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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