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Repetitive stress in mice causes migraine-like behaviors and CGRP-dependent hyperalgesic priming to a migraine trigger

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Introduction

Migraine is the second-most disabling disease worldwide [27], characterized by unilateral throbbing pain, cutaneous allodynia, often accompanied by nausea and sensitivity to light and sound. Attacks can be caused by triggers that include changes in hormones or sleep patterns, skipping meals, consumption of alcohol and certain foods [32]. Susceptibility to attacks following exposure to these common events suggests maladaptive changes have occurred within migraine-related nociceptive pathways. The most commonly-reported trigger for migraine is stress [32], which can increase the duration of headache [57] and may play a role in the development of chronic headache disorders [50]. Attacks often do not occur while stress is ongoing, but rather once the stressful event has passed [53; 54] with greatest susceptibility 6 to 18 hours following resolution of stress [38]. Additionally, multiple days of intense stress are more likely to trigger attacks than a single day [38]. Despite the close correlation between stress and migraine, mechanistic links between the two remain poorly understood.

Preclinical migraine studies have used methods of stress including restraint, bright light, unpredictable sounds, wet bedding, exposure to predators, or a combination of stressors. Acute or chronic stress decreases the threshold for cortical-spreading depression (CSD) [6; 58], increases nitric-oxide synthase expression [59], alters immune cell properties in the dura [44; 45], and regulates hypothalamic modulation of the trigeminal nucleus caudalis [48]. Behaviorally, restraint stress increases rat tail-flick responses to high-doses (10 mg/kg) of the nitric oxide (NO) donor nitroglycerin (NTG) [17] and increases eye blinking and shuddering behavior in a mouse model of familial-hemiplegic migraine [15]. Additionally, a 14-day social-defeat stress exposure and 40-day chronic-variable stress protocol caused anxiety-like responses, hindpaw hypersensitivity, and increased responses to high-dose NTG

(10 mg/kg) [31]. These prior studies were limited to the effects of stress either during or in the acute time frame following exposure, not whether stress causes lasting changes that may influence responses to future events i.e. hyperalgesic priming. Hyperalgesic priming (or latent sensitization) was shown previously following repeated exposure to sumatriptan which caused priming to subsequent bright-light stress or injection of PGE₂ [4; 20; 21]; priming was also caused by application of interleukin-6 (IL-6) to the dura, which primed rodents to dural stimulation with a pH 7.0 solution [11; 13]. However, these studies did not evaluate whether stress itself causes priming and thus whether exposure to stress might influence the threshold for future attacks. Given the major contributions of stress to migraine, more work is necessary to better understand how stress contributes to the disorder.

In the studies described here, we explored whether repeated stress in mice produces hyperalgesic priming to doses of NO donors much lower than those used in previous stress models (0.1 mg/kg here compared to 10 mg/kg in prior work) and whether it also caused priming to normally subthreshold stimulation of the dura. Additionally, we evaluated whether two common migraine therapeutics, a triptan and a monoclonal antibody to calcitonin gene-related peptide (CGRP), could decrease the response to the NO donor in stress-primed mice.

Methods

Animals

Female and male ICR mice ages 6–8 weeks (Envigo; Livermore, CA) were used for most of the experiments described here. ICR mice are used as they are outbred which provides increased genetic diversity between animals over standard inbred mouse strains. A mixed strain (C57BL6 and 129J background; bred in-house at UT Dallas) were also used to determine whether findings are specific to the ICR strain (see Supplementary Figure 1). Animal weights varied between 19 g up to a maximum of 30 g. Animals were housed on a 12-hour light-dark cycle and had access to food and water *ad libitum*. Animals were allowed a minimum of 72 hours in the animal facility to acclimate to their new environment after arrival from Envigo. The stage of the female estrous cycle was determined at the end of some experiments, but no influence of cycle was observed for any findings. In all experiments, investigators were blinded to treatment groups. All procedures were conducted with prior approval of the Institutional Animal Care and Use Committee at the University of Texas at Dallas.

Measurement of facial mechanical hypersensitivity

Mice were handled for a single 5-min session at 24-hours prior to habituation to the behavior chambers. During each session of habituation animals were placed in 4 oz paper cups (Choice) for 2 hours a day for 3 consecutive days as previously described [13]. Habituation was done in the rooms where all further behavioral testing occurred to acclimate animals to the room and light conditions. von Frey testing of the periorbital skin [13] was used to assess baseline values following habituation prior to stress as well as mechanical hypersensitivity that resulted from restraint stress and drug treatments. von Frey thresholds were not measured on the days that animals were subject to restraint stress. Testing began 24 hours

after the third day of restraint unless otherwise noted. Prior to stress, mice were subjected to baseline tests of cutaneous facial sensitivity for approximately 3–4 days. Baselined animals were defined as animals that exhibited a withdrawal threshold approximately 0.5 g - 0.6 g. Filaments greater than 0.6 g were not used. Mice with a baseline threshold lower than 0.5 g at the end of 4 days were excluded from experiments. Mechanical thresholds were determined by applying von Frey filaments to the periorbital region of the face (the midline of the forehead at the level of the eyes) in an ascending/descending manner starting from the 0.07 g filament. Briefly, if an animal did not respond, increasing filament forces were applied until the 0.6 g filament was reached or until a response was observed. If the animal responded to a specific filament, decreasing filament forces were applied until the 0.008 g filament was reached or until there were no responses. A response was defined as a mouse actually removing/swiping the filament away from its face during application. All animals were numbered and randomly allocated to experimental groups by drawing from pre-labeled paper slips.

Repetitive stress paradigm

Animals were subjected to restraint stress 24 hours following baseline. Sensory threshold values were determined with von Frey filaments. Animals were placed in cylindrical tail access rodent restrainers designed for animals 15–30 g (Stoelting 51338). Animals were placed in these restraint devices so that their tail was threaded through the moveable disk and their faces project out of the hole in the acrylic front face of the tube. Animals were introduced to the tube by placing the restrainer in front of the animals on the first day of stress and guiding them into the restrainer with the animal facing the acrylic front. Once the animal was in position the tail was threaded through the moveable disk, the disk was moved toward the animal and tightened to ensure that the animal was incapable of movement. Care was taken to avoid any trauma to the mice due to injuries from moving the disk or from threading the tail. Mice were also restrained at a level that still allowed normal respiration. Animals were placed in the restraint tube so that the moveable disk faced upward and an opening on the tube was on the bottom. The animals were restrained for 2 hours a day for 3 consecutive days unless otherwise noted. Restraint stress started no earlier than 9:30 am and stress ended prior to 12:00pm in all cases to account for natural rising in corticosterone levels that have been shown occur in rodents in the afternoon starting at 1:00 pm [18]. Sham animals were left in their home cages without access to water or food to ensure that water/food deprivation alone did not contribute to the stress responses. Sham animals were kept in a separate room from stressed animals for the duration of stress. Once animals were in restraint devices, checks were made every 15–20 minutes to ensure that animals had not altered their position; if an animal altered their position, they were readjusted by the experimenter by loosening the movable disk without completely removing the animal from the restrainer.

Animal weight was taken into consideration to ensure that all animals were restrained equivalently. Animals above 34 g were not used for stress due to the maximum weight of the restrainer and animals that weighed under 22 g had a custom 3D printed 1 mm thick plastic insert fit into the restrainer. Animals weighing 18 g or less were not used for restraint stress as in pilot studies, animals at this weight were capable of exiting through the hole in the

acrylic front of the restrainer. Animals subjected to stress were not co-housed with sham animals to avoid the transfer of a stressed phenotype between mice.

Measurement of grimacing pain behavior

Grimace was performed according to previously published methods [5; 13; 35] prior to von Frey testing for all time points. Assessment of 5 characterized pain behaviors on portions of the face (orbital tightening, nose bulging, cheek bulging, flattening of ears, and flattening of whiskers). These behaviors were scored on a scale of 0–2 (0 = not present, 1 = somewhat present, 2 = clearly present).

Dural Injections of reagents/drugs

For experiments where dural stimulation was applied, dural injections were performed according to previously published methods [13]. Briefly, animals were anesthetized for < 2 minutes under isoflurane using a nose cone. While under anesthesia, stimuli were administered onto the dura through the junction of the lambdoid and sagittal sutures via a modified internal cannula (Invivo1, part #8IC313ISPCXC, Internal Cannula, Standard, 28 gauge, fit to 0.5 mm) in a volume of 5 μ l. Following injection, animals were placed back in their respective cups in the testing chamber for 1 hour prior to von Frey testing.

Drugs

Drugs applied via dural injection were made in synthetic interstitial fluid (SIF) consisting of 135 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 10 mM glucose, 1 mM MgCl₂ (pH 7.4, 310 mOsm). Solutions at pH 7.0 were made using SIF and adjusting pH with HCl. Sodium Nitroprusside (Sigma Aldrich) and sumatriptan (suma; Sigma Aldrich) were dissolved in sterile 1 X PBS. Drug concentrations and administration routes are reported in Table 1.

Statistical analysis

All data here are shown as mean $\pm$ SEM. These data were analyzed among groups for each time point via two-way repeated measures (RM) ANOVA and then followed by Bonferroni post hoc analysis. All graphs were analyzed in two phases: 1. A two-way RM ANOVA was done on the initial acute phase starting from baseline to the return to baseline after stress. 2. Another two-way RM ANOVA was done only on the time points during the priming phase. Data was analyzed using Prism 8.0 (Graph-Pad Software). Significance was set at $p < 0.05$ for all analyses. The resulting F and P values are reported in Table 2.

Results

Repeated stress using restraint causes facial hypersensitivity and priming to an NO donor in mice

Prior studies using restraint as a model of stress show wide variations in protocols for mice with some using 30 minutes for 1 day [41] while others use 1 hour a day for 2 weeks [14], 2 hours a day for 3 days [33] or 5 hours a day for 14 days [52]. Due to this variation between stress protocols, we first aimed to determine the amount of stress required to produce facial

hypersensitivity in mice. Because the durations of a single session of stress are varied, we decided to use 2-hour sessions as an intermediate duration of each stress session. Because consecutive days of stress are more likely to trigger attacks in migraine patients than is a single day, we decided to test this stress across a 3 day paradigm [33; 38]. Males and females were restrained for 2 hours for 3 consecutive days, and von Frey testing was then conducted 24 hours after the end of the final stress session. The 24-hour time point was chosen as the earliest von Frey test after stress in order to avoid the potential of additional stress from behavioral testing as well as stress-induced acute analgesia, both of which have been documented immediately following restraint in rats [26]. Three consecutive days of stress produced significant facial hypersensitivity in both males (Fig. 1A) and females (Fig. 1B) that lasted from 24 hours following stress out to between 7 and 10 days in both males and females. Mice returned to baseline withdrawal thresholds typically by 14 days following the last session of stress. In a few instances, animals did not return to baseline until longer than 14 days, with a few animals even remaining hypersensitive out to 21 days after stress (data not shown as these animals were not used for the figures presented in this manuscript). In order to measure spontaneous non-evoked pain in these animals, we assessed grimace scores in female mice following stress. Females showed significant grimacing that lasted from 24 hours post stress to 7 days' post stress (Fig. 1C).

Prior studies have shown that in contrast to acute restraint, repeated restraint stress in rats is capable of inducing hypersensitivity to nitroglycerin in the tail-flick test [17]. In order to determine whether these repeated stress exposures caused priming to the NO-donor, animals were injected with the normally subthreshold dose of 0.1 mg/kg SNP following a return to baseline. Both male and female mice that received SNP had significantly reduced facial withdrawal thresholds at 1 and 3 hours following SNP injection. Female mice were also observed for facial grimace responses following SNP injection. While females that received the NO donor showed significantly reduced withdrawal thresholds, these animals showed no significant grimacing following SNP (Fig. 1C).

To ensure that stress responses to this paradigm were maintained across strains, mice with a mixed background of both C57BL6 and 129J were exposed to stress (Supplementary fig. 1). Both male and female mice of this background responded to initial stress from 24 hours following stress out to 14 days following stress. Additionally, male and female mice were primed to respond to 0.1 mg/kg SNP. These data demonstrate that this stress paradigm is not specific to a single mouse strain.

As previously mentioned, stress across consecutive days is more likely to cause migraine in migraine patients when compared with 1 day of stress [38]. In order to explore the potential differences in repeated sessions of stress in this model male and female mice underwent 1 session of 2-hour restraint stress. One 2-hour session of restraint stress failed to produce significantly reduced facial withdrawal thresholds in both male (Fig. 2A) or female mice (Fig 2B). Mice were tested out to 5 days following stress to ensure that delayed hypersensitivity did not occur. While the stress produced no acute facial hypersensitivity, our prior work demonstrated that a single stimulus (dural stimulation with IL-6 or CGRP) can cause priming to subthreshold doses of a common migraine trigger i.e. an NO donor [5; 12] so it was also important to test for potential priming following a single stress exposure. Prior

work using the tail-flick test in rats found that 1 session of acute stress caused stress-induced analgesia and resulted in no response to the NO-donor nitroglycerin [17]. We thus tested whether acute stress would cause priming to a subthreshold dose of the NO-donor sodium nitroprusside (SNP). Following the fifth day of testing, neither males nor females that received 0.1 mg/kg SNP showed facial hypersensitivity. These data demonstrate that a single session of stress is not sufficient to cause acute hypersensitivity nor is it sufficient to induce priming to SNP. The 3-day stress paradigm was used for all subsequent experiments.

The previous experiments show that 3 days of restraint stress cause priming to SNP when given relatively shortly after mice return to baseline. In order to determine whether stress-induced priming is extinguished over time, animals underwent the 3-day stress protocol and were tested with von Frey filaments until they returned to baseline at approximately 14 days after stress. Following a return to baseline, animals were then tested once weekly until they reached 35 days post stress (i.e. 35 days following the last session of restraint). Animals were then injected with 0.1 mg/kg SNP at this late time point. Neither males (Fig. 3A) nor females (Fig. 3B) that received SNP at 35-days post stress showed reduced facial withdrawal thresholds when compared with controls. These data indicate that while repeated stress causes priming to SNP, the primed state is not permanent.

Efficacy of human migraine therapeutics against priming to SNP following stress

CGRP has been strongly implicated in the pathology of migraine, and the use of monoclonal antibodies against CGRP and the CGRP receptor have now been demonstrated to be efficacious in the preventive-treatment of migraine [22; 23; 47; 49]. To determine the possible role for CGRP in stress-induced hypersensitivity, both male and female animals underwent the 3-day restraint stress protocol and were tested until they returned to baseline withdrawal thresholds. At 24 hours following return to baseline, animals received 10 mg/kg i.p. of ALD405, a monoclonal antibody against CGRP, or an isotype control. At 24 hours after dosing of the antibody, 0.1 mg/kg SNP was given systemically as described above. ALD405 significantly blocked the effects of SNP in females (Fig. 4A) from 1–72 hours following SNP injection. Whereas males (Fig. 4B) that received ALD405 only experienced decreased mechanical sensitivity at 48 hours following SNP administration. Females that received the active form of the antibody showed no significant differences from controls in response to SNP. These findings show that a monoclonal antibody against CGRP is capable of blocking the primed response to SNP in a sex-specific manner. This suggests that not only does CGRP have a prominent role in a stress induced priming to NO donors, but that this role may be sexually dimorphic.

Sumatriptan, as well as other triptans, are among the most commonly used acute migraine therapeutics. It was thus important to determine whether this stress priming model is sensitive to the effects of sumatriptan. To test this question, we examined the efficacy of sumatriptan against the hypersensitivity caused by SNP in stress-primed mice. Female mice underwent the restraint stress protocol and were allowed to return to baseline as described for the prior experiments. At 24-hours following return to baseline, mice received either vehicle (1X PBS), 0.1 mg/kg SNP, 0.6 mg/kg sumatriptan (the standard dose used throughout preclinical migraine studies [10; 20; 42; 46]), or a co-injection of SNP and

sumatriptan (Fig. 5). Animals that received only sumatriptan were not significantly different from baseline at any time point. As in prior experiments, animals that received SNP showed significantly reduced withdrawal thresholds when compared with controls out to 24 hours. Animals that received a co-injection of SNP and sumatriptan exhibited significantly reduced withdrawal thresholds when compared to either control animals or those that received sumatriptan alone out to 3 hours following injection. There were no significant differences between the SNP + sumatriptan group and animals that received only SNP at any time point. These data demonstrate that repeated stress leads to a state of priming where sumatriptan lacks efficacy against subsequent exposure to an NO donor.

Repeated stress primes mice to dural stimulation with a subthreshold pH

These studies demonstrate that repeated stress primes mice to subthreshold doses of NO-donors. However, it is not clear from these studies whether stress and NO donors have sensitizing actions within the meninges as both stimuli are non-specific to any location within the body. We thus investigated whether repeated restraint stress could prime animals to a direct dural stimulus. Female mice were subject to the repeated stress protocol and allowed to return to baseline withdrawal thresholds as in the prior experiments. At 24 hours after return to baseline, mice received dural injections of either physiological pH of 7.4 or a slightly decreased, but normally non-noxious pH 7.0, the latter a stimulus that we have previously shown animals become primed to by prior dural application of IL-6 or CGRP[5; 12]. Stressed females that received dural pH 7.0 experienced significant allodynia at 1 hour, 3hours, and 24 hours post injection when compared with stressed animals that received pH 7.4 and control non-stressed animals that received either dural pH 7.0 and pH 7.4 (Fig. 6A). In addition, these animals showed significant grimacing in response to stress out to 7 days (Fig. 6B), however none of the animals showed any significant grimace in response to any secondary stimulus. These data demonstrate that repeated stress primes mice to both systemic migraine triggers as well as direct stimuli applied to the dura.

Discussion

The mechanisms by which stress contributes to migraine are poorly understood. Better understanding of these mechanisms may lead to insights for new therapeutic targets as well as other potential approaches. Here we show that repeated restraint stress results in significant facial hypersensitivity and grimace in mice, starting approximately 24 hours after the end of stress, the earliest time point tested. Repeated stress also primes mice to subthreshold doses of the nitric oxide donor SNP as well as subthreshold dural stimulation with pH 7.0. Mice remained primed to SNP for 2 weeks following stress but priming was no longer present by 5 weeks. Responses to subthreshold doses of SNP in primed animals were blocked by a CGRP monoclonal antibody, implicating CGRP in the mechanisms that contribute to low-dose NO donors responses in the primed state. This was not the case when sumatriptan was used, demonstrating that priming due to stress may lead to a “triptan-unresponsive state.” Together, these data support the use of a stress priming model to help further study the mechanisms by which stress contributes to migraine. Importantly, this model can be induced without significant tissue injury.

Calcitonin gene-related peptide (CGRP) has long been implicated in the pathology of migraine [24; 30; 36]. The observation that the response to SNP following repeated stress is blocked by a CGRP monoclonal antibody (although to a greater extent in females) suggests that exposure to a subthreshold dose of SNP in the primed state is similar to migraine attacks. However, sumatriptan had no effect on SNP-donor induced responses in primed mice. This is in contrast to humans, where sumatriptan reduces nitroglycerin-induced headaches [1; 29]. In rodent studies, both sumatriptan and the monoclonal antibody ALD405 were shown to reduce pain from repeated glyceryl trinitrate administration [16]. In other rodent studies, sumatriptan was not efficacious in blocking NO donor induced facial hypersensitivity when given prior to repeated NO-donor administration across 5 days [19]. Neither of these studies used stress however. The inability of sumatriptan to prevent NO-donor mediated hypersensitivity following repeated stress may be able to provide insight into mechanisms that are present in the approximately 40% of migraine patients that take oral triptans and do not experience relief [25] and also how stress may contribute to triptan response/non-response. Further, this model may be helpful in identifying mechanisms by which NO donors contribute to migraine since only primed animals respond to low-dose SNP (thus mimicking the human observation that NO donors do not trigger attacks in controls). Our data show that the mechanism of NO in this model is dependent on CGRP since ALD405 blocked the response to SNP in primed mice. This suggests that stress increases NO donor-evoked CGRP release. Whether this is due to increased sensitivity to NO or increased CGRP expression is not clear but prior studies found increased CGRP expression or binding following stress in several locations throughout the body [28; 51; 56]. Since the increased sensitivity to SNP is not permanent i.e. it was not present 5 weeks following the end of the stress paradigm, this model may also be valuable for identifying mechanisms by which sensitivity to the effects of stress are extinguished.

Migraine affects women disproportionately to men; mechanisms underlying this dimorphism are not known. There were no sex differences in this study in the acute response to stress, nor were there sex differences in the response to SNP given after the resolution of the stress-induced behaviors. While we did not test stress-induced priming to dural pH 7.0 in both sexes, our prior work using dural IL-6 as a priming agent did not find any differences between males and females in the primed response to dural pH 7.0 [13]. We also did not test whether there are sex differences in the efficacy of sumatriptan against the SNP response in stress-primed mice, but there is little prior evidence to suggest that sex differences in the efficacy of sumatriptan exist in animals or humans. There are sex differences in the ability of sumatriptan to cause acute hyperalgesia or to cause priming [3; 4], but not in its ability to block behavior caused by other stimuli such as SNP. There was a sexually-dimorphic response observed here for efficacy of ALD405. We have recently shown that female mice are more sensitive than males to the pain-promoting effects of CGRP in the meninges [5]. Consistent with the prior finding of female-specific CGRP effects, ALD405 was more effective in blocking responses to SNP following stress in females than in males. While anti-CGRP therapeutics are effective in many migraine patients, both female and male, factors that predict efficacy are not currently known. Additionally, mechanisms by which stress and NO donors contribute to migraine are poorly understood. While far more investigation is needed, our work is a substantial advance and suggests that stress combined with NO donors

may act through CGRP-dependent mechanisms in females but not in males, that stress may increase NO donor-evoked CGRP release selectively in females, or that stress may exacerbate the hypersensitivity of female blood vessels to vasodilators [34]. In contrast, studies using traumatic-brain injury (TBI) in rodents have found greater efficacy of the CGRP monoclonal antibody in males compared to females [8; 9]. These latter studies highlight the potentially complex actions of CGRP in headache disorders supporting the need moving forward to examine all CGRP-based effects in both sexes. The model system that we have developed, and similar model systems, may start to draw mechanistic connections between psycho-social migraine findings, precipitating events like stress or TBI, and specific molecular mechanisms that can predict who will respond to certain types of therapeutics, like CGRP-targeting drugs or triptans.

How and where CGRP and NO contribute to migraine pathology is still unclear. We propose that the repeated stress-induced priming model is a valuable tool in addressing this question. In the case CGRP, the antibodies are highly unlikely to cross the blood-brain barrier and thus the location where this drug blocks the effect of SNP is within the peripheral compartment. Since NO freely diffuses throughout the body, there could be many potential locations where it causes behavioral responses at low doses in stressed mice (one of which is trigeminal ganglion neurons [7]). Nonetheless, we show that stress causes priming to stimulation of the dura, an important location for the development of headache. These data suggest that CGRP and/or NO could be acting in this tissue, but more work is needed to better address this question. Whether stress would cause sensitized responses to direct dural application of either CGRP or NO is not yet clear, but is the subject of future studies.

The findings described here demonstrate that repeated stress causes a grimace response in mice, lasting out to 7-days following the end of the stress protocol. Grimace is typically used in preclinical studies as a measure of the affective component of pain [2; 35; 37; 55]. The presence of grimace following stress suggests that a similar affective state is present to those that occur following surgical incisions and nerve injuries. The combination of grimace with cutaneous hypersensitivity is suggestive of pain, but it is naturally difficult to determine the exact state of the mice under these conditions. Grimace may nonetheless capture the state of unpleasantness that is caused by stress and thus offers a valuable additional endpoint to use in these studies. In contrast to the acute post-stress phase, there was no grimace response to SNP in the primed phase in these studies. While surprising, this may indicate that SNP causes cutaneous hypersensitivity (a characteristic present in migraine patients, particularly those with the greatest risk of progression to chronic migraine [39; 40]), but not “pain” or “unpleasantness”. Alternatively, the lack of grimace may rather be due to compensatory suppression of grimace over time during the early post-stress phase. Suppression of grimace over time as a protective mechanism to hide the presence of pain from predators has been suggested to occur in rodent pain models [43] and may occur here during the initial responses to stress. If this is indeed the case, mice may no longer exhibit a grimace response when presented with a subsequent noxious challenge as they have previously adapted to the presence of pain by suppressing grimace.

The data presented here support the use of repeated stress-induced priming as a new preclinical model of migraine in which to further investigate the mechanisms of the disorder

and their regulation by stress. It may also be a valuable tool to investigate efficacy of novel therapeutics, especially those that are likely to act within mechanisms or pathways regulated by stress. Finally, it offers the opportunity to study conditions related to migraine in the absence of tissue injury. While several preclinical behavioral models of migraine currently exist, it is unlikely that any single model will accurately capture the complexity that is human migraine. This model may be an important addition to the current list of preclinical tools, and may be able to capture aspects of the disorder not currently represented in the currently-available models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The repeated stress paradigm primes male and female mice to subthreshold doses of a NO donor.

Facial withdrawal thresholds were measured in male (A) and female (C) mice following repeated restraint stress. Upon returning to baseline thresholds at 14 days following the final day of stress, stressed mice were administered either 0.1 mg/kg SNP (n = 8 males, n = 8 females) or vehicle (n = 10 males, n = 6 females). All control mice were given SNP (n = 8 males, n = 7 females). (†) denotes statistical significance between stressed mice that received SNP and control mice; (*) denotes statistical significance between stressed mice that received vehicle and control mice. (§) denotes significance between stressed mice that received SNP and stressed mice that received vehicle. In a separate cohort of mice, grimace responses (B, D) were measured for stressed (n = 15) and control (n = 13) mice following acute stress and administration of 0.1 mg/kg SNP. Two-way RM ANOVA followed by Bonferroni multiple comparison analysis indicated significant differences between stressed and control mice following SNP in both males and females. Data are represented as means ±

SEM. $\dagger, \$p < 0.05$, $\dagger\dagger, \$\$p < 0.01$, $\dagger\dagger\dagger, ***p < 0.001$, $\dagger\dagger\dagger\dagger, ****p < 0.0001$. See Table 2 for additional results of analysis.

Figure 2. Single-restraint stress does not produce facial allodynia or priming to SNP.

(A) Facial withdrawal thresholds of male mice following a single session of stress and administration of either SNP (n=10) or vehicle (n=7) after 5 days of testing or naïve mice (n=9). (B) Facial withdrawal thresholds of female mice following a single session of stress and administration of either SNP (n=7) or vehicle (n=6) after 5 days of testing or naïve mice (n=6). Two-way RM with Bonferroni multiple comparison analysis indicates there was no statistical difference among control mice and all cohorts that were stressed in both the acute phase and the priming phase (mean $\pm$ SEM). See table 2 for additional statistical analysis.

Figure 3. Repeated stress induces transient priming to SNP in male and female mice.

Facial withdrawal thresholds were measured in (A) male and (B) female mice following acute stress ($n = 8$ male, 8 female). Following the final day of stress, animals were tested out to 35 days and received either 0.1 mg/kg SNP ($n = 8$ males, $n = 9$ females) or vehicle ($n = 9$ males, $n = 8$ females). All controls received SNP. (†) denotes statistical significance between stressed mice will receive SNP and control mice. (*) denotes statistical significance between stressed mice that will receive vehicle and control mice. (§) denotes statistical significance between stressed mice that received vehicle and stressed mice that received SNP. Two-way RM ANOVA with Bonferroni multiple comparison analysis indicated a significant difference between stressed and control mice in the acute phase, but revealed no significant differences following administration of SNP. Data are represented as means $\pm$ SEM.

* $p < 0.05$, ††††, **** $p < 0.0001$. See Table 2 for additional results of analysis.

Figure 4. Effects of CGRP monoclonal antibodies in repeated stress induced priming to SNP in mice.

Facial withdrawal thresholds were measured in male (A) and female (B) mice following acute stress ($n = 10$ males, $n = 7$ females). 15 days following the final day of stress, animals received either the anti-CGRP antibody ALD405 ($n = 10$ males, $n = 8$ females) or an isotype control ($n = 9$ males, $n = 9$ females). 24 hours following administration of antibody, all animals received 0.1 mg/kg SNP. In the acute phase, (†) denotes statistical significance between control mice and stressed mice that will receive ALD405 and SNP. (*) denotes statistical significance between control mice and stressed mice that will receive isotype control IgG. In the priming phase, (§) denotes statistical significance between stressed mice that received ALD405 and those that received the isotype control prior to SNP. Two-way ANOVA followed by Bonferroni multiple comparison analysis revealed significant differences in the priming phase between female stressed mice that received ALD405 and those that received the isotype. Statistical significance between these groups was observed in males at 48 hours following SNP. Data are represented as means $\pm$ SEM. § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$, ††††, **** $p < 0.0001$. See Table 2 for additional results of analysis.

Figure 5. Sumatriptan does not block SNP responses in stress-primed mice.

Facial withdrawal thresholds of female mice either naïve (n=8) or following acute stress and administration of either SNP (0.1 mg/kg; n=8), suma (0.6 mg/kg; n=7), or a coadministration of suma and SNP (n=7). In the acute phase, (*) denotes significance between control mice and stressed mice that will receive SNP; (^) denotes significance between control mice and stressed mice that will receive suma; (†) denotes significance between control mice and stressed mice that will receive suma and SNP. For 24hr-7DPS in the acute phase, the statistical significance is the same for each time point. Two-way RM with Bonferroni multiple comparison analysis indicates a statistical difference among control mice and all cohorts that were stressed. In the priming phase there was no statistical difference detected between stressed mice that received SNP and those that received suma and SNP (mean $\pm$ SEM, ^^ $p < 0.01$, *** $p < 0.001$, ****,^^^,†††† $p < 0.0001$). See table 2 for additional statistical analysis.

Figure 6. Repeated stress primes female mice to decreased dural pH.

(A) Facial withdrawal thresholds of female mice following acute stress and priming to dural pH 7.0 (n=12) or 7.4 (n=12) and control mice given pH 7.0 (n=12) and pH 7.4 (n=11). (B) Grimace scores of mice that were first stressed and then administered either pH 7.0 (n=8) or pH 7.4 (n=8) or mice that were naïve and then administered either pH 7.0 (n=8) or pH 7.4 (n=8). For the acute phase, (*) denotes significance between stressed and control mice that will receive dural pH 7.0 ; (^) denotes significance between stressed and control mice that will receive dural pH 7.4. For the priming phase, (†) denotes significance between stressed mice that received pH 7.0 and stressed mice that received pH 7.4. Dural pH stimuli are given at the time point indicated by the arrows in A; prior to dural pH injections, animals were only exposed to either stress or control. Two-way ANOVA RM with Bonferroni multiple comparison analysis indicate a significant difference between stressed and control mice and stressed mice that received pH 7.0 compared to stressed mice that received pH 7.4 (mean $\pm$ SEM, * $p < 0.05$, †† $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ****^ $p < 0.0001$, †††† $p < 0.0001$). See table 2 for additional statistical analysis.

Table 1

Drugs	Source	Doses	Administration
SNP	Sigma-Aldrich	0.1 mg/kg	Intraperitoneal
Sumatriptan	Bachem	0.6 mg/kg	Intraperitoneal
ALD405	Alder Biopharmaceuticals	10 mg/kg	Intraperitoneal
Isotype Control	Alder Biopharmaceuticals	10 mg/kg	Intraperitoneal

Table 2.

Statistical Analyses Results

Figure	Analysis	Statistics
Fig. 1A	Two-way repeated measure ANOVA	
BL-BL	Interaction Factor	$F_{(10, 115)}=12.04, p<0.0001$
	Time Factor	$F_{(3,008, 69,19)}=50.31, p<0.0001$
	Treatment Factor	$F_{(2, 23)}=28.16, p<0.0001$
	Bonferroni's multiple comparisons between treatments (see figure legend)	
Fig. 1A	Two-way repeated measure ANOVA	
SNP 1–24hr	Interaction Factor	$F_{(4, 46)}=3.862, p=0.0087$
	Time Factor	$F_{(1,854, 42,63)}=6.911, p=0.0031$
	Treatment Factor	$F_{(2, 23)}=1.685, p=0.2075$
	Bonferroni's multiple comparisons between treatments (see figure legend)	
Fig. 1B	Two-way repeated measure ANOVA	
BL-BL	Interaction Factor	$F_{(10, 89)}=36.19, p<0.0001$
	Time Factor	$F_{(2,822, 50,23)}=180.0, p<0.0001$
	Treatment Factor	$F_{(2, 18)}=149.7, p<0.0001$
	Bonferroni's multiple comparisons between treatments (see figure legend)	
Fig. 1B	Two-way repeated measure ANOVA	
SNP 1–24hr	Interaction Factor	$F_{(6, 54)}=3.952, p=0.0024$
	Time Factor	$F_{(1,799, 32,37)}=2.446, p=0.1074$
	Treatment Factor	$F_{(2, 18)}=14.38, p=0.0002$
	Bonferroni's multiple comparisons between treatments (see figure legend)	
Fig. 1C	Two-way repeated measure ANOVA	
BL-BL	Interaction Factor	$F_{(5, 130)}=35.53, p<0.0001$
	Time Factor	$F_{(2,574, 66,93)}=42.04, p<0.0001$
	Treatment Factor	$F_{(1, 26)}=124.6, p<0.0001$
	Bonferroni's multiple comparisons between treatments (see figure legend)	
Fig. 1C	Two-way repeated measure ANOVA	
SNP 1–24hr	Interaction Factor	$F_{(3, 78)}=1.571, p=0.2032$
	Time Factor	$F_{(2,6, 67,60)}=7.452, p=0.0004$
	Treatment Factor	$F_{(1, 26)}=4.448, p=0.0447$
	Bonferroni's multiple comparisons between treatments (see figure legend)	
Fig. 2A	Two-way repeated measure ANOVA	
	Interaction Factor	$F_{(12, 138)}=1.608, p=0.0961$
	Time Factor	$F_{(3,257, 74,91)}=4.811, p=0.0032$
	Treatment Factor	$F_{(2, 23)}=0.1607, p=0.8525$
	Bonferroni's multiple comparisons between treatments (see figure legend)	
Fig. 2B	Two-way repeated measure ANOVA	
	Interaction Factor	$F_{(12, 108)}=0.7945, p=0.6652$

Figure	Analysis	Statistics	
		Time Factor	Treatment Factor
	Time Factor	$F_{(3,763, 67,74)}=0.9845, p=0.4186$	
	Treatment Factor	$F_{(2, 18)}=2.249, p=0.1343$	
	Bonferroni's multiple comparisons between treatments (see figure legend)		
Fig. 3A	Two-way repeated measure ANOVA		
BL-35D	Interaction Factor	$F_{(16, 176)}=33.03, p<0.0001$	
	Time Factor	$F_{(4,232, 93,11)}=144.2, p<0.0001$	
	Treatment Factor	$F_{(2, 22)}=170.6, p<0.0001$	
	Bonferroni's multiple comparisons between treatments (see figure legend)		
Fig. 3A	Two-way repeated measure ANOVA		
SNP1-72hr	Interaction Factor	$F_{(6, 66)}=1.526, p=0.1834$	
	Time Factor	$F_{(2,334, 51,35)}=0.7346, p=0.5043$	
	Treatment Factor	$F_{(2, 22)}=0.2865, p=0.7537$	
	Bonferroni's multiple comparisons between treatments (see figure legend)		
Fig. 3B	Two-way repeated measure ANOVA		
BL-35D	Interaction Factor	$F_{(18, 198)}=16.84, p<0.0001$	
	Time Factor	$F_{(4,475, 98,44)}=76.59, p<0.0001$	
	Treatment Factor	$F_{(2, 22)}=78.94, p<0.0001$	
	Bonferroni's multiple comparisons between treatments (see figure legend)		
Fig. 3B	Two-way repeated measure ANOVA		
SNP 1-72hr	Interaction Factor	$F_{(8, 88)}=0.8713, p=0.5439$	
	Time Factor	$F_{(2,849, 62,69)}=2.019, p=0.1233$	
	Treatment Factor	$F_{(2, 22)}=0.5833, p=0.5664$	
	Bonferroni's multiple comparisons between treatments (see figure legend)		
Fig. 4A	Two-way repeated measure ANOVA		
BL-14D	Interaction Factor	$F_{(20, 130)}=59.77, p<0.0001$	
	Time Factor	$F_{(3,744, 97,34)}=222.8, p<0.0001$	
	Treatment Factor	$F_{(2, 26)}=392.2, p<0.0001$	
	Bonferroni's multiple comparisons between treatments (see figure legend)		
Fig. 4A	Two-way repeated measure ANOVA		
SNP 16D-72hr	Interaction Factor	$F_{(10, 130)}=3.467, p=0.0005$	
	Time Factor	$F_{(3,448, 89,64)}=15.26, p<0.0001$	
	Treatment Factor	$F_{(2, 26)}=10.56, p=0.0004$	
	Bonferroni's multiple comparisons between treatments (see figure legend)		
Fig. 4B	Two-way repeated measure ANOVA		
BL-14D	Interaction Factor	$F_{(10, 105)}=76.44, p<0.0001$	
	Time Factor	$F_{(3,171, 66,58)}=408.3, p<0.0001$	
	Treatment Factor	$F_{(2, 21)}=278.4, p<0.0001$	
	Bonferroni's multiple comparisons between treatments (see figure legend)		
Fig. 4B	Two-way repeated measure ANOVA		

Figure	Analysis	Statistics
SNP 16D-72hr	Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments (see figure legend)	$F_{(10, 105)}=5.245, p<0.0001$ $F_{(3,279, 68.86)}=6.289, p=0.0005$ $F_{(2, 21)}=17.62, p<0.0001$
Fig. 5 BL-14D	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments (see figure legend)	$F_{(18, 156)}=28.96, p<0.0001$ $F_{(3,536, 91.93)}=264.0, p<0.0001$ $F_{(3, 26)}=341.9, p<0.0001$
Fig. 5 SNP+Suma 1-72hr	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments (see figure legend)	$F_{(12, 108)}=4.287, p<0.0001$ $F_{(3,430, 92.62)}=8.103, p<0.0001$ $F_{(27, 108)}=, p=0.0105$
Fig. 6A BL-14D	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments (see figure legend)	$F_{(12, 172)}=26.66, p<0.0001$ $F_{(3,108, 133.6)}=94.04, p<0.0001$ $F_{(3, 43)}=64.93, p<0.0001$
Fig. 6A pH 1-24hr	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments (see figure legend)	$F_{(6, 86)}=3.244, p=0.0064$ $F_{(1,915, 82.33)}=1.063, p=0.3478$ $F_{(3, 43)}=27.50, p<0.0001$
Fig. 6B BL-14D	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments (see figure legend)	$F_{(12, 112)}=14.27, p<0.0001$ $F_{(2,616, 73.25)}=55.79, p<0.0001$ $F_{(3, 28)}=60.03, p<0.0001$
Fig. 6B pH 1-24hr	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments (see figure legend)	$F_{(6, 56)}=1.494, p=0.1970$ $F_{(1,929, 54.01)}=5.457, p=0.0075$ $F_{(3, 28)}=2.769, p=0.0478$
Suppl. Fig. A. BL-14D	Two-way repeated measure ANOVA Interaction Factor Time Factor Treatment Factor Bonferroni's multiple comparisons between treatments (see figure legend)	$F_{(7, 63)}=12.15, p<0.0001$ $F_{(2,811, 25.30)}=11.19, p<0.0001$ $F_{(1,9)}=80.78, p<0.0001$

Figure	Analysis	Statistics
Suppl. Fig. A.	Two-way repeated measure ANOVA	
SNP 1–72hr	Interaction Factor	$F_{(4, 36)}=1.241, p=0.3110$
	Time Factor	$F_{(2, 242, 20.18)}=1.263, p=0.3075$
	Treatment Factor	$F_{(1, 9)}=2.299, p=0.0011$
	Bonferroni's multiple comparisons between treatments (see figure legend)	
Suppl. Fig. B.	Two-way repeated measure ANOVA	
BL-14D	Interaction Factor	$F_{(7, 70)}=7.490, p<0.0001$
	Time Factor	$F_{(3, 211, 32.11)}=9.698, p<0.0001$
	Treatment Factor	$F_{(1, 10)}=55.15, p<0.0001$
	Bonferroni's multiple comparisons between treatments (see figure legend)	
Suppl. Fig. B.	Two-way repeated measure ANOVA	
SNP 1–72hr	Interaction Factor	$F_{(4, 40)}=7.378, p=0.0002$
	Time Factor	$F_{(2, 829, 28.29)}=2.544, p=0.0791$
	Treatment Factor	$F_{(1, 10)}=31.81, p=0.0002$
	Bonferroni's multiple comparisons between treatments (see figure legend)	