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Gabrielle Spagnuolo
R Vivian Allahyari
Simon Galer
Tyler Fox

See next page for additional authors
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Facial grimace testing as an assay of neuropathic pain-related behavior in a mouse model of cervical spinal cord injury

Nicolette M. Heinsinger *, Gabrielle Spagnuolo *, R. Vivian Allahyari, Simon Galer, Tyler Fox, David A. Jaffe, Samantha J. Thomas, Lorraine Iacovitti, Angelo C. Lepore

* Co-first authors

Department of Neuroscience, Vickie and Jack Farber Institute of Neuroscience, Sidney Kimmel Medical College at Thomas Jefferson University, Philadelphia, Pennsylvania, 19107, USA

Correspondence should be addressed to:
Angelo C. Lepore, Ph.D., Department of Neuroscience, Vickie and Jack Farber Institute for Neuroscience, Sidney Kimmel Medical College at Thomas Jefferson University, 233 South 10th Street, BLSB 245, Philadelphia, PA, 19107, U.S., angelo.lepore@jefferson.edu

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Abstract

A major portion of individuals affected by traumatic spinal cord injury (SCI) experience one or more types of chronic neuropathic pain (NP), which is often intractable to currently available treatments. The availability of reliable behavioral assays in pre-clinical models of SCI-induced NP is therefore critical to assess the efficacy of new potential therapies. Commonly used assays to evaluate NP-related behavior in rodents, such as Hargreaves thermal and von Frey mechanical testing, rely on the withdrawal response to an evoked stimulus. However, other assays that test spontaneous/non-evoked NP-related behavior or supraspinal aspects of NP would be highly useful for a more comprehensive assessment of NP following SCI. The Mouse Grimace Scale (MGS) is a tool to assess spontaneous, supraspinal pain-like behaviors in mice; however, the assay has not been characterized in a mouse model of SCI-induced chronic NP, despite the critical importance of mouse genetics as an experimental tool. We found that beginning 2 weeks after cervical contusion, SCI mice exhibited increased facial grimace features compared to laminectomy-only control mice, and this grimace phenotype persisted to the chronic time point of 5 weeks post-injury. We also found a significant relationship between facial grimace score and the evoked forepaw withdrawal response in both the Hargreaves and von Frey tests at 5 weeks post-injury when both laminectomy-only and SCI mice were included in the analysis. However, within only the SCI group, there was no correlation between grimace score and Hargreaves or von Frey responses. These results indicate both that facial grimace analysis can be used as an assay of spontaneous NP-related behavior in the mouse model of SCI and that the information provided by the MGS may be different than that provided by evoked tests of sensory function.

Keywords: spinal cord injury, neuropathic pain, cervical, SCI

Non-standard abbreviations:
CS (6, 7, etc.): cervical spinal cord level 5 (6, 7, etc.)
MGS: Mouse Grimace Scale
NP: neuropathic pain
SCI: spinal cord injury
ROC: receiver operating characteristic
AUC: area under the curve

Highlights:
- Cervical spinal cord injury mice exhibit increased facial grimace features.
- Grimace testing can be used to assess spontaneous neuropathic pain in SCI mice.
Introduction

Many spinal cord injury (SCI) patients suffer from one or more forms of debilitating neuropathic pain (NP) (Shiao & Lee-Kubli, 2018), which may manifest in the form of an increased response to a noxious stimulus (hyperalgesia), a painful sensation in response to a formerly innocuous stimulus (allodynia), and/or spontaneous pain in the absence of a peripheral stimulus (Shiao & Lee-Kubli, 2018). Current therapies for SCI-induced NP are relatively ineffective and can have severe adverse effects (Shiao & Lee-Kubli, 2018), making reliable preclinical rodent models of NP critical for the development of new treatment strategies (Deuis, Dvorakova, & Vetter, 2017). Commonly used tests for NP-related behavior in the rodent rely on reflexive withdrawal responses to an evoked stimulus, where the stimulus is applied directly to the plantar surface of the paw and the time, force or temperature needed to elicit a paw withdrawal response is measured (Deuis et al., 2017). Examples of such stimuli include a hot stimulus in the Hargreaves test or a mechanical stimulus in the von Frey filament test (Deuis et al., 2017). These tests are useful to measure a rodent’s sensitivity to pain modalities in specific anatomical areas where the stimulus is applied, such as the forepaw or hindpaw. These reflexive measurements, however, can be challenging to use as an assay of pain-like behavior, in part because an animal’s withdrawal from a stimulus may be mediated by hyperactivity of spinal reflex circuits and may not necessarily be due to a pain-like response (Deuis et al., 2017). Therefore, it would be useful to combine assays of evoked NP with other assays of supraspinal or cognitive aspects of NP in order to fully evaluate a rodent’s response to SCI.

Operant assays are one way to assess the cognitive or motivational aspects of NP (Shiao & Lee-Kubli, 2018). Examples of operant assays include place-escape avoidance paradigm, the Mechanical Conflict Avoidance System, and conditioned place preference (CPP). These assays often present the animal a choice to either stay in an aversive brightly-lit environment with no noxious stimulus or with pain-relief or move to the preferred dark environment but expose itself to a noxious stimulus or have no pain relief (Shiao & Lee-Kubli, 2018). Operant assays are useful to evaluate motivational and affective components of pain; however, results may be confounded by anxiety or depressive behavior as well as increased spasticity after SCI (Shiao & Lee-Kubli, 2018). Additionally, only a handful of studies have used these assays to measure NP-related behaviors.

Over the past decade, new behavioral assays measuring spontaneous pain, independent of reflexive withdrawal responses, have been developed. One example is the Mouse Grimace Scale (MGS), which uses mouse facial grimace features as a measurement of supraspinal and emotional aspects of pain (Langford et al., 2010). The advantage of the MGS is that it does not rely on a withdrawal from a stimulus. Therefore, the results may be more reliable about the pain-like state of the animal, as there would be reduced possibility of confounding influence from alterations to spinal reflex circuits. Additionally, the MGS can be used to assess spontaneous pain behaviors in the absence of an evoked stimulus, and is a reliable measure of cognitive and emotional aspects of pain (Flecknell, 2010). However, information about anatomical- and modality-specificity is lost, unless grimace assessment is paired with the presentation of a stimulus. While a similar test, the Rat Grimace Scale, has been characterized in the rat model of spinal cord injury (Schneider et al., 2017), the MGS has mostly been used to measure acute pain post-surgery or after injection of a chemical irritant, and has not been characterized in a mouse model of long-lasting SCI-induced NP (Deuis et al., 2017). In this study, we
sought to determine whether the MGS could be a reliable assay for chronic NP-related behavior in a mouse model of cervical contusion-type SCI.

As NP is an extremely complex phenomenon, a single behavioral test is not able to provide all of the necessary information to completely assess NP-related behaviors in a rodent. There are advantages and disadvantages of both evoked (e.g. Hargreaves and von Frey) and spontaneous (e.g. MGS) tests; however, when used in combination, they may provide a more complete appraisal of the NP-like state of the mouse. In this study, we characterized a novel assay for evaluating spontaneous supraspinal aspects of chronic NP-related behavior in a clinically-relevant model of cervical contusion SCI. First, we assessed whether mice with SCI-induced NP exhibited increased grimace facial features up to 5 weeks after SCI. We next analyzed the correlation between grimace score and withdrawal latency in the Hargreaves test or withdrawal threshold in the von Frey test to determine if mice that had a lower withdrawal latency or threshold in already-established assays of chronic NP also had increased grimace facial features. These data demonstrate for the first time that the MGS is effective in assessing long-term pain-like behaviors in a mouse model of SCI.

Material and Methods

Animal care. All experimental protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee (IACUC), complied with ARRIVE guidelines, and were carried out according to the NIH guide for the care and use of laboratory animals. Experiments were performed on 22 adult C57BL6 male (25-30g) and female (20-25g) mice, receiving ad libitum food and water, and maintained on a 12 hr light/dark cycle.

Surgeries. Mice received a unilateral C5/6 contusion SCI, as previously described (Watson, Hala, Putatunda, Sannie, & Lepore, 2014). Briefly, a unilateral laminectomy was performed, and animals received a unilateral contusion injury to the C5/6 spinal cord using the Infinite Horizon Impactor (Precision Systems and Instrumentation; Lexington, KY). SCI mice received an injury with an impactor tip of 0.7 mm in diameter, a force of 40 kilodynes, and a 2 second dwell time; control animals received a C5/6 laminectomy with no SCI.

Behavioral testing. A total of 22 mice were evaluated in 2 separate trials. The first experiment was performed with 8 mice (n=4 SCI, n=4 laminectomy-only controls); in this trial, we evaluated both Hargreaves and MGS assays. The second experiment was performed with 14 mice (n=8 SCI, n=6 laminectomy-only controls); in this study, we added the von Frey assay to the previously measured Hargreaves and grimace tests to more comprehensively evaluate different modalities of NP behaviors. Therefore, Hargreaves and MGS assays have a total of 22 mice in the analysis, and the von Frey assay has a total of 14 mice in the analysis. For behavioral testing, mice were acclimated to the testing room and handled for one hour per day for five days before baseline testing, and then again for one hour before each testing session.
measures were collected one week prior to surgery, and then once weekly beginning two weeks post-surgery. Week 1 post-surgery was excluded because SCI animals and laminectomy-only controls were still recovering from the surgery at this time point, and we wanted to avoid any false reductions in withdrawal responses or grimace features due to the surgery and not due to the group (SCI vs. laminectomy-only control). No more than one behavioral assay was performed per day.

The MGS (Langford et al., 2010) was used to measure spontaneous, non-evoked supraspinal aspects of NP after SCI. All MGS testing was performed in the morning with up to 2 mice recorded at once. The mice were placed in plexiglass "cubicles" about 9 x 5 x 5 cm high, the camera was placed directly in front of the animals, and the mice were recorded for 30 minutes using a RICH AV-01 Video Camera Camcorder. Videos were downloaded to a computer, identifying information was blinded to the researcher, and screenshots of the video clearly showing the mouse's face and ears were taken every 2-3 minutes. Screenshots were compiled in a Powerpoint presentation with identifying information in the notes section, and slides were randomized using a macro. Images were scored all at once at the end of the experiment by a researcher blinded both to group and timepoint of the study. For each image, orbital tightening, nose and cheek bulge, and ear position were scored 0-2 (0 = not present, 1 = somewhat present, 2 = obviously present). If a characteristic was not apparent, animals were given a score of 0 for that image feature. If the characteristic was obvious (eyes halfway closed or more, a visible bump or vertical ridges apparent down the nose, a noticeable bulge at the cheek, or ears oriented away from the face), that characteristic was given a score of 2. An intermediate phenotype was given a score of 1 (Langford et al., 2010). We omitted scoring change in whisker position because the whiskers were not always visible on the black mice in the video. The scores of the four categories for each image were averaged together. All of the images per timepoint were then averaged together to generate the grimace score for that mouse for each timepoint.

Modified versions of the Hargreaves and von Frey filament tests were used to measure thermal hyperalgesia and mechanical allodynia, respectively, in the ipsilateral forepaw, as previously described (Watson et al., 2014). For the Hargreaves test, mice were restrained by scruffing, with one forepaw held loosely on a glass pane directly above the infrared stimulus, and the time to withdrawal from the stimulus was recorded. We chose to loosely restrain the mice for technical reasons – when mice are placed in cubicles, they often balance on their hindpaws and place their forepaws up on the plexiglass walls of the cubicles. While this method works well to evaluate hindpaw pain behaviors (for example, after a thoracic SCI), when they are in this position with their forepaws up, it is impossible for the thermal or mechanical stimulus to reach their forepaws and it makes evaluating at-level NP behaviors after a cervical SCI very difficult. Therefore, we used this modified scruffing method to allow the stimulus to reach the anatomical area of interest. We and others have previously published several studies with an interval of 120 seconds between trials for behavioral assays (Detloff, Wade, & Houle, 2013; Watson et al., 2014). We do not see any within-day sensitization to the heat stimulus (i.e. Trial 3 is not significantly different from Trial 1 for each animal). We also do not see any chronic sensitization (i.e. in uninjured mice, Week 5 post-surgery is no different than baseline). Therefore, during each Hargreaves session, three trials were conducted for each forepaw with a 120-second interval between each trial. For the von Frey test, mice were placed on top of a wire mesh and loosely restrained by scruffing, and von Frey filaments were
applied to the plantar surface of the forepaw. 10 filaments were applied according to the up-down method, with a 120-second interval between each application. Forepaw withdrawal for Hargreaves and von Frey was defined by a quick removal of the paw from the infrared or mechanical stimulus, as well as a supraspinal or cognitive awareness of stimulus (i.e. licking or flapping of the forepaw, guarding of the forepaw, vocalization); spontaneous movements were not recorded and the trial was repeated. Cutoffs of 30 seconds for the Hargreaves test and 2g of force for the von Frey test were used to prevent damage to the animal’s paw.

**Statistics.** Experiments included both male and female mice, and no differences between sexes were observed. Behavioral assays were analyzed using a repeated measures two-way ANOVA, with comparisons made over time and between groups. Linear regression analyses were used to compare the relationship between the evoked and spontaneous behavioral assays. Statistics were calculated with Graphpad Prism 8. Assignment of mice to groups, surgical procedures, and behavioral analyses were conducted in a blinded manner.

Based on previous publications (Oliver et al., 2014; Reid et al., 2007; Schneider et al., 2017), we used a receiver operating characteristic (ROC) curve to evaluate the threshold for the analgesic intervention score. Diagnostic tests are useful to determine the presence or absence of a disease, but a binary yes/no diagnosis is difficult when the test uses a continuous variable as a reporter of the disease (Hoo, Candlish, & Teare, 2017). In these cases, an ROC curve is often used as a statistical tool to determine the binary threshold value (Hoo et al., 2017). Using GraphPad Prism, an ROC curve was generated by tabulating all of the possible values for the threshold, as well as the sensitivity (true positive rate) and 1-specificity (false positive rate) (Hoo et al., 2017). The area under the curve (AUC) of the ROC curve is a measure of how accurate the ROC curve can distinguish between the disease and non-disease groups, with an AUC of 1.0 meaning perfect distinction between the two groups and an AUC of 0.5 meaning the test cannot distinguish between the two groups at all. We then chose the optimum cutoff value that maximized both sensitivity and 1-specificity, or the value that was closest to the upper left corner of the ROC curve.

**Results**

**Mice exhibited increased spontaneous grimace features after cervical SCI.** To test whether the MGS can be used as an assay for chronic NP-related behavior in a model of SCI, we performed MGS analyses at multiple time points in cervical contusion SCI and laminectomy-only controls. Representative images from laminectomy-only control (Figure 1A) and SCI mice (Figure 1B) two weeks after surgery show that SCI mice had increased nose and cheek bulge, eyes halfway closed, and ears positioned back away from the face. These characteristics were absent in the laminectomy-only mice. In the absence of an evoked stimulus, SCI mice exhibited increased spontaneous grimace features of orbital tightening, nose or cheek bulge, or change in ear position compared to laminectomy-only control mice beginning at 2 weeks post-SCI and
persisting to 5 weeks post-SCI (Figure 1C; SCI compared to laminectomy at 2-5 weeks: p<0.0001; SCI compared to baseline at 2-5 weeks: p<0.0001; Laminectomy compared to baseline at 2-5 weeks: p=ns). These results indicate that following cervical SCI, mice experience increased NP-like grimace features even at chronic time points and that the MGS can be used to evaluate spontaneous chronic NP-related behaviors.

A pain rating increases in clinical utility if there is an intervention “threshold”, meaning if a patient (or animal in this case) has a pain score above this threshold then they would require treatment with an analgesic (Reid et al., 2007). An analgesic intervention score is used as a diagnostic tool to inform decisions about when to provide pain treatment or not to nonverbal animals (i.e. when the animal is in “pain” or “no significant pain”) (Oliver et al., 2014). In order to determine the percentage of SCI mice that exhibited spontaneous grimace features, we also determined the analgesic intervention score (Oliver et al., 2014; Schneider et al., 2017). We generated a ROC curve, similar to previous studies (Oliver et al., 2014; Schneider et al., 2017). The ROC curve had an area under the curve (AUC) of 1.0 (p<0.0001), sensitivity of 100% (95% confidence interval: 92.59% to 100.0%), and 1-specificity of 100% (95% confidence interval: 91.24% to 100.0%), indicating perfect distinction between the laminectomy-only and SCI groups (Figure 1D). The threshold value of 0.68 was chosen as the analgesic intervention score because it both maximized the sensitivity and 1-specificity on the curve as well as was not a grimace value included in either group (laminectomy range: 0.2273-0.6750; SCI range: 0.6818-1.100). Therefore, any mouse with a grimace score above 0.68 would be classified as a “pain” state and any mouse with a score below 0.68 would be classified as a “no significant pain” state. We analyzed the grimace scores of all mice in both the laminectomy-only and SCI groups at 2 weeks post-SCI, which is when we observe initiation of NP-related behaviors in this model (Watson et al., 2014), as well as a more chronic time point at 5 weeks post-SCI. We found that 100% of the mice in the SCI condition were in a “pain” state and 100% of the mice in the laminectomy-only condition were in a “no significant pain” state at both 2 weeks (Figure 1E) and 5 weeks (Figure 1F) after injury.

**SCI mice exhibited NP-related behaviors of thermal hyperalgesia and mechanical allodynia.** We also measured the well-established NP-like phenotypes of thermal hyperalgesia and mechanical allodynia using the Hargreaves and von Frey filament tests, respectively. Similar to our previously published findings (Watson et al., 2014), we observed a decrease in the withdrawal latency in the Hargreaves test (Figure 1G; SCI compared to laminectomy: p=0.0199 at 2 weeks, p=0.0497 at 3 weeks, p<0.0001 at 4 weeks, p=0.0005 at 5 weeks; SCI compared to baseline at 2-5 weeks: p<0.0001) and a decrease in the 50% withdrawal threshold in the von Frey test (Figure 1H; SCI compared to laminectomy: p=0.0242 at 2 weeks, p=0.0041 at 3 weeks, p=0.0013 at 4 weeks, p<0.0001 at 5 weeks; SCI compared to baseline at 2-5 weeks: p<0.0001). These results indicate that our model exhibited both a spontaneous, supraspinal pain phenotype in the form of grimace facial features, as well as evoked thermal and mechanical NP-related phenotypes beginning at 2 weeks post-SCI and persisting to at least 5 weeks post-SCI.

**Grimace score was correlated with Hargreaves withdrawal latency.** The grimace test measures spontaneous, supraspinal aspects of pain, but the location of the pain is not well defined. In contrast, the Hargreaves and von Frey tests measure pain-related behavior due to an evoked stimulus and provide information about sensory function at an
anatomically defined region (i.e. the location of stimulus presentation). Because these assays measure different aspects of NP-related behaviors, we next evaluated the relationship between how mice performed on the grimace test and their performance on either the Hargreaves or von Frey test to determine whether mice that showed spontaneous pain-related behaviors also showed increased evoked, at-level NP-related behaviors. We chose to evaluate the initiation of NP in all three tests (2 weeks post-SCI) and a more chronic time point after SCI (5 weeks post SCI).

Linear regression analysis was performed to test whether mice with increased spontaneous grimace facial features also had decreased withdrawal latency on the Hargreaves test. When evaluating all mice from both the SCI and laminectomy-only groups at the 2 week timepoint, we observed a significant negative relationship between grimace score and Hargreaves withdrawal latency, indicating that mice that exhibited more grimace features also had the lowest withdrawal latency on the Hargreaves test (Figure 1; $r^2=0.2155$, $p=0.0295$). However, when the laminectomy-only mice were removed from the analysis, we did not observe a significant correlation within only the SCI animals (Figure 1J; $r^2=0.04162$, $p=\text{ns}$); these results indicate that, within the SCI group, there was not a relationship between grimace score and Hargreaves withdrawal latency. At the chronic 5 weeks post-SCI, when we included all animals in both the SCI and laminectomy groups, we again observed a significant negative relationship between grimace score and withdrawal latency (Figure 1K; $r^2=0.5503$, $p<0.0001$). Consistent with the data at 2 weeks post-SCI, in SCI-only mice, there was no relationship between Hargreaves and grimace score (Figure 1L; $r^2=0.0004$, $p=\text{ns}$). Overall, when comparing both injured and uninjured mice at both the time of the initiation of NP-related behaviors and at a more chronic time point, mice with a higher grimace score generally had a lower Hargreaves withdrawal latency. However, this effect was lost when the laminectomy-only controls were removed, indicating that within the injured mice, there was no intra-animal relationship between the magnitudes of the grimace score and response on the Hargreaves test.

**Grimace score was correlated with von Frey withdrawal threshold.** We also tested mechanical allodynia using the von Frey filament test. Again, we performed a linear regression analysis to test whether mice with increased grimace score also had increased mechanical sensitivity, as measured by the 50% withdrawal threshold on the von Frey test. We found that at the onset of NP-related behaviors at 2 weeks post-SCI, there was no relationship between grimace score and withdrawal threshold, either when comparing both injured and uninjured mice (Figure 1M; $r^2=0.1859$, $p=0.1238$) or in SCI mice alone (Figure 1N; $r^2=0.0049$, $p=\text{ns}$). At 5 weeks, however, we found a significant negative correlation when evaluating both SCI and laminectomy-only groups (Figure 1O; $r^2=0.7969$, $p<0.0001$), indicating that mice with a higher grimace score also had lower 50% withdrawal thresholds. There was no correlation at 5 weeks in the SCI alone group (Figure 1P; $r^2=0.08879$, $p=\text{ns}$). In summary, at the 5 week time point, there was a significant relationship between von Frey withdrawal threshold and grimace score when comparing both SCI and laminectomy-only mice, but this intra-animal relationship was not observed at the 2 week timepoint. Additionally, there was no relationship between grimace score and withdrawal threshold when comparing only injured mice at either timepoint.
Discussion

This study characterizes a novel behavioral assay to test spontaneous, supraspinal aspects of chronic NP in a clinically relevant model of cervical SCI. We found that mice with a cervical contusion SCI had more grimace facial features and a higher MGS score compared to laminectomy-only uninjured controls beginning at 2 weeks and persisting through at least 5 weeks post-injury. These data suggest that injured mice had significantly higher levels of spontaneous NP compared to their uninjured counterparts; therefore, the MGS can be used as an assay to test spontaneous chronic NP-related behavior in mice with SCI.

Evoked measures of NP-related behaviors typically rely on reflexive withdrawal responses after stimulation in a localized, anatomically-defined region of the body, such as the forepaw. In contrast, the MGS measures supraspinal and emotional aspects of spontaneous, non-evoked NP, but with no indication of the anatomical location of the pain. Both types of tests yield different, yet complementary, pieces of important information about the NP-related behavioral status of the mouse. Therefore, we sought to test whether there was a correlation between evoked measures of NP behaviors and grimace score in order to ask whether animals with increased hyperalgesia or allodynia also had greater spontaneous pain. We found that Hargreaves withdrawal latency and grimace score were negatively correlated at both 2 weeks and 5 weeks after SCI when linear regressions were performed including both injured and uninjured groups. We also found that the von Frey 50% withdrawal threshold and grimace score were negatively correlated at 5 weeks. These results demonstrate that mice that exhibited NP-related behaviors in reflexive withdrawal tests also exhibited NP-related grimace behaviors. However, we found no correlation between evoked pain responses and MGS score when analyzing the SCI mice alone, suggesting that within the SCI group mice with the most pronounced thermal hyperalgesia or mechanical allodynia did not necessarily have the most robust spontaneous grimace behaviors. One reason for this could be that the scoring system rates the four grimace categories on a scale of 0, 1, or 2, so there is not a wide range of possible scores. Since most mice in a “pain” state are receiving very similar scores, there may not be enough variability to determine a relationship. Another explanation could be that each of these behavioral assays reveals distinct aspects of the NP-related behavioral status in the mouse that are not necessarily related to each other. In this case, multiple behavioral assays would be necessary to comprehensively evaluate the NP-like state of the mouse and these tests may be used as complementary analyses to fully appreciate the animal’s sensory behavioral status.

In this study, the MGS was used to test spontaneous, non-evoked grimace facial features. However, to enhance the evoked reflexive withdrawal behavioral assays, in future studies, we may pair the grimace assay with the evoked measures of NP by recording facial expressions at the moment of withdrawal from the stimulus. Pairing the two assays would allow us to avoid measuring reflexes after an evoked stimulus that occur in the absence of a supraspinal response.

Our results showing that mice with cervical contusion SCI have increased facial grimace scores are consistent with a previous study where rats with cervical SCI had elevated grimace scores on the Rat Grimace Scale at 5 weeks post-SCI compared to laminectomy-only controls (Schneider et al., 2017). We determined an analgesic intervention score in order to determine the percentage of mice in a “pain” or “no significant pain” state after SCI. An analgesic
intervention score may be useful in future studies if an experimental treatment is given to the SCI group in an attempt to attenuate the NP phenotype. By including the analgesic intervention score, the SCI treatment group could be compared to the SCI-controls, laminectomy-only uninjured animals, the animal’s own baseline, and now the analgesic intervention score. Therefore, it gives researchers one more tool to analyze the therapeutic potential of the experimental pain treatment. Our analgesic intervention score of 0.68 is also consistent with previously published scores of >0.67 in rats post-surgery (Oliver et al., 2014) and 0.7 (rounded) in a rat SCI model (Schneider et al., 2017), confirming inter-rater reliability in our model. However, while Schneider et al. found that only 50% of rats were in a “pain” state 5 weeks after SCI, we found that 100% of mice were in a “pain” state starting 2 weeks after SCI and persisting to 5 weeks post-SCI. These results may indicate that there is more variability in rats in chronic NP states, while mice may show a more robust and reproducible NP-related phenotype.

In addition to species differences that may contribute to NP-related behaviors, there may be differences in NP based on whether the injury site has spared fibers (e.g. contusion, compression) or no spared fibers (e.g. complete transection), the location on the spinal cord, injury severity (e.g. force of contusion or compression), species, sex, or the anatomic pain level (e.g. above-level, at-level, below-level) (Kramer et al., 2017). Several studies have shown that only a subset of rats develop a NP phenotype following SCI (Detloff et al., 2013; Schneider et al., 2017); however, the current study showing that 100% of mice were in a “pain” state is consistent with our previous studies that show the majority of mice exhibit an at-level NP phenotype after a cervical contusion-type SCI (Watson et al., 2014). In future studies, we may assess whether location, pain level, or injury severity changes the percentage of mice that exhibit a NP-like phenotype or if this is a species-specific phenomenon.

Conclusions. In conclusion, we found that the MGS is a novel behavioral assay that can be used to assess long-lasting spontaneous NP-related behavior in a mouse model of cervical SCI. MGS reveals information about the mouse’s chronic NP-related state distinct from other well-established measures of chronic NP like the Hargreaves or von Frey tests. Therefore, the grimace test can be used in combination with these well-established measures to more fully characterize chronic NP behaviors after SCI. Importantly, the use of the MGS in models of SCI allows researchers to take advantage of the experimental power afforded by mouse genetics.
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**Figure Legend**

**Figure 1. Mice exhibited increased facial grimace features after SCI.** Representative images of grimace facial features of laminectomy-only (A) and cervical contusion SCI mice (B) two weeks after surgery. Grimace scores of SCI (n=12) and laminectomy-only control (n=10) mice (C). ROC curve panel of grimace scores (D). Scatterplot showing distribution of mice above or below the anesthetic intervention score of 0.68 at 2 weeks (E) and 5 weeks (F) post-surgery. Withdrawal latency for Hargreaves test for thermal hyperalgesia of SCI (n=12) and laminectomy (n=10) mice (G). Withdrawal threshold for von Frey test for mechanical allodynia of SCI (n=8) and laminectomy (n=6) mice (H). Linear regression showing the relationship between grimace score and Hargreaves withdrawal latency at 2 weeks with both SCI and laminectomy mice (I) and with only SCI mice (J), and at 5 weeks with both SCI and laminectomy mice (K) and with only SCI mice (L). Linear regression showing the relationship between grimace score and von Frey withdrawal threshold at 2 weeks with both SCI and laminectomy mice (M) and with only SCI mice (N), and at 5 weeks with both SCI and laminectomy mice (O) and with only SCI mice (P).