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TITLE

Dural Stimulation and Periorbital von Frey Testing in Mice as a Preclinical Model of Headache

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SUMMARY

The most notable symptom of migraine is severe head pain, and it is hypothesized that this is mediated by sensory neurons innervating the meninges. Here, we present a method to locally apply substances to the dura in a minimally invasive manner while using facial hypersensitivity as an output.

ABSTRACT

The cranial meninges, comprised of the dura mater, arachnoid, and pia mater, are thought to primarily serve structural functions for the nervous system. For example, they protect the brain from the skull and anchor/organize the vascular and neuronal supply of the cortex. However, the meninges are also implicated in nervous system disorders such as migraine, where the pain experienced during a migraine is attributed to local sterile inflammation and subsequent activation of local nociceptive afferents. Of the layers in the meninges, the dura mater is of particular interest in the pathophysiology of migraines. It is highly vascularized, harbors local nociceptive neurons, and is home to a diverse array of resident cells such as immune cells. Subtle changes in the local meningeal microenvironment may lead to activation and sensitization of dural perivascular nociceptors, thus leading to migraine pain. Studies have sought to address how dural afferents become activated/sensitized by using either in vivo electrophysiology, imaging techniques, or behavioral models, but these commonly require very invasive surgeries. This protocol presents a method for comparatively non-invasive application of compounds on the dura mater in mice and a suitable method for measuring headache-like tactile sensitivity using periorbital von Frey testing following dural stimulation. This method maintains the integrity of the dura and skull and reduces confounding effects from invasive techniques by injecting

substances through a 0.65 mm modified cannula at the junction of unfused sagittal and lambdoid sutures. This preclinical model will allow researchers to investigate a wide range of dural stimuli and their role in the pathological progression of migraine, such as nociceptor activation, immune cell activation, vascular changes, and pain behaviors, all while maintaining injury-free conditions to the skull and meninges.

INTRODUCTION

Migraine pain remains a major public health issue worldwide. The World Health Organization ranks it as the sixth-most prevalent disease in the world, afflicting just under 15% of the Earth's population[1] and causing a substantial socioeconomic burden on society[2,3]. Treatment options and their efficacy have been suboptimal and only provide symptomatic relief but do not significantly modify pathophysiological events that underly migraine occurrence[4,5]. The lack of treatment success is likely due to migraine being a multifactorial disorder whose pathology is poorly understood, leading to a limited number of therapeutic targets. Migraine is also challenging to fully capture in animal models, especially given that migraine diagnosis is made based on verbal communication with patients who describe their experience with migraine hallmarks such as aura, headache, photophobia, and allodynia. Notwithstanding, it is important to note that recent advances in migraine treatments are currently outperforming treatments for many neurological conditions that have been well validated by preclinical models. For instance, monoclonal antibodies and small molecules that target calcitonin gene-related peptide, or its receptor have been very successful in improving the quality of life of migraine sufferers and can potentially transform the clinical management of migraine. While there has been advancement in understanding this disorder, there continues to be much yet to be elucidated.

Based on preclinical animal models and human studies, it is widely accepted that migraine headaches are initiated by aberrant activation of nociceptive fibers within the meninges that signal through the trigeminal and upper cervical dorsal-root ganglia[6–10]. Despite this theory, many studies still use systemic administration of drugs to understand underlying contributing mechanisms in migraine. While systemic dosing of drugs has substantially strengthened our understanding, these findings do not directly assess whether local actions within the target tissue of interest play a role in migraine. Conversely, several studies have taken an approach to stimulate the dura; however, these experiments require cannula implantation via an invasive craniotomy and extended recovery times[11,12]. Because of these limitations, we developed a minimally invasive approach to locally stimulate the dura where the lack of a craniotomy eliminates post-surgical recovery and allows for immediate testing in awake animals[12–14]. These injections are performed under light isoflurane anesthesia and administered at the junction of the sagittal and lambdoid sutures in mice.

Several approaches have been developed to evaluate nociceptive behavioral responses in rodents[15]. Cutaneous allodynia has been reported in approximately 80% of migraine sufferers[16,17] and represents a potential translational endpoint for use in rodents. In preclinical models, the application of von Frey filaments to the plantar region of the rodent paw has been used to assess pain behaviors in preclinical migraine models. The primary limitation of this approach is that it does not test the cephalic region. Facial grimace scoring has been used to capture pain behaviors

in rodents by analyzing facial expressions after induction of pain stimuli[18,19]. However, its limitations include only capturing responses to acute stimuli and not chronic orofacial pain conditions. Facial grooming and decreased rearing are also considered outputs of behavioral responses in preclinical models of migraine[20,21]. Limitations of the former include difficulty in differentiating pain responses from normal routine grooming and other sensations such as itch. In the case of the latter, rearing behaviors typically decrease quickly after the introduction of rodents to novel environments. Although each of these behavioral endpoints is valuable in the understanding of various mechanisms that contribute to pain conditions, there is a critical need for preclinical models of pain disorders such as migraine to include endpoints that specifically capture cephalic hypersensitivity responses. Assessing tactile hypersensitivity of the periorbital skin following dural stimulation is a method that may provide better insight into mechanisms contributing to migraines where sensory symptoms are predominantly cephalic in nature. Here, we describe a method to administer substances onto the mouse dura as a preclinical model of migraine. Following dural application, we also present a detailed method for testing periorbital tactile hypersensitivity using calibrated von Frey filaments applied in the Dixon up-down method.

PROTOCOL

All procedures were conducted with prior approval of the institutional Animal Care and Use Committee at the University of Texas at Dallas. ICR (CD-1) (30–35 g) and C57/BL6 (25–30 g) mice aged 6–8 weeks were used in this study.

1. Dural infuser

1.1. Create the mouse infusers/injectors by modifying a commercially available internal cannula and infuser for unilateral injections with a non-metallic fused silica plastic cap that is adjustable and inserts into/extends below a 28 G guide cannula with inner diameter (I.D.) of 0.18 mm and an outer diameter (O.D.) of 0.35 mm (**Figure 1A**).

1.2. Use a caliper or other measuring device to adjust the fused silica plastic cap on the infuser to a length of 0.6 mm; measured from the tip of the infuser to the edge of the silica plastic cap.

1.2.1. Take caution not to bend or dull the infuser when adjusting the plastic cap.

1.2.2. For other mouse strains that have not been previously used for dural injections, determine the optimal infuser length by setting the length to 0.6 mm and conducting pilot injections with ink or dye, adjusting the infuser length until it is observed that the dye is only in the dura mater and not on the brain or skull.

1.3. Attach the long end (or the end that was not measured to be 0.6 mm) of the adjusted infuser to plastic tubing (pump tubing, 2-stop, I.D. 0.19 mm, length 406 mm).

1.3.1. Cut the tubing to a minimum length of 8 in to ensure there is sufficient line to hold a volume of 5 µL.

1.3.2. Make sure the tubing covers the metal part and the top of the plastic stopper located on the infuser. This will help prevent air bubbles from accumulating in the line.

1.4. Attach the other end of the tubing to a 10 µL glass microsyringe (gas-tight; cemented needle; 21 G with a 10 mm projection), again, making sure to have a tight seal over the metal part of the syringe (**Figure 1A**).

1.5. Once the line is connected, backfill the syringe with 5 µL of phosphate-buffered saline (PBS), synthetic interstitial fluid (SIF), or other vehicles of choice to prevent air bubbles from forming.

1.5.1. If air bubbles are observed in the line, flood the line with the vehicle until the bubbles have dissipated.

NOTE: It may help fill the syringe with the vehicle prior to attaching it to the line and then push the fluid through the line once connected.

1.6. After the line is backfilled with 5 µL of the vehicle and is working efficiently, load 5 µL of the drug/solution into Hamilton syringe (ink or dye may be used as an alternative to the drug/solution if learning or practicing this technique).

1.6.1. Ensure that all vehicle solutions administered onto the dura is maintained at pH 7.4 and measured to an osmolality of 310. This reduces the potential activation of acid-sensing ion channels and other osmosensitive channels within the dura.

1.7. The maximum volume tested in mice that did not cause leaking into the brain is roughly 10 µL. The behavioral effects after injections with this volume have not been tested. For this reason, administer only 5 µL of the solution onto the dura.

NOTE: These observations are based on the mouse strains/ages/weights of 6–8 week old CD1/ICR mice.

2. Dural injections

2.1. Once the syringe is prepared and the drug is loaded, position a mouse flat onto its abdomen and anesthetize it under brief 3% isoflurane with an oxygen flow rate of 0.5–1 L/min via nosecone.

2.1.1. After the mouse no longer displays a pinch reflex, adjust the anesthesia and sustain it at a 1.5% isoflurane.

2.2. Once anesthetized, get in a position conducive to a successful injection.

2.3. Use one hand to steady the head of the animal and hold the infuser with the other hand.

2.4. Carefully probe and locate the junction of the sagittal and lambdoidal sutures on the skull of the mouse (**Figure 1B**,**C**).

2.4.1. To locate this discreet junction through the skin, use the topographical features of the skull and gently probe the general location of the junction with the infuser.

2.4.2. Verify the position of the junction by re-positioning the infuser along the skull and feeling for the exact location.

2.5. Once the suture is located and the infuser is in place, very slowly and gently wiggle the infuser back and forth until it pierces through the skin and drops down into the junction all the way up to the plastic stopper.

NOTE: Ensure to insert the entire 0.6 mm tip of the infuser into the junction.

2.6. To verify the accuracy, use ink or dye as an injection solution and euthanize and decapitate the mouse.

2.6.1. Remove the skull cap to visualize the dye within the dura mater (**Figure 1C**).

NOTE**:** Dye should not be observed on the brain or exterior of the skull. Likewise, mice in any experiment should be checked post-mortem to verify the accuracy of the injection as well as to ensure the integrity of the dura mater was undamaged.

2.7. Following the injection, remove the mouse from anesthesia, wait for it to regain consciousness and then return to its cage or place it into a testing chamber to begin the desired assays.

NOTE: Allow the mouse to recover from anesthesia for a minimum of 30 min prior to doing any behavioral experiments.

3. Periorbital von Frey

3.1. Begin the study with a cohort of approximately 16–20 mice.

3.2. One the day prior to habituation, handle each mouse for at least 5 min.

3.3. Approximately 24 h after handling, habituate the mice to the testing room conditions and the von Frey testing apparatus.

NOTE: The acrylic testing apparatus consists of individual compartments with lids that are approximately 3 in x 3.5 in x 5 in (W x H x D) and are supported by aluminum stands connected via 0.25 in 19 G square galvanized steel chicken wire.

3.3.1. Place the mice inside a horizontally placed 4-oz white paper cup that is odorless and does not contain polythene or paraffin wax.

NOTE: These types of cups are preferred because it reduces gastrointestinal upset in the mice if ingested (**Figure 2B**).

3.4. While the animals are in their respective chambers, place a pellet of the normal chow diet in the individual chamber of each mouse to calm the animals and avoids any unnecessary stress to the animals. Do this for 3 days prior to any von Frey behavior testing.

3.4.1. Ensure that every time the mice are in the chamber that there is access to food.

3.4.2. Number and assign each animal to the same space in the testing rack. Place the mouse in the same cup every day of the testing period to ensure that each animal becomes acclimated to its testing environment.

NOTE: Mice will gnaw on the cups and subsequently destroy the cups. If this should happen, replace the cup and label it with the corresponding mouse number.

3.5. Following the initial 3 days of habituation, place the mice in their individual chambers.

3.5.1. Allow the animals to acclimate to the testing room and chambers for at least 1 h prior to any von Frey testing to allow the mice to calm down and subsequently are easier to test.

3.6. After acclimation to the room on the testing day, remove one mouse while still in its cup from its respective chamber.

3.6.1. Maintain the cup in the horizontal position so that the mouse is on both forepaws and hind paws to help keep their weight evenly distributed.

NOTE: Unequal weight distribution may alter animal's responses and even prevent animals from responding.

3.7. Place the cup with the mouse inside on the table below the testing rack on the absorbent pad.

3.8. For periorbital von Frey testing, place the 0.07 g von Frey filament directly in the center of the face and between the eyes.

3.9. Apply enough pressure on the filament to cause the von Frey hair to bend into a "C" shaped formation.

3.9.1. Maintain contact with the region at least 3 s but no more than 5 s or until the mouse withdraws its head and swipes at the filament with its paw.

NOTE: If the filament slips or more than the tip of the filament touches the animal during testing, any responses should not be counted. These responses may be in response to the brush which are activated by different mechanoreceptors and therefore may not reflect accurate results.

3.9.2. Apply von Frey filaments according to the Dixon "up-down" method[22,23].

3.9.2.1. Initially, apply the von Frey filament which has a weight of 0.07 g. The lowest possible filament and the highest tested filament in this study are filaments with weights of 0.008 g and 0.6 g, respectively.

3.9.2.2. Use the filaments of weight 0.008 g, 0.02 g, 0.04 g, 0.07 g, 0.16 g, 0.4 g, and 0.6 g to perform this assay.

3.9.2.3. In this method, if an animal does not exhibit a response to the filament, apply the filament of the next higher gram weight.

3.9.2.4. If the mouse does respond to a filament, consider that mouse responsive to that filament. If this is the case, apply the filament of the next lower gram weight.

3.9.2.5. Repeat this pattern until the animal is tested 4 times after the initial response or the animal is determined to be unresponsive to any filaments tested in the assay.

NOTE: Refrain from applying any additional pressure stemming from the arm or wrist. A scale may be used to practice the application of the filament.

4. Testing for baseline withdrawal thresholds

4.1. Prior to inclusion in an experiment, ensure that the mice reach a baseline withdrawal threshold between 0.5–0.6 g.

4.1.1. A mouse reaches baseline if they fail to respond to any filament tested in the series mentioned in step 3.9.2.2 (0.07 g, 0.16 g, 0.4 g, and 0.6 g).

4.2. Test mice daily when establishing baseline withdrawal thresholds.

4.2.1. Testing allows animals to acclimate to the testing conditions and the pressure of the von Frey filaments.

4.2.2. If the mice are still extremely hypersensitive after the third day of testing, try waiting 1 or 2 days before testing again.

NOTE: Too much time between testing days may result in the animal failing to adjust to the weight of the filament on their periorbital region, thus not reaching the targeted withdrawal threshold.

4.3. Test mice for approximately 7 days before determining which animals do not meet the inclusion criteria for an experiment.

NOTE: Approximately 70% of mice will reach the targeted baseline level.

4.3.1. Prior to dural stimulation, analyze the baseline data to exclude any mouse that has not reached a baseline value of 0.5–0.6 grams or higher.

4.3.2. After exclusion, randomly allocate each remaining mouse to a testing group. Achieve this by drawing out of a cup or writing a script on a spreadsheet to randomize numbers to a group.

5. Analysis of von Frey results

5.1. Once the series of responses have been obtained, determine the delta, k value, the 50% threshold, and the withdrawal threshold in grams according to previously published methods[24].

5.2. Calculate the withdrawal threshold using this formula WT = $10^{(x*F+B)}$, where WT= withdrawal threshold, F = paw withdrawal threshold calculated via the Chaplan method, and B = linear regression of log (bending force) = x*Filament number + B.

5.3. Plot the data as either 50% withdrawal threshold or average withdrawal threshold in grams.

REPRESENTATIVE RESULTS

This injection method is used to administer stimuli onto the dura of mice so that subsequent behavioral testing may occur. The most common behavioral output measured with this model is cutaneous facial hypersensitivity assessed via von Frey[12–14]. Here we show how this model can be used to assess potential sex-specific contributions to migraine pathology (**Figure 3**).

This procedure has been used to examine the effects of dural prolactin (PRL) on mechanically evoked facial hypersensitivity[14] (**Figure 3**). The results of this study demonstrated that female ICR mice show significantly reduced facial withdrawal thresholds in response to 5 µg of dural prolactin (**Figure 3A**). A ten-fold lower dose of 0.5 µg of prolactin (PRL) also showed responses similar to high dose of PRL (**Figure 3B**).

These injections have also been shown to produce spontaneous pain-related behaviors assessed via grimace. Dural 0.5 µg of PRL caused significant grimacing in female mice (**Figure 3C**), further

demonstrating a clear role for dural PRL in female migraine-like behaviors. We performed grimace assays prior to all testing with von Frey filaments.

FIGURE AND TABLE LEGENDS

Figure 1: Dural infuser and injection placement. (**A**) The injectors/infusers consist of a modified cannula adjusted to the length of ~0.5 mm- 0.65 mm and attached to a needle cemented on a 10 µL gas-tight syringe via tygon tubing. (**B**) Aerial view of marked injection site location on the head of the mouse. (**C**) (Left panel) Diagram of the location of the dural injection. Placement of the injection is on the junction of the lambdoid and sagittal sutures at approximately 4.8 mm posterior to bregma. (Middle panel) Post-mortem aerial view of a mouse skull following dural injection of 5 µL of blue injection dye. (Right panel) Separation of the mouse skullcap from the brain. There was no observable leakage of blue injection dye on the brain.

Figure 2: von Frey Testing chambers. (**A**) von Frey testing chamber composed of 3.5 in x 3.5 in individual acrylic chambers with lids placed on a wire mesh rack. These are connected via columns of 10 chambers organized in 2 rows. (**B**) Example of mice in their individual cups housed inside the von Frey testing chambers.

Figure 3: Dural application of prolactin induces behavioral responses in mice. Mechanical withdrawal thresholds were assessed following dural application of PRL (5 µg or 0.5 µg) in female mice. (**A**) Application of 5 µg of PRL (n = 7 PRL, n = 6 vehicle) induced facial hypersensitivity compared to vehicle. (**B**) Application of 0.5 µg of PRL (n = 5 PRL, n = 4 vehicle) induced long-lasting facial hypersensitivity. (**C**) Grimace was also assessed in the same mice treated with 0.5 µg of PRL at each time point. These mice exhibited significantly higher grimace scores compared to the mice treated with vehicle. Statistics: Two-way ANOVA followed by Bonferroni multiple comparison post-hoc analysis. Data are represented as means ± SEM. $*p < 0.05$, $****p < 0.0001$.

DISCUSSION

Maladaptive changes in the local nociceptive system in the dura are considered a key contributor to the headache phase of migraine attacks despite a lack of tissue injury[25,26]. Here the study presents a method whereby minimally invasive stimulation of the dura can induce facial tactile hypersensitivity. Elucidating the mechanisms and events involved in dural nociceptor activation without causing damage to the cranium and tissues may more accurately reflect migraine mechanisms in a preclinical model.

Craniotomy and cannula implantation have long been used to assess functions and mechanisms that contribute to migraine pain[11,12]. However, it has been reported that a craniotomy can induce activation of dural mast cells and increase pial vascular permeability in rodents[27]. Given that mast cell activation in the dura is highly implicated in migraine[7,8,28,29], this technique has major caveats that may skew the interpretation. Administering substances through the junction of the sagittal and lambdoidal sutures effectively diminishes the activation of nociceptors mediated by craniotomy-induced mast cell activation. Moreover, non-invasive dural stimulation does not require post-surgical recovery and administration of analgesics which may alter the interpretation of results. Local application of substances onto the dura allows researchers to

focus on this specific target tissue, as opposed to systemic administration of drugs where the site of action is not easily determined[12–14]. While systemic administration of substances such as nitroglycerin and calcitonin gene-related peptide trigger experimental attacks in humans that are similar to migraines, they do not allow for assessment of the location of action in rodent models; more targeted tissue-specific models offer an alternate approach.

This technique described here involves injecting a drug or other solution directly onto the dura mater of the meninges through the junction where the sagittal and lambdoidal sutures of the skull meet. For best results, ICR (CD-1) or C57/BL6 mice aged 6–8 weeks should be used for these experiments. Younger mice may be used; however, the use of ICR (CD-1) mice that are older than 8 weeks are not recommended as their skull plate sutures are typically completely fused by this age, making it impossible to inject without damaging the skull. It is also critical to consider the weight/size of each mouse that will undergo this procedure. It is recommended that these injections are performed on animals that have a weight greater than 19 g as the skull is typically very thin at lower weights and may not withstand the pressure applied during the injection. Of importance, there are also likely factors that contribute to the age/weight at which skull plate fusion occurs (e.g., the composition of lab chow used in animal facilities). Therefore, experimenters may need to determine the age/weight range suitable under their own conditions. Different age ranges and animal weights may be required for other mouse strains or genotypes, depending on when the skull plates fuse in those animals, and may also require optimization of the injection itself.

When learning or practicing this technique, it is highly recommended that a level of comfort is obtained with locating the suture junction in euthanized mice. It may be best to first practice with the scalp excised or peeled back in these mice and slowly advance to locating the junction through the skin. Once establishing the precise location, inks and dyes can be injected into the dura to verify location accuracy and depth of the injection. This technique was developed and optimized using ICR (CD-1) mice (30–35 g) and C57/BL6 mice (25–30 g). An infuser length of 0.5–0.6 mm is sufficient to inject a mouse weighing within the range of 25–35 g. However, the length of the infuser may need to be calibrated if injecting mice that significantly differ from the mice used to optimize this technique. For example, a mouse smaller than 25 g would likely result in the use of an infuser that has a length less than 0.5 mm.

Alterations in tactile sensitivity are an important measurement when assessing pain behaviors in rodents. Here we demonstrate the use of periorbital von Frey testing to assess these behaviors in a preclinical migraine model. A major advantage of using this technique in migraine models is that we can assess hypersensitivity of the head, which has more relevance than other non-cranial locations such as paws. The critical step to ensure reproducible results is to make certain that the mice are fully baselined. This will require a well-trained experimenter that can apply von Frey filaments precisely. It is likely that it will take approximately 7 days for an animal to reach baseline. However, it is possible that not every animal will reach the targeted baseline. In our experience, after about 7 days of working with mice, only 60%–70% of animals will reach a baseline of 0.6 g in the periorbital region, but this is dependent on the cohort of animals. This timing should be considered prior to beginning an experiment to ensure sufficient numbers are

used to account for dropout and that animals are the proper age post-baseline for using this non-invasive method to stimulate the dura. The steps for determining a baseline are outlined in protocol section 4.

A limitation to von Frey testing is that it can be difficult to distinguish between pain responses and routine grooming/itch. To help distinguish pain from grooming, it is important to notice the length of time this behavior occurs. Usually, a pain response is one swipe following the filament application, while grooming behaviors tend to be prolonged and can last for several seconds to minutes. If the grooming/itch behavior cannot be distinguished from a hypersensitive response, it is best not to record this as a response. Additionally, improper filament placement (e.g., filament slipping) can result in prolonged grooming of the animal, making it difficult to test properly. If this happens, the experimenter should wait until grooming has stopped and the mouse is calm enough to test. Continue from the same filament used prior to the beginning of the grooming behavior. If the mouse continues for very long bouts of time, place the mouse back in the testing chamber for approximately 5 min. Once the 5 min have passed, try testing the mouse again. If this behavior continues with no resolve, the mice have to be removed from the study. Of importance, it is not recommended to shave the fur on the face as it is unclear whether mouse skin retains the same sensitivity after hair is removed, and the process of hair removal (shaving, depilatory creams) may also influence skin sensitivity.

In most situations, it is ideal for administering substances onto the dura no more than 24 h after the mouse has reached baseline. It is recommended that mice are subjected to von Frey filament testing once per hour. If possible, testing every other hour gives enough time for the animals to calm down after testing. Additionally, experiments should be timed as not to interfere with their circadian patterns. Alterations to the circadian rhythm in mice may alter behavioral phenotypes and ultimately result in irreproducible results.

Periorbital von Frey testing can be used in combination with other behavioral assays to strengthen experimental conclusions. The grimace scale relies on spontaneous facial expressions in rodents rather than evoked responses[18,19]. This method has high accuracy and reliability when assessing and quantifying acute pain behaviors and has been used in many preclinical models of migraine[12,30]. When using both grimace and periorbital von Frey assays, the experimenter should consider scoring for grimace prior to application of von Frey filaments to the periorbital region of the mouse. This ensures that the grimacing behavior is spontaneous and not evoked by filament application. Hindpaw mechanical hypersensitivity can also be used in conjunction with periorbital von Frey testing. Contrary to grimace scoring, it is best to test facial hypersensitivity prior to assessing hind paw hypersensitivity. Hindpaw testing requires that the mouse is placed back in the chamber without the cup after periorbital von Frey testing is completed.

In conclusion, periorbital von Frey testing and non-invasive dural stimulation in mice add valuable options to the current range of preclinical models of migraine. The dural stimulation protocol can easily be modified to use with several drug applications. Periorbital von Frey testing paradigms can also be modified to best fit the experimental specifications. Additionally, periorbital von Frey

testing can be used in other orofacial pain disorders. These techniques are an important tool to help further understand the complex underlying mechanisms of migraine pain.

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DISCLOSURES

The authors have nothing to disclose.

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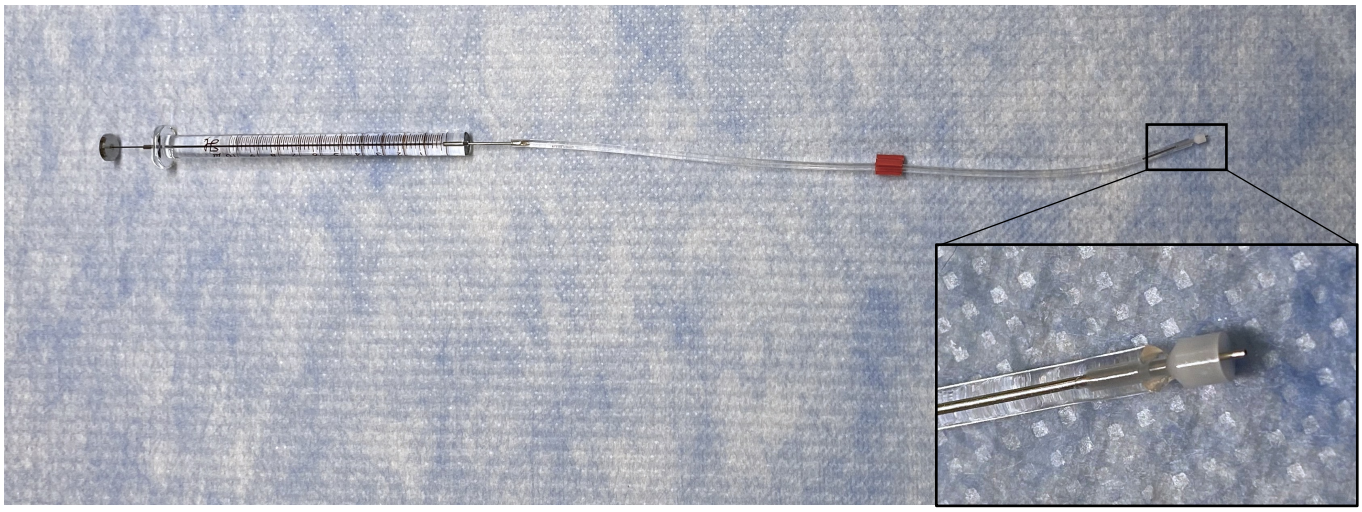
Figure 1

A



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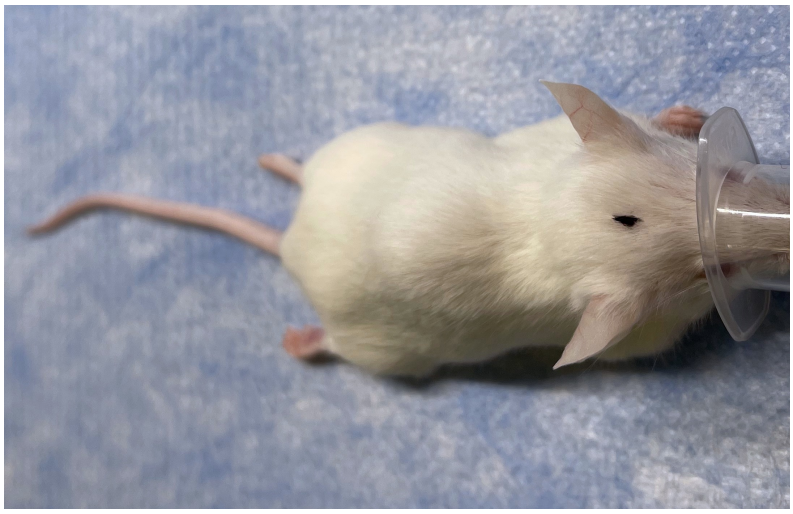

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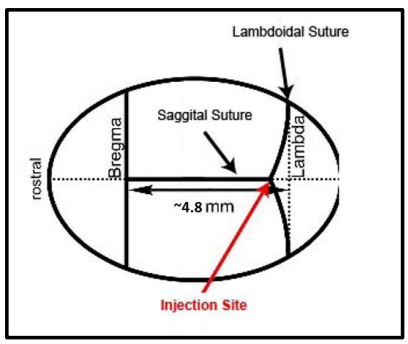


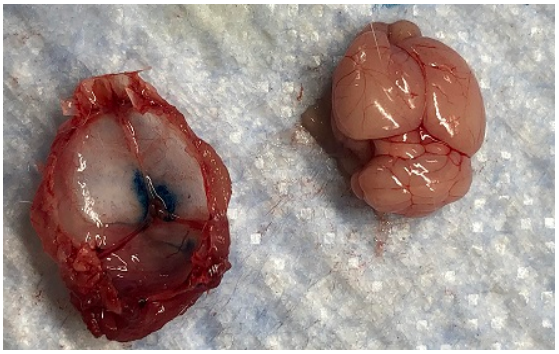
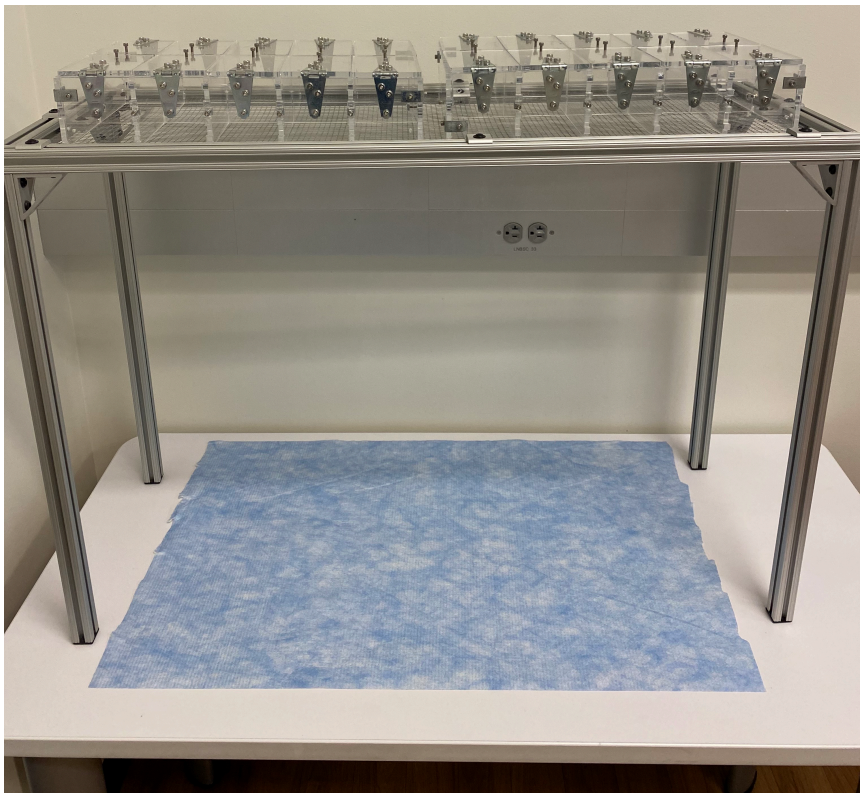


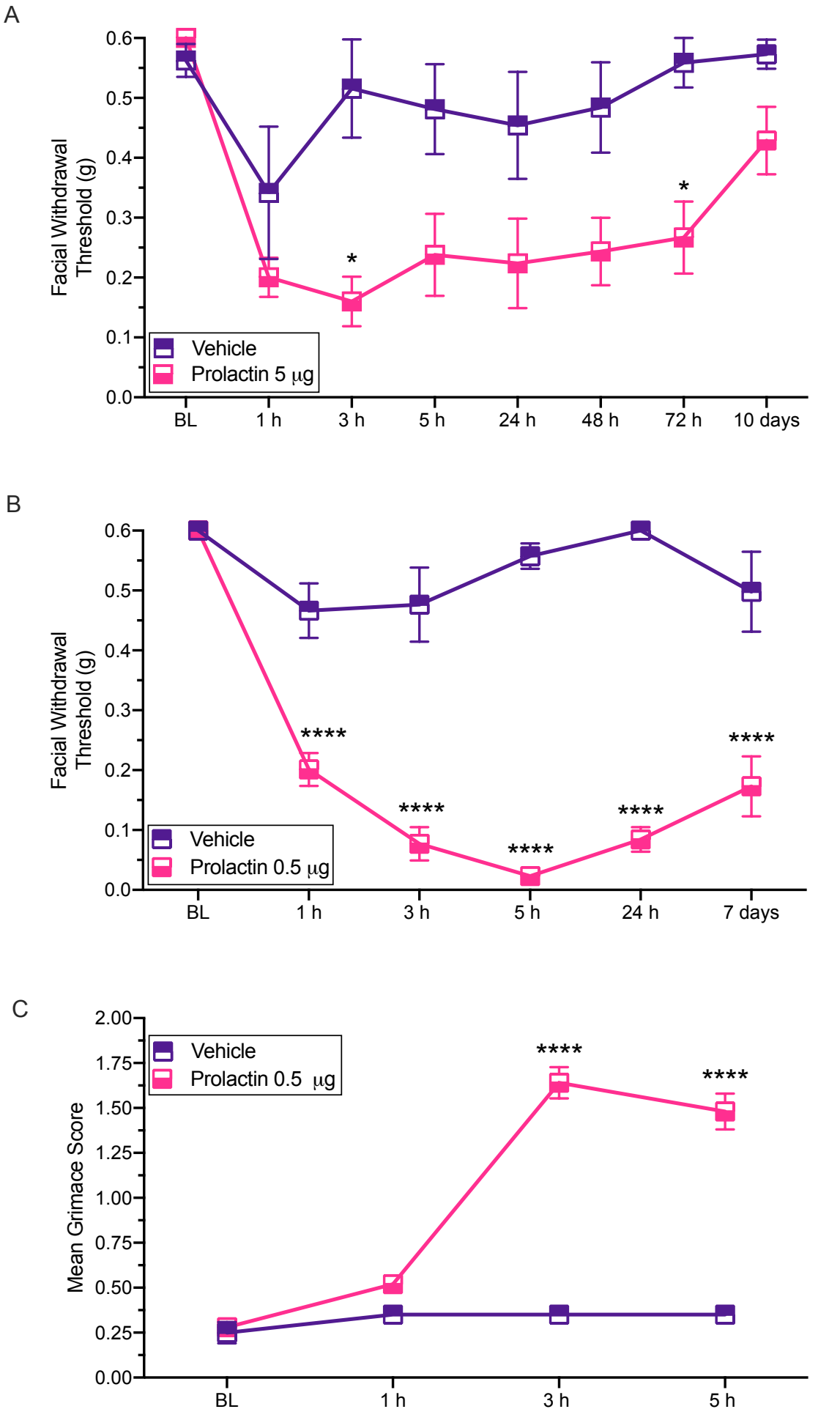
Figure 2

A



B

Figure 3



We thank the Editor and Referees for their insightful comments that have greatly improved the quality of the manuscript. We hope to have addressed all the concerns. Responses are detailed below and all modifications are indicated by track changes in the text.

Editor comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have taken the opportunity to rectify spelling and grammar issues.

2. Please provide at least 6 keywords or phrases.

We have added to the manuscript the keywords: migraine, dura mater, facial hypersensitivity, non-invasive stimulation, behavior to the manuscript. Please see line 45.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed personal pronouns from the manuscript.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: PlasticsOne, Cole-Parmer, Hamilton Gastight Model 1701 SN, Choice Paper Company, etc.

We have modified the text to contain more generic descriptions of products used in this protocol.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

The text has been changed to be written in the imperative tense and “Notes” are used accordingly.

6. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added more detail to the protocol throughout the manuscript.

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

The ethics statement “All procedures were conducted with prior approval of the institutional Animal Care and Use Committee at the University of Texas at Dallas” has been added after the Introduction section. Please see Line 105.

8. Line 91-110: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

The items in line 91-110 have been moved to the discussion. Please see line 424.

9. Line 144-146: Is there any specific concentration or any desired concentration?

This protocol is using prolactin as an example of substances that can be administered onto the dura. There is an abundance of stimuli that may have relevance for migraine that can be applied to the dura. The desired concentrations are based on those compounds and will require titration.

10. Line 193-200: Please move the lines to the discussion or add it as a brief note under “Periorbital Von Frey testing”.

This text has been moved to section 3, line 224 as a “NOTE”.

11. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
12. Please include some limitations of the study in the discussion.

We have now placed a space between each protocol step and highlighted essential steps of the protocol video.

13. Please include a Disclosures section, providing information regarding the authors’ competing financial interests or other conflicts of interest. If authors have no competing financial interests, then a statement indicating no competing financial interests must be included.

A disclosures statement has been added to the manuscript. Please see line 514.

14. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

The manuscript does not contain any reused figures from previous publications. We have used data from Avona et al 2021 and created new graphs of the data for this manuscript. We have also added to the data sets compared to what was published in Avona et al 2021 by extending the time courses.

15. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

The references section has been reformatted.

16. Figure 3: Please revise the X axis scales to “1 h, 3 h, 5 h, etc.” instead of “1 hr, 3 hr, etc.”. Please leave “7 days” as it is. Please maintain a single space between the numeral and (abbreviated) unit.

The X-axis scales of Figure 3 have been modified as described above.

17. Please ensure that the table of materials include all the essential supplies, reagents, and equipment used in the study. The table should include the name, company, and catalog number and sort the table in alphabetical order.

We have now added a table of materials and have sorted in alphabetical order.

Reviewer #1:

Manuscript Summary:
This manuscript presented a method for dural application of substances in a minimally invasive manner while using facial hypersensitivity as an output. Dural application of substances allows for the preferential activation of dural afferent neurons, which is critical in studying headache mechanisms and therapy. A minimally invasive approach to locally stimulate the dura eliminates post-surgical recovery and allows for immediate testing in awake animals. Assessing tactile hypersensitivity of the periorbital skin following dural stimulation provides a robust endpoint for elucidating the mechanisms underlying headache generation. A detailed description of these two techniques is timely for labs that are interested in headache research. The methods are clearly described, equipment/material list is included. Critical steps/parameters are emphasized. I just have one minor comment.

Minor Concerns:
For people planning to use mice different from those in this study, it would be useful to discuss how to determine the length of the infuser in 2.2.

We have added “This technique was developed and optimized using ICR (CD-1) mice (30-35 g) and C57/BL6 mice (25-30 g). An infuser length of 0.5-0.6 is sufficient to inject a mouse weighing within the range of 25-35 grams. However, the length of the infuser may need to be calibrated if injecting mice that significantly differ from the mice used to optimize this technique. For example, a mouse smaller than 25 grams would likely result in use of an infuser that has a length less than 0.5 mm.” to the Discussion. Please see line 445.

Reviewer #2:

Manuscript Summary:
The most notable symptom of migraine is severe head pain and it is hypothesized that this is mediated by sensory neurons innervating the meninges. Here, we present a method to locally apply substances to the dura in a minimally invasive manner while using facial hypersensitivity as an output. I thought this was a really good methods paper. It was easy to follow along, and I don't see any problems with the design. They also covered a lot of the limitations of dural injection.

Major Concerns:
NONE

Minor Concerns:
Can you clarify line 205 if the mice are handled for 5 minutes 24 hours prior to habituation or are they handled for five minutes for 3 days prior to habituation.

We agree that this sentence is vague. On the day prior to habituation (24 hours before), mice are handled for approximately 5 minutes. This has been clarified in the protocol in section 3, line 219.

In the introduction, the authors mentioned that facial grooming by mice has been a limitation for von Frey testing as it's difficult to distinguish between pain responses and routine grooming/itch. I agree, it's sometimes easy to confuse this behavior with a response to the applied filament. Does the authors had any solution to this that they didn't included in the protocol.

If grooming/scratching cannot be distinguished from a hypersensitive response, it is best to not record this as a response. We prefer to wait until grooming has stopped and the mouse is calm to test for responses. Continue from the same filament used when the mouse started grooming. If the mouse continues to groom for long bouts of time, place the mouse back in the testing chamber for approximately 5 minutes (if there are other mice that still need to be tested continue testing them). Once 5 minutes have passed (or other animals have been tested) try testing the mouse again. If this continues with no resolve, the mice may have to be removed from the study. This has been added to the Discussion, line 466.

Also, if the filaments move and ruffle the fur, which causes the mice to groom their heads, is there a way to

control this, as to ruffled fur could affect sensitivity and the grooming habits and result in incorrect measurements. Do you suggest shaving the fur in the test are of the animal?

Shaving fur on the face is not recommended. Exposed skin that is typically covered by fur may have differential sensitivity to touch compared to fur-covered skin. Additionally, the shaving or hair removal process can influence the sensitivity of the skin, particularly if depilatory creams are used. We agree that there are some strains of mice that have fur that is more oily than other strains (e.g. C57/BL6). The best way to circumvent this is to ensure the experimenter is properly trained and the animal is calm. This has been added to the Discussion, line 479.

Reviewer #3:
Manuscript Summary:
Mason and colleagues have conducted a very good description of periorbital von Frey testing after dural stimulation as a preclinical model of migraine headache. It is a very elegant technique.

Major Concerns:
Concerning the dural stimulation:

1) It is an elegant how the authors show sex dependent changes in sensitivity with prolactin. However, how does prolactin induce periorbital hypersensitivity? And why only in females for longer duration?

We have taken out the male data because we feel it detracts from the overall point of the paper. We intend for the current manuscript to be focused on the process of dural stimulation and periorbital von Frey testing and not on mechanisms of sex differences in response to prolactin. For more discussion of sex differences, please see Avona et al. “Meningeal CGRP-Prolactin Interaction Evokes Female-Specific Migraine Behavior”. Ann Neurology. 22 March 2021. PMID: 33749851

2) The female mice seem to become more sensitive with 0.5 compared to 5 µg prolactin at 3 and 5 hours. Is there an explanation for this?

We believe this may be an effect of desensitization of prolactin receptors at the higher dose, however, this explanation requires further testing. We discussed this point in our recent paper, please see Avona et al. “Meningeal CGRP-Prolactin Interaction Evokes Female-Specific Migraine Behavior”. Ann Neurology. 22 March 2021. PMID: 33749851

3) An essential part of this methods is the location of the point of injection. The authors write:
The easiest way to locate this discrete junction through the skin is by using topographical features of the skull and gently probing the general location of the junction with the infuser. It would be beneficial for the article if the authors could go into even more details on how they find the junction of the sagittal and lambdoidal sutures.

The Reviewer raises an excellent point but one that is not easily addressed with text in a manuscript. In our experience, this is one aspect of the technique that is only learned by manually palpating the skull plates to feel for the intersections. We hope that the videos associated with the article can appropriately capture this process.

While the dural stimulation sections is well described, the section regarding von Frey testing is less well described.
1) The authors do not go into the details concerning how they convert their von Frey data into quantitative data? There are several methods to do this. See e.g. PMID: 31889375

We have now added a section on von Frey analysis. Please see section 5, line 343.

2) I the discussion, the authors mentioned that it is critical to ensure reproducible results is to make certain that the mice are fully baselined. Moreover, they mention that it is likely that it will take approximately 7 days for an

animal to reach baseline and that it is not every animal will reach the targeted baseline. They mentioned that only 60-70% of animals will reach a baseline of 0.6 grams in the periorbital region. It would strengthen the article considerately, if they could mention these steps in the protocol.

This has been added to the protocol. Please see section 4, line 333.

3) From (2) one understands that the animals are selected for how well they reach a particular baseline during a pre-dural stimulation protocol. This would suggest that the authors are selecting certain animals before the dural stimulation. If this is correct, the authors should comment on this.

This is a good point. While we do only include mice that pass baseline in our dural stimulation experiments, those mice that are used are randomly allocated to each testing group. This can be done by drawing numbers out of a cup or a script can be written in excel to randomize numbers to groups. This has now been added to the Discussion. Please see section 4, line 338.

That said, we think the Reviewer may be noting that there is a selection bias towards animals that pass baseline. While true, this is common practice in behavioral testing in rodents. It also creates additional challenges to separately test mice that did not pass baseline given that they would start experiments already being defined as “hypersensitive” using our standard criteria. It would be interesting to know more about differences between mice that do and do not pass baseline e.g. are there simply psychological differences between them, are there true differences in nociceptive thresholds, is this mediated in the transcriptome/proteome? This is clearly beyond the scope of the present study but is an interesting idea for future work.

We have added the following text to address the randomization of animals post-baseline. Please see section 4, line 335:

Prior to dural stimulation, baseline data is analyzed to exclude any mouse that has not reached a baseline value of 0.5 g or higher. After the exclusion, mice are randomly selected to a testing group. It can be done by drawing out of a hat or a script can be written in excel to randomize numbers to groups.

Minor Concerns:
At linie 127 there seem to miss a unit "minimum length of 8 (?) in to".

The line now states that the “tubing is cut to a minimum of 8 in. to ensure…”.

Reviewer #4:
The authors present a methodological manuscript exploring a preclinical model of headache that utilizes dural stimulation and periorbital mechanosensitivity testing. Overall the manuscript is of a high quality and comes from a well-respected group in the field which is a strength.

Below I have a number of comments that I feel would help further improve/clarify the protocol/article.
* In the introduction the treatment success is portrayed in a rather negative way. Migraine is currently outperforming many neurological conditions with novel therapies that have been well validated in preclinical models. Perhaps highlight the recent advances and suggest that further models are still needed to aid in future development.

We agree that the success in treatments should be highlighted more. This has now been added to the introduction, line 59.

* The discussion regarding systemic administration is a complex one. Systemic administration of NTG and CGRP for example in patients leads to the triggering of experimental attacks that are similar to spontaneous migraines. As such, while they do lack tissue specificity one could argue they are one of the closest thing to translational work that exists. Suggest, highlighting that while they have been useful, more targeted tissue specific models offer an alternate approach.

Excellent point. This has been added to the discussion on systemic administration. Please see line 420.

* Section 2.3.1: please confirm tubing length unit as this is lacking.

The line states that the “tubing is cut to a minimum of 8 in. to ensure…”.

* Would the authors propose any special housing instructions for the mice. For example, if the mice have cephalic hypersensitivity, it is likely they may also be photophobic. Do you house mice under specific dim lighting conditions.

Following induction of facial hypersensitivity, mice are housed with the same conditions as they were before dural stimulation. It is also a possibility and likely that they will be experiencing light sensitivity as well, however, we do not test for this behavior.

* Points 5.7-5.10 could be reduced and restructured as the response is noted early but what constitutes a response is not made clear until later, along with detail about ensuring the filament bends appropriately.

We agree and have restructured that section accordingly.

* I would personally like to see more detail regarding how the baseline is established as this is not immediately clear, more detail is in the discussion. When doing this are mice tested daily or every other day as repetitive periorbital testing can cause sensitization in our experience. Could this be leading to the high drop out rate.

Periorbital von Frey testing is inherently challenging in mice, much more so than in rats. Mice are much more active than rats and this is the rationale for using the paper cup method of restricting movement around an open chamber. This cup approach is not necessary in rats. This is almost certainly a primary reason why there are relatively few papers with this technique in mice while there are many more publications in rats. Our opinion is that the high dropout rate is related to this issue where some mice are just not amenable to periorbital von Frey testing because they are not calm enough to be still in the cups. If one is willing to continue attempting to baseline mice, an increasing % of them will eventually pass baseline, but then it often becomes a matter of waiting for more mice vs proceeding with experiments in a timely manner.

Importantly, and to address the next comment from the Reviewer, we do not simply euthanize mice that fail to pass baseline. These mice are still used, just not for behavioral testing. We commonly use them to do pilot experiments for techniques like immunohistochemistry, patch clamp, or western blots, to dial in experimental conditions before running actual experiments. We remain concerned about using tissue from mice that cannot be baselined for critical studies because we do not know what might be different about these mice compared to those that pass baseline. But we always use them for other purposes.

To address the specific details, the mice are tested for baseline for the first three consecutive days. After three days, if the mice are unusually hypersensitive they are allowed to rest 1-2 days and then resume testing. However, it is not recommended to wait more than 4 days between testing periods as this can result in the animal failing to adjust to the weight of the filament on their periorbital region.

* The complexity of this baseline is shown in the fact that as many as 40% of mice do not make baseline and therefore will be excluded from the study. This appears a very high attrition rate, especially if combining this methodology with transgenic mice in the future. What are the authors views on the ethics of an up to 40% increase in animal usage to get a fully powered study.

We thank the Reviewer for this important point and have hopefully addressed this in the response to the prior comment.

* Please also comment on the fact that the baseline appears to be set differently compared to the up-down

method used post-treatment. Can the authors not use the up-down method throughout to establish a true 50% withdrawal threshold and simply counterbalance mice to prevent high dropout.

We have historically used the 0.6 g filament alone as the cutoff to test for baseline. Similarly, we have always used the 8 g filament in rats for periorbital testing in the same manner. Others have taken a similar approach in rats. Arguments could certainly be made that finding the actual threshold with the up-down method could be used to determine baseline, then graphs are plotted using responses as a delta value from a normalized baseline. We have simply chosen to use a single filament as the cutoff threshold to attempt to minimize testing of the animals prior to the start of experiments with administration of stimuli. As discussed above, we do not think the high dropout rate will be addressed by testing the 50% threshold for baseline because part of the reason for drop out is inconsistencies in the daily thresholds and challenges in determining responses in specific mice.