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Research paper

Engaging pain fibers after a spinal cord injury fosters hemorrhage and expands the area of secondary injury

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ABSTRACT

In humans, spinal cord injury (SCI) is often accompanied by additional tissue damage (polytrauma) that can engage pain (nociceptive) fibers. Prior work has shown that this nociceptive input can expand the area of tissue damage (secondary injury), undermine behavioral recovery, and enhance the development of chronic pain. Here, it is shown that nociceptive input given a day after a lower thoracic contusion injury in rats enhances the infiltration of red blood cells at the site of injury, producing an area of hemorrhage that expands secondary injury. Peripheral nociceptive fibers were engaged 24 h after injury by means of electrical stimulation (shock) applied at an intensity that engages unmyelinated pain (C) fibers or through the application of the irritant capsaicin. Convergent western immunoblot and cyanmethemoglobin colorimetric assays showed that both forms of stimulation increased the concentration of hemoglobin at the site of injury, with a robust effect observed 3–24 h after stimulation. Histopathology confirmed that shock treatment increased the area of hemorrhage and the infiltration of red blood cells. SCI can lead to hemorrhage by engaging the sulfonylurea receptor 1 (SUR1) transient receptor potential melastatin 4 (TRPM4) channel complex in neurovascular endothelial cells, which leads to cell death and capillary fragmentation. Histopathology confirmed that areas of hemorrhage showed capillary fragmentation. Co-immunoprecipitation of the SUR1-TRPM4 complex showed that it was up-regulated by noxious stimulation. Shock-induced hemorrhage was associated with an acute disruption in locomotor performance. These results imply that noxious stimulation impairs long-term recovery because it amplifies the breakdown of the blood spinal cord barrier (BSCB) and the infiltration of red blood cells, which expands the area of secondary injury.

1. Introduction

After an insult to the spinal cord (primary injury), cellular processes can lead to cell death in the surrounding tissue (secondary injury). These secondary processes unfold over the course of hours-to-days after injury and may double the area of tissue loss (Beattie et al., 2002; Ducker et al., 1971; McVeigh, 1923; Hausmann, 2003). Recent work has shown that the development of secondary injury is amplified by pain input below the injury site (Grau et al., 2004, 2017). This is clinically important because SCI is often accompanied by additional tissue damage (polytrauma) that provides a source of pain input (Chu et al., 2009; Hasler et al., 2011; Saboe et al., 1991; Sekhon & Fehlings, 2001; Wang et al., 2001).

We have explored the effect of pain (nociceptive) input using an animal (rat) model (Grau et al., 2004, 2017). In rats that have undergone an upper thoracic transection, intermittent electrical stimulation

Abbreviations: ANOVA, Analysis of variance; ANCOVA, Analysis of covariance; BBB, Basso, Beattie and Bresnahan (locomotor scale); BSCB, Blood-spinal cord barrier; ECL, Electrochemiluminescence; H&E, Hematoxylin & eosin; IL-1ß, Interleukin-1ß; IL-18, Interleukin-18; PHN, Progressive hemorrhagic necrosis; PVDF, Polyvinylidene difluoride; RRID, Research resource identifier; SCI, Spinal cord injury; SUR1, Sulfonylurea receptor 1; T, Thoracic; TBST, Tris-buffered saline tween-20; TNF, Tumor necrosis factor; TRPM4, Transient receptor potential melastatin 4 (TRPM4); TRPV1, Transient receptor potential cation channel subfamily V member 1

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(shock) at an intensity that engages unmyelinated pain (C) fibers impairs adaptive plasticity (Baumbauer et al., 2008; Crown et al., 2002; Ferguson et al., 2006). Application of capsaicin, which engages C-fibers that express the transient receptor potential cation channel subfamily V member 1 (TRPV1), to one hind paw has the same effect (Hook et al., 2008). In animals that have received a contusion injury of the lower thoracic spinal cord, engaging pain fibers below the injury expands tissue loss and undermines long-term locomotor recovery (Grau et al., 2004; Turtle et al., 2018). Nociceptive stimulation also fosters the development of spasticity, impairs the recovery of bladder function, and enhances the development of chronic pain (Grau et al., 2004, 2017; Garraway et al., 2014). These adverse effects are most evident when stimulation occurs within a few days of injury (Grau et al., 2004) and are associated with the activation of pro-inflammatory cytokines [e.g., tumor necrosis factor (TNF), interleukin-1ß (IL-1ß), and interleukin-18 (IL-18)] and indices of cell death (e.g., caspase 1, 3, and 8) (Garraway et al., 2014; Turtle et al., 2018).

In spinally transected rats the adverse effect of noxious electrical stimulation is blocked by intrathecal application of the anesthetic lidocaine (Joynes et al., 2003). In contused rats, lidocaine given by means of lumbar puncture prior to noxious electrical stimulation blocked its effect on recovery and tissue loss (Turtle et al., 2017). It also attenuated the activation of pro-inflammatory cytokines (IL-1ß and IL-18), indices of cell death (e.g., caspase 3), and the deposition of red blood cells (hemorrhage) at the site of injury. Because blood borne contents are neurotoxic, nociception-induced hemorrhage could increase tissue loss (secondary injury), which would undermine long-term recovery (Mautes et al., 2000).

The observation that noxious stimulation induces hemorrhage after SCI helps to explain why pain input has an adverse effect and suggests new targets for treatment, related to alterations in the BSCB and blood flow. However, our evidence for this effect is limited, based on one type of stimulation, a single time point, and one experiment (Turtle et al., 2017). The present study is designed to address these issues by assessing the extent of hemorrhage at multiple time points, after alternative forms of noxious stimulation (shock or the irritant capsaicin), and employing multiple assays (spectrophotometry, immunoblotting, histopathology, and a cyanmethemoglobin colorimetric assay). Our results show that noxious stimulation induces acute hemorrhage and that this effect emerges within hours of treatment. We further show that the development of hemorrhage is accompanied by an acute decline in locomotor performance.

2. Experimental procedures

2.1. Animal subjects

Subjects were adult male Sprague-Dawley rats (RRID: RGD 5508397; 100–120 days old, 300–350 g) obtained from Envigo in Houston, TX. Rats were maintained under a 12-h light-dark cycle, with all experimental procedures taking place during the light portion. All experiments were approved by the Institutional Animal Care and Use Committee at Texas A&M University and were performed in accordance with NIH standards for the use of laboratory animals (NIH publication No. 80–23). The number of animals used was limited to that which was absolutely necessary for the experiment, and every effort was made to minimize suffering.

2.2. Surgery

Rats received a contusion injury at the level of the T11–12 vertebrae using the MASCIS device. Animals were anesthetized with 5% isoflurane gas and a surgical level of anesthesia was maintained with 2–3% isoflurane. A longitudinal incision extending approximately 2 cm rostral and caudal to the injury site was made on both sides of the vertebral column. The T11–12 vertebrae were then palpated and exposed. A laminectomy was performed, exposing the spinal cord while keeping the dura intact. The vertebral column was held steady and a 10 g impactor was dropped onto the spinal cord from a height of 12.5 mm. Following surgery, the incision was closed with Michel clips and rats were administered 100,000 units/kg of penicillin and three ml of saline to prevent infection and replace lost fluids.

For the 24 h following surgery, animals were housed individually in a temperature-controlled room and allowed to recover overnight. After the first day, animals that were maintained for an additional day were returned to standard housing. After surgery, animals had free access to food and water. Bladders were checked at regular intervals (every 8–10 h after surgery and 2–4 h during testing) and expressed. While prior work has shown that nociceptive stimulation delays the recovery of bladder function (Grau et al., 2004), these effects were observed weeks after injury. Because animals in the present study were sacrificed within 48 h of injury, nearly all (91%) required expression after surgery.

Prior to shock or capsaicin treatment, locomotor performance was assessed using the scale developed by Basso, Beattie and Bresnahan (BBB) (Basso et al., 1995). As in prior studies, animals that exhibited a partial injury (defined as a BBB score > 8) were excluded (1 rat). Across the experiments, there were no group differences in locomotor performance a day after injury, prior to noxious stimulation (all F's < 1.0, $p > .05$).

2.3. Nociceptive stimulation

Prior work has established that a brief (6 min) exposure to intermittent electrical stimulation can induce a form of maladaptive plasticity and impair recovery after a contusion injury (Grau et al., 2004; Hook et al., 2008). An advantage to this procedure is that both the duration and intensity of stimulation can be readily controlled. Further, there is no tissue damage at the intensity used. In the present study, stimulation was applied to the tail while the rat was loosely restrained in an opaque Plexiglas tube. The electrodes were coated with electrode gel and taped to the tail centered approximately 3.5 cm from the tip. Using a constant current shocker, animals (Shocked) received 180 shocks, 100-msec in duration on a variable inter-stimulus interval that ranged from 0.2–3.8 s (mean 2 s). After the last shock, the electrodes were removed and the rat was returned to a holding bin. Unshocked controls were treated the same, except they received no electrical stimulation.

To evaluate the generality of our results, we also assessed the effect of chemically activating peripheral pain fibers using the irritant capsaicin. Rats were loosely restrained in Plexiglas tubes with their hind limbs and tail exposed. With a 27-gauge needle, 50 µL of a 3% capsaicin solution was injected into the dorsal surface of the hind paw. Control subjects were administered an injection of vehicle (7% Tween-20 in normal saline). In contrast to a brief (6 min) exposure to intermittent electrical stimulation, peripheral treatment with capsaicin tonically activates nociceptive fibers for a period of hours (Huang et al., 2016; Willis, 2001).

We chose these two forms of stimulation to demonstrate the generality of our results. Both forms of stimulation have been shown to sensitize nociceptive processing and impair long-term recovery (Grau et al., 2004; Huang et al., 2016; Turtle et al., 2018). They differ in duration, temporal character (phasic versus tonic), and site of application. In addition, capsaicin provides a natural stimulus that selectively engages one class of C-fibers whereas shock engages a broad range of sensory fibers.

2.4. Cellular assays

2.4.1. Tissue collection

Rats were euthanized with 100 mg/kg of pentobarbital at 1, 3, or 24 h after the application of nociceptive stimulation. One centimeter of

spinal cord tissue centered at the lesion was dissected and flash frozen in liquid nitrogen. Protein was extracted using the Qiagen RNeasy Lipid Tissue Mini kit according to manufacturer's instructions. A Bradford (BioRad, Hercules, CA) assay was performed to determine the concentration of protein extracts. Protein samples were diluted in 4× Laemmli buffer to a final concentration of 3 mg/mL.

2.4.2. Spectrophotometry for hemoglobin

Spectral analyses for free hemoglobin were conducted from protein extracts from lesion tissue. Spectrophotometric absorbance was measured from 200 to 800 nm from 1.0 µl of protein extract (NanoDrop, Thermo Scientific). Absorbance at 420 nm was used as a measure of hemoglobin content (Prahl, 1999).

A second photometric assay based on cyanmethemoglobin colorimetry was conducted to verify that the absorbance reflected the infiltration of blood. Briefly, 10 µl of protein extract was added to 40 µl of Drabkin's reagent (Ricca Chemical, #RC266032) and incubated at room temperature for 20 min. 1 µl of each sample was loaded onto a spectrophotometer and absorbance was assessed at 540 nm (as per the manufacturer's instructions), against a standard curve generated with known concentrations of native rat hemoglobin (Lifespan Biosciences, #LS-G11201-10, Seattle, WA) in Drabkin's reagent.

2.4.3. Immunoblotting

Samples were analyzed using SDS PAGE on 12% pre-cast Tris-HCl gels (BioRad, Hercules, CA). Protein samples in sample buffer were thawed at 96 °C for 10 min. Pre-cast 12% Tris-HCL gels (BioRad) were inserted into a Criterion cell and loaded with a standard Laemmli running buffer solution. Equal amounts of protein (30 µg) were loaded into each well and 5 µL of a biotinylated ladder (BioRad) was added to the first well. Gels were run at 180 V for 1 h. Ice packs were used to prevent overheating during the run cycle. The gels were then equilibrated in cold transfer buffer and transferred onto a methanol-activated polyvinylidene difluoride (PVDF; Millipore, Bedford, MA) membrane. The transfer apparatus was assembled according to the manufacturer's instructions and placed on ice. The proteins were transferred at 100 V for 1 h. Following transfer, the membrane was blocked with 5% blotting-grade milk (BioRad, Hercules, CA) in Tris-buffered saline with Tween-20 (TBST) for 1 h on an orbital shaker. The blots were then incubated with either anti-hemoglobin α primary antibody [1:1000; Abcam (Cambridge, MA) ab92492; RRID: AB10897054] or anti-lamin B primary antibody (1:1000; Abcam ab16048; RRID: AB443298) at 4 °C on an orbital shaker overnight. The next day, the blots were washed three times in TBST for 10 min each at room temperature and then incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000; #31460; Pierce, Rockford, IL) for 1 h. Blots were washed three more times with TBST then developed with an electrochemiluminescence (ECL) substrate kit (BioRad) and imaged with Fluorchem HD2 (ProteinSimple, Santa Clara, CA). Ratios of the integrated densitometry of each protein of interest to the loading control were calculated and normalized to a control group (run on the same blot) that did not received nociceptive stimulation.

2.4.4. Co-immunoprecipitation

Protein was extracted from whole tissue lysate under non-denaturing conditions and prepared for co-immunoprecipitation using an agarose bead preparation. Briefly, protein samples were diluted to a final concentration of 1 µg/µl. Then, 500 µL of protein extract was incubated with 50 µl of TrueBlot anti-rabbit immunoprecipitation beads (Rockland Immunochemicals, Limerick, PA) on ice for 30 min for preclearing. Beads were spun down and the supernatant was retained. Precleared samples were next incubated with the primary antibody against SUR1 (QED Biosciences, 11567) on ice for 1 h. After incubation, 50 µL of TrueBlot beads were added and samples were incubated for 1 h on a rocking platform. Next, samples were spun down and washed with lysis buffer. Finally, 100 µL of 4× Laemmli buffer was added to each sample and the samples were heated for 10 min at 96 °C. After heating, samples were spun down and the supernatant retained. The co-immunoprecipitated proteins were then run on 26-well pre-cast Criterion gels (BioRad) according to manufacturer's instructions. Samples were boiled for 10 min in 4× Laemmli buffer prior to loading. 10 µl of each sample was loaded into each well and electrophoresis was performed at 180 V for 1 h and 15 min. Proteins were then transferred onto a PVDF membrane for 1 h at 100 V and blocked in milk for 1 h prior to overnight incubation in primary antibody for TRPM4 (Novus, Cat# NB110–74949, RRID:AB_2208611) at 4 °C. Following a series of washes in tris-buffered saline tween-20 (TBST), blots were incubated in secondary antibodies for 1 h at room temperature. Finally, blots were imaged using ECL.

2.5. Microscopy

2.5.1. Tissue collection and sectioning

3 h after nociceptive stimulation, rats were injected with a lethal dose of pentobarbital. After the heartbeat had terminated, subjects were perfused transcardially with ~300 mL of glacial phosphate-buffered saline (PBS) (pH 7.3) followed by ~400 mL of 4% paraformaldehyde (PFA). Spinal cord tissue at the site of injury was collected and incubated in 4% PFA for 2 h at 4 °C. The tissue was rinsed with PBS before cryoprotection in a solution of 30% sucrose in PBS for at least 72 h.

To quantify the extent of hemorrhage and capillary fragmentation, a 1 cm portion of spinal cord tissue was collected that encompassed the site of injury. After fixation, 20 µm coronal sections were collected and mounted on Fisherbrand Superfrost Plus (Fisher Scientific) microscope slides.

2.5.2. Assessment of hemorrhage extent

To reconstruct the area of hemorrhage, the tissue was stained with hematoxylin and eosin (H&E) using the procedure described by Lillie (1947). Ten sections spaced 800 µm apart were washed with distilled water then incubated in hematoxylin for five minutes. Sections were rinsed with sequential changes of distilled water until the water ran clear. Slides were then dipped in 1% hydrochloric acid (HCl) in 70% ethanol (EtOH) twice, and rinsed with distilled water. Next, slides were incubated in Scott's tap water (Sigma-Aldrich, Cat# S5134) substitute before distilled water rinses. Finally, slides were incubated in eosin for two minutes, and dehydrated with ethanol and xylene before mounting with Permount. Sections were imaged using light microscopy at 40× magnification and were analyzed by blinded observers using ImageJ software. Ten sections spanning the injury site were selected for each subject and the perimeter of the spinal cord was traced to determine the total area for each section. Areas of hemorrhage appeared much redder than surrounding tissue, which was confirmed by the presence of red blood cells at a higher magnification. The hemorrhaged area for each section was traced by a blinded observer to determine the total area of hemorrhage. The amount of hemorrhage was quantified as a percentage of the total section area. After a preliminary analysis, the region of greatest damage was identified and seven sections (lesion center ± 3) were selected to represent the area of hemorrhage.

2.5.3. Assessment of capillary fragmentation

To examine whether the areas of hemorrhage in rats that had received electrical stimulation were associated with capillary fragmentation, a set of coronal sections were stained with a marker for endothelial cells. The tissue was allowed to thaw for one hour at room temperature. The film of mounting medium was removed with tweezers and a ring was made around each section with a PAP pen and allowed to dry. The slides were washed three times in PBS for ten minutes on an orbital at room temperature. Sections were covered in 200–300 µl of blocking solution (30 mL PBS + 30 µl Triton-X + 900 µl normal goat serum) and placed in a humidified box for one hour. The slides were then blotted on a paper towel and incubated in 300 µL of fluorescein

isothiocyanate (FITC) – tomato lectin (1:200 concentration; Sigma Aldrich RRID: AB_2307440) in a humidified dark box on an orbital shaker for 24 h at room temperature. After incubation, the slides were washed three times in PBS for ten minutes and cover-slipped with Prolong Gold with 4′,6-Diamidino-2-phenylindole (DAPI) (Invitrogen, Cat# P36935). Areas of hemorrhage on the tomato lectin stained slides were identified using adjacent H&E stained slides. Two regions of equal area, one with hemorrhage and one without, were selected by a blinded observer. Care was taken to select areas that were positioned symmetrically across the medial axis. Capillaries were identified as tubular structures larger than 5 μm with a length to width ratio greater than one. Capillary length was analyzed at the lesion epicenter and 0.5 cm rostral and caudal to the epicenter using the ImageJ plugin, NeuronJ (Meijering et al., 2004). Using NeuronJ, the beginning and end of each fragment with the region was identified by a blinded observe. The plugin then traces the length of the fragment. Average capillary length was calculated and compared between areas with and without hemorrhage.

2.6. Statistics

All data were analyzed using analysis of variance (ANOVA) or analysis of covariance (ANCOVA). In all cases, a criterion of $p < .05$ was set as the threshold for statistical significance. For our primary measures, we also report the proportion of variance accounted for [eta squared (η^2)].

3. Results

3.1. Experiment 1: engaging sensory fibers with electrical stimulation induces hemorrhage after SCI

In a study examining whether intrathecal lidocaine can block the adverse effect of nociceptive stimulation (Turtle et al., 2017), we observed that the tissue from contused rats that had received noxious electrical stimulation (shock) appeared to have a higher concentration of red blood cells. This effect was evident from both the coloration of the protein samples collected 3 h after treatment and western blotting for a key component of red blood cells, alpha hemoglobin. The present study sought to replicate this effect and evaluate its time course by collecting tissue 1, 3 and 24 h after shock treatment.

Rats ($N = 36$) received a lower thoracic contusive spinal cord injury. The next day, half of the animals were restrained and given intermittent electrical stimulation to the tail (Shocked). The remaining animals were restrained but received no stimulation (Unshocked). Rats were returned to their home cages and sacrificed 1, 3, or 24 h later. These treatments yielded a 2 (Shocked or Unshocked) × 3 (tissue collected at 1, 3, or 24 h) factorial design with six rats per group. For protein assays, a 1 cm section of spinal cord tissue centered at the lesion epicenter was collected. Levels of alpha hemoglobin were then assessed using spectrophotometry and western blotting.

Spectrophotometry revealed a peak absorbance at 420 nm (Fig. 1A), the absorption peak for hemoglobin (Prahl, 1999). Quantitative assessment showed that shocked contused rats exhibited greater absorbance at 420 nm with a maximal effect at 3 h (Fig. 1B). An ANOVA revealed a significant effect of shock treatment ($F_{(1,\ 30)} = 10.91$, $p < .005$, $\eta^2 = 0.102$), time ($F_{(2,\ 30)} = 28.07$, $p < .0001$), and a Time x Shock interaction ($F_{(2,\ 30)} = 5.41$, $p < .01$). The interaction indicates that noxious stimulation initiates a time-dependent process that peaks at three hours.

To verify that the change in coloration was due to the infiltration of blood, we assessed the levels of alpha hemoglobin within the protein samples using immunoblotting. In circulating red blood cells, hemoglobin exists as a heterotetramer made of 2 alpha subunits and 2 beta subunits. After hemorrhage, extravascular red blood cells are lysed, releasing hemoglobin and other cell contents into the spinal cord. Hemoglobin subunits then dissociate in the extracellular space and are subsequently degraded over time. Western blotting showed greater labeling at 16 kDa and 32 kDa after shock treatment (Fig. 1E), the regions associated with the monomer and dimer forms of hemoglobin, respectively. Independent ANOVAs confirmed that shock treatment had a significant effect (both $Fs > 7.91$, $p < .001$, $\eta^2 = 0.170$). In both cases, neither the effect of time nor the Time x Shock interaction were statistically significant (all $Fs < 2.30$, $p > .05$).

3.2. Experiment 2: engaging sensory fibers with capsaicin induces hemorrhage after SCI

Peripheral application of the irritant capsaicin 24 h after a spinal contusion injury engages an acute up-regulation of signal pathways associated with cell death and impairs long-term recovery (Turtle et al., 2018). The present experiment examines whether this treatment also promotes hemorrhage after injury and how this effect varies over time. We also incorporated an additional assay of blood infiltration, which is based on the conversion of hemoglobin to a cyanoderivative (Stadie, 1920). Drabkin and Austin (1935) showed that this process could be assessed using a (Drabkin) reagent that contains alkaline ferricyanide and cyanide, which in the presence of hemoglobin forms a complex (cyanmethemoglobin) that absorbs light within a narrow range (530–550 nm). A quantitative measure of hemoglobin content can then be derived using spectrophotometry.

The design was identical to that employed in the previous experiment assessing the effect of noxious electrical stimulation, except capsaicin (or its vehicle) was applied instead. Briefly, animals received a lower thoracic contusive spinal cord injury, and 24 h later, half of the rats received a single injection of capsaicin to the left or right hind paw (counterbalanced across subjects). Controls received an injection of vehicle to a hind paw. Rats were sacrificed 1, 3, or 24 h after injection and 1 cm of spinal tissue centered at the lesion epicenter was collected. The 2 (Capsaicin or Vehicle) × 3 (1, 3, or 24 h) factorial design used 33 rats (5–6 per group).

Protein samples from animals that were treated with capsaicin were tinted red. Spectrophotometry confirmed that samples from capsaicin treated rats exhibited greater absorbance at 420 nm. An ANOVA showed that noxious stimulation had a significant effect, ($F_{(1,\ 26)} = 7.93$, $p < .01$, $\eta^2 = 0.199$). Neither the main effect of time, nor the interaction between Time and Capsaicin treatment, were significant (both $Fs < 1.44$, $p > .05$). This indicated that pharmacologic activation of C-fibers increased hemoglobin within the lesion to roughly the same extent across all three time points.

A cyanmethemoglobin colorimetric assay provided further evidence that capsaicin treatment increases hemoglobin content at the site of injury (Fig. 2B). An ANOVA showed that capsaicin had a statistically significant effect ($F_{(1,\ 26)} = 5.82$, $p < .05$, $\eta^2 = 0.087$). While the magnitude of the capsaicin effect did not vary over time ($F_{(2,\ 26)} < 1.0$, $p > .05$), overall levels were higher at 24 h after treatment ($F_{(2,\ 26)} = 17.38$, $p < .0001$).

Immunoblotting revealed labeling at 16 and 32 kDa, the regions associated with the monomer and dimer forms of alpha hemoglobin, respectively (Fig. 2E). Assessment of labeling over time revealed that capsaicin treatment increased the content of both the monomer (Fig. 2C) and dimer (Fig. 2D). For both, there was a statistically significant effect of capsaicin treatment (both $Fs > 4.27$, $p < .05$). In neither case, was there a significant effect of time or Time x Capsaicin interaction (all $Fs < 1.0$, $p > .05$).

3.3. Experiment 3: histological evidence for hemorrhage and capillary fragmentation

The results reported above indicate that nociceptive stimulation after injury increases hemoglobin content within the injured region of the spinal cord. Here, we verified that this effect was associated with

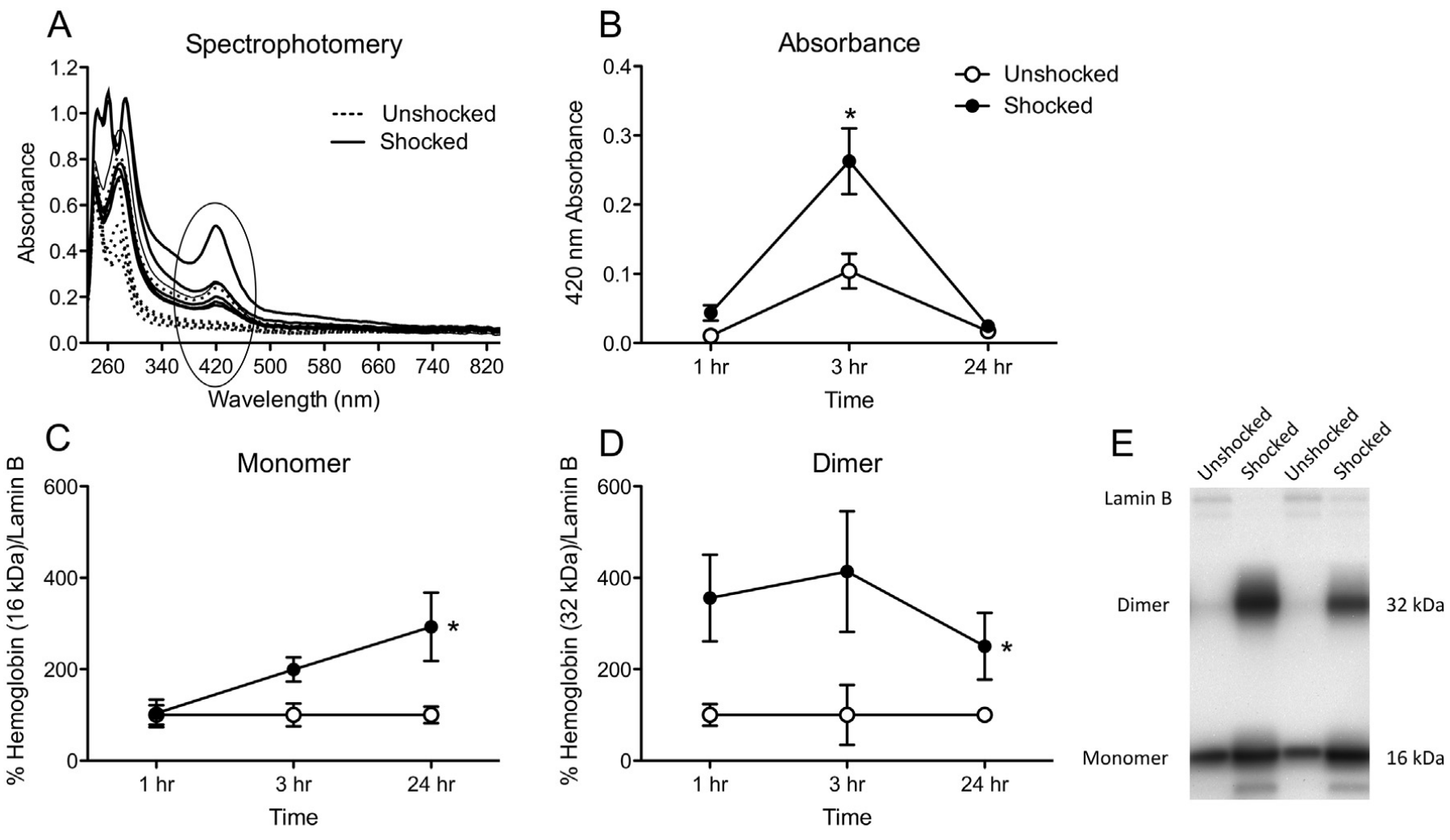

Fig. 1. Noxious electrical stimulation increased hemoglobin content at the site of a spinal cord contusion injury. Stimulation was applied a day after animals received a thoracic contusion injury and tissue was collected 1, 3, or 24 h later. (A) Spectrophotometry revealed that protein isolates exhibited a peak absorbance at 420 nm, the region associated with hemoglobin. (B) Quantification of peak absorbance showed that rats that had received electrical stimulation (Shocked) exhibited greater absorbance at 420 nm three hrs after treatment relative to contused animals that had not received stimulation (Unshocked). Immunoblotting showed that the spinal cord tissue from shocked rats had higher concentrations of the monomer (C) and dimer (D) forms of alpha hemoglobin. (E) A representative immunoblot illustrating greater labeling after shock treatment at 32 kDa (dimer) and 16 kDa (monomer). The lower bands are associated with the degraded form of hemoglobin (12 kDa). * Indicates statistical significance ($p < .05, n = 6$). An asterisk placed over a time point indicates that the groups differ at that time point; an asterisk placed to the side indicates that the main effect of treatment, across time, was statistically significant. Error bars represent the standard error of the mean (SEM).

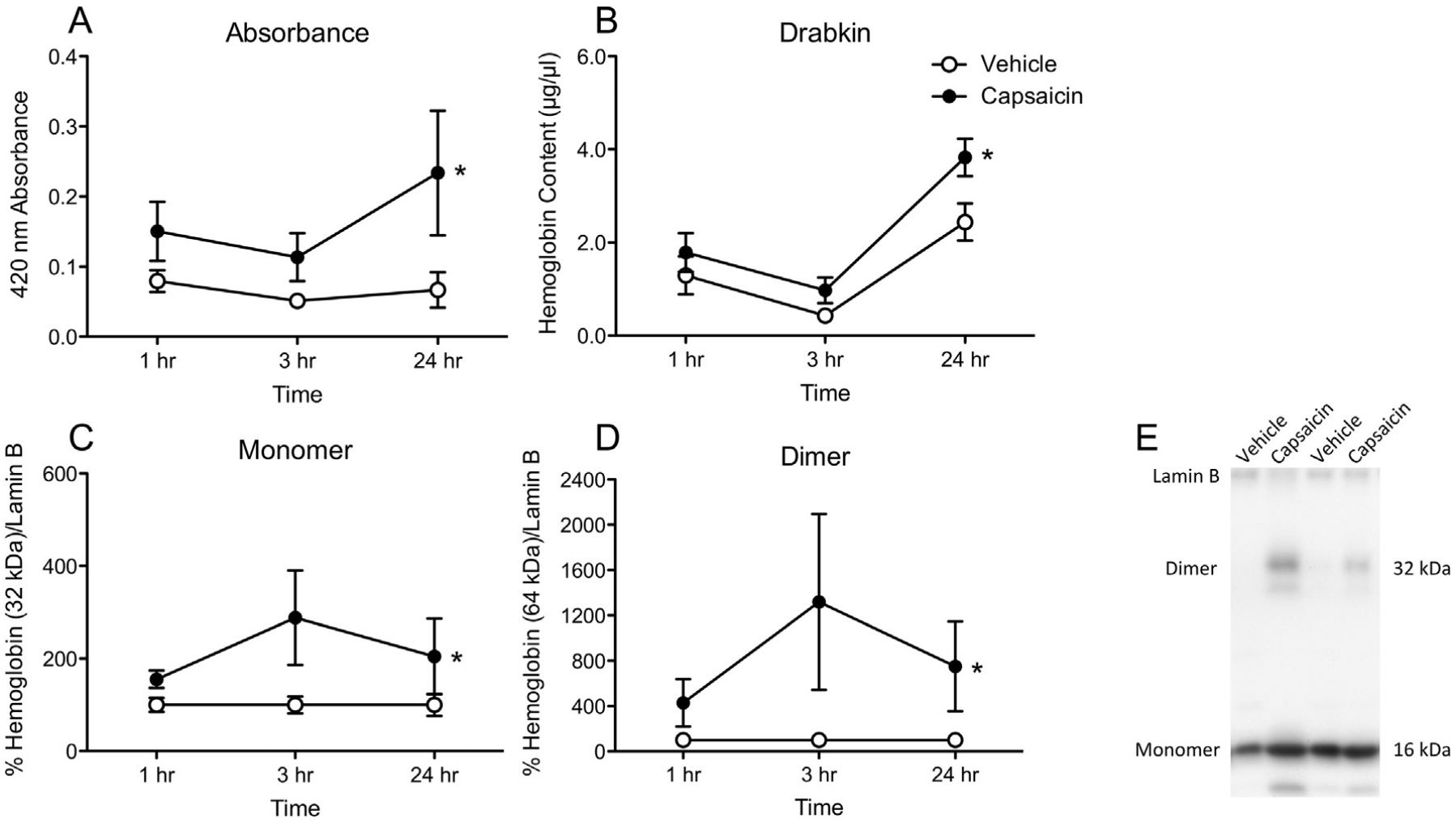

Fig. 2. Peripheral treatment with the irritant capsaicin increased hemoglobin content at the site of a spinal cord contusion injury. Capsaicin, or its vehicle, was applied to one paw a day after rats received a moderate contusion and tissue was collected 1, 3, or 24 h later. (A) Protein samples from capsaicin treated rats exhibited greater absorbance at 420 nm, the wavelength associated with hemoglobin. (B) A cyanmethemoglobin colorimetric assay, which provides a quantitative measure of hemoglobin content based on the formation of cyanmethemoglobin, confirmed that tissues samples from capsaicin treated animals had a higher concentration of hemoglobin. Immunoblotting showed that capsaicin treatment increased the concentration of the monomer (C) and dimer (D) forms of alpha hemoblobin. (E) A representative immunoblot revealed greater labeling at 32 kDa (dimer) and 16 kDa (monomer) in samples from capsaicin treated rats. The lower band (12 kDa) is associated with the degraded form of hemoglobin. * Indicates statistical significance ($p < .05$, n = 6). An asterisk placed to the side indicates that the main effect of treatment, across time, was statistically significant. Error bars represent SEM.

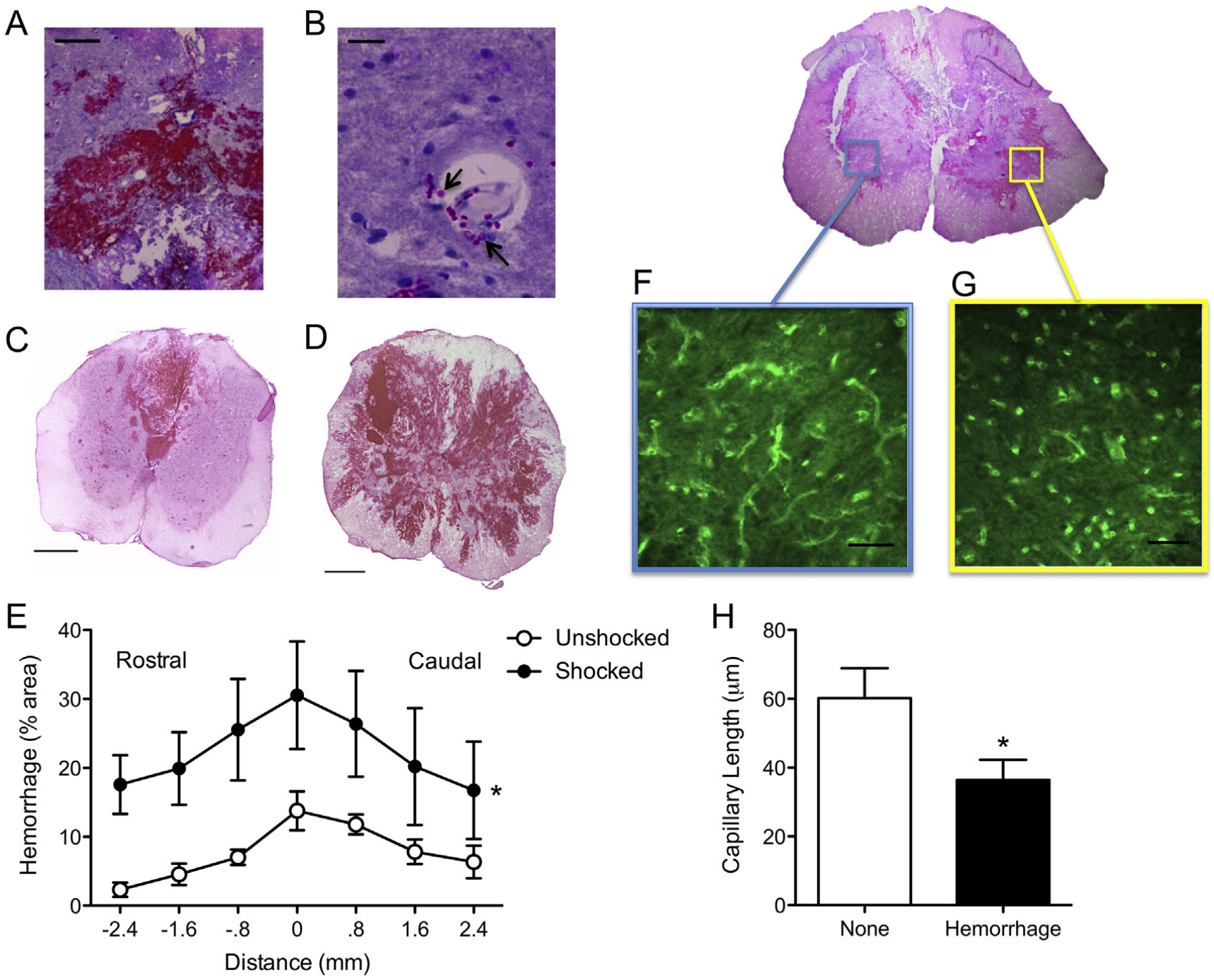

Fig. 3. Noxious electrical stimulation increased the area of hemorrhage. Stimulation was applied a day after rats received a contusion injury and tissue was collected 3 h later. (A) Low power images (10×; bar = 10 μm) of coronal sections stained with H&E showed extravascular red blood cell infiltration in the spinal cord parenchyma. (B) High power images [40×; bar = 5 μm] revealed identifiable red blood cells. Typical sections from animals that were unshocked showed evidence of hemorrhage (C), which was enhanced by shock treatment (D) (4×; bar = 500 μm). (E) Quantification of the extent of hemorrhage over a 4.8 mm section of tissue showed that it was increased by shock treatment. (F) Adjacent sections stained with the endothelial cell label tomato lectin revealed that capillaries had a rod-like appearance in non-hemorrhagic areas (20×; 50 μm). (G) In areas of hemorrhage, capillaries exhibited punctate labeling indicative of capillary fragmentation. (H) Quantification of capillary length confirmed that capillaries were foreshortened in areas of hemorrhage. * Indicates statistical significance ($p < .05$, $n = 4$). Error bars represent SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the infiltration of red blood cells and quantified hemorrhage extent by performing histopathological analyses of tissue sections.

To examine hemorrhage and capillary fragmentation after nociceptive stimulation, rats underwent a moderate contusion injury and, 24 h later, were placed in restraining tubes and given electrical stimulation (Shocked; $n = 4$) or nothing (Unshocked; n = 4). Animals were euthanized three hours later and a one-cm portion of the spinal cord that encompassed the injury site was collected and prepared for histological imaging. Tissue was sectioned coronally and mounted on sides. Sections spaced 800 μm apart were then stained with H&E and the extent of hemorrhage was quantified as described above.

H&E stained sections from shocked animals revealed areas of hemorrhage (Fig. 3A) and extravascular red blood cells (Fig. 3B). Typical sections from unshocked controls showed areas of hemorrhage (Fig. 3C), which was amplified by shock treatment (Fig. 3D). Quantification of the area of hemorrhage confirmed that it was increased by nociceptive stimulation (Fig. 3E). An ANOVA revealed main effects of stimulation ($F_{(1,\ 10)} = 5.92$, $p < .05$) and section ($F_{(6,\ 60)} = 4.47$, $p < .05$). The interaction between stimulation and section was not significant ($F_{(6,\ 60)} < 1.0$, $p > .05$), which indicated that stimulation had a comparable effect across the entire region.

Having established that nociceptive stimulation increases the area of hemorrhage, we evaluated whether the hemorrhage was associated with capillary fragmentation. Sections from the site of injury were stained with the endothelial cell label tomato lectin. The sections were from the same shocked animals used to assess hemorrhage extent with H&E staining, which allowed us to demarcate the area of hemorrhage. Capillary length was assessed within a window of standard size that contained an area of hemorrhage. To gain an internal control, we analyzed regions exhibiting unilateral hemorrhage. This permitted comparison to a non-hemorrhaged region on the contralateral side of equivalent size.

As expected, capillaries in sections from non-hemorrhagic areas had a rod-like appearance (Fig. 3F). In contrast, in areas of hemorrhage, capillaries appeared shorter (Fig. 3G). Quantification of capillary length (Fig. 3H) confirmed that capillaries were generally shorter in areas of hemorrhage ($F_{(1,\ 14)} = 41.28$, $p < .0001$, $\eta^2 = 0.746$).

In summary, our results are consistent with prior work demonstrating that injury per se induces hemorrhage, which is associated with capillary fragmentation (Simard et al., 2013). Pain input appears to fuel this process, expanding the area of hemorrhage and capillary fragmentation.

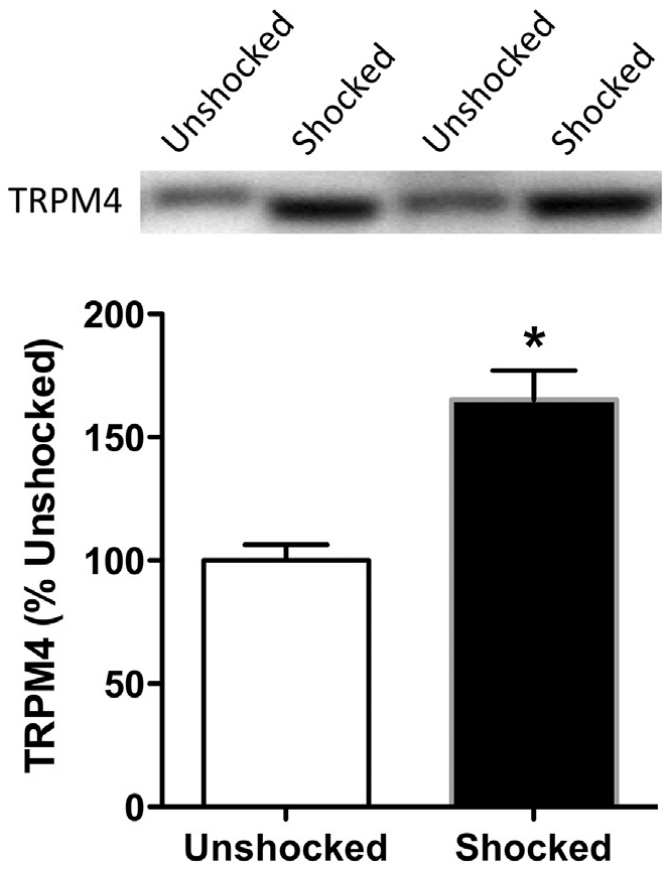

Fig. 4. Nociceptive stimulation activates SUR1-TRPM4. Stimulation was applied a day after rats received a contusion injury and spinal cord tissue was collected 1 h later. Immunoprecipitation of the SUR1-TRPM4 complex, followed by immunoblotting for TRPM4, showed that shock treatment increased the formation of SUR1-TRPM4 protein complexes. * Indicates statistical significance ($p < .05$, n = 4). Error bars represent SEM.

3.4. Experiment 4: noxious stimulation increases the expression of SUR1-TRPM4

Evidence suggests that injury leads to capillary fragmentation, and the breakdown of the BSCB, because it activates SUR1-TRPM4 channels on the endothelial cells that form the BSCB (Simard et al., 2013). The present experiment examines whether the expression of this channel is amplified by nociceptive stimulation.

Rats ($N = 12$) received a contusion injury and, 24 h later, were placed in restraining tubes where they received noxious electrical stimulation (Shocked; $n = 6$) or nothing (Unshocked; n = 6). Animals were euthanized one hour later and 1 cm of the spinal cord encompassing the injured region was collected and prepared for co-immunoprecipitation and western blotting as described above. Briefly, samples were immunoprecipitated using the SUR1 antibody and agarose beads. The protein complexes were then denatured and examined by gel electrophoresis and immunoblotting against TRPM4.

We found that exposure to noxious electrical stimulation led to an increase in SUR1-TRPM4 protein complexes in the region of injury (Fig. 4). An ANOVA confirmed that this difference was statistically significant ($F_{(1,\ 10)} = 23.11$, $p < .001$, $\eta^2 = 0.698$).

3.5. Experiment 5: noxious stimulation induces an acute disruption in motor performance

Prior work has shown that noxious stimulation causes a disruption in locomotor recovery that is evident within 24–48 h of treatment (Grau et al., 2004). The present results imply that pain input has this effect because it leads to a breakdown of the BSCB and hemorrhage. The accompanying alteration in the extracellular milieu, and subsequent cell death, would be expected to bring about a rapid deterioration in neural function and behavior. To explore this possibility, we assessed motor performance 0–3 h after shock treatment. We also reinforced our earlier observations using a cyanmethemoglobin colorimetric assay, which provides a quantitative measure of hemoglobin content. Finally, to evaluate the extent of the hemorrhage effect, we collected tissue rostral and caudal to the site of injury.

Twelve rats received a contusion injury as described above. A day after injury, half of the animals received 6 min of noxious electrical stimulation (Shocked). The remaining rats with contusion SCI were treated the same, but received no stimulation (Unshocked). Hind limb motor performance was assessed prior to stimulation, and 0, 1, 2, and 3 h after, by observers blinded to the animal's treatment condition using the BBB scale. After the last assessment of motor performance, animals were euthanized and a 1 cm section of spinal cord tissue encompassing the injury was collected. We also collected 1 cm rostral/caudal to injury. Tissue from each region was then prepared as described above.

Average scores on the BBB scale prior to treatment were 3.25 (± 0.52) and 3.50 (± 0.94) for the animals assigned to the unshocked and shocked conditions, respectively. This difference was not significant ($F_{(1,\ 10)} < 1.0, p > .05$) (Fig. 5A). Exposure to shock produced an immediate disruption in motor performance that was evident across the three hours of testing (Fig. 5A). To control for variation in baseline performance, an analysis of covariance (ANCOVA) was performed with the pretreatment BBB score entered as the covariate. As expected, this covariate accounted for a significant proportion of the variance ($F_{(1,\ 9)} = 28.16$, $p < .0005$, $\eta^2 = 0.476$). More importantly, shock treatment had a statistically significant effect on locomotor performance ($F_{(1,\ 9)} = 22.04$, $p < .0011$, $\eta^2 = 0.372$). The small fluctuations observed across time points were not statistically significant (all $Fs < 1.07$, $p > .05$).

A cyanmethemoglobin colorimetric assay confirmed that shock treatment increased hemoglobin content and that this effect was most robust at the site of injury (Fig. 5B). An ANOVA showed that the extent of hemorrhage varied across region ($F_{(2,\ 20)} = 23.15$, $p < .0001$) and that the magnitude of this effect depended upon shock treatment ($F_{(2,\ 20)} = 4.73$, $p < .05$). The overall effect of shock treatment, collapsed across regions, was not significant ($F_{(1,\ 10)} = 3.17$, $p > .05$). This pattern of results indicates that shock treatment has a local effect that increases hemorrhage at the site of injury.

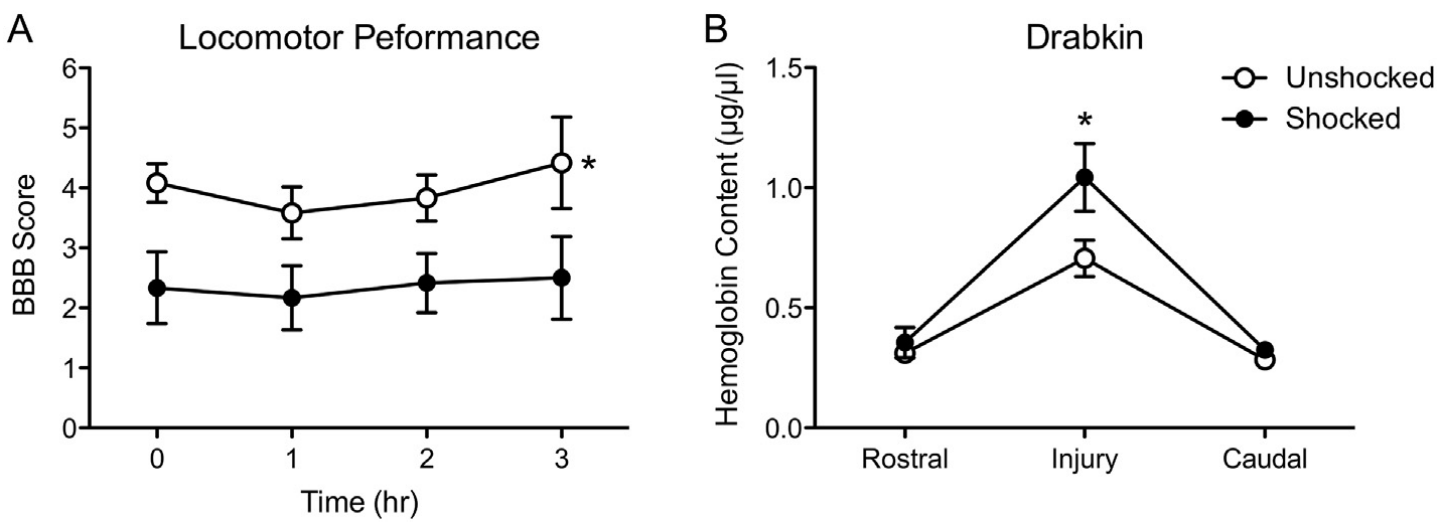

Fig. 5. Nociceptive stimulation impairs motor performance and increases hemorrhage at the site of injury. Stimulation was applied a day after rats received a moderate contusion injury. (A) Shock treatment produced an acute impairment in locomotor performance. (B) A cyanmethemoglobin colorimetric assay showed that hemoglobin content was higher 3 h after shock treatment at the site of injury, but not within an equivalent (1 cm) region of tissue rostral or caudal to injury. * Indicates statistical significance ($p < .05$, n = 6). An asterisk placed over a time point indicates that the groups differ at that time point; an asterisk placed to the side indicates that the main effect of treatment, across time, was statistically significant. Error bars represent SEM.

4. Discussion

Prior work has shown that nociceptive stimulation soon after a contusive SCI increases lesion volume and impairs recovery of locomotor function (Grau et al., 2004). The present results imply that engaging pain fibers has this effect because it increases the infiltration of red blood cells at the site of injury, expanding the extent of hemorrhage. Hemorrhage was inferred from both the discoloration of protein samples and western blotting for alpha hemoglobin. We found that the application of noxious electrical stimulation increased absorbance of protein samples at 420 nm, the absorption peak for hemoglobin (Prahl, 1999). Immunoblotting showed that shock treatment increased labeling for the monomer and dimer forms of alpha hemoglobin. A similar of pattern of results was obtained when pain fibers were activated using capsaicin, which engages the TRPV1 receptor found on C-fibers. We reinforced these observations using a Drabkin assay, which provides a measure of hemoglobin content based on the formation of cyanmethemoglobin. The results indicate that nociceptive stimulation begins to induce hemorrhage within an hour of treatment and that this effect is still evident 24 h later.

We bolstered our cellular results with histopathology, demonstrating that shock treatment increases the area of hemorrhage and that this effect is evident over a 4.8 mm region of the injured tissue. Sections stained with the endothelial cell label tomato lectin revealed capillary fragmentation in areas of hemorrhage.

Due to the neurotoxicity of blood borne cells and proteins, nociception-induced hemorrhage would expand the area of cell death at the site of injury (Mautes et al., 2000). This increase in secondary injury could produce an acute disruption in motor function. Supporting this, we found that hind limb motor performance was disrupted soon after shock treatment. This experiment also showed that nociceptive stimulation has little effect on blood infiltration rostral and caudal to the site of injury.

4.1. Processes contributing to the breakdown of the BSCB

The BSCB is derived from a specialized system of nonfenestrated endothelial cells and associated structures that include the basement membrane, pericytes, and astrocytic end feet processes (Bartanusz et al., 2011; Beggs et al., 2010). The paracellular diffusion pathways are restricted by tight junctions between enthothelial cells. Astrocytes surround the outer surface of the capillaries and play a critical role in the maintenance of the endothelium barrier. Together, these cells form a unit that restricts/regulates what can pass from the blood into the surrounding tissue. Nociceptive input has been shown to increase the permeability of the BSCB (Echeverry et al., 2011; Sauer et al., 2017), inducing a local modification that allows relatively small molecules (e.g., Evans Blue) to pass through. The fact that nociceptive stimulation allows large blood cells to enter the injured region implies a different category of effect, a catastrophic breakdown in the integrity of the endothelial cell wall known as PHN (Simard et al., 2010).

PHN is associated with the formation of the SUR1-TRPM4 channels in endothelial cells (Simard et al., 2013). This ion channel is not constitutively expressed, but is formed de novo following upregulation of SUR1 after spinal cord trauma. The channel is engaged in metabolically compromised cells [e.g., in response to low adenosine triphosphate (ATP)] and is thought to provide a form of negative feedback to Ca^{++} influx, promoting depolarization and reducing Ca^{++} overload (Gerzanich et al., 2009). In the injured nervous system, ATP depletion can lead to prolonged activation of the SUR1-TRPM4 channel and the unrestricted influx of Na^{+}, causing the cell to swell and initiating oncotic cell death. This endothelial cell death leads to a breakdown in the BSCB, a fragmentation of capillaries, and the influx of blood-borne cells into the damaged region. The current study suggests that nociceptive stimulation can amplify these processes, promoting the upregulation of the SUR1-TRPM4 channel and capillary fragmentation.

Elsewhere, we have shown that noxious stimulation can activate signal pathways linked to neural over-excitation and cell death (e.g., caspase 1, 3 and 8) (Ferguson et al., 2008; Garraway et al., 2014; Huie et al., 2015; Turtle et al., 2018). These processes could also contribute to the breakdown of the BSCB by initiating cell death within endothelial cells or indirectly by depleting energy stores (ATP). Further work will be needed to elucidate how nociceptive stimulation triggers endothelial cell death and capillary fragmentation. Additional studies are also being conducted to explore the generality of these phenomena. We hypothesize that electrical stimulation and capsaicin treatment have a common effect because both engage C-fibers. How does this input trigger a breakdown in the BSCB? Past work suggests that nociceptive input can drive glutamatergic over-excitation and the release of proinflammatory cytokines [e.g., tumor necrosis factor (TNF)], which can fuel cell death (Beattie et al., 2002; Garraway et al., 2014; Hermann et al., 2001; Huie et al., 2015). In addition, C-fiber activity would be expected to drive the release of substance P and nitric oxide (NO), which are known to impact capillary function (Conti et al., 2007; Freire et al., 2009; Khasabov et al., 2002; Moncada, 1991; Steinhoff et al., 2014). Further work is also needed to establish whether PHN, and the up-regulation of SUR1-TRPM4, represents the cause of capillary fragmentation or a down-stream consequence. Finally, further work is needed to clarify how systemic effects triggered by pain input (e.g., a rise in blood pressure) influence the development of hemorrhage after injury. Prior work has shown that a period of hypertension can expand tissue loss after SCI (Nielson et al., 2015). Given this, we hypothesize that pain input after injury may (1) induces neurochemical processes that weaken the BSCB and (2) produce a rise in blood pressure that fuels hemorrhage.

Our research shows that nociceptive input within a day of injury adversely affects tissue sparing, fosters the development of chronic pain, and impairs the recovery of locomotor function (Grau et al., 2004; Garraway et al., 2014). Interestingly, these effects appear strongest when nociceptive stimulation is given relatively soon (24 h) after injury (Grau et al., 2004). In some regards, this is surprising because injury per se can inhibit neural/reflex function caudal to injury, a phenomenon known as spinal shock (Bach-y-Rita et al., 1993; Dietz, 2010; Ditunno et al., 2004). While there is no doubt that this phenomenon can affect the performance of integrated motor tasks, we routinely observe that simple reflexes (e.g., paw withdrawal in response to mechanical stimulation) can be elicited within hours of injury. Further, our prior work has related the development of maladaptive plasticity to alterations in nociceptive processing within the dorsal horn (Garraway et al., 2014), which may be less affected by spinal shock. Finally, recent evidence suggests that injury can engage compensatory processes within the dorsal horn that foster neural excitation soon after injury, a modification that may enable nociceptive-induced over-excitation (Huang et al., 2016, 2017, 2018). Further work is needed to detail how these processes interact.

4.2. Implications for treatment

We have previously shown that inhibiting cellular activity at the site of injury, using the Na^{++} channel blocker lidocaine, blocks the development of acute hemorrhage and the adverse effect noxious stimulation has on long-term recovery (Turtle et al., 2017). Further work will be needed to determine whether treatments that selectively target processes related to cell death, or the activation of the SUR1-TRPM4 channel, can attenuate the development of hemorrhage in response to noxious stimulation. Of particular interest, other work has shown that the SUR1-TRPM4 inhibitors (e.g., riluzole, glibenclamide) can attenuate the development of hemorrhage after injury and promote recovery (Simard et al., 2007, 2012). On-going studies are examining whether these treatments also attenuate the effect of nociceptive stimulation. A key issue in examining this question concerns the temporal sequence of events, which will inform the optimal treatment regime after injury.

Does injury trigger an up-regulation of SUR1-TRPM4 that is fueled by nociceptive stimulation or is a second wave of expression engaged by pain input? Noxious stimulation may also engage other forms of cell death (e.g., apoptosis; Beattie et al., 2002) that weaken the BSCB and amplify hemorrhage. In this case, increased activation of SUR1-TRPM4 could reflect a down-stream effect, stemming from the loss of supporting tissue and metabolic deficiency. Here, a combinational therapy that targets multiple cellular processes may be most effective.

As noted above, a period of high blood pressure (hypertension) after injury can increase tissue loss and undermine long-term recovery (Guha et al., 1989; Nielson et al., 2015). Nociceptive stimulation may promote hemorrhage because it drives a prolonged increase in blood pressure. This suggests that carefully monitoring blood pressure after injury, and taking steps to maintain it within a normal range, could foster tissue sparing and recovery.

The vast majority of cases of SCI occur following traumatic accidents (National Spinal Cord Injury Statistical Center, 2016), producing peripheral damage that can engage pain signals. Our work has shown that engaging nociceptive fibers will promote the loss of tissue at the site of injury and undermine long-term recovery. The link to pain would seemingly imply that treatment with an analgesic, such as morphine, could reduce tissue loss at the site of injury and promote recovery. However, an analgesic dose of morphine that blocks behavioral reactivity to noxious stimulation in contused rats does not reduce the adverse effects of pain input on long-term recovery, including tissue loss (Hook et al., 2009) or shock-induced hemorrhage (Turtle et al., 2017). Of greater concern given that opiates are widely prescribed after injury (Alpen and Morse, 2001) is that morphine per se can have an adverse effect (Hook et al., 2007, 2009) and exacerbate stimulation-induced hemorrhage (Mahinda et al., 2004). Local anesthesia induced using the Na^+ channel blocker lidocaine may provide a safe alternative, blocking shock-induced activation of proinflammatory cytokines, indices of cell death, and hemorrhage (Turtle et al., 2017). Most importantly, lidocaine blocked the adverse effect noxious stimulation has on long-term recovery and tissue loss.

In summary, the present study explored how pain input expands tissue loss after SCI. Our work indicates that nociceptive stimulation leads to a breakdown in the BSCB and the infiltration of red blood cells into the injured tissue. Treatments that target these effects could reduce nociception-induced secondary injury and promote long-term recovery.

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