlier studies, based on the *Horridge paradigm* (Horridge, 1962), typically measured response number or the time that the animals spent with their leg in an extended position (Buerger and Chopin, 1976; Chopin and Buerger, 1976). The problem is that a master-yoke paradigm can produce behavioral differences on these measures without learning (Church, 1964; Church and Lerner, 1976; Church, 1989). To see this, consider time in solution. If an animal's leg falls in a probabilistic manner after shock, the yoked rat's leg will contact the underlying solution first on half the trials and remain there until its master partner receives a shock. As soon as the master animal touches the solution, it receives a shock that elicits a flexion response. As a consequence, the master animal would spend less time with its leg in an extended position. A mechanical (reactive) system would produce a behavioral difference in this situation in the absence of learning. We addressed this issue by developing a new set of procedures to determine whether spinal cord neurons can support instrumental conditioning. These include (1) adopting a different index of learning (increased flexion duration), (2) testing animals under common conditions, and (3) experimentally manipulating the R-O relation, by independently delaying the onset or offset of shock (Grau et al., 1998). The last manipulation revealed that learning is reinforced by shock onset, not offset. From this, we can conclude that spinal neurons can encode R-O relations. To distinguish this approach from Horridge's studies of invertebrate learning, Hansen suggested a descriptive label: the *instrumental learning paradigm* (ILP; Hansen et al., 2016).

4.5. Building upon common processes

Just as brain-dependent Pavlovian conditioning exhibits a level of flexibility not evident in spinally-mediated examples of this phenomenon, brain-dependent R-O learning shows greater versatility. Brain systems allow the organism to quickly modify performance in response to a change in the R-O contingencies and outcome value (Domjan, 2015). There is no evidence that spinally-mediated instrumental learning can exhibit this level of flexibility. This parallels Skinner's distinction between respondent and operant behavior (Skinner, 1938; Grau, 2014). At the time Skinner outlined his views, others were characterizing instrumental behavior in terms of stimulus-response reflexes (Hull, 1943). From this perspective, a rat reinforced with food for pressing a bar acquires a kind of $S_{\text{bar}}-R_{\text{press}}$ habit that drives the behavior. Skinner saw little evidence to support this view, arguing instead that the organism could operate on its environment in a variety of ways. Rejecting accounts based on S-R reflexes, he argued that these flexible patterns of behavior seemed emitted, not elicited, and distinguished this form of learning with the term *operant* behavior. While it is clear that operant behavior often seems more biologically constrained than Skinner originally imagined (Timberlake and Lucas, 1989; Timberlake, 1990), it also evident that the brain allows for a level of flexibility that the spinal cord lacks. At the level of the spinal cord, R-O relations impact pre-existing S-R reflexes, that Skinner would consider an example of respondent conditioning. Minus input from the brain, there is no evidence that spinal cord neurons can respond to a R-O relation in a

flexible manner—that it can support operant learning.

The data reviewed above show that behavioral control regulates how nociceptive stimulation affects spinal cord function. The critical outcome (O) in this situation has been linked to the onset of the nociceptive stimulus, which occurs when the leg falls to a particular position. Because leg position and movement is signaled by proprioceptive cues, shock onset (the O) would always occur in the presence of a particular pattern of afferent input—indicative of current leg position. These proprioceptive cues could function as a kind of Pavlovian CS, which would be paired with the onset of nociceptive stimulation (the US). As a consequence of this CS-US pairing, the CS (leg position) may gain the capacity to drive a flexion response (the CR). The implication is that instrumental learning within the spinal cord may be built upon a form of Pavlovian conditioning, wherein a CS (proprioceptive cues)-US (shock onset) relation strengthens a pre-existing S-R pathway that augments the motor output (flexion duration). At this level of the nervous system, the distinction between Pavlovian and instrumental conditioning may be relevant to how learning is studied (i.e., whether the experimenter or subject controls the presentation of the outcome/US), but not to our analysis of the underlying neurobiological processes and the functional mechanisms involved.

4.6. Instrumental learning within the context of neurorehabilitation

Common processes also appear to contribute to alternative methods for applying stimulation in studies of neurorehabilitation. As discussed above, others have used a form of Pavlovian conditioning to strengthen synaptic connections between spared descending fibers and a motor response (Mishra et al., 2017; Urbin et al., 2017). Here, the stimulation-induced motor response served as the US, which was paired with cortical stimulation. With pairing, the cortical stimulation (the CS) gained the capacity to elicit a stronger motor response (the CR). A common feature of this form of stimulation and Pavlovian conditioning is that the stimuli are applied independent of whether they evoke a motor response (*open-loop stimulation*).

An alternative form of stimulation (*closed-loop*) links its application to a behavioral/electrophysiological response, effectively establishing an instrumental (R-O) relation. Here, the subject, rather than the experimenter, controls the delivery of stimulation. For example, Bonizzato et al. (2018) recorded neural activity in the motor cortex, seeking neural signatures that were related to the movement of an impaired limb after SCI. This neural signature (the R) was then used to trigger electrical stimulation of the spinal cord (the O). Establishing a R-O relation in this manner allowed a form of “self-training” that improved performance (Fig. 6D).

A R-O relation can also be established using a proxy for descending neural activity. McPherson accomplished this by monitoring electromyographic (EMG) activity within a muscle of the impaired limb (McPherson et al., 2015). Whenever EMG activity was detected, an electrical pulse [the outcome (O)] was sent to an intraspinal electrode that had been implanted in a region of the cervical spinal cord that elicited a muscle response in the impaired forelimb. They found that this training fostered behavioral recovery and had a lasting effect that was evident 3 weeks after stimulation was terminated. Again, this learning was related to a form of spike-timing-dependent plasticity that fostered synaptic connectivity between surviving corticospinal fibers and motoneurons. This is consistent with work in monkeys demonstrating that activity dependent stimulation can strengthen terminal connections with motoneurons (Nishimura et al., 2013).

5. Beyond conditioned reflexes: Metaplasticity

One of the most intriguing discoveries from work exploring the capacity for relational learning is that enlisting this form of processing can do more than modify a S-R reflex: relational processing can have a long-term effect that influences the capacity to learn in the future. Here,

learning (a form of plasticity) is impacting plastic potential, a kind of plasticity of plasticity that Abrahams called *metaplasticity* (Abraham, 2008; Hulme et al., 2013; Schmidt et al., 2013). In the sections that follow, we describe how exposure to uncontrollable and controllable stimulation can have opposing effects on the capacity to learn. We also review how spinal cord neurons can abstract temporal regularity and the implications of this work for locomotor training and recovery.

5.1. Uncontrollable stimulation disables learning

Evidence that a R-O relation can do more than strengthen the performance of a particular behavior response first emerged from studies designed to address a common criticism of past work. As noted above, it is often impossible to draw strong conclusions based on group differences during training. The problem is that animals may differ in two ways: their prior experience and the current behavioral contingency. For example, with the master-yoke paradigm, animals differ in both their training history and whether shock is currently controllable or uncontrollable. To eliminate this confound, animals must be tested under common conditions. In the present case, this can be achieved by testing animals with controllable stimulation. Initially, there was little reason to believe that animals that had previously received uncontrollable stimulation (yoked) would differ from the naïve (untrained) controls. Of course, it was known that exposure to uncontrollable stimulation can induce a learning/performance deficit (learned helplessness) in uninjured animals (Maier and Seligman, 1976). However, this behavioral phenomenon is typically ascribed to brain-dependent processes (Maier and Seligman, 2016). To our surprise, when spinally-transected rats that had received uncontrollable stimulation were tested with controllable shock, they failed to learn (negative transfer).

Of course, animals could exhibit a behavioral deficit because they have lost the ability to perform the target response. Alternatively, prior exposure to uncontrollable stimulation could potentially induce a permanent change in the central nervous system (e.g., apoptotic cell death; Beattie and Bresnahan, 2000) that removes the cellular machinery needed to support learning. The latter appears unlikely because the deficit fades over 2 to 4 days (Crown et al., 2002b), implying it reflects a reversible process. In addition, we routinely equate flexion force across groups and verify that stimulation elicits a comparable flexion response at the start of training. Further, the deficit is evident independent of whether animals are tested on the pretreated or opposite leg, which suggests that it does not reflect a form of muscle fatigue. Finally, animals that have received uncontrollable stimulation, and fail to learn, routinely exhibit the highest number of flexion responses during testing (Grau et al., 1998), which again implies that performance is not impaired. Taken together, our results show that prior exposure to uncontrollable stimulation *disables* the capacity to learn.

To study the learning impairment observed after uncontrollable stimulation, we created a computer program that emulated the pattern of intermittent electric shock produced by a typical master rat, applying shocks on a variable schedule from 0.2 to 3.8 s apart (Fig. 5A) (Crown et al., 2002b). We found that just 6 min of variable intermittent shock induced a learning impairment that lasted 24–48 h. Moreover, this effect was evident when animals were tested on the opposite (contralateral) leg, implying that the phenomenon reflected a central (spinally-mediated) modification (Joyner et al., 2003).

Further work showed that exposure to uncontrollable, but not controllable, stimulation sensitizes reactivity to mechanical stimulation, producing an effect analogous to that observed after treatments that sensitize nociception. This observation led Ferguson to hypothesize that uncontrollable stimulation interferes with the capacity to learn because it induces a diffuse state of over-excitation that saturates NMDAR-dependent plasticity (Ferguson et al., 2006). Supporting this, pretreatment with an NMDAR antagonist prior to uncontrollable stimulation blocks the development of the learning impairment.

Treatments that induce nociceptive sensitization (e.g., capsaicin) also impair instrumental learning (Hook et al., 2008).

These results show that a bout of uncontrollable noxious stimulation induces an alteration in neural function (a form of plasticity) that disables the capacity to learn (plasticity). This metaplastic effect has been linked to an up-regulation of the pro-inflammatory cytokine tumor necrosis factor (TNF), which appears to induce a state of over-excitation through a modification of AMPARs, increasing their permeability to Ca^{++} (Huie et al., 2012a; Huie et al., 2015).

Prior work has shown that serotonergic (5HT) fibers that descend through the DLF can quell nociceptive sensitization and the development of long-term potentiation (Gjerstad et al., 2001; Huang and Grau, 2018). These same processes appear to block the development of the spinally-mediated learning impairment. Supporting this, if applied prior to a spinal transection, uncontrollable stimulation does not affect plastic potential within the spinal cord (Crown and Grau, 2005). This protective effect can be eliminated by lesions limited to the DLF or i.t. application of a 5HT-1A antagonist. Conversely, i.t. application of a 5HT-1A agonist blocks the induction of the learning impairment in spinally transected rats.

As noted earlier, spinally-mediated instrumental learning is reinforced by the onset of nociceptive stimulation. Results from the master-yoke paradigm show that the consequence of this stimulus is gated by behavioral control; if no behavioral relation is detected, noxious stimulation appears to induce a state of over-excitation that enhances reactivity to mechanical stimulation and impairs adaptive learning. Does the emergence of these effects depend upon the phasic (intermittent) character of the stimulation or net exposure to noxious stimulation? If what matters is the duration that nociceptive fibers are engaged, a period of continuous electrical stimulation should have a comparable effect. To evaluate this possibility, we compared the effect of 180 intermittent (80 ms) shocks applied over a period of 6 min to continuous shock given for 14.4 s (180×0.8) or 360 s (Crown et al., 2002b). We found that changing the pattern of stimulation induced a qualitative shift in how it affected nociceptive reactivity and learning. Exposure to continuous shock did not enhance nociceptive reactivity or impair adaptive learning. Instead, it induced a robust antinociception. Further, when intermittent and continuous shocks were applied to distinct dermatomes, exposure to continuous shock blocked the induction of the learning impairment. Likewise, Mitchell and his colleagues have reported that exposure to continuous and intermittent hypoxia have divergent effects on respiratory function; only intermittent hypoxia appears to induce a sensitizing effect that enhances respiration (Mitchell et al., 2001).

5.2. Controllable stimulation enables learning

While exposure to uncontrollable stimulation appears to disable the capacity to learn, exposure to controllable stimulation has the opposite effect. This was shown by training rats with controllable stimulation and testing them with a higher response criterion, accomplished by requiring the animal to lift its leg 4 mm higher to terminate shock (Crown et al., 2002a). Raising the response criterion made the task so difficult that naïve animals failed to learn. But previously trained rats were able to learn and this was true independent of whether they were tested on the same or opposite leg. The fact that learning was enabled (positive transfer) when animals were tested on the contralateral limb again implies a central (spinally-mediated) effect that fosters the capacity to learn. Research has linked this effect to an up-regulation of BDNF (Gomez-Pinilla et al., 2007). Supporting this, TrkB-IgG, which scavenges free BDNF, blocks the enabling effect when applied prior to testing. Conversely, pretreatment with BDNF (i.t.) can substitute for training and enable learning.

Training with controllable stimulation also induces a protective effect that counters the effects of uncontrollable stimulation or capsaicin, blocking both the induction of a learning impairment and

enhancement in mechanical reactivity (Crown and Grau, 2001). Conversely, behavioral training can have a restorative effect when given after noxious stimulation. Both of these have been linked to an up-regulation of BDNF (Huie et al., 2012b).

Our findings indicate that training with controllable stimulation can have two effects, inducing both (1) a limb-specific increase in the flexion response (the target behavior), and (2) a general process that enables learning and reverses the learning impairment produced by uncontrollable stimulation. The latter effect suggests that the initial learning impacts the capacity to learn in future situations, a kind of learning to learn (positive transfer) (Grau et al., 2014; Grau and Huang, 2018). This example of behavioral metaplasticity (Schmidt et al., 2013) is linked to BDNF. A similar effect has been found in studies of intermittent hypoxia, which appears to promote adaptive plasticity by up-regulating BDNF (Mitchell et al., 2001).

Metaplasticity may be especially important to neurorehabilitation. Ideally, a training paradigm will do more than enhance the performance of a particular response—it will induce a lasting effect that enables adaptive plasticity in the future when the individual is challenged in new ways. Importantly, our work suggests that this will counter the development of maladaptive plasticity.

5.3. Learning about temporal relations

Other studies have shown that spinal neurons are sensitive to the temporal relationship between stimuli and that this learning can modulate the consequences of uncontrollable stimulation. Baumbauer discovered this in a series of experiments examining how the adverse effect of intermittent electrical stimulation varies with frequency and intensity (Baumbauer et al., 2008). To study these effects, he made what seemed a trivial change to the procedure—instead of presenting the shocks in an irregular manner, with the temporal spacing between shocks varying in a random manner [a variable time (VT) schedule], he presented the stimuli with the inter-stimulus interval fixed [a fixed time (FT) schedule] (Fig. 5A). For both schedules, the overall frequency of stimulation was the same (0.5 Hz). He reasoned that shifting to a FT schedule should have no effect because timing is the province of the brain (Mauk and Buonomano, 2004). As expected, when animals were exposed to 180 shocks, a learning impairment was observed irrespective of whether the stimuli were given in a regular or irregular manner (Baumbauer et al., 2009). But when shock number was increased 5-fold (720–900 shocks), only irregular (VT) stimulation induced a learning impairment. It appears that continued exposure to FT stimulation has a restorative effect that eliminates the learning impairment induced by a shorter bout (180) of shocks.

What distinguishes FT and VT stimulation is that the former introduces a kind of temporal predictability, where the occurrence of one shock predicts when the next shock will occur (Lee et al., 2015; Lee et al., 2016). Research suggests that an internal process can abstract this relation, providing a temporal cue (a Pavlovian CS) that predicts the occurrence of the next shock (US), a phenomenon known as temporal conditioning.

Further work revealed that an extended exposure to FT stimulation has an effect analogous to behavioral control. For example, exposure to FT shock has a lasting protective effect that blocks the learning impairment and enhances mechanical reactivity induced by VT shock or capsaicin (Baumbauer et al., 2006; Baumbauer and Grau, 2011; Baumbauer et al., 2012). Again, these protective/restorative effects depend upon an up-regulation of BDNF (Baumbauer et al., 2009).

Interestingly, spinal cord neurons can abstract regularity when the bouts of stimulation are given across days (Lee et al., 2015; Lee et al., 2016). This additive effect emerges independent of whether the frequency of stimulation varied across days, suggesting that the key component of training involves stimulus regularity rather than learning about a particular temporal duration. Moreover, regularity can be derived when the locus of the stimulation is randomly varied across

dermatomes and when some of the stimuli are randomly omitted (Lee et al., 2016). These observations imply that FT stimulation engages a central oscillator, a timing-like device that is entrained to the frequency of stimulation. Lee et al. (2016) posited that this capacity could be linked to the central pattern generator that drives hind-limb stepping. Indirect support for this came from the observation that the frequency range that supports timing within the spinal cord mirrors the frequency range of stepping (Baumbauer et al., 2017). More direct evidence came from a study designed to surgically separate the CPG from the neural processes that mediate instrumental learning (Lee et al., 2016). Past work had shown that neurons within the lower lumbosacral (L4–S2) region mediate instrumental learning (Liu et al., 2005). In contrast, the CPG that drives stepping depends upon neurons in the rostral lumbar (L1–2) spinal cord (Magnuson et al., 1999). If a CPG within the L1–2 spinal cord plays a role in the abstraction of regularity, surgically disconnecting the rostral lumbar region (via a L3 transection) should eliminate the capacity to time while having no effect on the ability of the system to learn or the induction of the learning impairment. Under these conditions, an extended exposure to regular (FT) stimulation should induce a learning impairment, and that is indeed what Lee et al. (2016) found.

5.4. Relational processing during step training

Spinally transected animals exposed to treadmill training recover the capacity to step and demonstrate considerable flexibility in performance, adjusting their gait to variation in treadmill speed and flexion force to reduce contact with an obstacle (Edgerton et al., 2004). Stepping involves a form of relational processing, wherein sequenced motor outputs are adjusted based on sensory signals. Like other forms of relational processing, step training up-regulates the expression of BDNF (Gomez-Pinilla et al., 2002; Boyce et al., 2007; Cote et al., 2011; Boyce and Mendell, 2014). Further, pretreatment with BDNF or over-expression of BDNF using a viral vector foster the recovery of locomotor performance.

In addition to improving locomotor performance, step training attenuates spasticity and behavioral indices of chronic pain and these effects have been related to an up-regulation of KCC2 (Cote et al., 2014). These observations are nicely illustrated by the work of (Tashiro et al., 2015), who showed that treadmill training up-regulates the expression of both BDNF and KCC2 in the lumbar spinal cord of rats that had received a T10 contusion injury. Training also attenuated spasticity and allodynia, and these effects were correlated with the up-regulation in KCC2. Further, blocking BDNF signaling with TrkB-IgG attenuated the effect of training on spasticity, allodynia, and KCC2. Similar results were reported by Cote (Cote et al., 2011; Cote et al., 2014), who showed that hyperreflexia emerged 7–28 days after a complete SCI and that this effect was accompanied by a reduction in rate dependent depression (an index of spasticity; Thompson et al., 1992). These effects were also accompanied by a reduction in KCC2 and BDNF within the lumbar spinal cord. Here too, exercise reversed the down-regulation of KCC2 and BDNF, attenuated hyperreflexia, and re-established rate dependent depression.

The findings reviewed here suggest that locomotor training, instrumental conditioning, and exposure to regular stimulation have a common effect after SCI, up-regulating the expression of BDNF, which increases the expression of membrane-bound KCC2. The consequent increase in GABA-dependent inhibition attenuates neural excitability and behavioral signs of pain. Uniting these effects is a dependence on relational processing. This can involve the abstraction of the relation between a response and an outcome, environmental stimuli, or internal cues tied to the regularity of stimulation over time. Indeed, passive cycling (Nardone et al., 2017), treadmill training, and FT stimulation (temporal conditioning) could up-regulate BDNF for the same reason—all provide a form of regular stimulation that entrains the CPG. If true, randomly varying the rate of rotation during cycling would

eliminate its beneficial effect. Temporal regularity could also play a role in step training in another way, through rhythmic stimulation of the perineum, a stimulus used to encourage locomotor performance (Alluin et al., 2015; Rossignol, 2017). Surprisingly little is known regarding this component of the training paradigm. Indeed, work on FT stimulation suggests that perineum stimulation per se could engage the CPG and up-regulate BDNF, yielding some benefit independent of treadmill training.

We noted earlier that enthusiasm regarding the potential benefits of BDNF treatment must be tempered by the adverse effect BDNF has on nociceptive processing in uninjured animals, where research suggests that it down-regulates KCC2 and fosters nociceptive processing (Coull et al., 2005; Merighi et al., 2008; Beggs and Salter, 2013). A potential benefit of behavioral treatments that impact BDNF is that they appear to auto-regulate how BDNF affects cellular function, to assure a homeostatic effect that augments adaptive plasticity. To our knowledge, there is no evidence that behavioral training fosters chronic pain. Indeed, studies suggest the opposite (Tashiro et al., 2015). This auto-regulatory function may be related to the regulation of phospholipase C gamma (PLC γ), which is thought to regulate how BDNF affects KCC2 (Fig. 4B)—when PLC γ is high, BDNF down-regulates KCC2, whereas when PLC γ is low, BDNF up-regulates KCC2 (Rivera et al., 2004; Rivera et al., 2005). Given this, it is noteworthy that Tashiro et al. (2015) found that SCI led to a down-regulation in PLC γ , which could explain why the training-induced increase in BDNF up-regulated (rather than down-regulated) KCC2. These observations suggest that physical therapy may prove beneficial to recovery without risk, to promote adaptive plasticity and counter the development of chronic pain and spasticity.

After SCI, animals can also be trained to stand (Edgerton et al., 2004), which would seemingly require a kind of relational processing, with the motor output adjusted given on-going sensory signals. While less work has been done on the effects of stand training, it does not appear to have the same benefit as locomotor training. To our knowledge, there is no evidence that it up-regulates BDNF or attenuates spasticity and pain. Further, stand training interferes with both learning to step and instrumental conditioning (Bigbee et al., 2007). What may distinguish locomotor and stand training is that only the former engages rhythmic neural activity within the spinal cord.

Interestingly, the down-regulation in membrane-bound KCC2 after a spinal cord transection is accompanied by an increase in the GABA synthetic enzyme glutamic acid decarboxylase (GAD)₆₇ (Tillakaratne et al., 2002). This could enhance GABA release and fuel a diffuse state of over-excitation that interferes with locomotor function. Supporting this, pretreatment with a GABA-A antagonist (bicuculline) promotes locomotor function in transected animals (Robinson and Goldberger, 1986). In addition, the up-regulation of KCC2 observed with step training is associated with a normalization of GAD₆₇, re-establishing pre-injury levels of GABAergic inhibition.

5.5. Relational learning and recovery

Our work suggests that spinal cord systems are wired to detect whether the onset of a nociceptive stimulus is related to limb position, a predisposition that facilitates the performance of an adaptive response designed to minimize tissue damage (Grau et al., 2012). From this perspective, it is only after the system fails to detect a behavioral relation that the alternative mode of processing is engaged, leading to a diffuse state of over-excitation that enhances both psychological relevance (by increasing pain signals relayed to the brain) and behavioral reactivity independent of the locus of stimulation (Ferguson et al., 2006; Ferguson et al., 2012). Lacking a prepared response, the system appears to assume a general defensive state. In the short-term, this could yield an evolutionary advantage; in the long-term, the increased energy expenditure exacts a biological cost. This appears true across the neural axis: within both the brain and spinal cord, uncontrollable stimulation impairs adaptive instrumental learning and induces a state of

over-excitation (Rosen and Schulkin, 1998). In the clinic, there may be little reason to worry whether nociceptive input is being processed by the brain or spinal cord. In either case, providing behavioral control should bring a therapeutic benefit.

The gating action of behavioral control is nicely illustrated by work examining the effect of nociceptive stimulation on secondary injury and recovery. As noted above, engaging nociceptive fibers soon after injury promotes hemorrhage in contused rats and impairs long-term recovery. These adverse effects are not observed if the stimulation is applied in a controllable manner.

The view outlined here assumes that incoming nociceptive signals are processed by two competing systems. One reflects an unconditioned response to intermittent nociceptive stimulation, that grows as a function of stimulus intensity and drives neural excitability. The second detects whether the stimulus occurs within a regular proprioceptive context (controllable stimulation), counters the development of neural over-excitation and promotes adaptive plasticity/learning. We further assume that the protective effect of controllable stimulation is limited (increases to an asymptote) and, for this reason, may be overwhelmed if intense noxious stimulation is applied (Crowe et al., 2002b). If this is true, optimal learning may be observed at intermediate stimulus intensities; further increasing intensity can induce an unconditioned state of over-excitation that could interfere with the capacity to learn. Research has shown that the capacity for learning does indeed decline as shock intensity is increased beyond an optimal level (Grau et al., 1998). In a clinical setting, this would suggest some caution is warranted when adjusting the intensity of electrical stimulation used to encourage adaptive function. While the natural inclination may be to increase stimulus intensity when less than optimal performance is observed, in some situations, a reduction may promote learning and improve performance (Rejc and Angeli, 2019).

As we noted above, noxious input can trigger unregulated sympathetic activation (autonomic dysreflexia) weeks to years after injury (Krassioukov et al., 2009). Research is needed to determine whether a down-regulation in membrane-bound KCC2, and the resultant increase in neural excitability, contributes to this effect. In addition, our results suggest that noxious stimulation may be less likely to trigger this phenomenon when it is provided in a controllable/predictable manner.

In spinally transected animals, we have seen that an extended (24–30 min; 720–900 shocks) exposure to temporally regular (FT) intermittent electrical stimulation can reverse the learning deficit induced by a brief exposure to noxious stimulation (Baumbauer et al., 2009). Further, FT stimulation has a lasting protective effect that counters the adverse effect of subsequent noxious stimulation. Introducing temporal regulation does not, however, mitigate the acute effect of noxious stimulation. The reason for this is that acute hemorrhage in the spinal cord can be triggered by a relatively brief (e.g., 72 s) exposure to nociceptive stimulation, far less than that needed to engage the spinal cord systems that encode regularity (Turtle et al., 2019; Strain et al., 2019). Later in recovery, when stimulation no longer affects tissue loss at the site of injury (Grau et al., 1998), introducing temporal regularity could have therapeutic value.

6. A neurofunctionalist approach to recovery

We have outlined a neurofunctionalist approach to the study of spinal cord plasticity (Grau and Joynes, 2005a). As the name implies, we see value in describing the processes involved in learning at both a functional and neurobiological level. To translate discoveries to clinical practice, a language is needed that readily relates observable events to behavioral recovery. A system is required that allows us to characterize how alternative forms of stimulation and behavioral contingencies impact performance, providing the tools needed to elucidate the conditions that promote learning and yield a lasting benefit.

6.1. Summary: Learning within the spinal cord

After disrupting communication with the brain by means of a thoracic transection, exposure to a noxious stimulus can induce a NMDAR-dependent increase in neural excitability within the lumbosacral dorsal horn (Willis, 2001; Ji et al., 2003). Further work revealed that SCI fosters the development of nociceptive sensitization, an effect tied to a reversal in how GABA impacts neural excitability (ionic plasticity; Rivera et al., 2005), causing GABA to drive rather than inhibit neural excitation (Huang et al., 2016). These findings suggest that nociceptive sensitization may be especially relevant to the development of chronic pain after SCI; therefore, treatments that re-establish GABAergic inhibition could have therapeutic value.

Studies examining the relevance of these observations to recovery after a contusion injury have shown that pain input can expand the area of hemorrhage, increasing tissue loss (secondary injury) and undermining long-term recovery (Turtle et al., 2019). Noxious stimulation also fosters the development of chronic pain and spasticity (Grau et al., 2004; Garraway et al., 2014; Grau et al., 2017). These detrimental effects can be safely blocked through local application of the anesthetic lidocaine (Turtle et al., 2017).

Using spinally transected rats, researchers have shown that neural circuits caudal to injury can support Pavlovian, instrumental, and temporal conditioning (Grau, 2014). This work revealed that exposure to controllable/predictable stimulation has a metaplastic effect that enables learning and counters the learning impairment and nociceptive sensitization induced by uncontrollable/unpredictable stimulation (Grau et al., 2014). The beneficial effect of controllable/predictable stimulation has been related to an up-regulation of BDNF.

Others have shown that locomotor training and exercise also increase the expression of BDNF (Boyce and Mendell, 2014). We suggested that these treatments have a similar effect because they involve the application of rhythmic stimulation that engages the CPG that drives stepping behavior. Further work has revealed that locomotor training and exercise up-regulate KCC2 (Cote et al., 2014; Tashiro et al., 2015). While i.t. BDNF in uninjured animals can reduce KCC2 and foster the development of nociceptive sensitization (Coull et al., 2005), there is no evidence that behavioral training produces this effect. Indeed, behavioral training has been shown to reduce both spasticity and pain. The implication is that locomotor training and exercise engage both BDNF release and a neurochemical signal (e.g., PLC γ) that mitigates the pronociceptive effect of BDNF (Tashiro et al., 2015).

Relational learning also impacts the effectiveness of neurorehabilitation. Engaging proprioceptive neurons with epidural stimulation can promote motor performance, but the effect typically fades soon after the stimulus is terminated (Rejc et al., 2017). A more robust and longer-lasting effect can be induced by pairing epidural stimulation with activity in surviving corticospinal neurons, engaged by stimulating the motor cortex (Harel and Carmel, 2016). A similar effect is observed when an instrumental relation is established between a proxy for the behavioral response (EMG activity in the target muscle) and focal stimulation of the relevant spinal motoneurons (the outcome) (Bonizzato et al., 2018; Nishimura et al., 2013; McPherson et al., 2015; Rascoe et al., 2018).

6.2. Rethinking the traditional view

We began by asking whether circuits within the spinal cord can learn without input from the brain, an issue that is routinely explored using animals that have received a complete SCI (Groves et al., 1969; Patterson, 2001a; Grau, 2014). New findings show that SCI enables plasticity and neural excitation caudal to injury by reducing GABA-dependent inhibition (Drew et al., 2004; Cramer et al., 2008; Boulenguez et al., 2010; Medina et al., 2014). These observations imply that ionic plasticity regulates the capacity for learning within the spinal cord (Fig. 4B). Based on these data, it could be argued that the spinal

cord in uninjured animals is immutable, as assumed within the traditional view. There are, however, a number of observations that temper this conclusion. First, membrane-bound KCC2 within the dorsal horn is regulated by the brain and exhibits a rapid shift (within 30 min of treatment) in response to pharmacological treatments that target the 5HT-1A receptor (Huang and Grau, 2018). Second, the regulation of plasticity within the spinal cord could reflect a general phenomenon within the CNS; under normal circumstances, much of the adult brain may be relatively immutable, with neural connectivity preserved over time through GABAergic inhibition (e.g., see Ostroumov and Dani, 2018). It should also be recognized that an inhibition of plasticity does not necessitate an inhibition of relational processing. Indeed, GABAergic inhibition presumably functions to maintain neural circuits, to help assure learned relations are processed in the appropriate manner.

The perspective offered here is consistent with the model outlined by Krishnan (2006) and Grossberg (2013), wherein a neural network (hidden layer) encodes environmental relations and structures behavior. From this perspective, adaptive learning depends upon the maintenance of a *stability* $\leftrightarrow$ *plasticity balance*. From our perspective, the over-excitation caused by a down regulation in membrane-bound KCC2 disrupts this balance, inducing a form of maladaptive plasticity that interferes with learning. This could contribute to the *stability* $\leftrightarrow$ *plasticity dilemma* that Krishnan suggests fosters *catastrophic forgetting* and motor paralysis. Re-establishing the balance through training or drug treatment would uncover the latent motor patterns and promote behavioral function. Such a view suggests that the synaptic connections needed to support hind limb stepping after a thoracic injury are present but inaccessible—training allows locomotor function to re-emerge by reducing over-excitation, re-establishing the *stability* $\leftrightarrow$ *plasticity balance*.

Our perspective is also consistent with the view that neurobiological processes that mediate learning and memory within the brain (e.g., NMDAR-mediated plasticity, long-term potentiation, spike-timing dependent plasticity, spine formation) contribute to plasticity within the spinal cord (Sandkuhler, 2000; Ji et al., 2003). What a reference to learning adds is an interface with behavior that helps to characterize the circumstances under which these systems will be engaged and how this plasticity affects behavioral/psychological function. From this perspective, training regimens that encourage relational learning should up-regulate BDNF and foster enduring structural modifications (e.g., spine formation) (Bennett and Lagopoulos, 2014; Gomez-Pinilla et al., 2007; Malcangio and Lessmann, 2003; Vavrek et al., 2006). Further, the molar principles derived from the study of learning may help us to characterize how systems interact. For example, it well known that learning about a particular cue depends upon the value of co-occurring cues; if an untrained cue is presented in compound with one that has been previously trained (blocking), or that is more salient (overshadowing), learning will be impeded. This basic relation is captured by the Rescorla-Wagner model (Rescorla and Wagner, 1972) and has been used to describe the circumstances that engage plastic modifications within the amygdala, nucleus accumbens, and cerebellum (Kim et al., 1998; McNally et al., 2011; Schultz, 2006). Such a competitive process may also be at work within the spinal cord (Illich et al., 1994). For example, neural injury can initiate a form of plasticity in the spinal cord that rebalances the degree to which corticospinal fibers versus proprioceptive afferents drive motor circuits. Recent work has shown that the relative sprouting in these two pathways emerges in a competitive manner (Jiang et al., 2016). Supporting this, driving proprioceptive afferents using electrical stimulation favors sprouting of this input whereas inhibiting proprioceptive afferents favors corticospinal fiber sprouting.

Throughout this review, we have acknowledged the limits of learning within the spinal cord, detailing how it is biologically prepared and built upon pre-existing neural circuits. While biological constraints impact brain-dependent learning (Timberlake and Lucas, 1989), their influence often appears weaker, enabling more flexible behavioral

Spinally-mediated learning and memory

- Brain-dependent learning can induce a lasting alteration (memory) in the spinal cord (Wolpaw, 2010).
- Neurons within the spinal cord can learn in response to stimulation and behavioral relations without input from the brain (Grau, 2014).
- Learning within the spinal cord depends upon pre-existing (biologically prepared) networks and (S-R) habits (Grau, 2014).
- Encouraging change within the spinal cord typically requires higher levels of stimulation and/or extended training (see text).

Nociceptive stimulation

- SCI removes a brain-dependent brake on neural excitation and plasticity (Huang et al., 2016).
- After SCI, noxious stimulation sensitizes nociceptive circuits within the spinal cord (Huang et al., 2016).
- The consequences of stimulation depends upon its temporal pattern (e.g., intermittent vs. continuous) (Crown et al., 2002).
- After an incomplete SCI, acute pain input increases tissue loss and impairs recovery (Grau et al., 2004).
- Pain input during the chronic phase of injury can disrupt locomotor performance (Bouffard et al., 2014).

Pavlovian conditioning

- Neurons within the spinal cord can encode Pavlovian (S1 → S2) relations (Durkovic, 2001).
- Learning about stimulus relations varies with the temporal relationship between S1 and S2 (forward > backward) (Durkovic, 2001).
- Presenting S1 in compound with a pretrained stimulus (blocking) or a more salient cue (overshadowing) disrupts learning (Illich et al., 1994).
- Unpaired presentation of S1 weakens the acquired response (extinction) (Durkovic, 2001).

Instrumental conditioning

- Neurons within the spinal cord can encode Instrumental (R → O) Relations (Grau et al., 1998).
- Spinally-mediated instrumental learning is reinforced by the onset of noxious stimulation (Grau et al., 1998).
- Prior exposure to uncontrollable stimulation disrupts instrumental learning (Crown et al., 2002).
- Training with controllable stimulation enables adaptive learning (Crown et al., 2002).
- Training with controllable stimulation can prevent, and reverse, maladaptive plasticity (Crown & Grau, 2001).
- Optimal learning is observed at intermediate levels of electrical stimulation; inferior learning is observed with intense stimulation (Grau et al., 1998).
- Behavioral control can mitigate the adverse effect noxious stimulation has on tissue loss and recovery (Grau et al., 1998).

Temporal conditioning, locomotor training, and exercise

- Neurons within the spinal cord can abstract that a sequence of stimuli occur at regular (fixed) intervals (temporal conditioning) (Lee et al., 2015).
- Spinal cord neurons can abstract stimulus regularity across gaps in time (savings) (Lee et al., 2015).
- Temporal conditioning can prevent, and reverse, maladaptive plasticity (Baumbauer et al., 2009).
- Locomotor training and cycling promotes adaptive plasticity after SCI (Cote et al., 2011).

Neuromodulation

- Stimulation that engages proprioceptive afferent fibers promotes spinal function (Harkema, 2011).
- Greater gain is observed when cortical stimulation is paired with epidural stimulation (open-loop stimulation) (Harel & Carmel, 2016).
- Greater gain is observed when spinal cord stimulation is given in a response-contingent manner (closed-loop stimulation) (McPherson et al., 2015).

Fig. 7. Principles derived from experimental work examining how stimulation and training affects behavior and function after SCI. In all cases, further work is needed to establish the generality of these principles and their clinical relevance. Additional references are provided within the text.

solutions. Brain-mediated processes allow the organism to span gaps in time, abstract complex stimulus relations, and rapidly modify performance. In terms of classic learning approaches, brain-dependent processes allow for a form of cognitive inference (Tolman, 1949), whereas learning within the spinal cord seems more akin to S-R habit learning (Hull, 1943).

6.3. A neurofunctionalist approach

A neurofunctionalist approach finds value in describing the abstract relations that govern learning across situations (Grau and Joynes, 2005a). Such a view recognizes that environmental puzzles can be solved in multiple ways, by a variety of processes and neural structures. From this perspective, our scientific task is to discover when these processes are engaged and the neurobiological systems involved. For treatment, the task is to describe the events and relations that engage these systems with the aim of promoting adaptive plasticity. This framework is consistent with recent trends emphasizing a hypothesis-driven approach based on computer simulations (e.g., Moraud et al., 2016; Formento et al., 2018).

In neurorehabilitation, treatment can bring about a modification in performance through compensation or a restoration of function (Fouad et al., 2019). At a behavioral level, the loss of a particular ability (e.g., being able to pick up an object with the right hand) can engage a form of *compensation* (use the left hand) that accomplishes the goal.

Restoration of function would require re-learning to execute the affected behavior. In the present example, this could potentially be achieved through forced use of the disabled limb (Taub, 2012). Notice that, from a neurofunctionalist perspective, a particular behavioral function may be subserved by multiple biological mechanisms (Fig. 2B). As a result, the capacity to perform a specific action after injury (behavioral restoration) could be achieved through a form of neural restructuring (e.g., sprouting) that compensates for an injured biological system (loss fibers). What is critical to rehabilitation is behavioral restoration and, at a practical level, whether this involves a form of biological compensation may be of little concern. Indeed, true biological restoration (i.e., re-establishing the pre-injury pattern of neural connectivity) is likely fleetingly rare.

Additionally, a functionalist approach to learning considers the ultimate goal of behavior, assuming that this will shed light on the circumstances under which a processing module is engaged. This assumes that biological predispositions have been tuned by evolution and shaped by development—that the adult nervous system brings an inherent structure, and that we need to understand this structure to make advances (Courtine and Bloch, 2015). It is further presumed that learning occurs within a behavioral system, a structure that functions as a sort of lattice hierarchy (in contrast to a traditional, military-like, hierarchy; Gallistel, 1980). Again, it is assumed that the organism can utilize multiple solutions when faced with an environmental puzzle, solutions that are coordinated and integrated across different levels of

the nervous system. Within this lattice, learning can function in multiple ways, to maximize biological advantage and minimize the predictive error, gravitating towards a negotiated equilibrium (Wolpaw, 2018).

Recent advances in neurorehabilitation have been inspired by neurobiological data demonstrating spike-timing-dependent plasticity (Quilgars and Bertrand, 2019). While this conceptual link can help us understand how a treatment impacts spinal cord function, the net effect of training likely depends upon the additive consequence of dozens of processes. Given the limits of current technology, the state of these processes cannot be tracked during clinical treatment. Neurofunctionalism offers an alternative framework, seeking the abstract principles that link neurobiological processes to definable stimuli and behavioral relations. To illustrate this approach, Fig. 7 provides a preliminary draft based on the work reviewed above. These should be treated as a rough road map that will need to be refined through further work designed to identify the generality, limits, and clinical usefulness of the principles.

We have suggested that concepts from the field of learning are relevant to recovery after SCI and noted parallels to recent work in the area of neurorehabilitation. Research in this area has emphasized the importance of factors such as stimulus pairing and response-contingent (closed-loop) stimulation (Mishra et al., 2017; Courtine and Sofroniew, 2019). These concepts have a long history in the field of learning and recognizing the parallels could help researchers identify the most effective treatment regimes.

We have focused upon the analysis of learning using a model system that allows us to experimentally manipulate key relations. This work has revealed a host of principles that appear to guide the development of adaptive plasticity within the spinal cord and the factors that impede this learning. As we have shown, the work is consistent with on-going research using training paradigms designed to emulate key features of rehabilitation (e.g., locomotor training). While these distinct lines of work complement each other, a simple model system has some advantages, allowing precise control over key training parameters and a methodology that can be used to uncover the capacities and limits of spinal learning. Likewise, Wolpaw (2010, 2018) and his colleagues have adopted a similar approach to uncover how brain-dependent learning modulates neural function within the spinal cord.

In developing a framework to characterize spinal cord function, we have leaned upon concepts developed within the field of learning, suggesting some benefit can be gained from decades of work that details how sensitization and relational learning develop and impact neural function. At the same time, it is recognized that a broader view could contribute additional value. Indeed, recovery of stepping with locomotor training would appear to have much in common with the acquisition of bird song, where experience fosters the performance of a motor pattern based upon an internal neural model. The key points, however, remain the same: neurons within the spinal cord can learn, and characterizing the nature of this capacity will shed light on spinal cord function and clarify the circumstances under which neurorehabilitation and physical therapy will be most beneficial.

Declaration of Competing Interest

The authors declare no competing financial interests.

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Nociceptive (pain) input after spinal cord injury (SCI) enhances secondary injury: Identifying treatments that can be translated to clinical practice

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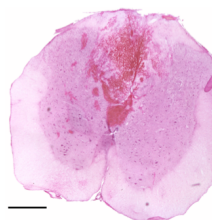
Study/Product Aim(s)

- **Aim 1:** When does nociceptive input have an adverse effect?
- **Aim 2:** Promoting recovery using general anesthesia.
- **Aim 3:** Promoting recovery using local anesthesia.

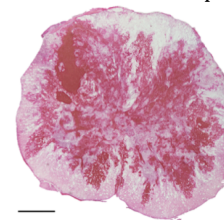
Approach

Pain input after spinal cord injury drives tissue loss by increasing the area of secondary injury/hemorrhage. Three aims were proposed that will define when pain input affects injury processes and how to block this effect. It is proposed that general anesthesia, or blocking spinal activity with lidocaine, will reduce pain-induced tissue loss and thereby promote recovery. These treatments could be rapidly translated to practice.

Contusion Alone



Contusion+Pain Input



Typical sections from contused rats that received noxious stimulation after injury (right) or nothing (left). Pain input dramatically increases the area of hemorrhage (dark red). (4x; bar = 500µm).

Accomplishments: Prior work had shown that noxious electrical stimulation induces hemorrhage at the site of injury and undermines long-term recovery. This effect is eliminated by a rostral transection. Research funded by this grant has shown that an irritant that engages pain (C) fibers has a similar effect, that comparable results are observed in both male and female rats, and that the pain input induces greater damage 6-24 hr after injury, and that hemorrhage fades 24 hrs after pain input (Aim 1). We also showed that administration of anesthesia after painful stimulation does not attenuate hemorrhage (Aim 2, Task 1), that anesthesia blocks the effect of capsaicin treatment (Aim 2, Task 2), and that rostral lidocaine blocks the effect of nociceptive stimulation (Aim 3, Task 2). New data suggest anesthesia after surgery has a protective effect (Aim 2, Task 3).

Timeline and Cost

Activities	Year 1	Year 2	Year 3
Major Aim 1: Determine when does nociceptive input has an adverse effect?			
Major Aim 2: Determine when general anesthesia has a protective effect.			
Major Aim 3: Determine when the local application of lidocaine has a protective effect.			
Estimated Budget (\$K):	\$162,423	\$166,165	\$170,031

Goals/Milestones

Mos. 1-2: Complete animal protocols

- IACUC approval; ■ ACURO approval

Mos. 3-16: Aim 1—When does nociceptive input affects recovery?

- Task 1 (100%); ■ Task 2 (100%); □ Task 3 (75%); □ ■ Task 4

Mos. 17-26: Aim 2 – Evaluate effectiveness of general anesthesia.

- Task 1; ■ Task 2; □ Task 3 (40%);

Year 3/Aim 3 – Evaluate effectiveness of local lidocaine.

- Task 1; ■ Task 2 (100%); □ Task 3

Concerns

- To date, no problems have been encountered.

Budget Expenditure to Date

Expenditures: \$383,865 (direct; 77% of the net budget)

Updated: January 28, 2020