Establishing a Mouse Model for Neuropathic Pain Elicited by Thoracic Contusion Spinal Cord Injury

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Abstract:

With a high occurrence of spinal cord injuries (SCIs) per year in the United States, and a significant number of patients living with SCI, innovations in modeling, treating, and managing these injuries is in high demand. An often overlooked aspect of chronic SCI is the management of neuropathic pain. Although the subject of neuropathic pain carries a substantial focus within current literature, a surprisingly small fraction focuses on SCI-induced neuropathic pain. Most notably, the field lacks a well-defined, clinically relevant mouse model for SCI-induced neuropathic pain. Here we tested the hypothesis that a moderate contusion SCI in mice would cause neuropathic pain, and that this pain might be alleviated by inactivating astrocytes. We report significant inductions of acute-to-chronic hyperalgesia and allodynia in both male and female mice, with female mice exhibiting lower thermal nociceptive thresholds than males. Furthermore, locomotor recovery occurred primarily within the acute phase of injury recovery, with little improvement during the chronic phase. We hypothesized astrocytes would play a critical role in the induction of neuropathic pain with SCI based on this timeline. To test this, mice were injected with an inhibitory DREADD viral vector (pAAV-GFAP-HA-hM4D(Gi)-IRES-mCitrine) then subjected to a thoracic contusion SCI. Astrocytes were inactivated by systemic application of clozapine-N-oxide (CNO). Our data suggest that DREADD-mediated astrocyte inactivation may have been ineffective, as there were few behavioral changes and these mice had little GFP expression around the injury epicenter. Although the exact role may be unclear, astrocytes appear to be critical players in mediating neuropathic pain in post-SCI recovery. The model for neuropathic pain developed here also provides a base for developing and refining methods to treat and manage SCI in humans.

Introduction:

In 2016, spinal cord injury (SCI) afflicted 17,000 new individuals (National Spinal Cord Injury Statistical Center). A major challenge with SCIs is that there is no known treatment that enables complete, functional recovery. SCI affects many aspects of quality-of-life; for example one study found that 66% of individuals with chronic SCI reported moderate to severe neuropathic pain, and that, combined with bowel/bladder related difficulties, the overall quality of life was significantly reduced [1]. While it is still unknown how to effectively treat traumatic SCI to maximize functional recovery, one particularly important challenge for quality of life is chronic neuropathic pain. SCI contusion models in mice have been used extensively to study some of the underlying mechanisms of SCI[2,3], but few have utilized contusion models to exclusively study SCI-induced neuropathic pain. Neuropathic pain can be caused by several potential mechanisms. SCI could alter synaptic signaling, such as GABA signaling, in the spinal cord to drive nociceptive sensitivity[4]. SCI could also strengthen glial cell signaling/amplification of nociception; microglia and astrocytes become activated in peripheral injury-induced neuropathic pain models [5]. One clinical trial determined that neuroinflammation in post-SCI recovery plays a significant role in generating neuropathic pain, suggesting pro-inflammatory factors as a target for attenuation [6]. However, further studies are required to determine the specific role of spinal glial cells, such as astrocytes, in functional recovery after a traumatic SCI.

Astrocytes are cells within the central nervous system (CNS) that have a variety of functions, ranging from glutamate re-uptake, regulation of ion concentration, and glial scar formation after CNS injury [7,8]. Their role in post-CNS injury recovery, however, is more dynamic than just scar formation. Astrocytes release both pro-inflammatory factors and anti-inflammatory factors, and are themselves subject to context-dependent morphological changes

[8,9]. One notable post-injury response is the activation and hypertrophy of astrocytes ("reactive astrogliosis") which can worsen functional recovery, induce secondary injury, and exacerbate neuropathic pain [7,8,10,11]. Glial scar formation by astrocytes also aides in axonal regrowth after CNS injury[12,13]. Reactive astrogliosis seems to have the greatest clinical interest for improving the recovery of CNS-injuries. However, a considerable amount of literature has focused on astrogliosis within CNS disease models[8], but not within CNS injury models. This prevents us from truly understanding the role astrocytes play in traumatic injury recovery. A key step in understanding this role is developing a sufficient animal model for SCI-induced neuropathic pain.

One innovative strategy for manipulating cell-specific activation is via designer receptors exclusively activated by designer drugs (DREADDs). DREADDs are a research tool with remarkable temporal resolution and *in vivo* applications. By synthesizing a G-protein-coupled receptor (GPCR) that is exclusively activated by a pharmacologically inert ligand – commonly clozapine-N-oxide (CNO) – and expressing it under a cell-specific promoter, researchers can elicit a massive change in activity by altering intracellular Ca^{2+} concentration across all cells within a system that express that promoter. Three different types of DREADDs are used most commonly: hM3Dq, which is coupled to a Gαq GPCR, increases cellular activity; hM4Di, which is coupled to a Gαi GPCR, effectively silences activity; rM3Ds, which is coupled to a Gαs GPCR, modulates activity by generating more sporadic firing [14]. However, their application is not limited only to neurons. Considering their mechanism of action, other cells, such as glia (specifically astrocytes) could be over-activated, silenced, or burst-activated with excellent spatial and temporal precision.

Here we sought to develop a mouse model of SCI-induced neuropathic pain. We hypothesized that a thoracic contusion SCI would evoke below-level neuropathic pain in the form of hyperalgesia and allodynia. We assessed these metrics of neuropathic pain through an acute phase of post-injury recovery (up to 28 days post injury). In addition, we hypothesized that SCI-induced neuropathic pain could be attenuated by inhibiting astrocyte activity. We utilized inactivating DREADDs (hM4Di) with a CNO agonist to silence astrocyte activity and assessed hyperalgesia and allodynia in acute and chronic phases of post-injury recovery.

Materials/Methods:

Animals:

C57BL/6J strain mice were used for all experiments. All housing, surgery, and postoperative care was approved by the University of Colorado Boulder Institutional Animal Care and Use Committee. All animals were fed standard chow and filtered tap water *ad libitum*. Animals were maintained on a standard 12:12 light/dark cycle with all surgeries being conducted at interspersed times during the light cycles.

For establishment of a neuropathic pain model:

21 male and 24 female mice from Jackson Laboratories were each separated into SCI (female n= 14, male n=9) and Sham (female n=11, male n=12) groups. Each subject was shaved, cleaned, and given 0.1mL of gentamycin subcutaneously while being anesthetized with isofluorane. A T9 laminectomy was performed followed by either immediate aftercare (sham group) or a 60 kilodyne impact with no dwell to generate a moderate spinal cord contusion (SCI group). An Infinite Horizon impactor was used to deliver the spinal cord contusion.

For DREADD inhibition of astrocytes:

20 male and 20 female mice from Jackson Laboratories were separated into groups of similar averages of pre-surgery thermal and mechanical nociceptive thresholds. A subset of the animals (female n=9, male n=8) were subjected to infusion of an AAV viral vector containing a GFAP-promoter tagged inhibitory DREADD (pAAV-GFAP-HA-hM4D(Gi)-IRES-mCitrine) three weeks prior to surgery via L4/L5 intrathecal (IT) injection. Another subset of mice (female n=6, male n=7) were exposed to a control treatment consisting of the same vector with a GFAP-promoter tag but no inhibitory DREADD (pAAV-GFAP-HA-IRES-mCitrine). Each animal was shaved, cleaned, and given 0.1mL of gentamycin subcutaneously while being anesthetized with isofluorane. Subjects of these cohorts were subjected to a T9 laminectomy followed by a 60 kilodyne impact with no dwell to generate a moderate contusion to the thoracic spinal cord. A third subset (female n=5, male n=5) of mice received the control vector (pAAV-GFAP-HA-IRES-mCitrine) but were subjected to the T9 laminectomy without the 60 kilodyne impact. All subsets of animals were then given intraperitoneal injections of CNO one hour post-surgery as well as throughout the behavioral testing time course. An Infinite Horizon impactor was used to deliver the spinal cord contusion.

Behavior:

For establishment of a neuropathic pain model:

Sensorimotor recovery was assessed using the Basso Mouse Scale[15] (BMS) while thermal hyperalgesia and mechanical allodynia were assessed using Hargreaves[16] and von Frey simplified up-down method (SUDO) thresholds[17] respectively. Prior to surgeries, all animals were subjected one round of BMS assessment (to determine group placement)

and two rounds of each Hargreaves and von Frey to establish baseline nociceptive thresholds. BMS was assessed at 1, 4, 7, 10, 14, 21, and 28 days post injury (dpi). Thermal hyperalgesia and mechanical allodynia were assessed weekly through 28 dpi.

For DREADD inhibition of astrocytes:

Sensorimotor recovery was assessed using the Basso Mouse Scale[15] (BMS) while thermal hyperalgesia and mechanical allodynia were assessed using Hargreaves[16] and von Frey simplified up-down method (SUDO) thresholds[17] respectively. Prior to surgeries, all animals were subjected one round of BMS assessment (to determine group placement) and two rounds of each Hargreaves and von Frey to establish baseline nociceptive thresholds. BMS was assessed at 1, 4, 7, 10, 14, 21, and 28 days post injury (dpi). Thermal hyperalgesia and mechanical allodynia were assessed weekly through 28 dpi. Hargreaves and von Frey assessments were administered at time points around CNO injection; von Frey testing was conducted 1 hour before CNO injection, 30 minutes after CNO injection, and 4 hours after CNO injection, while Hargreaves testing was conducted 30 minutes before CNO injection, 1 hour after CNO injection, and 5 hours after CNO injection on each testing day.

Tissue Collection:

All subjects were sacrificed using a 0.2mL injection of Pentobarbitol at approximately 32 days post injury. All animals were perfused with 0.9% saline solution, followed by 0.4M paraformaldehyde at physiologic pH. The following tissues were collected from each animal: brain, segments of the spinal cord, dorsal root ganglia, liver, and spleen. The spinal cord segments were collected in reference to the T9 contusion. The contusion epicenter and L4/L5 lumbar spinal cord regions were extracted. All tissues were stored in

0.4M paraformaldehyde at physiologic pH for approximately 24 hours before being transferred to 30% sucrose in PBS. At earliest convenience, the spinal cord regions extracted from each animal were transferred to, and oriented in, a cryo-mold filled with Tissue-Tekk O.C.T. Compound and frozen at -80° C. The gel blocks were then sliced into 10 micrometer thick slices using a Leica CM1850 Cryostat and mounted on Permafrost+ glass slides for immunohistochemical analysis.

IHC/Microscopy:

For establishment of a neuropathic pain model:

Slides containing tissue from the contusion epicenter and lumbar spinal cord were allowed to thaw from storage temperature (-20° C) and washed with 0.1M PBS. They were then blocked with 10% neutral goat serum (NGS) and treated with a 1:1000 dilution of rabbit derived anti-Iba1 primary antibodies in 0.1M PBS + 0.2% Triton X. After 24 hours of incubation with the primary antibodies, the were washed with 0.1M PBS and treated with a 1:500 dilution of Alexa Fluor 488 donkey derived anti-rabbit secondary antibodies in 0.1M PBS + 0.2% Triton X. 1.0 microliter of DAPI was also added to the secondary antibody dilution prior to application. After 2 hours of incubation, the slides were washed with 0.1M PBS and a cover slip with Immumount was applied. The slides were sealed, left to dry in the dark, and then stored at (4° C). Slides were examined for astrocyte morphology using the *CAPTAIN AMERICA* – WF and *DANTE* – Olympus IX81 fluorescent microscopes in the MCDB Core Facility. Images were captured using SlideBook ver. 6 and analyzed using FIJI.

For DREADD inhibition of astrocytes:

Slides containing tissue from the contusion epicenter were allowed to thaw from storage temperature (-20° C) and washed with 0.1M PBS. They were then blocked with 10% neutral donkey serum (NDS); slides were treated with a primary antibody solution consisting of a 1:100 dilution of mouse derived anti-GFAP and a 1:1000 dilution of rabbit derived anti-GFP antibodies in 0.1M PBS + 0.2% Triton X. After 24 hours of incubation with the primary antibodies, the slides were washed with 0.1M PBS and treated with a secondary antibody solution consisting of a 1:500 dilution of Alexa Fluor 488 goat derived anti-rabbit and a 1:500 dilution of Alexa Fluor 594 goat derived anti-mouse antibodies in 0.1M PBS + 0.2% Triton X. 1.0 microliter of DAPI was also added to the secondary antibody dilution prior to application. After 2 hours of incubation, the slides were washed with 0.1M PBS and a cover slip with Immumount was applied. The slides were sealed, left to dry in the dark, and then stored at (4° C). Slides were examined for astrocyte/DREADD colocalization using the *CAPTAIN AMERICA* – WF and *DANTE* – Olympus IX81 fluorescent microscopes in the MCDB Core Facility. Images were captured using SlideBook ver. 6 and analyzed using FIJI.

Results:

SCI in female and male mice causes chronic mechanical allodynia and thermal hyperalgesia

Mechanical allodynia and thermal hyperalgesia constitute two important metrics of pain sensitivity that present in peripheral neuropathies and often have associations with morphological and molecular changes in the spinal cord[18–20]. Furthermore, these metrics offer a

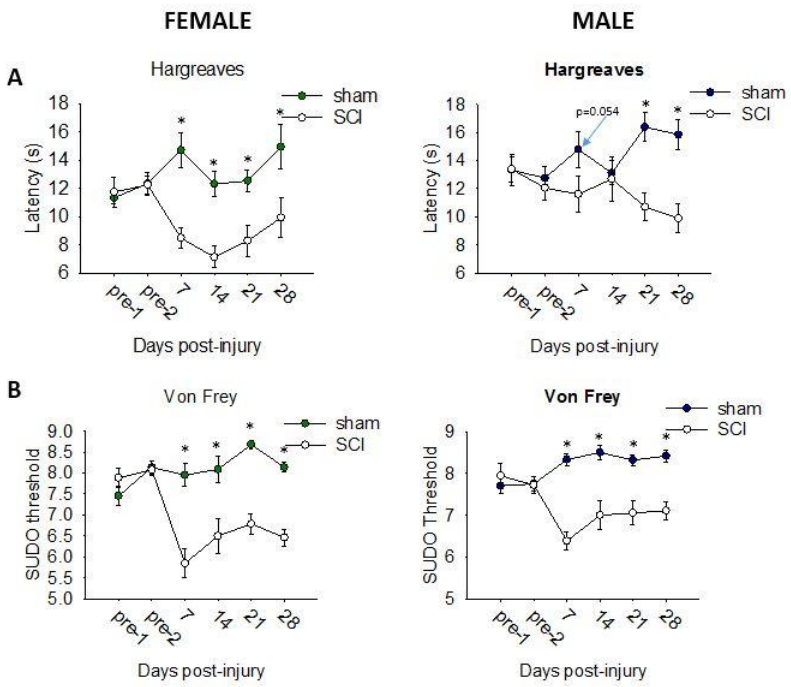

Figure 1: T9 contusion SCI in female and male mice causes neuropathic pain symptoms.

(A): Thermal hyperalgesia in male and female mice with moderate T9 contusion. Female mice (left) and male mice (right) were both tested twice before SCI and at weekly intervals through 28 days post injury. Females in the SCI group had consistently significant reductions in response latency at all post-SCI testing periods ($p<0.05$). Males in the SCI group showed significant reductions in response latency at 21 and 28 dpi time points ($p<0.05$).

(B): Mechanical allodynia in male and female mice with moderate T9 contusion. Female (left) and male (right) were tested twice before SCI and at weekly intervals through 28 days post injury. Both male and female SCI groups exhibited significantly enhanced mechanical sensitivity throughout post injury testing ($p<0.05$).

significant clinical value in assessing neuropathic pain during SCI recovery [21]. Female and male mice were tested for thermal nociceptive thresholds (Fig. 1A). Both female and male mice with SCI displayed thermal hyperalgesia (decreased response latency) compared to control mice (Fig. 1A). Both male and female SCI animals exhibited this increased sensitivity at later time points, but only females exhibited thermal hyperalgesia throughout the post-injury recovery period. Mechanical hypersensitivity was tested in female and male SCI mice (Fig. 1B). Both males and females exhibited significant decreases in SUDO threshold relative to the sham group, implying an increased sensitivity to mechanical force to the foot pads of the hind limbs. Thus, contusion SCI caused neuropathic pain symptoms in both female and male mice. As females showed expedited thermal hyperalgesia development, these data suggest a sex bias in the development of neuropathic pain after SCI in a mouse model.

Female and male mice with SCI gradually recover partial locomotor function over 28 days

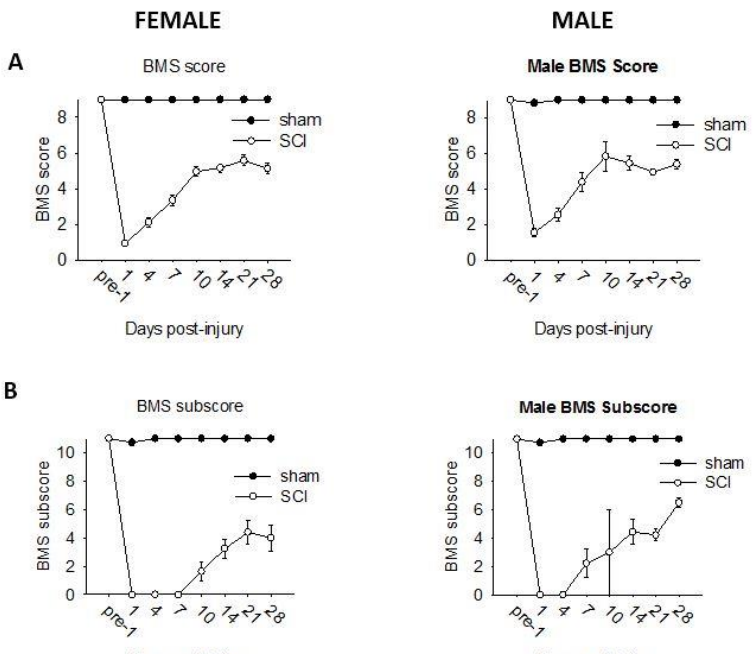

Figure 2: Female and male mice with SCI gradually recover partial locomotor function over 28 days.

(A): Hind-limb motor recovery measured using the Basso Mouse Scale (BMS). Females (left) and males (right) were tested once before injury and again at seven intervals through 28 days post injury. SCI groups of both males and females exhibit significant reductions in motor function (1 dpi) with recovery patterns of increasing motor function.

Figure 2b: The BMS sub-scores provide a more refined metric of locomotor recovery once the animal begins to frequently take plantar steps. At the point of frequent plantar stepping (post 4 dpi) the animals display a similar recovery pattern as depicted by the main BMS scoring.

The Basso Mouse Scale (BMS) offers a comprehensive means of assessing post-SCI locomotor recovery specifically in mice[15]. Compared to sham mice, both male and female SCI mice show a fairly similar post-surgery recovery of BMS locomotor scores (Fig. 2A). Males regained plantar stepping sooner than females and exhibited more refine motor recovery at chronic phase (Fig. 2B). Chronic-phase locomotor function in SCI animals remains constantly below that of sham animals, suggesting that animals with SCI can only regain partial hind-limb locomotor function with a T9 contusion.

SCI mice show increased astrocyte density in deep dorsal horn

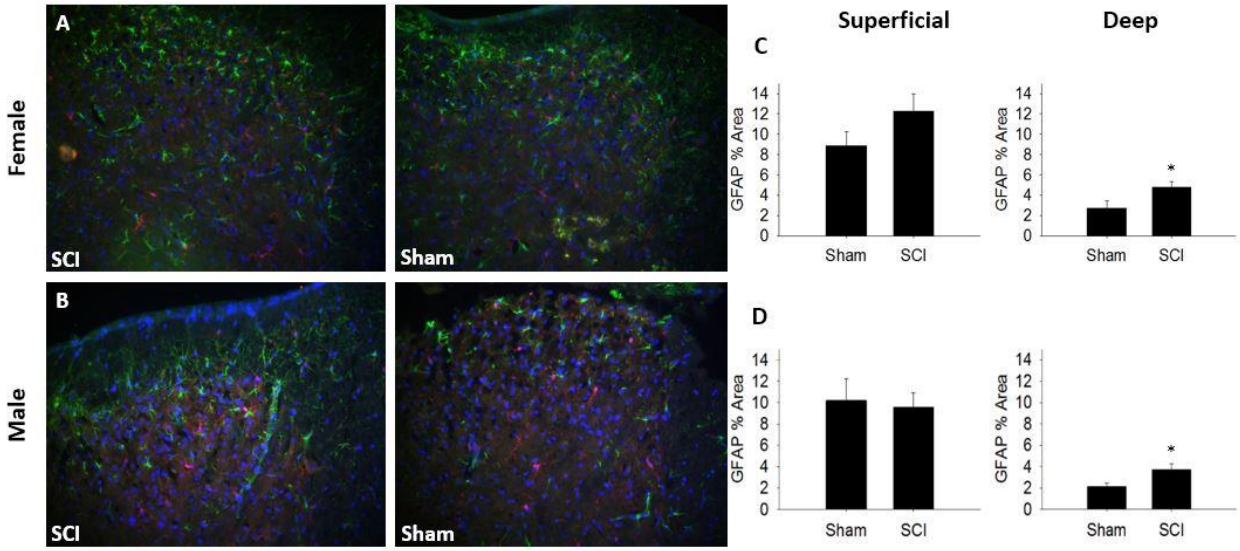

Figure 3: Female and male mice with SCI display increased GFAP+ astrocyte density in the deep (but not superficial) dorsal horn laminae.
(A): Fluorescent micrographs of left dorsal horns in the lumbar spinal cord at 20X in female SCI and sham animals at chronic time points (28dpi and later). Iba1 is shown in red, nuclei are shown in blue, and GFAP is shown in green.
(B): Fluorescent micrographs of left dorsal horns in the lumbar spinal cord at 20x in male SCI and sham animals at chronic time points (28dpi and later). Iba1 is shown in red, nuclei are shown in blue, and GFAP is shown in green.
(C): Quantification of GFAP expression in superficial and deep laminae of the dorsal horns in female sham and SCI animals (n=6). Using One-Way ANOVA it was determined that the percent area GFAP+ cells was significantly higher in the deep laminae of the dorsal horns of females with SCI compared to sham ($p<0.05$).
(D): Quantification of GFAP expression in superficial and deep laminae of the dorsal horns in male sham and SCI animals (n=4). Using One-Way ANOVA, it was determined that the percent area GFAP+ cells was significantly higher in the deep laminae of the dorsal horns of males with SCI compared to sham ($p<0.05$).

A special focus was placed on astrocyte density within the dorsal horns due to their role in neuropathic pain initiation/maintenance [5,7,20]. While reactive astrogliosis is expected within acute phases of post-SCI recovery (1 dpi – 28 dpi)[7] it remains relatively unclear whether this astrogliosis persists in the chronic phase of recovery (post-28 dpi). Figures 3A and 3C depict relative density of GFAP+ cells (shown as green) within superficial and deep laminae of the dorsal horn laminae in sham and SCI female mice at chronic time points (28 dpi and later). There was a significant increase in GFAP+ cell density in the deeper laminae of the dorsal horn in

females and males with SCI ($p<0.05$). There was no significant difference in GFAP+ cell density in the superficial dorsal horn. Figures 3B and 3D depict relative densities of GFAP+ cells (shown as green) within superficial and deep laminae of the dorsal horn in sham and SCI male mice at chronic time points (28 dpi and later). Our data suggest that T9 contusion SCI causes chronic reactive astrogliosis in the deep dorsal horn laminae within the lumbar spinal cord.

SCI-induced mechanical allodynia and thermal hyperalgesia are not significantly reversed with DREADD inhibition of astrocytes

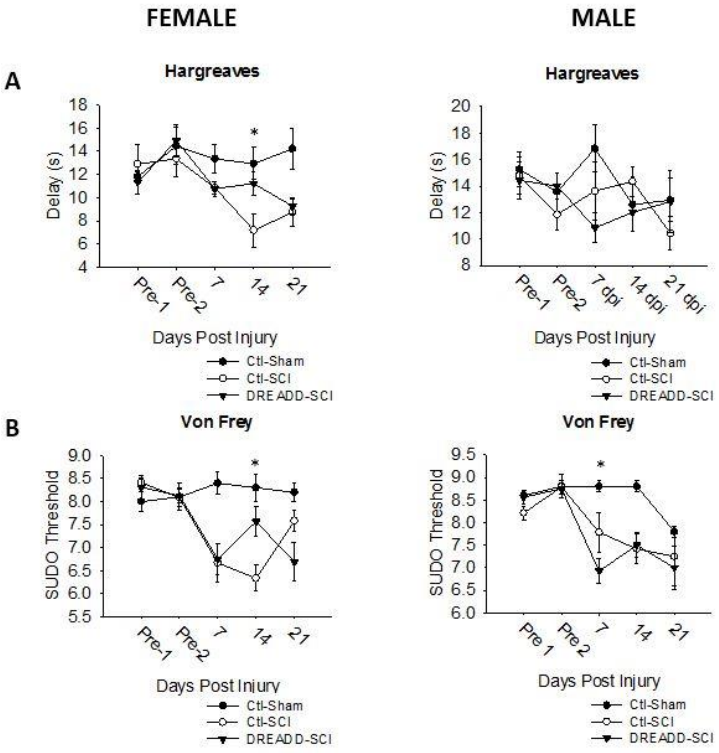

Figure 4: Inhibiting astrocyte activation using DREADDs in mice with SCI to limit SCI-elicited pain.
(A): Thermal hyperalgesia in male and female mice with moderate T9 contusion and transfection with astrocyte specific inhibitory DREADD. Female mice (left) and male mice (right) were both tested twice before SCI and at weekly intervals through 21 days post injury. Neither males nor females treated with the inhibitory DREADD exhibited significant recovery in thermal hyperalgesia as compared to the SCI control groups overall. Although at 14 dpi, DREADD treated females showed a significant improvement in hyperalgesia over their SCI control counterparts ($p<0.05$).
(B): Mechanical allodynia in male and female mice with moderate T9 contusion and transfection with astrocyte specific inhibitory DREADD. Female (left) and male (right) were tested twice before SCI and at weekly intervals through 21 days post injury. Neither males nor females treated with the inhibitory DREADD exhibited significant recovery in mechanical allodynia as compared to the SCI control groups. Although at 14 dpi, DREADD treated females showed a significant improvement in mechanical allodynia over their SCI control counterparts ($p<0.05$).

A myriad of previous studies have utilized chemogenetic interventions for modulation of neuron activity,[14] yet few have explored chemogenetic modulation of support cells. One study documented both induction of allodynia and attenuation of allodynia following transfection

of stimulatory and inhibitory DREADD respectively in microglia[22]. However, DREADD modulation of astrocytes is still relatively unexplored for potential therapeutic benefits. Figure 4 depicts mechanical allodynia and thermal hyperalgesia assessment of male and female mice given sham, control – SCI, and DREADD – SCI treatments. Comparing DREADD – SCI to control – SCI groups, there are few patterns of significant difference. This lack of significance suggests DREADD inhibition of astrocytes as an ineffective intervention against the development of acute post-SCI neuropathic pain. Furthermore, there are also few significant differences between male and female groups. This observation is worth noting as a stark contrast from the data exhibited in Figure 1 and may constitute sex differences in DREADD efficacy.

Female mice show a minor reversal of SCI-induced locomotor deficit when treated with DREADD inhibition of astrocytes

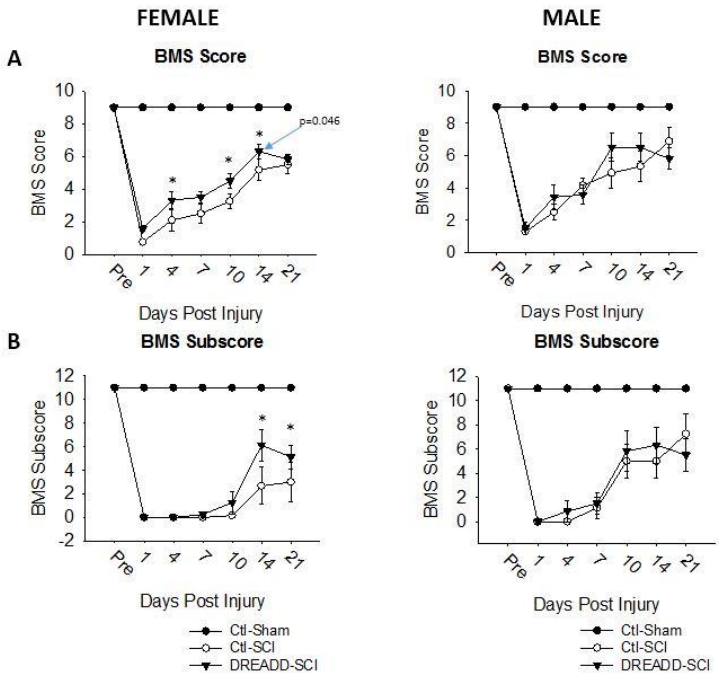

Figure 5: Inhibiting astrocyte activation using DREADDs in mice with SCI to improve locomotor recovery over 28 days. **(A)**: Hind-limb motor recovery measured using the Basso Mouse Scale (BMS). Females (left) and males (right) were tested once before injury and again at six intervals through 21 days post injury. Differences in recovery between DREADD and SCI control groups begin to manifest in females at 1 dpi and persist through 14 dpi, with significant differences at 4, 10, and 14 dpi ($p<0.05$). **(B)**: The BMS sub-scores provide a more refined metric of locomotor recovery once the animal begins to frequently take plantar steps. At the point of frequent plantar stepping (post 4 dpi) the animals display a similar recovery pattern as depicted by the main BMS scoring. DREADD treated females exhibit significant improvement in locomotor recovery at 14 and 21 dpi ($p<0.05$).

Further examination of DREADD modulation of astrocytes requires observation of locomotor recovery after SCI as well as development of hyperalgesia and allodynia. Differences in hind-limb locomotor recovery were compared between control-sham, control-SCI, and DREADD-SCI animal groups utilizing the Basso Mouse Scale (BMS) (Fig. 5). Although all three groups are compared here, the key focus is on differences between the control-SCI and DREADD-SCI. Although we did not observe significant improvements in locomotor recovery in males within the DREADD-SCI group, we observed moderate augments to locomotor recovery in female DREADD-SCI groups. Furthermore, the lack of differences in males reinforces the potential for a sex-specific effect of DREADD inhibition suggested by Figure 4.

Density of GFP+ cells suggests ineffective DREADD integration into astrocytes at injury epicenter

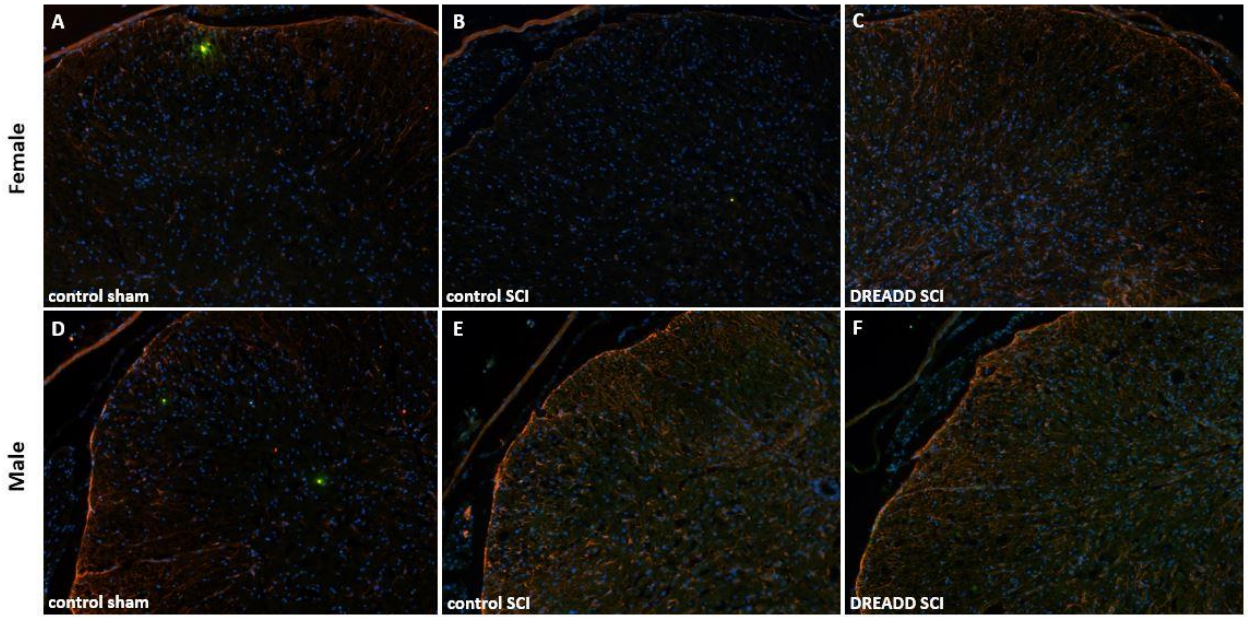

Figure 6: Density of GFP+ cells in dorsal horns of the injury site suggests ineffective DREADD integration
(A-C): Fluorescent micrographs of the dorsal horns in the injury epicenter at 10X in female animals at chronic time point (28dpi and later). Nuclei are shown in blue, GFAP is shown in red, and mCitrine is shown in green.
(D-F): Fluorescent micrographs of the dorsal horns in the injury epicenter at 10X in male SCI animal at chronic time point (28dpi and later). Nuclei are shown in blue, GFAP is shown in red, and mCitrine is shown in green.

The therapeutic value of chemogenetic modulation of astrocytes has yet to be seen with the behavioral data thus far. Therefore, immunohistochemical analysis was conducted to determine whether the viral transfection of the hM4Di Gαi was successful. The dorsal horns of female mice transfected with a control vector (pAAV-GFAP-HA-IRES-mCitrine) before a sham or SCI surgery show little-to-no GFP+ cells (Fig. 6A-B). Tissue was collected after sacrifice at 28 dpi. The dorsal horns of female mice transfected with the DREADD vector (pAAV-GFAP-HA-hM4D(Gi)-IRES-mCitrine) before SCI surgery also show no GFP+ cells (Fig. 6C). Tissue was collected after sacrifice at 28 dpi. The dorsal horns of male mice transfected with a control vector (pAAV-GFAP-HA-IRES-mCitrine) following a sham or SCI surgery show little to no GFP+ cells (Fig. 6D-E). Tissue was collected after sacrifice at 28 dpi. The dorsal horns of male mice transfected with the DREADD vector (pAAV-GFAP-HA-hM4D(Gi)-IRES-mCitrine) following SCI surgery also show no GFP+ cells (Fig. 6F). Tissue was collected after sacrifice at 28 dpi. As shown, there is virtually no colocalization of the red and green fluorescent antibodies, implying no integration of the vector in the astrocytes. Percent area quantification of GFP+ cells was excluded due to their nonoccurrence. The lack of transfection (at least using this histological analysis) suggests that the DREADD delivery method (intrathecal injection) and/or timing may not have effectively targeted astrocytes throughout the neuraxis. Thus, although we saw minimal changes with DREADD-mediated astrocyte inhibition, more effective delivery of this vector may still constitute some post-SCI therapeutic value.

Discussion:

Here, we showed that clinically relevant contusion SCI in female and male mice caused chronic below-level neuropathic pain symptoms, and that astrocyte activation may play a role in

SCI-elicited locomotor deficits and pain. Female mice tend to exhibit more consistent hind-limb neuropathic pain after SCI as evidenced by continuous depressions in mechanical and heat withdrawal thresholds. Where males also exhibit mechanical allodynia and thermal hyperalgesia indicative of neuropathic pain development, their reductions in mechanical and heat withdrawal thresholds are less profound than what was exhibited in females. Furthermore, females displayed a more consistent pattern of significant allodynia and hyperalgesia compared to males. Locomotor recovery was more comparable between male and female groups, with both displaying positive trends before reaching an asymptotic plateau of recovery by 10 dpi. However, looking more specifically at the BMS sub-scores reveals an earlier incidence of recovery in males than in females, with males exhibiting plantar stepping earlier in the recovery process. This observation is consistent with the resilience to SCI-induced allodynia and hyperalgesia recorded in males. It is worth noting that the error bars within the male BMS constitute a potential bias in the data; we had an unfortunate and irreparable loss of BMS data at 10 dpi for a number of the subjects. However, the consistency between the locomotor recovery and expression of allodynia and hyperalgesia within the males is expected to outweigh the error associated with loss of data for the single time point. An interesting observation in the tissue morphology of both male and female mice with SCI was the increase in astrocyte densities in the deep laminae of the dorsal horns, but not in the superficial laminae. Considering the role astrocytes play in mediating C-fiber synaptic potentiation in lamina I of the spinal cord[20,23], and dynamic range of innervation mediated by lamina V in neuropathic pain processing[23], astrocyte density was expected to increase in deep and superficial dorsal horn laminae with SCI. However, since the animals were sacrificed at a chronic time point (28 dpi) the expected saturation of astrocytes could have subsided, limiting the reactive astrogliosis process to the acute phase of

post-SCI recovery. This would be consistent with pro-inflammatory-cytokine-dependent mechanism that indirectly governs Lamina I synaptic potentiation[20].

Behavioral and morphological changes with SCI were observed with some minor sex-specific differences. However, to solidify the observed patterns, additional analyses, such as quantitative polymerase chain reaction (qPCR), would benefit the model. Considering their roles in peripheral neuro-inflammation and systemic inflammation, analyzing relative mRNA levels of Il-1β and TNF-α could contribute to a greater understanding of acute phase neuro-inflammation in a spinal cord injury model[20,24,25] which may contribute to acute-phase neuropathic pain. Specifically, there have been correlations between NLRP2 and NLRP3 inflammasome formation and neuropathic pain[18,26], although these correlations have not been documented specifically after SCI.

In addition to insignificant attenuations of the allodynia and hyperalgesia in DREADD-SCI animals, a certain abolishment of previously-observed patterns occurred. We observed fewer significant differences between control-sham and control-SCI groups than shown with previous animal cohorts. Assuming that the transfection of the hM4Di Gαi vector was truly unsuccessful, there are other factors that could have affected the behavioral data. One consideration is the timeline on which these animals were assessed. Animals within this portion of the study received allodynia assessment quickly followed by hyperalgesia assessment three times within a day. Coupling these assessments with separation from their cage mates could induce a sense of anxiety within the animals. This anxiety instilled within the animals could have exacerbated the neuropathic pain experienced by some of these animals[27,28], contributing to uncontrolled variations within the data that produced the observed patterns. A potential observation of this phenomenon can be seen when comparing the females in Figures 4 and 5. The significant

differences, with the exception of one, fall on days in which animals were not assessed for allodynia and hyperalgesia (4 dpi and 10 dpi specifically). Although we observed significant improvements in locomotor function in DREADD-SCI animals at 14 dpi, these improvements were only just significant ($p=0.046$).

Interestingly, some of the sex differences established in the first cohort of animals appeared to reverse in the second cohort. Where females of the first cohort generally exhibited more neuropathic pain than males, females in the second cohort appeared to show more reversal of neuropathic pain symptoms with the DREADD treatment. However, this potential sex bias is at most a potential finding. Our final number of male subjects in the DREADD cohort of animals was significantly reduced due to the inability to carry out certain aspects of animal care, such as micturition assistance. This had been due to a widespread occurrence of penile prolapse with a delayed onset in the male population. Although there are no direct connections between the penile prolapse and neuropathic pain enhancement, the phenomenon could still significantly alter behavioral results[29]. Ruling out the effect of the hM4Di Gαi DREADD (as exhibited in Figure 6) and assuming the aforementioned chronic stress confound would leave us to consider a sex-specific response to chronic stress that may impact neuropathic pain management. Despite sex differences potentially playing a significant role in both chronic and acute stress models[30,31], whether or not these differences affects management of neuropathic pain (either peripheral or SCI-induced) is unexplored in the current literature, and is well beyond the design/controls of this study.

The perceived failure of the DREADD intervention to attenuate neuropathic pain symptoms could still be attributed to a myriad of reasons. Where there could be a failure in detecting the DREADD integration (ineffective GFP antibody), there could have also been a

failure in the IT application of the DREADD injection, or even a failure in the DREADD to target the appropriate regions. Assuming the transfection was only off-target, DREADD inactivation of other tissues, or inactivation of astrocytes of a different region, could potentially explain the deviations we observed in the behavioral data. Furthermore, we only assessed regions of the injury epicenter; lumbar regions were not histologically assessed after presuming the failure of the DREADD transfection. We also only conducted immunohistochemical analysis at the injury epicenter. Tissue had not been extracted for quantitative PCR, which would have confirmed whether or not the Gαi protein was being produced. Despite the novelty of the technology, let alone the concept of modulating the activity of neural "support" cells, there have been significant behavioral and morphological differences observed in other studies [22,32]. However, few of these studies, if any, have addressed the clinical potential of chemogenetic modulation of supportive cells as a means of improving neuropathic pain management – specifically elicited by SCI. This lack of literature constitutes an unresolved demand for further research into chemogenetics as a tool for understanding the underlying mechanisms that contribute to development and persistence of SCI-induced neuropathic pain.

SCI-induced neuropathic pain constitutes a significant oversight in the treatment and management of SCI. Here, we established a mouse model for SCI-induced neuropathic pain and explored the role astrocytes play in initiation and maintenance of neuropathic pain. Our data suggests potential sex differences between male and female mice in perception of neuropathic pain elicited by SCI. In addition, our data suggests that locomotor function and nociceptive sensory thresholds are only partially regained during the acute phase of post-SCI recovery, with little additional recovery during the chronic phase. Our work here also shows a likely correlation between post-SCI astrocyte activation and SCI pathology and pain. This work has established an

effective mouse model for post-SCI neuropathic pain and identified astrocytes as a potential target for future therapeutic efforts. Future studies could use this model to better understand the role of astrocytes in the initiation/maintenance of post-SCI neuropathic pain, and subsequently, to develop new methods for treating and managing neuropathic pain in human SCI patients.

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