ANALYSIS OF BEHAVIORAL ASSAYS USED FOR ASSESSMENT OF MIGRAINE-LIKE PAIN IN MICE

By

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Abstract

Migraine is a common pain disorder that severely impedes daily life for many individuals. To address the evident prevalence and burden of migraine, there is a growing need for the development of new therapeutic interventions. However, there has been a hindrance in the discovery of new analgesics for chronic pain in general due to a translational gap that exists between preclinical advances and clinical interventions. This gap is partially the result of an over-reliance on acute models of pain and experimenter-evoked outcome measures. It has been proposed that the study of spontaneous, elective behaviors may be more translationally relevant measures of chronic pain. For instance, changes in cage hanging, a natural behavior displayed by rodents, has been shown to be an indicator of sustained pain. Rearing behavior, which is an important exploratory component of locomotion in rodents, can also be reduced during a painful event. The purpose of the present study is to determine if cage hanging and/or rearing are useful dependent measures of migraine pain specifically. We found that induction of migraine-like pain through supradural injection of inflammatory mediators reduced cage hanging and rearing in mice. Currently, we cannot conclusively state that these behaviors are reliable measures of pain specific to migraine headache, but we show promising results that will be expanded on in the future.

Introduction

According to data from the American Migraine Study II, an estimated 23.6 million Americans suffer from migraine headaches, making it one of the most common pain disorders.[1] In fact, various other studies indicate that the prevalence of migraine is steadily increasing.[2,3] Additionally, it has been found that migraine is associated with substantial disability.[1] Despite

the evident burden of migraine for many Americans, migraine remains underdiagnosed and undertreated in the United States, with a sizable proportion of patients meeting criteria for preventive measures not receiving treatment.[4]

One factor that may contribute to a lack of adequate treatment for migraine sufferers is an unmet need for discovery and development of more effective analgesics. This is partially due to the translational gap that evidently exists between preclinical progress and clinical interventions for chronic pain.[5] At present, many animal models being utilized to study pain responses are not sufficiently representative of the human experience of chronic pain. For instance, models in which acute pain is induced do not adequately address the nociception produced by chronic pain, as the neural mechanisms underlying these types of pain are anatomically and physiologically distinct.[6] While methods of inducing pain have recently advanced, outcome measures of pain need improvement. Presently, the most common behavioral measure used in pain research is hypersensitivity, namely hyperalgesia and allodynia. However, the most prevalent symptom reported by pain patients is not hypersensitivity, but rather spontaneous, ongoing pain.[7]

As a result of this common complaint among pain patients, there has been a shift of focus to spontaneous behaviors as dependent measures in pain assays.[5,8] Of particular interest is elective behaviors, which are natural activities that are not essential to survival. In rodents, some of these behaviors, such as burrowing, have previously been found to indicate well-being and can be impacted by poor health as well as the experience of pain.[9,10] Analysis of such behaviors may be beneficial in a preclinical setting, as chronic pain has been shown to affect various elective behaviors displayed by humans.[11,12] Two elective behaviors that have been observed in rodents, and may be useful as outcome measures of ongoing pain, are cage hanging and rearing.

When housed in wire cages, rodents will naturally grasp the cage's wire bars with their forelimbs and/or hindlimbs and hang (Fig. 1B).[13] A recent study conducted by researchers at the University of Toronto found that cage hanging behavior may be a translationally relevant measure of pain in a mouse model. In this study, multiple assays involving sustained pain, including a systemic inflammation assay, were utilized to ensure that the results obtained were generalizable to various types of nociception. Mice were observed in their natural homecage environment and behavioral changes were analyzed. It was found that cage-lid hanging was the only behavior studied that was a reliable indicator of pain: with prolonged pain, there was a reduction in the time mice spent hanging on the modified lids of their cages. Furthermore, researchers found that the depression in cage hanging behavior was dependent on the intensity of the stimulus and could be reversed with analgesics.[14]

Rearing behavior is yet another nonessential behavior expressed in rodents in which the animal will stand on its hindlegs with its head elevated (Fig. 1A). Rearing is a form of exploratory behavior and a key component in locomotion.[15,16] This is important to note because locomotion has been evaluated as an outcome measure in various headache models. In these studies, induction of migraine-like pain (via inflammatory mediators) resulted in an increase in inactivity as well as a reduction in exploratory behavior.[17,18] These effects could be partially reversed by administration of anti-migraine drugs.[18] Additionally, exposure to TRPA1 agonists, which are thought to contribute to signaling pathways in headaches, reduced the number of rears in rats.[19] Administration of CGRP, a critical component of migraine pathophysiology, also resulted in a depression of rearing behavior relative to mice treated with vehicle.[20] Thus, rearing behavior can be reduced in response to a painful event, and may be used as an indirect marker of noxious experience.[16]

While reliable and relevant outcome measures of migraine are limited, there has nevertheless been a great deal of progress in animal models of migraine. At present, there are various means of inducing pain similar to the nociception experienced during migraines. One of these methods is dural administration of inflammatory mediators. Local inflammatory processes result in the excitation and sensitization of nociceptors of the intracranial meninges.[21,22] It is thought that the activation of sensory neurons that innervate the intracranial meninges as well as their associated blood vessels contribute to the pain of migraine.[23] This innervation is provided by neurons that have cell bodies located primarily in the trigeminal dorsal root ganglia.[24] These cells are thought to be nociceptors that innervate the dura mater.[25,26] The activation of intracranial meningeal nociceptors that occurs in these central processes is believed to promote the throbbing pain that often is experienced during migraines.[21] Thus, the central changes promoted by application of inflammatory mediators, along with various peripheral changes, are thought to produce sensory effects analogous to those seen in migraines.[27]

In our study, we utilized supradural injection of inflammatory mediators in order to produce migraine-like pain in mice. We then measured behavioral changes in the time spent lid-hanging, the number of hangs, and the number of rears. The purpose of the study was to investigate the efficacy of cage hanging and rearing behaviors as outcome measures of migraine pain. We found that there was a reduction in both behaviors in mice treated with inflammatory mediators. Overall, further research into the use of cage hanging and rearing as dependent variables is necessary. Nonetheless, our results indicate that a focus on these two behaviors may be valuable in the future of migraine studies.

Methods

Animals

Subjects were C57BL/6J female mice. The animals were housed in a climate-controlled room with a reversed light/dark cycle of 12 hours for the duration of the experiment. Food and water were freely available. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Arizona.

Hanging cages

Each mouse was caged separately during experimentation and had their own designated "hang box." These holding chambers included a clear Plexiglas box with a modified lid composed of steel wires at an upward-facing angle to facilitate hanging behavior. To limit distraction, no food, water, or other environmental enrichment was present in the hanging cages during the testing. A limited amount of corn cob bedding was included. The experiments were conducted during the dark cycle because mice are nocturnal. Red lamps were used as the only source of light in the testing room to support recording because rodents lack red-light cones in the retina and are dichromats. Therefore, it is assumed that they cannot see red light.[28,29] Lamps were placed at a sufficient distance from the hang boxes to avoid temperature changes, and animals were not exposed to any other source of light to maintain the reversed cycle.

Acclimation and baseline collection

The acclimation period was 4 days. Each day, animals were placed in their designated cages for 1 hour. Acclimation began at roughly the same time each day. One day after the acclimation periods, baseline data was collected. Animals were placed in their designated hang boxes for 30 minutes to acclimate, then their behavior was recorded for 4 hours.

Supradural administration of inflammatory mediators

Isoflurane was used as an anesthetic during the supradural injections. Animals in the control group received supradural injection of 5 µL synthetic interstitial fluid (SIF), which contained 10mM HEPES, 5mM KCl, 135mM NaCl, 1mM MgCl2, 2mM CaCl2 and 10mM glucose. Animals in the treatment group received supradural administration of 5 µL inflammatory mediators (IM), which contains 1 mM serotonin, 1 mM bradykinin, 1mM histamine and 100 µm prostaglandin E2. SIF was our control because it is the vehicle used to dilute the IM. Therefore, any behavioral change seen in the treatment group would be attributed to the IM rather than the vehicle. A modified 0.7 mm depth injector (Invivo1, part #C313I/SPC, Internal Cannula, Standard, 28 gauge, I.D. 0.18 mm, O.D. 0.35 mm) connected to a polyethylene tube and Hamilton syringe was utilized for administration. The day before the experiment, injectors were inserted into a vial with 70% alcohol and then cold sterilization was performed by placing the injectors into a 20˚C freezer. The site of injection was located at the intersection between the sagittal and lambdoidal sutures of the skull.

Behavior testing

The day following baseline, animals were again placed in their respective hang boxes. Prior to injection, animals were given 30 minutes to acclimate in the hanging cages. Immediately following supradural injections, animals were re-placed in their boxes and their behavior was recorded for 4 hours.

Data analysis

The recorded behavior was rated based on amount of time spent hanging on the cage lid, the number of hangs, and the number of rears. Only the first 2 hours of the recordings were analyzed by a single examiner. This is because 2 hours is the point at which the animals

experience the peak of evoked pain induced by IM. Behavioral changes from baseline to treatment day within groups were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey post-hoc multiple comparison test. Difference in rearing behavior (test-baseline) for control and IM treatment groups was analyzed with an unpaired t-test.

Results

Mice treated with IM showed a significant decrease in time spent hanging from baseline to testing ($P < 0.05$). On average, mice spent 52.8 mins or 44% of their time (SE: 5.9 mins) hanging prior to IM injection. After treatment, IM-treated mice spent only 24.1 mins or 20.1% of their time (SE: 7.0 mins) hanging on average (Figs. 2A and B). Mice in the control group also showed a decrease in the time spent hanging from baseline to testing. Control mice spent an average of 32.6 mins or 27.1% of their time (SE: 8.5 mins) hanging at baseline, and 20.4 mins or 17% of their time (SE: 4.2 mins) hanging during testing. This difference, however, was not significant ($P > 0.05$, Figs. 2A and B).

Similar to the time hanging results, mice showed a decrease in the number of hanging behavior from baseline to testing. Prior to IM admininstration, mice in the treatment group hung 294 ± 34 times on average within the first 2 hours of baseline recordings. After treatment, the mice hung 91.3 ± 22.3 times on average within the 2-hour period. This decrease reached significance ($P < 0.05$, Fig. 2C). Over the first 2 hours of recordings, the mean number of times control mice hung decreased from 159 ± 37 at baseline to 95.5 ± 22.5 at testing (Fig. 2C).

Rearing behavior was also analyzed. Mice injected with the vehicle showed no significant difference in recorded number of rears from baseline to testing ($P > 0.05$). At baseline, these mice reared an average of 483 ± 12 times over the first 2 hours of recording. After

vehicle injection, the mice reared 518 ± 60 times (Fig. 2D). Over the 2 hour period, mice reared 562 ± 33 times on average at baseline prior to supradural administration of IM. However, after IM injection, the average number of rears decreased to 348 ± 12.4 times. This decrease is considered significant ($P < 0.05$). Furthermore, there was a significant difference between number of rears by control mice and IM-treated mice at testing ($P < 0.05$, Fig. 2D).

For both groups of mice, the difference in rears was calculated by subtracting the number of rears at baseline from the number of rears at testing. These values were graphed in order to further emphasize the changes in rearing behavior seen in the treatment groups. The difference in number of rears was 35 and -214 in control and IM-treated mice respectively. In other words, mice in the control reared 35 more times during testing than they did during baseline, and mice treated with IM reared 214 less times. The difference between these values was found to be statistically significant ($P < 0.05$, Fig. 2E).

Figures and Graphs

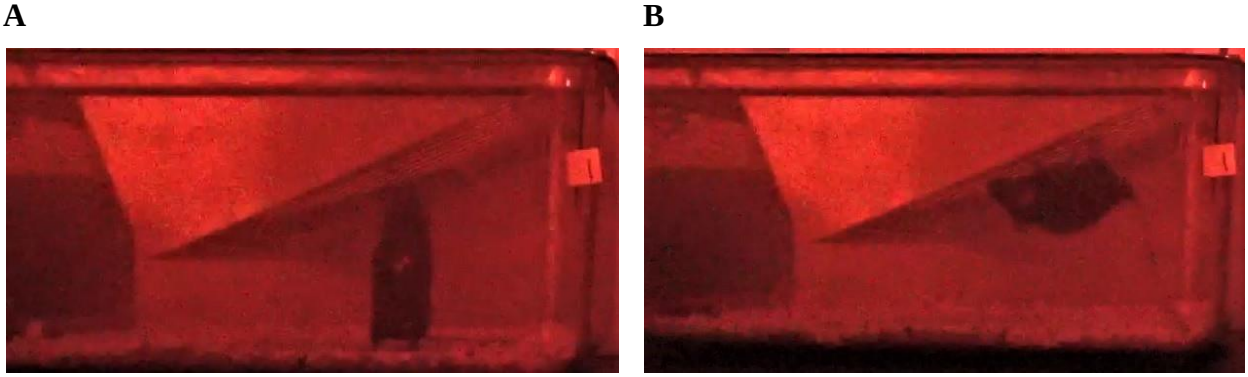

Figure 1. Rearing and cage-hanging behaviors. This image shows the representative behaviors being analyzed in our experiment. (A) The mouse shown on the left is displaying rearing behavior. (B) The mouse on the right is displaying cage lid-hanging behavior.

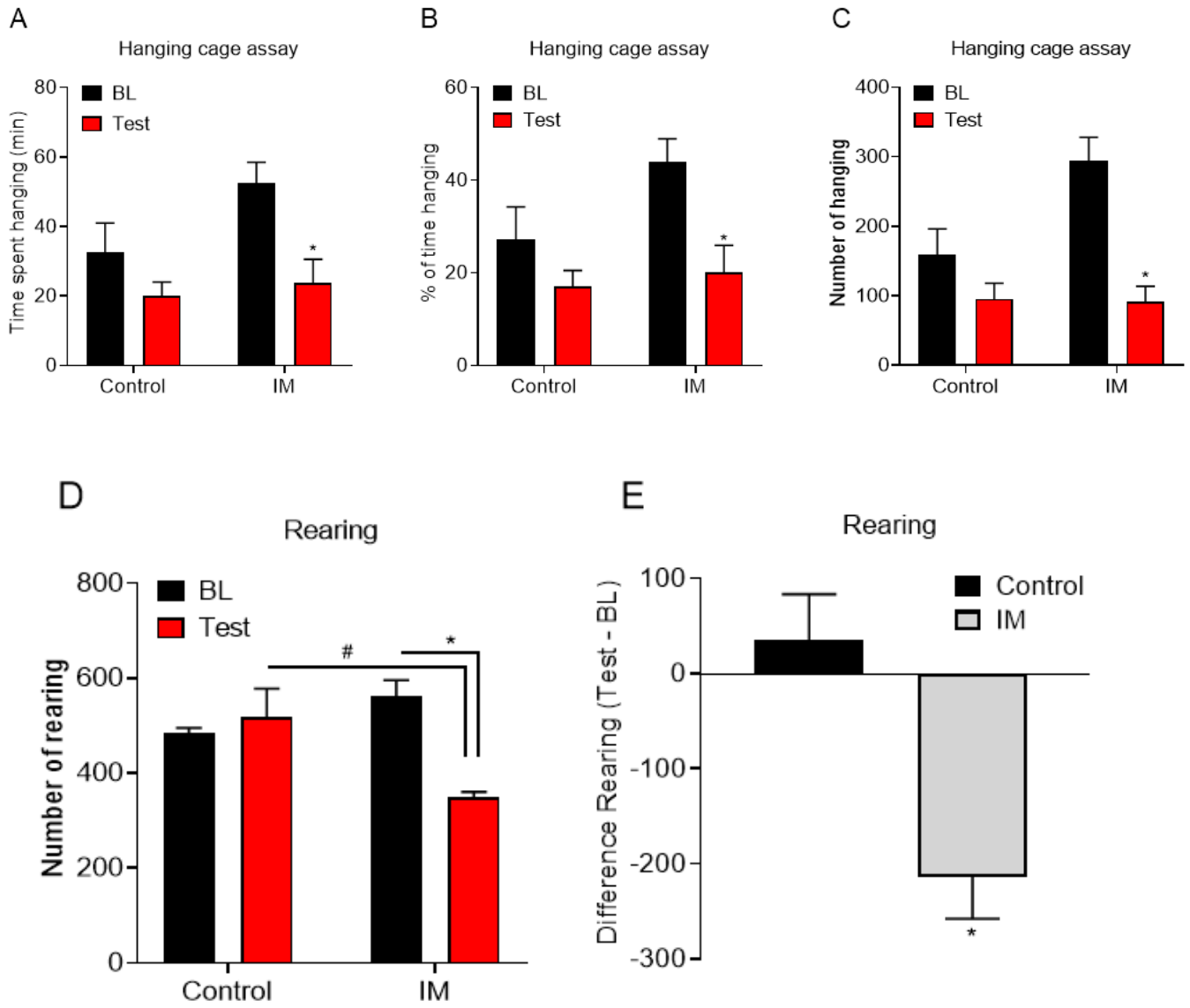

Figure 2. Administration of IM into the dura mater induced decrease of hang and rearing behaviors in C57BL/6J female mice. Mice were given an acclimation period of 4 days prior to baseline collection in which they were placed in their designated hanging cages for an hour a day. On day 5, four hours of baseline recordings were collected after a 30-minutes acclimation period. After baseline collection, on day 6, mice acclimated into their designated hanging cage for a 30-minutes acclimation, followed by supradural injection of inflammatory mediators (IM; 5 µL) or vehicle (control; 5 µL) and four hours of recordings were collected. The data represent the rearing and hanging behavior analyzed for the first two hours of the baseline and testing recordings. This data was analyzed using One-Way ANOVA followed by Tukey post-hoc multiple comparison test with * and # representing $p < 0.05$ in comparison to BL - test and control – IM, respectively (n = 2 - 4). Difference in rearing behavior was calculated and analyzed using an unpaired t-test with * representing $p < 0.05$ in comparison to control (n = 2 – 4).

Discussion

The aim of our study was to evaluate two nonessential behaviors as potential measures of migraine-like pain. Mice treated with inflammatory mediators exhibited a significant impairment in both the time spent hanging and the number of hangs relative to baseline. Similarly, the amount of rearing behavior was impaired in IM-treated mice after injection. Through analysis of these behavioral changes, it seems that both cage hanging and rearing may offer promising prospects as objective migraine pain indicators.

Although IM-treated mice did exhibit reductions in both behaviors, we cannot draw definite conclusions from the present data. Regarding cage hanging behavior, it must be noted that there was no significant difference between the two groups of mice at testing for either the time spent hanging or the number of hangs. This would indicate that, at testing, mice experiencing pain had similar hanging behavior as those not experiencing pain. This could potentially be accounted for by the fact that control mice had slightly lower averages at baseline. This latter observation further emphasizes that there was a great deal of variance in the results. Standard error values were relatively high, likely due to a small sample size, indicating that accuracy was low.

As stated, our results represent that migraine-like pain reduced rearing behavior in IM-treated mice. For number of rears, there was a significant difference in the two groups of mice at testing, indicating that mice experiencing pain did in fact rear less than those treated with the vehicle. Nonetheless, variance in rearing behavior was still non-negligibly high, and a larger sample size is needed for conclusiveness.

Currently, there are plans for this project to continue. The sample size will be increased in order to reduce variability in the data. To streamline the time-consuming process of video

analysis, we could implement automated systems of counting. The University of Toronto has already developed an electronic device that accurately quantifies hanging behavior.[14] Additionally, other automated systems have been produced that are capable of analyzing rearing behavior and have been found to be at the same standard as human scoring.[13] In fact, it could even be said that the use of an automated system would minimize the subjectivity of a human examiner, while also increasing reproducibility.

The way pain is perceived is subjective and can vary widely based on gender and age. This notion is supported by the results of the University of Toronto study, which indicate that cage hanging does in fact vary between different genders and ages. For instance, female mice displayed more hanging behavior than males.[14] Rearing behavior has also been shown to differ based on gender, with female mice generally being more active when exposed to a novel environment.[30] Moreover, gender-related hormonal levels can result in activity changes in mice.[31] Activity has also been shown to differ with age, with an increase in age resulting in a decrease in exploratory behaviors, including rearing.[32] In addition to gender and age, pain-related behaviors may also vary between strains. This is supported by the University of Toronto study, which sited differences in cage hanging behavior between multiple strains of mice.[14] The latter factors must be taken into consideration while choosing future subjects for experimentation. Analysis of differences in pain perception may also provide insight into mechanisms underlying the prevalence of migraines in certain genders and ages.

Another key factor that can influence behavior is stress.[31] While the hanging cages utilized in our experiment functioned to facilitate hanging behavior, their use removed the added benefit of observing the mice in their home cage environment. It is possible that the added stress of moving the mice caused alterations in their behavior. Allowing for acclimation may have

minimized this stress. Still, it may be advantageous to adjust housing conditions of the mice during future experimentation in such a way that moving them is avoided.

There are many other directions that future experimentation could go, assuming the two behaviors in question are valid measures of migraine pain. For one, the physiological and neurological processes underlying pain-related changes in these two behaviors remains unclear. To truly understand the relationship between these behaviors and nociceptive processing, more research would need to be conducted on the physiological factors at play.

The goal of this research is to translate preclinical models of migraine into safe and effective clinical interventions. Thus, future experimentation should involve the use of analgesics. If these two behavioral outcomes are reliable measures of migraine pain, then it is possible that any pain-induced changes in hanging and/or rearing could be reversed by analgesics. This could be determined by administering anti-migraine medications after testing, and observing if baseline hanging and rearing behaviors are restored. Though, it should be kept in mind that some analgesics may have a sedative effect, and have been shown to decrease rearing behavior and locomotion in general.[33]

Our findings support that cage hanging and rearing are two promising behaviors that could potentially function as measures of migraine pain in a preclinical mouse model. It is our hope that, with further research, we can collect more conclusive results that validate our current conjectures. The ultimate aim of our work is to minimize the gap that exists between preclinical progress and clinical interventions. Through analysis of translationally relevant, spontaneous pain-related behaviors, we may be able to develop an efficient model for screening migraine medications. In this way, research on behavioral indicators of pain may someday give rise to the

development of new migraine treatments. Thus, work in this area could contribute to a decrease in the prevalence and burden of migraine.

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