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A rat model of full thickness thermal injury characterized by thermal hyperalgesia, mechanical allodynia, pronociceptive peptide release and tramadol analgesia

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ABSTRACT

Opioid related side effects are problematic for burn patients. Dual mechanism therapeutics targeting opioid and non opioid mechanisms may have reduced side effects with similar analgesic efficacy. Tramadol combines mu opioid receptor agonism with norepinephrine reuptake inhibition and has been effective in treating some types of pain. The effectiveness of tramadol in treating pain associated with burns is unclear. We hypothesized that tramadol is effective in reducing thermal injury evoked pain behaviors in a rat model. Rats were anesthetized and a 100 °C metal probe was placed on the hindpaw for 30 s to induce a full thickness thermal injury. A subset of rats was perfusion fixed and hindpaw tissue and spinal cord collected for anatomical analysis. Rats received morphine (5 mg/kg; i.p.), tramadol (10 30 mg/kg; i.p.) or vehicle and latency to paw withdrawal from a noxious thermal or non noxious mechanical stimulus was recorded every 10 min over 70 min and again at 2 h. We report that pain behaviors developed within 48 h and peaked at 1 week; paralleled by enhanced expression of pronociceptive neuropeptides in the spinal cord. Morphine and tramadol significantly attenuated hyperalgesia and allodynia, while not significantly altering motor coordination/sedation. These data indicate dual mechanism therapeutics may be effective for treating pain associated with burns.

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1. Introduction

Blast and burn insults account for over half of modern warfare casualties [1] and advances in battlefield medical techniques, protective armor, and medical care during evacuation have led to an impressive >90% survival rate [2]. Concurrent with this improvement in battlefield survival is an increase in the number of patients needing treatment for substantive pain evoked by traumatic injuries as evidenced by a cohort of 162 soldiers receiving treatment at Walter Reed Army Medical Center who reported an average visual analogue scale (VAS) pain score of 5.9 out of 10 [3]. Burned Service Members represent one patient population in the military medical care

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system in need of optimal pain control, reduced incidence of chronic pain and reduced risk of tolerance and addiction [4].

Opioid based narcotics are the most prevalent therapeutics for the management of severe pain in civilian and military inpatient settings [5]. Because traumatic injuries, including burns, require multiple painful treatments, including wound debridements, dressing changes and lengthy rehabilitation, tolerance to opioids resulting in dose escalation during treatment is common [4]. This may lead to addiction, as evidenced by the near tripling of prescription drug abuse among active duty military personnel between 2005 and 2008 (Department of Defense Health Behavior Study, 2008). Reducing reliance on traditional opioid based narcotics is one way to improve pain management and outcomes in burned Service Members and civilians.

Antidepressants that target the neurotransmitters serotonin (5HT) and norepinepherine (NE), such as amitriptyline and duloxetine, have been successful for a variety of pain conditions [6 8], with the potential added benefits of mood elevation, sleep pattern normalization and muscle relaxation. However, antidepressants have not been successful for all pain conditions and the pain relieving properties of antidepressants alone may not be efficacious for pain experienced with severe trauma. Alternatively, pain therapeutics that target dual mechanisms simultaneously may improve pain management in this population. The dual mechanism therapeutic tramadol combines opioid receptor activation and 5HT/NE reuptake inhibition [9 11]. Both preclinical and clinical research has reported that tramadol reduces acute, postoperative, neuropathic and cancer pain [9,10,12 14] and may have a lower propensity to induce addiction [15] with little to no adverse events compared to morphine [14].

Because of the complexity and severity of pain experienced by burn patients, this population receives multiple pain therapeutics simultaneously; thus, it is difficult to determine the efficacy of a single analgesic in this population. We have developed an animal model of thermal hyperalgesia and mechanical allodynia evoked by full thickness thermal injury that shares pathological characteristics with full thickness burns in patients. We then used this model to test the hypothesis that tramadol is effective in reducing full thickness thermal injury evoked hyperalgesia and allodynia.

2. Methods

2.1. Subjects

A total of 117 adult (250 400 g) intact male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were used in these experiments. Rats were pair housed in a 12:12 h light:dark cycle with *ad libitum* access to food and water. All studies were approved by the U.S. Army Institute of Surgical Research Institutional Animal Care and Use Committee and conform to federal guidelines and guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. This study was conducted in strict compliance with the Animal Welfare Act, implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals.

2.2. Full thickness thermal injury

A rat model of thermal injury was adapted from previous reported models [16 19] for use in these studies. Male rats were inhalation anesthetized with 4% isofluorane. Rats were laid ventrally and a 100 °C slanted soldering tip connected to a temperature controlled super soldering station (RX 80HRT 5.4D; Goot, Hiroshima, Japan) was steadily applied to the right hindpaw for 30 s to induce a full thickness thermal injury of <1% of the total body surface area. The temperature was chosen based on previous reports [53]. Five minutes following injury and once daily for the next 4 days, 1% silver sulfadiazine cream (Watson Laboratories, Corona, CA, USA) was applied to the injured hindpaw to prevent infection. A subset of rats received silver sulfadiazine cream on the uninjured hindpaw to control for potential effects on pain behaviors.

2.3. Skin histology

Rats (n 4) received thermal injury to the hindpaw and were euthanized by lethal injection of sodium pentobarbital (160 mg/kg; i.p.; Lundbeck Inc., Deerfield, IL, USA) within 5 min post injury to examine burn depth. The collected paws were fixed in formalin, decalcified, and the plantar tissue was paraffin embedded. The tissue was then sectioned sagittally and cross sectioned at the center of injury at 4 μM onto glass slides and stained with hematoxilin and eosin for visualization. Images were captured at 40× magnification with a Nikon Eclipse 80i microscope equipped with a DS Fi1 camera head and depth at the focal point of injury was measured with NIS Elements Advanced Research v3.22.00 software. Three depth measurements were collected from each of 3 fields of view from the same section for a total of 9 measurements providing an average depth of the total burn area per rat. An average of three measurements taken at the deepest points of injury of each paw was also recorded. Paws were also collected at 24, 72 h, 1 week and 4 weeks (n 2 per time point) post injury to visualize burn pathology. A board certified veterinary pathologist characterized the degree of burn across the time course based on tissue morphology.

2.4. Pain behavior testing

To determine pain behaviors following thermal injury, rats were acclimated to the testing apparatuses and baseline measurements were recorded prior to thermal injury. Paw withdrawal latencies to a noxious thermal stimulus were determined using the Paw Thermal Stimulator (Univ. California, San Diego, CA, USA) as previously described [20]. For this test, rats were placed in a clear Plexiglass box resting on an elevated glass plate maintained at 30 °C. Following acclimation, a radiant beam of light was positioned under the hindpaw and the average time over three trials for the rat to remove the paw from the thermal stimulus was electronically recorded in seconds as the paw withdrawal latency (PWL). The intensity of the beam was set to produce basal PWLs of approximately 10 12 s. A maximal PWL of 20 s was used to prevent excessive tissue damage due to repeated application of the thermal stimulus.

To test mechanical allodynia, the force (in grams) required to elicit a paw withdrawal from a non noxious mechanical stimulus was determined using a Dynamic Plantar Anesthesiometer (Ugo Basile; Collegeville, PA, USA) as previously described [21]. Rats were placed in a Plexiglass box on an elevated grid platform and a blunt mechanical stimulus was aimed at the plantar surface of the hindpaw. The force of the mechanical stimulus was increased with a ramp of 3 g/s over 10 s with a cutoff of 30 s to avoid mechanical lifting of the paw by the device.

Rats (n 9) then received a thermal injury and the thermal and mechanical stimuli were applied either approximately 5 mm proximal to the focal point of injury or at the focal point of injury. PWLs and force to withdrawal were recorded from both the injured and uninjured hindpaws at 24, 48 and 72 h, then once per week up to 4 weeks to characterize the development of pain behaviors. An image of the injured hindpaw was recorded at each time point to document the injury formation and progression. A separate group of rats (n 7) were examined for responses to the thermal and mechanical stimuli applied to the focal point of injury (primary site) versus peripheral to the injury (secondary site) for comparison of magnitude of pain behaviors.

To examine analgesia, a separate group of rats received one injection of either morphine sulfate at its ED_{50} dose for both inflammatory pain [22,23] and burn pain [24] (5 mg/kg; i.p.; Baxter Healthcare, Deerfield, IL, USA) as a positive control, tramadol hydrochloride (10, 20, or 30 mg/kg; i.p.; Sigma Aldrich; St. Louis, MO, USA) or vehicle (n 6 per group) one week following thermal injury, the time point when pain behaviors peak. Morphine was chosen for positive control as a dose response analysis has been previously reported in a rat burn model with 4.6 mg/kg providing analgesia two weeks following burn [24]. The tramadol doses were chosen based on previous studies in other models [9 11,13] and limited to $\leq$30 mg/kg to avoid sedation/motor coordination effects [10,13] which would confound our behavior results and are unwanted side effects in burned Service Members. Immediately following injection, the latency to withdraw the paw from a noxious thermal (n 8 morphine; n 6 per dose tramadol) or non noxious mechanical stimulus (n 6 morphine; n 5 tramadol) was recorded every 10 min over 60 70 min and compared to saline treated rats (n 6 per group) to control for potential changes in sensitivity to the behavioral assays with repeated testing [25]. To limit the number of animals used, 3 doses of tramadol were tested for effects on thermal hyperalgesia, while only the highest dose was tested for effects on mechanical allodynia. In studies not involving drug administration, responses to both a thermal and mechanical stimulus were recorded in the same rats with at least 3 h between testing to avoid sensitization. For studies involving drug administration, separate rats were used for each behavior test to avoid multiple injections of analgesics. Rats were randomized to treatments and behavioral testing was conducted by an observer blind to which drug the animal received.

2.5. Rotarod test

To examine the effects of tramadol on motor coordination/sedation, a separate group of 12 rats were trained to run on the rotarod (Med Associates Inc., St. Albans, VT, USA) according to a previously reported protocol [26]. On the first day of training, the rats underwent three 5 min training sessions at speeds of 8, 12 and 16 revolutions per minute (rpm). The next day rats underwent one 5 min refresher session at 16 rpm and two sessions with acceleration from 4 to 40 rpm over 5 min with 30 min separating each session. The following day, the number of seconds each rat remained on the rotarod as it accelerated from 4 to 40 rpm over 5 min was recorded as baseline. Rats then received either tramadol (30 mg/kg; i.p.; n 6) or saline (i.p.; n 6) and 30 and 60 min post injection the rats were again placed on the rotarod and the number of seconds (300 s cut off) the rats remained on the rotarod was recorded. Rats were randomized to treatments and behavioral testing was conducted by an observer blind to which drug the animal received.

2.6. Perfusion fixation

A subset of rats that received thermal injury were euthanized (sodium pentobarbital; 160 mg/kg; i.p.) at 24 h, 72 h, 1, 2, 3 and 4 weeks (n 4 5 rats per time point) for analysis of spinal cord pronociceptive peptide expression by immunohistochemistry. Heparin sodium (0.1 mL; 1000 USP Units/mL; APP Pharmaceuticals, Schaumburg, IL, USA) was injected into the apex of the heart and rats were transcardially perfused with 200 250 mL of 0.9% sodium chloride containing 2% sodium nitrite as a vasodilator followed by 300 mL 4% paraformaldehyde in 0.1 M phosphate buffer. A final rinse with 200 250 mL sodium chloride/sodium nitrite solution was perfused to remove residual paraformaldehyde. The lumbar segment of the spinal cord was extracted and placed in 30% sucrose solution and stored at 4 °C.

2.7. Immunohistochemistry

Perfusion fixed lumbar spinal cords were sectioned into 30 μM sections directly onto slides with a Microm HM 560 cryostat (ThermoFisher Scientific; Rockford, IL, USA) at 20 °C. Tissue was fixed to the slide by a 10 min incubation in 4% paraformaldehyde. A 1:4 series through the rostrocaudal axis of the lumbar spinal cord was processed for CGRP or substance P immunoreactivity using standard immunohistochemical techniques as previously described [27]. Briefly, sections were rinsed in potassium phosphate buffered saline (KPBS) and incubated in primary antibody solution rabbit anti CGRP (1:10,000; Immunostar; Hudson, WI, USA) [28] or rabbit anti substance P (1:50,000; Immunostar) [29] in KPBS containing 1% Triton X for 1 h at room temp followed by 48 h at 4 °C. Tissue was then rinsed and incubated for 1 h in biotinylated goat anti rabbit IgG (1:600; Jackson Immunoresearch; West Grove, PA, USA), rinsed again and incubated for 1 h in avidin biotin peroxidase complex (1:10; ABC Elite Kit; Vector Laboratories; Burlingame, CA, USA). Tissue was then rinsed, and staining was visualized using nickel sulfate intensified 3,3′ diamino benzidine solution (2 mg/10 mL) containing 0.08% hydrogen peroxide in sodium acetate buffer. Slides were then air dried, dehydrated in a series of graded alcohols, cleared with HistoChoice (Sigma) and glass coverslipped using Permount (Fisher Scientific; Fair Lawn, NJ, USA).

2.8. Densitometry

For immunohistochemistry data, bilateral images at 10× magnification of the dorsal horn quadrant, as the region of interest to be quantified that included the superficial lamina I III, were captured with a Nikon Eclipse 80i microscope equipped with a DS Fi1 camera head. Three representative sections each from caudal L2, caudal L3, mid L4 and rostral L5, based on known innervation of the spinal cord from the plantar surface of the hindpaw [30,31], from 3 to 5 different rats at each behavioral time point were chosen for quantification. All tissue was processed in parallel and for the same time duration to ensure stain homogeneity. Images were imported into NIH Image J 64 (http://rsb.info.nih.gov/ij/), converted to 16 bit grayscale to be sampled for average gray scale pixel value (sum gray values/number pixels) following background correction (set a ~150 pixels)[23,32,33]. Following quantification, images for illustration were adjusted for brightness/contrast to optimize visualization for publication.

2.9. Data analysis

All data were analyzed using GraphPad Prism software version 5 (GraphPad, San Diego, CA, USA). For pain behavior data analysis, data are expressed as either mean ± standard deviation PWLs or percent maximal possible effect (%MPE) calculated as [(PWL baseline)/(20 s cutoff baseline)] × 100. Rotarod data are expressed as seconds on rotarod. Significant outliers were identified for exclusion with the Grubbs' test (GraphPad Quick Calcs Online, the extreme studentized deviate method; [(mean value)/standard deviation]) to detect outliers over 2 standard deviations from the mean. Behavioral data and anatomical data were analyzed by one way or two way ANOVA and rotarod data was analyzed by repeated measures two way ANOVA across all time points. Individual groups were compared using Bonferroni *post hoc* tests. The effect of silver sulfadiazine was examined by unpaired *t* test. The statistical significance was tested at $p < 0.05$.

3. Results

3.1. Thermal injury evoked a full thickness burn characterized by damage to the dermis

The area of the burn developed edema, erythema and blister formation during the initial 3 days following injury, followed by scab development from 1 to 3 weeks, with the wound closed by 4 weeks post injury (Fig. 1A). The uninjured rat hindpaw was approximately 5.59 ± 0.28 mm in diameter from the plantar surface to the dorsal surface. Exposing the plantar surface of the right hindpaw to 100 °C for 30 s produced an average burn depth of 2.04 ± 0.50 mm across the field injury as measured within 5 min post burn, with an average depth of 2.45 ± 0.44 mm at the deepest point in each paw. At the time of injury, the burn is characterized as a full thickness thermal injury by observation of damage to the epidermis, dermis and extending into the subcutis and underlying muscle. Unburned tissue was observed as intact dermis and epidermis (Fig. 1B), while the burn tissue showed evidence of denatured collagen (Fig. 1C, asterisk). The epithelium directly under the burn area was necrotic with streaming nuclei, especially affecting basal cells (Fig. 1D, solid arrow), and multifocal detachment from the underlying dermis (open arrow). Muscle bundles deep to the thermal wound contained contraction band degeneration. The necrotic epidermis and denatured dermis remained intact for 72 h following injury and there was mixed inflammatory infiltrate at the interface of necrotic and viable areas with phagocytosis of myocytes (Fig. 1D and E). At 3 days post injury there are bacterial colonies within the necrotic epidermis and neutrophilic inflammation within the hyalinized dermis (Fig. 1E, solid arrow). The interface between viable and necrotic areas is expanded by infiltration by fibroblasts and neovascularization (open arrows). At one week, the necrotic tissue was sloughed and the wound was covered with a scab observed as necrotic epithelium and hyalinized dermis replaced by serocellular crust containing large bacteria colonies (Fig. 1F, solid arrow). By 3 weeks, the wound was re epithelialized and the defect filled by granulation tissue. At 4 weeks, the epithelium appears normal with a thick layer of orthokeratotic keratin on the hindpaw surface (Fig. 1G, solid arrow). Inflammation is resolved and granulation tissue is replaced by mature collagen (asterisk).

3.2. Thermal injury to the rat hindpaw evokes significantly greater hyperalgesia and allodynia secondary to the primary site of thermal injury

Thermal injury to the right hindpaw was inflicted with a 30 s exposure to a metal probe set to 100 °C. As thermal injury is thought to produce extensive nerve damage at the focal site of injury, potentially reducing thermo and mechanosensation, we compared thermal and mechanical sensitivities at the focal point (primary site) of injury to the secondary site approximately 5 mm proximal to the focal point of injury (locations illustrated in Fig. 2A). There was no significant reduction in paw withdrawal latency to a thermal stimulus when applied to the primary site of injury [$F(3,27)$ 2.87; n.s.] (Fig. 2B) and there was no significant reduction in mechanical threshold at the primary site of injury [$F(3,19)$ 1.93; n.s.] (Fig. 2C). While mechanical sensitivity developed in the secondary site [$F(3,24)$ 6.58; $p < 0.05$], mechanical sensitivity of the primary site was not significantly different from the secondary site [$F(1,24)$ 1.25; n.s.] (Fig. 2C). However, thermal hyperalgesia was significantly more robust at the secondary site as compared to the primary site [$F(1,36)$ 27.74; $p < 0.05$] (Fig. 2B) supporting the secondary site as the testing site to examine potential reductions in pain sensitivities following thermal injury.

3.3. Thermal injury evokes unilateral thermal hyperalgesia and mechanical allodynia

Thermal and mechanical stimuli were applied approximately 5 mm proximal to the focal point of injury following thermal injury to detect changes in pain behaviors. Thermal injury resulted in the development of thermal hyperalgesia in the injured paw as observed by a significant reduction in paw withdrawal latency to a noxious heat stimulus at 24 h post injury; hyperalgesia peaked at 72 h to one week and lasted up

to 2 weeks post injury [$F(7,71)$ 1.91; $p < 0.05$] (Fig. 3A). The same rats also developed mechanical allodynia in the injured paw as observed as reduction in force (in grams) required to elicit a paw withdrawal. Significant mechanical allodynia was observed as early as 48 h following injury and lasted up to 2 weeks post injury [$F(7,47)$ 7.00; $p < 0.05$] (Fig. 3B). Rats were also examined for the potential development of thermal and mechanical hypersensitivity in the uninjured (left) hindpaw as compared to the injured hindpaw. There was no significant reduction in paw withdrawal latency to a thermal stimulus [$F(7,47)$ 2.06; n.s.] or a mechanical stimulus [$F(7,55)$ 0.48; n.s.] in the uninjured left hindpaw (data not shown).

In a subset of rats, silver sulfadiazine was also applied to the left hindpaw to detect potential independent analgesic effects. There was no significant effect of silver sulfadiazine on thermal sensitivity one week following thermal injury [t 0.98; df 5; n.s.] (data not shown). Also, there was no significant weight loss over the first two weeks following thermal injury [$F(5,29)$ 1.81; n.s.] and no signs of distress or alterations in mobility or weight bearing were observed

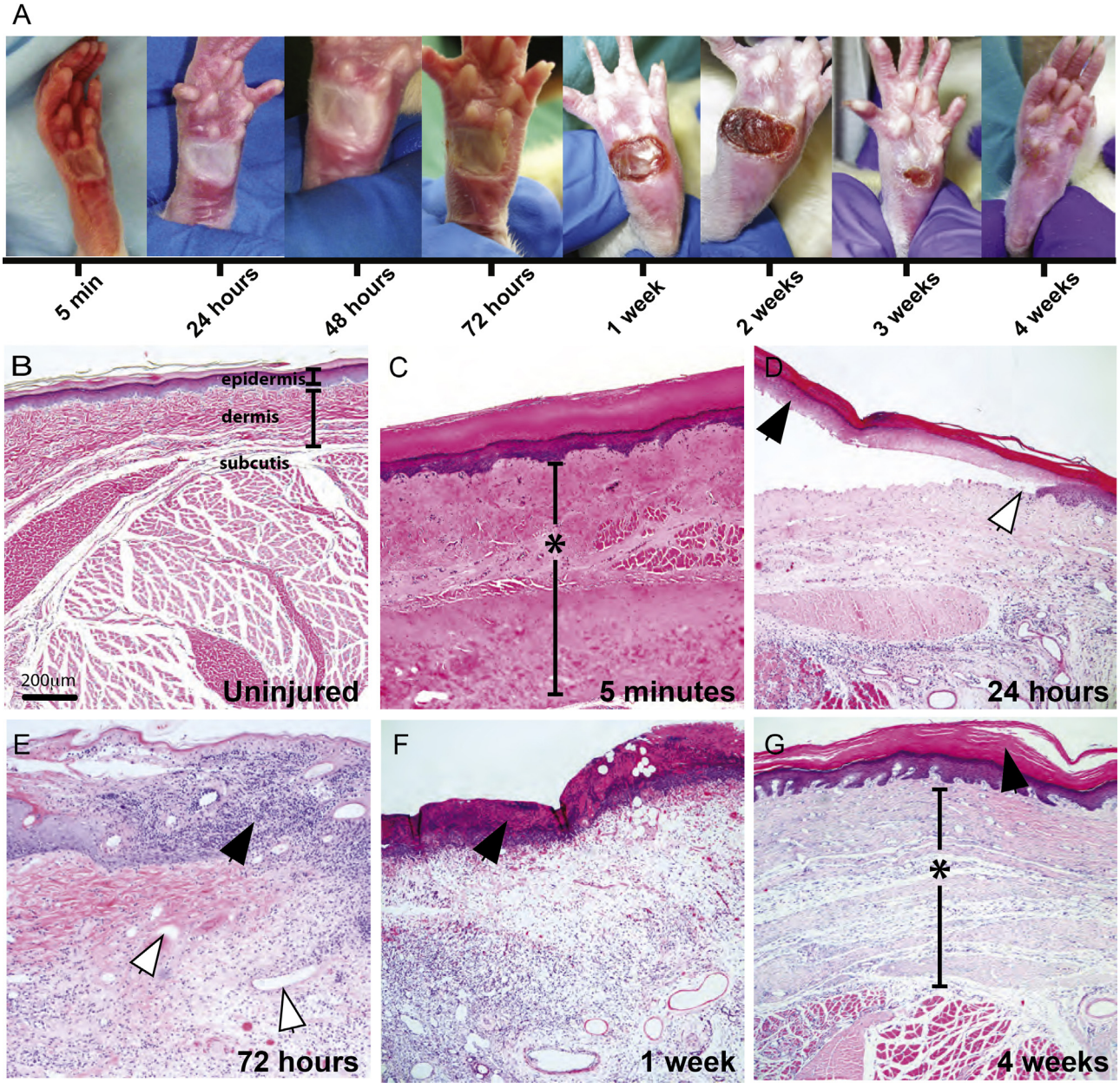

Fig. 1 – Full thickness thermal injury pathology. Time course photographs of the plantar surface of the rat hindpaw following thermal injury (A) and photomicrographs of the underlying epidermis, dermis and subcutis in uninjured hindpaw (B), damaged collagen denoted by the asterisk following thermal injury at 5 min (C), necrotic basal cells (solid arrow; D) and detachment from the dermis at 24 h (open arrow; D), inflammatory infiltration (solid arrow; E) and neovascularization at 72 h (open arrows; E), serocellular crust present at 1 week (solid arrow; F) and development of an orthokeratotic keratin surface (solid arrow; G) and granulation tissue replacement with mature collagen denoted by the asterisk 4 weeks (G) post-injury.

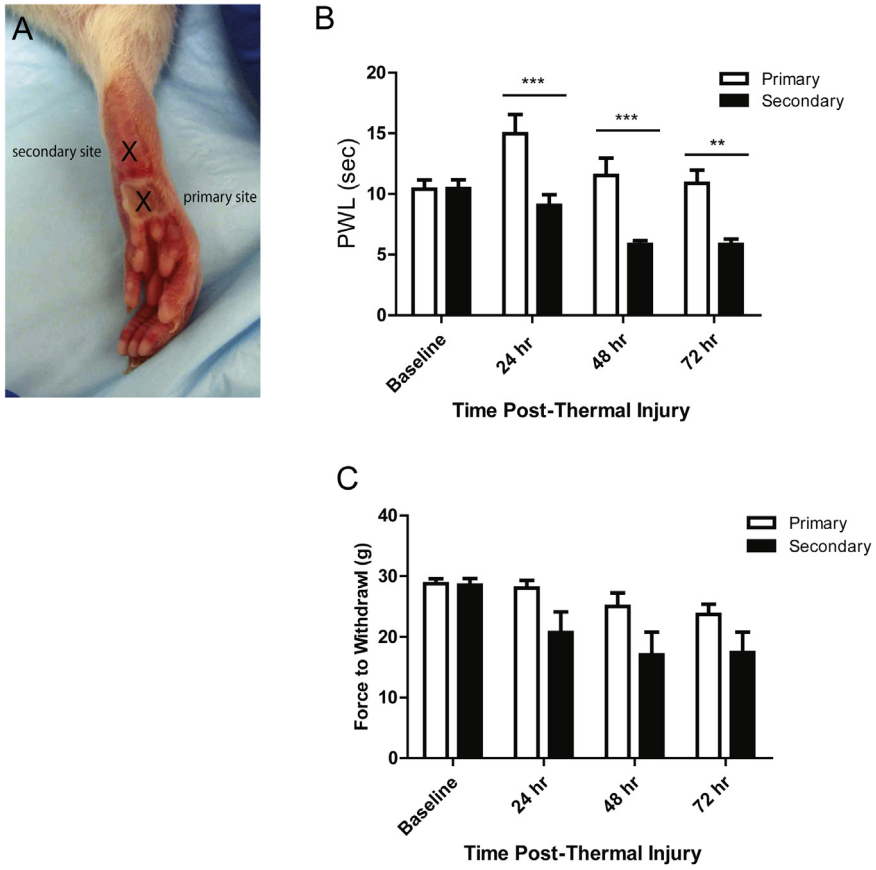

Fig. 2 – Pain behaviors elicited from the primary vs. secondary sites. Image of the thermal injured hindpaw with X's labeling the primary site of injury, denoting the focal burn area, versus the secondary site, denoting the testing site peripheral to the focal point of burn (A). Paw withdrawal latencies (PWLs) recorded in seconds to a thermal stimulus (B) or a mechanical stimulus (C) elicited from the primary site of injury (open bars) or the secondary site of injury (closed bars). PWL were recorded prior to thermal injury (baseline) and 24 h, 48 h and 72 h following thermal injury. *Denotes significance at $p < 0.05$, ** at $p < 0.01$ and * at $p < 0.001$ by two-way ANOVA; $n = 6–7$ rats per group.**

during daily in cage assessment observations prior to behav ior testing.

3.4. *Pronociceptive neuropeptides are upregulated in the spinal dorsal horn following full thickness thermal injury to the rat hindpaw*

We next analyzed the expression of pronociceptive neuropep tides, CGRP and substance P, in the dorsal horn of the spinal cord to provide a neuroanatomical characterization of our model. CGRP immunoreactivity was observed at all behavioral time points (Fig. 4A F) and was significantly more dense at 24 h, 72 h, and 1 week post thermal injury compared to baseline CGRP immunoreactivity [$F(3,35)$ 49.42; $p < 0.05$] (Fig. 4G). In the same tissues run in parallel, substance P immunoreactivity was also observed at all behavioral time points (Fig. 5A F) and was significantly more dense at 24 h, 72 h, and 1 week post thermal injury compared to baseline substance P immunoreactivity [$F(3,35)$ 22.17; $p < 0.05$] (Fig. 5G). There was no significant difference in CGRP or substance P immunoreactivity between the ipsilateral and contralateral dorsal horns at any time point examined nor was there an effect of level of spinal cord analyzed ($p > 0.05$ in all comparisons; CGRP differences ranged between 3.04 and 11.18 and substance P differences ranged between 1.07 and 10.13 at all time points and levels) so these data were combined for presentation.

3.5. *Morphine and tramadol significantly attenuate thermal injury-evoked thermal hyperalgesia and mechanical allodynia*

We next analyzed whether morphine at its ED_{50} dose for inflammatory and burn pain [22 24] could effectively reduce pain behaviors associated with thermal injury as a positive control in our animal model. Morphine (5 mg/kg) signifi cantly attenuated thermal hyperalgesia when administered one week following thermal injury, a time point when thermal hyperalgesia is robust, compared to vehicle con trols [$F(1,136)$ 5.98; $p < 0.05$]. Morphine analgesia was significant at 30 40 min post injection (Fig. 6A). In a separate group of rats, morphine significantly attenuated mechanical sensitivity [$F(1,63)$ 16.62; $p < 0.05$], which peaked at 30 min post injection, compared to vehicle controls (Fig. 6B) when administered one week following thermal injury.

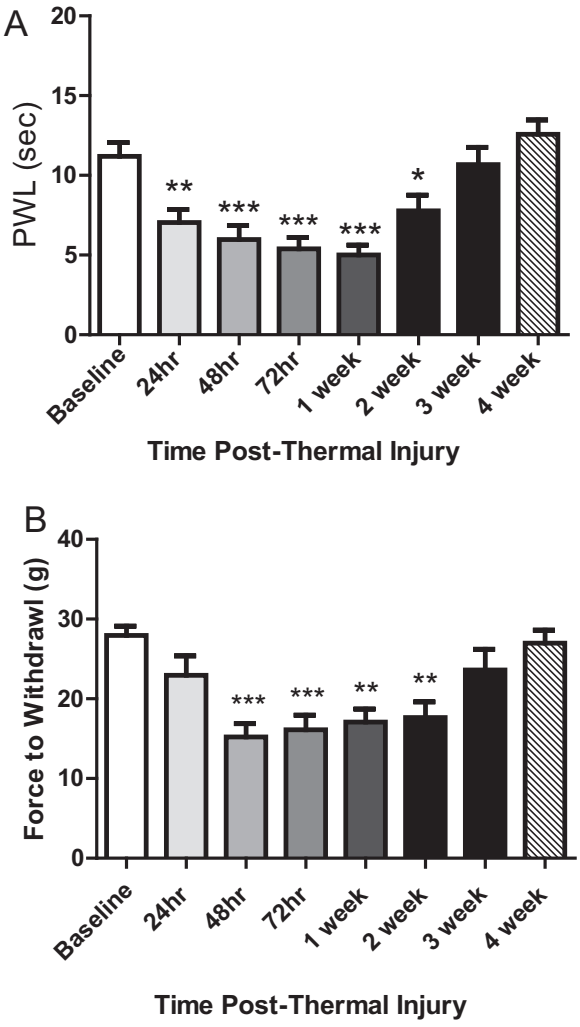

Fig. 3 – Full thickness thermal injury evokes thermal hyperalgesia and mechanical allodynia. Paw withdrawal latencies (PWLs) to a thermal stimulus recorded in seconds (A) or a mechanical stimulus (B) recorded prior to thermal injury (baseline) and 24 h, 48 h, 72 h, 1 week, 2 weeks, 3 weeks and 4 weeks following thermal injury. *Denotes significance at $p < 0.05$, ** at $p < 0.01$ and * at $p < 0.001$ by one-way ANOVA; $n = 6–9$ rats per group.**

As the dual mechanisms therapeutic tramadol targets both opioid and non opioid systems simultaneously, we next examined whether tramadol could attenuate burn evoked pain behaviors in our rat model of full thickness thermal injury. Tramadol attenuated thermal hyperalgesia when administered one week following thermal injury, a time point when pain behaviors peak in this model (Fig. 7A). The 30 mg/kg dose significantly attenuated thermal sensitivity as early as 20 min post injection and lasted for 70 min [$F(3,176)$ 6.27; $p < 0.05$]. The 30 mg/kg dose of tramadol that significantly attenuated thermal hyperalgesia also significantly attenuated mechanical allodynia in a separate group of rats [$F(1,80)$ 22.25; $p < 0.05$]. Tramadol significantly attenuated mechanical sensitivity from 50 to 70 min following injection (Fig. 7B). While tramadol significantly reversed both thermal hyperalgesia [$F(3,23)$ 36.51] and mechanical allodynia [$F(3,23)$ 20.11] to pre injury baseline levels 1 hr following injection, analgesia fully subsided to post injury thermal [$p > 0.05$] and mechanical [$p > 0.05$] sensitivity by 2 h post injection.

Tramadol 30 mg/kg did not significantly alter motor coordination/sedation in rats compared to saline controls at 30 min or 60 min post injection as examined by the rotarod test [$F(1,20)$ 0.57; $p > 0.05$] (Fig. 8). While there was a significant effect of time on the rotarod test [$F(2,20)$ 7.30], Bonferroni *post hoc* analysis indicates no significant differences between treatment groups at each time point [$p > 0.05$]. Prior to injection, rats spent an average of 191 ± 58 SD s on the accelerating rotarod (4 40 rpm over 300 s). Thirty minutes following injection, saline rats spent an average of 164 ± 45 SD s while tramadol rats spent 124 ± 52 SD s on the rotarod. Sixty minutes following injection, saline rats spent an average of 164 ± 54 SD s while tramadol rats spent 131 ± 51 SD s on the rotarod.

4. Discussion

We have adapted a rat model of full thickness thermal injury to screen potential therapeutics that may be effective in reducing pain associated with burn. This model, and the testing of both mechanical allodynia and thermal hyperalgesia, may provide a more clinically relevant mechanism for testing the efficacy of acute administration of analgesics in a burn population. Because the most common source of pain in the burn patient is repetitive debridements and dressing changes, testing evoked pain in an animal model of thermal injury pain may provide data to support the use of particular analgesics to relieve the evoked pain experienced by the burn patient during wound care.

As dual mechanism therapeutics, such as tramadol, have weak opioid receptor agonism combined with norepinephrine and serotonin reuptake inhibition, their use in burned patients may be beneficial in treating pain while reducing negative side effects associated with traditional opioid based analgesics. However, their efficacy as a pain therapy for burn is unclear. We hypothesized that tramadol is effective in reducing thermal injury evoked pain behaviors in a rat model of thermal injury. Here we report that (1) full thickness thermal injury produces significant, unilateral thermal hyperalgesia and mechanical allodynia within 24 48 h that lasts for 2 weeks, (2) this thermal injury enhances CGRP and substance P immunoreactivity in the spinal dorsal horn up to 1 week following injury, and (3) pain behaviors evoked by full thickness thermal injury are attenuated by morphine and tramadol.

Applying a 100 °C metal probe for 30 s to the plantar surface of the rat hindpaw produced a reliable full thickness thermal injury pathologically characterized based on burn depth and tissue morphology. The depth of the burn was on average 2.5 mm at the deepest focal point of injury, with evidence of denatured collagen extending through the epidermis, dermis and subcutaneous tissues, indicative of a full thickness thermal injury. The structural integrity of the gross lesions remained stable for the first 72 h as observed by the

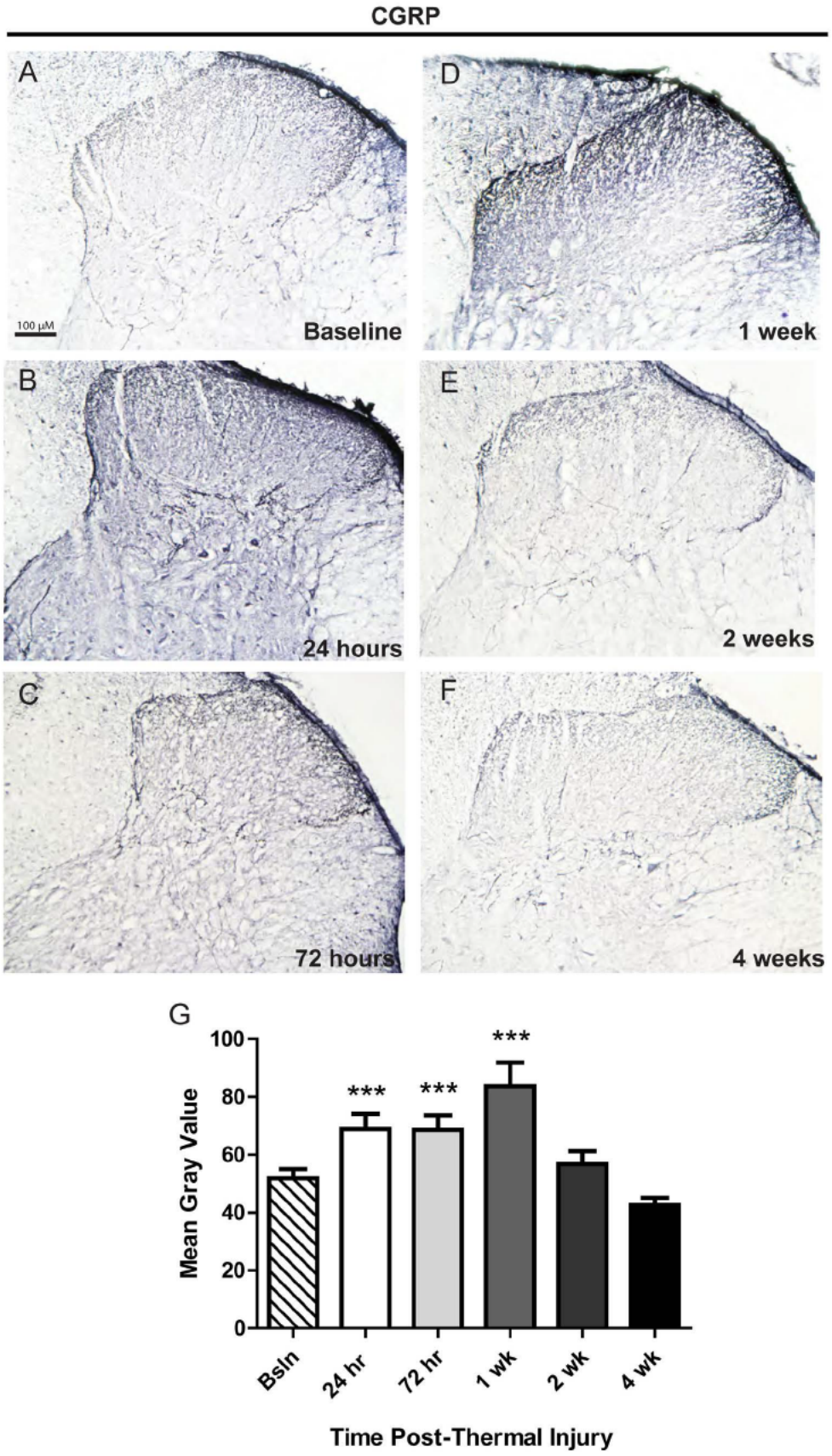

Fig. 4 – CGRP immunoreactivity in the spinal dorsal horn during thermal injury. Photomicrographs of CGRP immunoreactivity in the dorsal horn of the spinal cord prior to injury (A) and post-injury at 24 h (B), 72 h (C), 1 week (D), 2 weeks (E) and 4 weeks (F). Mean Gray Value as measured by densitometry is graphed across these time points (G). ***Denotes significance at $p < 0.001$ by one-way ANOVA; $n = 3–5$ rats per group. Scale bar = 100 μM.

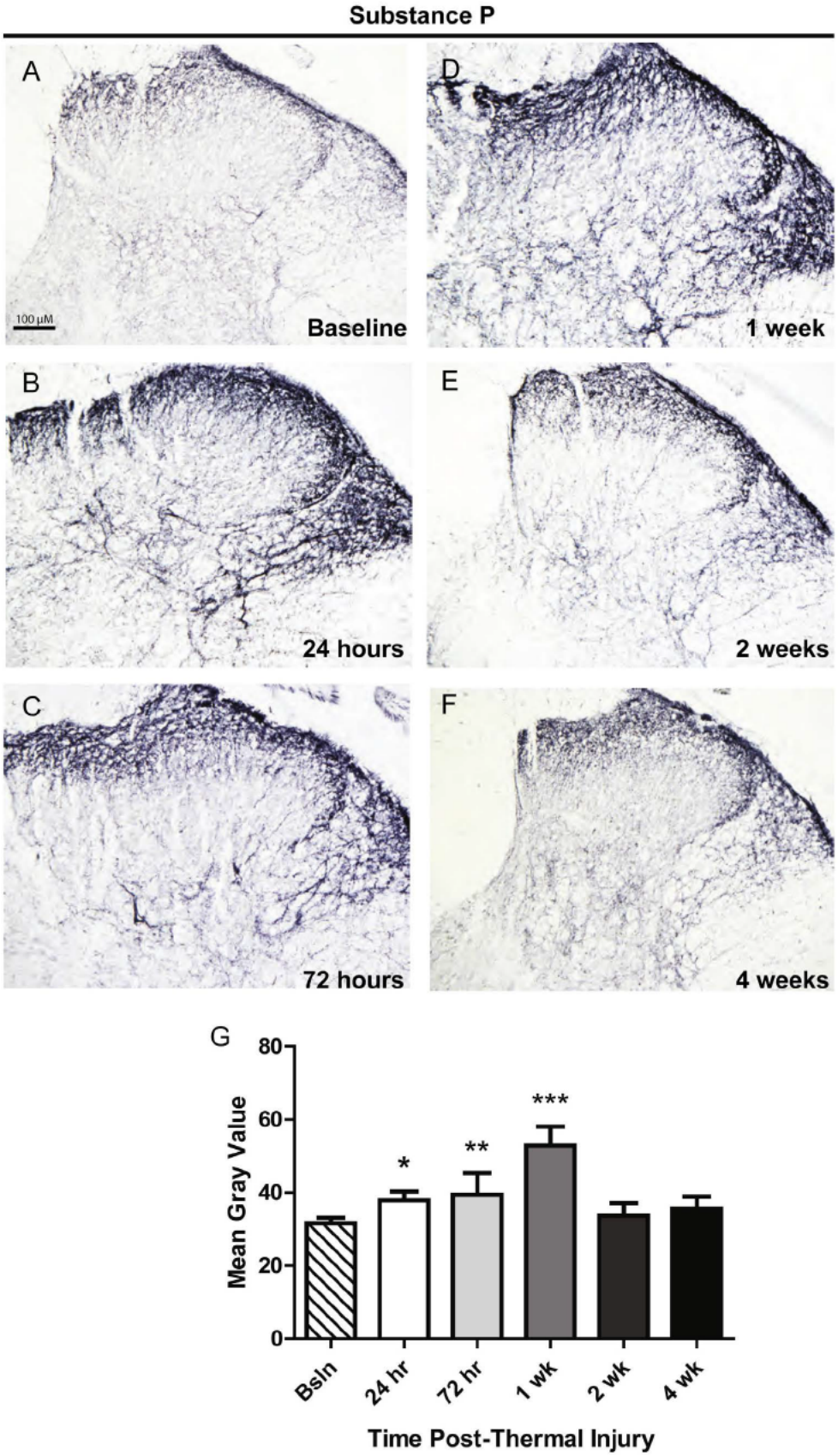

Fig. 5 – Substance P immunoreactivity in the spinal dorsal horn during thermal injury. Photomicrographs of substance P immunoreactivity in the dorsal horn of the spinal cord prior to injury (A) and post-injury at 24 h (B), 72 h (C), 1 week (D), 2 weeks (E) and 4 weeks (F). Mean Gray Value as measured by densitometry is graphed across these time points (G). *Denotes significance at $p < 0.05$, ** at $p < 0.01$ and * at $p < 0.001$ by one-way ANOVA; $n = 3–5$ rats per group. Scale bar = 100 µM.**

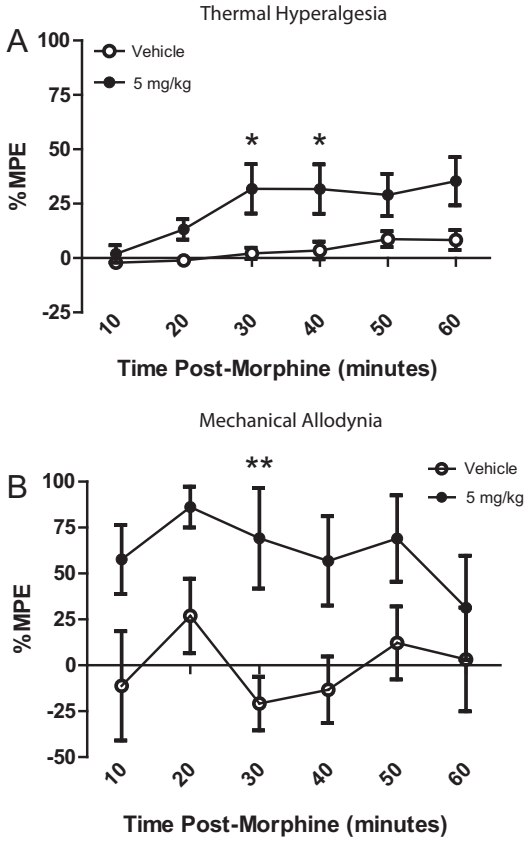

Fig. 6 – Morphine analgesia following full thickness thermal injury. Paw withdrawal latencies to a thermal stimulus (A) or a mechanical stimulus (B) recorded in seconds are reported as percent maximal possible effect (%MPE) calculated from baselines. Responses were detected every 10 min for 60 min following i.p. injection of 5 mg/kg morphine administered one week following thermal injury. *Denotes significance at $p < 0.05$ and ** at $p < 0.01$ by two-way ANOVA; $n = 6–8$ rats per group.

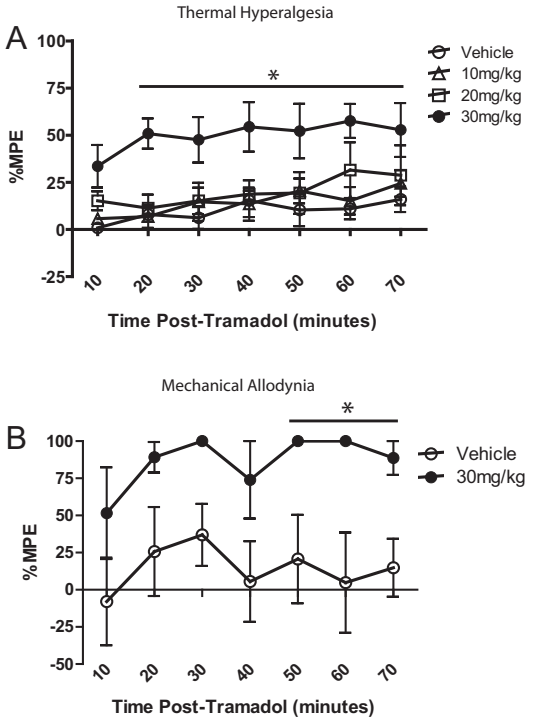

Fig. 7 – Tramadol analgesia following full thickness thermal injury. Paw withdrawal latencies to a thermal stimulus (A) or a mechanical stimulus (B) recorded in seconds are reported as percent maximal possible effect (%MPE) calculated from baselines. Responses were detected every 10 min for 70 min following i.p. injection of tramadol (10–30 mg/kg) administered one week following thermal injury. *Denotes significance at $p < 0.05$ by two-way ANOVA; $n = 5–6$ rats per group.

characterized denatured collagen replacing the function of the epithelium. Once the denatured collagen subsided one week post injury, further ulceration into deeper structures contin ued until re epithelialization at week 3 post injury.

Thermal injury resulted in robust thermal hyperalgesia that was evident within 24 h post injury, similar to a previous study utilizing hot water immersion [24]. Thermal hyperalge sia peaked at 72 h to 1 week post injury and was resolved by 3 4 weeks post injury. As previous studies have reported a lack of thermal hyperalgesia following mild or partial thickness thermal injury in the rat hindpaw [18,34], thermal hyperalge sia may be a characteristic specific to full thickness thermal injury as observed in this study. It may be possible that mechanosensitive and thermosensitive cutaneous nocicep tors are differentially sensitized by burn such that partial thickness burn increases sensitivity of Aβ fibers, which normally respond to innocuous stimuli [18], while full thickness burn also increases the sensitivity of Aδ and C fibers. Further studies are warranted to delineate these potential effects. In the present model, the time course of thermal hyperalgesia parallels histological characterization of the time course of depth and degree of burn.

Thermal injury also produced significant mechanical allodynia observed as a significant reduction in the force in grams required to solicit paw withdrawal from a non noxious

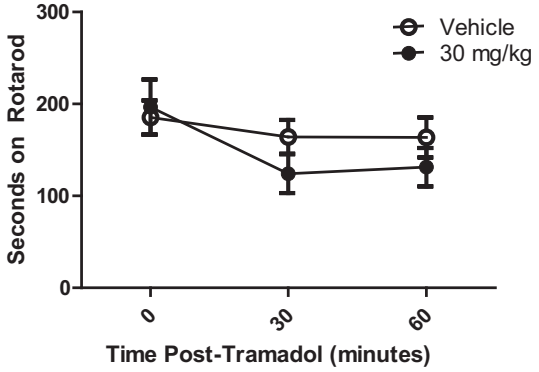

Fig. 8 – No significant effect of tramadol on the rotarod test. Time in seconds (cutoff of 300 s) spent on the rotarod prior to (0 min) and following 30 mg/kg i.p. tramadol vs. saline vehicle injection (30 and 60 min); $n = 6$ rats per group.

mechanical stimulus, similar to previous studies [18,19,24,34,35]. Mechanical sensitivity peaked at 48 72 h post injury, a time point when burn significantly enhances spontaneous and evoked activity in dorsal horn neurons [18], and is resolved by 3 4 weeks post injury. No changes in sensitivity were noted in the uninjured hindpaw similar to previous reports [17,19,24,34,36], however bilateral sensitivity has also been reported [16,18]. This difference may be due to different temperatures and lengths of exposure to a heating device [24], highlighting the importance of pathological characterization of burn tissue in these models.

As a full thickness burn is expected to produce nerve damage at the focal site of injury, the thermal and mechanical stimuli were applied to the area approximately 5 mm proximal to the focal point of the burn, termed here as the secondary site, to capture robust pain behaviors. Previous studies using a rat model of superficial to partial thickness thermal injury have reported primary thermal hyperalgesia and secondary mechanical hyperalgesia [34,35,37,38], while other studies report primary mechanical hyperalgesia [17,19] and no primary thermal hyperalgesia [16,18]. In comparison, experi mental superficial to partial thickness burn in humans produce primary thermal and mechanical hyperalgesia, while only mechanical hyperalgesia is noted in the secondary site [39 43]. Our rat model of full thickness thermal injury exhibits secondary thermal hyperalgesia and mechanical allodynia, while there are no significant pain behaviors detected at the primary burn site. Instead, there appeared to be an increased thermal threshold at the primary site 24 h following thermal injury indicating that thermosensation may be dampened at the focal point of injury due to nerve damage. Alternatively, as a fluid filled blister is present following thermal injury, the nerve endings may not be accessible to the testing stimuli. This could be examined by immunohistochemical analysis of nerve fibers in the burn tissue in future studies.

The pronociceptive neuropeptides CGRP and substance P are released from primary afferents innervating the spinal dorsal horn, and their expression is enhanced during noxious stimulation and inflammation [44]. Both neuropeptides were significantly increased in the dorsal horn of the spinal cord following unilateral full thickness thermal injury of the rat hindpaw. CGRP and substance P expression increased within 24 h of thermal injury, and their expression peaked at 1 week following injury. This increased expression of CGRP and substance P occurs along the same time course as the development and resolution of pain behaviors following thermal injury providing anatomical evidence of the persis tent engagement of the ascending pain system in this rat model of thermal injury. Similarly, increased NMDA receptor and PKCγ and decreased mu opioid receptor and nNOS have been observed in the dorsal horn following thermal injury [24,36] corresponding with burn evoked pain behaviors. Enhanced expression of CGRP and substance P was observed in both the ipsilateral and contralateral dorsal horn. While no changes in thermal hyperalgesia or mechanical allodynia were detected in the contralateral uninjured paw, the upregulation of CGRP and substance P in the contralateral dorsal horn may reflect central sensitization occurring without a behavioral manifestation in the uninjured paw. Alternatively, the ipsilateral upregulation of these pronoci ceptive peptides may be occurring due to contralateral projections of CGRPergic fibers via the dorsal commissure [45,46].

Interestingly, opioids evoke an *increase* in substance P and CGRP release from cultured DRGs [47] and at the spinal dorsal horn [48]. This increased pronociceptive peptide release is positively correlated with a decline in opioid analgesia [49] and is thought to be a mechanism involved in the development of tolerance [50]. As opioid potency has been shown to decline over the development of burn [24], future studies utilizing our rat burn model to examine this mechanism for burn are warranted.

Systemic morphine has been previously shown to reduce burn evoked thermal hyperalgesia and mechanical allodynia when administered one week following injury [24]. We first examined the effects of systemic morphine in our rat model of full thickness thermal injury as a positive screen for analgesia. Indeed, morphine significantly attenuated both thermal hyperalgesia and mechanical allodynia associated with burn at one week post injury, with analgesia peaking at 30 min post injection. At 5 mg/kg, morphine does not alter motor coordination/sedation [51]. Interestingly, a previous study reported that morphine's analgesic effects are reduced when given 2 weeks post thermal injury [24], suggesting a require ment for additional therapeutics to produce an acceptable level of analgesia. This animal data is further validated by a study of experimental burn in healthy human volunteers which indicated that morphine alone is often ineffective in treating pain associated with burn [40]; effective analgesia in this population required synergistic combined therapy with another analgesic or anesthetic such as ketamine [39,41]. Similarly, morphine reduces self reported pain in burn patients but to a greater degree when combined with another therapeutic [52].

Preclinical research has reported that tramadol reduces postoperative [10], acute and neuropathic pain [9,13]. Similarly, clinical studies have reported that high dose tramadol is effective for the treatment of post operative pain [14] and cancer pain [12] with little to no adverse events as compared to low dose morphine [14]. In our rat model of full thickness thermal injury, tramadol dose dependently attenuated thermal hyperalgesia within 20 min and lasted up to 70 min post injection. Tramadol also significantly attenuated mechanical sensitivity at 50 70 min post injection. Previous studies have shown that tramadol exerts analgesic effects via weak agonism of the mu opioid receptor and reducing the reuptake of 5HT and NE [9,11]. In support, microdialysis studies report that tramadol treatment increases 5HT and NE levels in the spinal dorsal horn within 30 min following 20 mg/kg i.p. tramadol [10].This study administered one dose of tramadol at the peak expression of pain behaviors in this model to examine the analgesic efficacy of tramadol on burn pain. Burn patients repeatedly receive analgesics for extended periods of time during treatment. Future studies examining the effect of repeated dosing of tramadol or combined with other analgesics in a rat model of pain associated with full thickness thermal injury is warranted to further inform clinical care of burn pain.

The dose of tramadol required to elicit an analgesic effect in thermal injured rats (30 mg/kg) was higher than the dose required to elicit analgesia in a rat model of neuropathic pain

(2.5 mg/kg) [9] and a rat model of postoperative pain (10 mg/kg) [10]. The tramadol dosage was limited to ≤30 mg/kg in this study to avoid sedation/motor coordination effects [10,13] which would confound our behavior results and are unwanted side effects in burned Service Members. While tramadol does not alter motor coordination/sedation at 5 20 mg/kg dosage in rats [13], there are mixed reports on the effects of 30 mg/kg tramadol. These differences in findings may be due to differences in the particular strain being tested as our report and the report from Kimura et al. found no significant effect of 30 mg/kg tramadol on motor coordination/sedation in Sprague Dawley rats, while Guneli et al. report an effect in Wistar rats. We report that 30 mg/kg tramadol did not significantly alter motor coordination/sedation in rats examined on the rotarod, but we do expect that based on our findings and others [10,13] that doses above 30 mg/kg will produce effects on motor coordination/sedation.

Overall, thermal injury to the rat hindpaw evoked unilateral thermal hyperalgesia and mechanical allodynia, paralleled by enhanced pronociceptive neuropeptide release at the spinal dorsal horn. Burn evoked pain behaviors were significantly attenuated by systemic morphine and tramadol. The ability of high dose tramadol to reduce pain behaviors associated with full thickness thermal injury in the rat indicate that dual mechanism therapeutics may be an effective analgesic option over low dose opioids or in combination with lower dose opioids for treating burn pain in humans while reducing adverse events. As tramadol has weak opioid receptor agonism combined with norepinephrine and serotonin reuptake inhibition, the use of tramadol for treating pain associated with burns has the potential to reduce reliance on traditional opioid based therapeutics in aims of reducing negative side effects [14] and the development of tolerance and addiction [15] in burned Service Members and civilians.

Conflict of interest statement

The authors report no conflicts of interest. The opinions or assertions contained herein are the private views of the author and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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