UNPREDICTABLE SOUND STRESS MODEL CAUSES MIGRAINE-LIKE BEHAVIORS IN MICE WITH SEXUAL DIMORPHISM

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Abstract

Background and purpose Migraine represents one of the major causes of disability worldwide and is more prevalent in women, it is also related to anxiety symptoms. Stress is a frequently reported trigger in migraine patients, such as sound stress, but the underlying mechanisms are not fully understood. However, it is known that patients with migraine have higher levels of plasma inflammatory cytokines and calcitonin gene-related peptide (CGRP). Stress mediated by unpredictable sound is already used as a model of painful sensitization, but migraine-like behaviors and sexual dimorphism have not yet been evaluated. This study characterized the nociception and anxiety-related symptoms after the induction of unpredictable sound stress in mice. Experimental approach C57BL/6 mice (20-30 g) were exposed to unpredictable sound stress for 3 days. Mainly, after 7 days of the last stress session mice developed hind paw, periorbital mechanical allodynia, grimacing pain behavior, anxiety-like, and reduced exploratory behavior. Key results These nociceptive and behavioral alterations detected in this model were shown mostly in female stressed mice. Besides, 7th-day post-stress nociception, these behaviors were consistently abolished by CGRP receptor antagonist olcegepant (BIBN4096BS, 100mg/kg by intraperitoneal route) until 3 h after treatment in stressed mice. In addition, we demonstrated an increase in levels of IL-6 and TNF-α and CGRP levels in stressed mice plasma, with female with higher levels when compared to male mice. Conclusions and implications This stress paradigm allows further preclinical investigation of mechanisms contributing to migraine pain, which appear to be distinct in male and female mice.

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Author's contributions. FTV and GT: designed the research (research plan formulation, and study oversight); FTV, PR, JF, NA, MF, AS, GP, MP, GB, RN, PG and JF performed experiments, analyzed and interpreted data. FTV and GT interpreted data and contributed to the writing of the manuscript. All authors read and approved the final manuscript.

Data availability. The data that support the findings of this study are available from the senior author (gabrielatrevisansantos@gmail.com) upon reasonable request.

Bullet point summary

What is already known:

* Stress is a common trigger to a migraine

* CGRP antagonist was effective to reduce periorbital allodynia in different models of migraine-like pain

What this study adds:

* unpredictable sound stress model in mice in addition to causing periorbital/hind paw mechanical allodynia, lead anxiety-like symptoms.

* female mice after stress induction presented higher levels of nociceptive parameters and anxiety-like symptoms

* plasmatic levels of inflammatory cytokines (IL-6 and TNF-α) and CGRP were increased in ST mice, particularly in female ST mice.

Clinical significance:

* CGRP antagonist had a antinociceptive effect in male and female ST mice and reduced the anxiety-like behavior

* stress priming model to the study of the mechanisms by which stress contributes to migraine-related pain and anxiety-like symptoms

ABSTRACT

Background and purpose

Migraine represents one of the major causes of disability worldwide and is more prevalent in women, it is also related to anxiety symptoms. Stress is a frequently reported trigger in migraine patients, such as sound stress, but the underlying mechanisms are not fully understood. However, it is known that patients with migraine have higher levels of plasma inflammatory cytokines and calcitonin gene-related peptide (CGRP). Stress mediated by unpredictable sound is already used as a model of painful sensitization, but migraine-like behaviors and sexual dimorphism have not yet been evaluated. This study characterized the nociception and anxiety-related symptoms after the induction of unpredictable sound stress in mice.

Experimental approach

C57BL/6 mice (20-30 g) were exposed to unpredictable sound stress for 3 days. Mainly, after 7 days of the last stress session mice developed hind paw, periorbital mechanical allodynia, grimacing pain behavior, anxiety-like, and reduced exploratory behavior.

Key results

These nociceptive and behavioral alterations detected in this model were shown mostly in female stressed mice. Besides, 7th-day post-stress nociception, these behaviors were consistently abolished by CGRP receptor antagonist olcegepant (BIBN4096BS, 100mg/kg by intraperitoneal route) until 3 h after treatment in stressed mice. In addition, we demonstrated an increase in levels of IL-6 and TNF-α and CGRP levels in stressed mice plasma, with female with higher levels when compared to male mice.

Conclusions and implications

This stress paradigm allows further preclinical investigation of mechanisms contributing to migraine pain, which appear to be distinct in male and female mice.

INTRODUCTION

Migraine is a pain disorder characterized by atypical neurological symptoms that occur in the absence of tissue injury. It is classified as a type of primary headache, which represents one of the highest causes of disability worldwide. Migraine has a major female prevalence under 50 years and has a challenging treatment [23]. Also, it has been showed that migraine may be related to psychiatric disorders, including anxiety [27]. The migraine attacks are elicited by a variety of agents, such as stress induction and others [30]. Stress is the most common trigger reported in migraine patients, also it can raise the duration of the crisis and the induction of chronic migraine [30,53].

Thus, different stress models have been used to induce migraine-like behaviors (periorbital/hind paw mechanical allodynia, grimacing pain behavior, and anxiety-related symptoms) in mice to better study the mechanisms involved in this painful disease. These previous studies used acute or chronic stress caused by repetitive restrain stress paradigm, social defeat, chronic variable, and early life stress [8,20]. Besides, sound stress seems to be a relevant mediator of headache induction in patients [26]. Previous studies using an unpredictable sound stress model have observed the development of plantar nociception (hind paw allodynia and chemical hyperalgesia) in male rats [32]. Nevertheless, the development of migraine-like behaviors using this stress model has not been evaluated yet.

The relationship among calcitonin gene-related peptide (CGRP) signaling and stress-mediated migraine-like behaviors has been investigated in only one preclinical study [8]. CGRP has been implicated in the pathology of migraine for several decades [31]. Recent clinical studies have further confirmed a protagonist of CGRP in migraine due the use of inhibitors of CGRP signaling [24]. CGRP antagonist (olcegepant, BIBN4096BS) was effective to reduce periorbital allodynia in different models of migraine-like pain [16]. Besides, CGRP injection to the trigeminovascular system causes periorbital mechanical allodynia and anxiety like-behavior, that was prolonged in female rats [5].

CGRP may lead to the release of pro-inflammatory cytokines [44], and the levels of interleukine-6 (IL-6)

and tumor necrosis factor alpha (TNF-α) were increased in the plasm of migraine patients [14]. In fact, the application of IL-6 in dura mater and cisterna magna causes both periorbital and hind paw cutaneous hypersensitivity [9]. Also, TNF-α injection was able to sensitize the dural meningeal nociceptors [57]. Previously, skeletal muscle hyperalgesia caused by unpredictable sound stress model was reduced by the treatment with antisense targeting the IL-6 or TNF-α receptors [17].

Thus, the mechanisms involved in stress induction of migraine-related pain need to be studied to reach a better treatment for this painful disease. Here, we initially characterized the periorbital/hind paw mechanical allodynia, grimacing pain behavior, and anxiety-related symptoms after the induction of unpredictable sound stress in male and female mice and the effect of a CGRP antagonist. Second, we detected the plasmatic levels of pro-inflammatory cytokines and CGRP.

METHODS

Animals

Male and female adult C57BL/6 mice (20–30 g) were maintained in a humidity level (55–65%) and temperature-controlled room on a 12-hours light/dark cycle with food (pelleted form) and water *ad libitum* . Animals were housed 8 per cage, with nesting material. All experiments were carried out in the light phase (between 07:00 a.m. and 7:00 p.m) and the animals were acclimatized to the laboratory room for at least 1 hour before the experiments. The protocols employed in our study were approved by the Institutional Committee for Animal Care and Use of the Federal University of Santa Maria (UFSM; #9818180820).

The experimental protocols followed the guidelines of Animal Research Reporting In Vivo Experiments (ARRIVE) [39]. Also, experiments were performed using the current ethical guidelines for the investigation of experimental pain in conscious animals, and the minimum necessary number of animals and the intensity of noxious stimuli were used to demonstrate the consistent effects of the treatments. Group size of n=8 animals for behavioral experiments was determined using G*Power (v3.1).

All measurements of animal behavior were always performed by the same experimenter and blinded to drug administration or the group to be tested. ST (stressed) and NST (non-stressed) groups (both female and male) were divided and studied on the same day, and experiments were replicated on different days to generate results from the required number of mice (See on Fig 1. schematic representation of experimental design).

Reagents

All experimental reagents, if not specified in the text, were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Stress induction

Animals were handled and acclimatized to the experimenter and all apparatuses for 3 days before testing to reduce anxiety-like symptoms or stress. Exposure to unpredictable sound stress occurred over 3 days as described previously (Khasar et al., 2009; Khasar et al., 2008). Animals were placed 4-5 per cage and the cage placed 25 cm from a speaker that emitted 4 pure tones (5, 11, 15, and 19 kHz), whose amplitudes varied through time independently from 20-110 dB sound pressure level at random times each minute, lasting 5 or 10 seconds. Non-stressed (NST) animals were placed in the sound chamber for 30 minutes but without exposure to the sound stimulus. Following sound or non-stress, mice were returned to their home cages in the animal care facility.

Treatment protocol

C57BL/6J mice were injected intraperitoneal and performed unilaterally on the right side. BIBN4096BS (olcegepant) (i.p., 100 mg/kg) or its vehicle (4% dimethyl sulfoxide, DMSO, and 4% tween 80 in 0.9% NaCl) [37] were used.

Behavioral experiments

Firstly, baseline grimace scale, periorbital and hind paw thresholds to von Frey filaments were recorded for each animal. Once periorbital thresholds had been tested, animals were placed back in hind paw testing chambers to habituate for approximately 5 minutes before beginning hind paw testing [10]. After, post-stress induction the behavioral experiments were evaluated on days 1, 3, 7, 10, and 14 days. Subsequently, on the nociceptive peak after stress induction (7 days) the treatment protocol with CGRP antagonist or the different plasma or trigeminal ganglion analyses were performed.

Nociceptive tests

The sequence of the tests was carried out following the rationale from the less stressful to the most stress-inducing test as follows: grimace, hind paw mechanical allodynia, followed by periorbital mechanical allodynia (PMA). In the latter, it was performed the open field test. All nociceptive measurements were performed on the same animal, to reduce the number of animals used in the study.

Evaluation of grimacing pain behavior

The grimace scale quantifies changes in a few "action units" including orbital tightening, nose-cheek bulge, whisker tightening, and ear position, and orbital tightening, nose bulge, cheek bulge, ear position, and whisker change for mice. Then, animals were placed in a Plexiglas chamber (5x5) of von Frey test for 60 minutes. This time, we capture one image every 2 minutes. Thus, face images were screened, labeled, randomly scrambled, and scored, with the experimenter blinded to the treatment groups and identity of each image. 30 images were blinded selected for each animal—per treatment condition or time-point—and on each image, each action unit was given a score of 0, 1, or 2, as previously described [36]. Mean grimace scores were calculated as the average score across all the action units. This behavior scale was evaluated on days 1-, 7-, and 14-days post-stress induction or in NST animals. Grimace was also measured before ST or NST induction (Basal), after ST or NST induction (7 days after), and 1 and 3 hours after BIBN4096BS/vehicle treatment.

Hind paw mechanical allodynia

To evaluate the development of mechanical allodynia, the mice were individually placed in transparent boxes on a wire mesh platform allowing easy access to the right hind paw plantar surface. Filaments of different stiffness were applied to the plantar surface of the hind paw, ranging from 0.07 to 2.0 g (0.07, 0.16, 0.40, 0.60, 1.0, 1.4, 2.0 g). The mechanical threshold was obtained according to the up-and-down paradigm [51].

This paradigm continued for a total of six measurements, or until four consecutive positive or four consecutive negative responses occurred. The mechanical paw-withdrawal threshold (in g) response was then calculated from the resulting scores [18]. To determine the baseline thresholds, the animals were acclimatized for 60 minutes before the test and all animals were assessed before stress induction (baseline values). The mechanical threshold was evaluated on days 1, 3, 7, 10 and 14 days. Hind paw mechanical allodynia was also measured before ST or NST induction (Basal), after ST or NST induction (7 days after), and 1 and 4 hours after BIBN4096BS/vehicle treatment.

Periorbital mechanical allodynia (PMA)

The measurement of PMA was performed by using the up-and-down paradigm as described previously [18]. Animals were allocated in a restraint apparatus designed for the evaluation of periorbital mechanical thresholds. The apparatus consists of an individual clear three-walled plexiglass box (5x5) with an opening for the tail and one for the head and front paws, located on a platform to allow the operator to access the periorbital area. The box size allowed for head and forepaw movements but prevented the animal from turning around inside it. One day before the first behavioral observations, mice were habituated to the apparatus. On the day of the experiment, after 20 minutes of adaptation inside the chamber, a series of 7 Von Frey filaments in logarithmic increments of force (0.02, 0.04, 0.07, 0.16, 0.4, 1.0, and 1.4 g) were applied to the periorbital area perpendicular to the skin, with sufficient force to cause slight buckling, and held for approximately 5 seconds to elicit a positive response. The response was considered positive by the following criteria: mouse vigorously stroked its face with the forepaw, head withdrawal from the stimulus,

or head shaking. The stimulation was initiated with the 0.16 g filament. The absence of response after 5 s led to the use of a filament with increased weight, whereas a positive response led to the use of a weaker (i.e. lighter) filament. Six measurements were collected for each mouse or until four consecutive positive or negative responses occurred. The 50% mechanical withdrawal threshold (expressed in g) was then calculated from these scores.

The mechanical threshold was evaluated in the periorbital region over the rostral portion of the eye (i.e., the area of the periorbital region facing the sphenoidal rostrum) initially on basal (before stress) and on 1, 3, 7, 10 and 14-days post-stress induction. PMA was also measured before ST or NST induction (Basal), after ST or NST induction (7 days after), and 1 and 4 hours after BIBN4096BS/vehicle treatment.

Open Field Test

The open-field test was used to analyze the locomotor activity, pain, and anxiety-like behaviors, such as grooming (s), rearing (s), sniffing (s), time into the peripheral zone, and crossings (number of times the line of a square is crossed with all 4 legs)[4]. The apparatus is made of light-grey polyvinyl chloride (PVC) (50 x 50 x 25 cm). This test was performed for 30 minutes in the 1, 7, and 14 days after stress induction and in the first h after the treatment on day 7 post-stress. Illumination (about 40 lux) was provided by a light bulb hanging 60 cm above the apparatus. The temperature of the room was maintained at 22 °C and was a recorded test using AnyMaze® 7.0 software. Between each trial, the apparatus was cleaned with a 30% ethanol solution to avoid odor cues.

Sample collection and analysis

On day 7 post-stress induction or in NST group, mice were anesthetized with ketamine–xylazine (100:20) intraperitoneal (i.p) plus maintenance dose of isoflurane (2.5%) and euthanized by cardiac puncture. The plasma was collected to determine the levels of corticosterone, cytokines, and CGRP.

Corticosterone plasma levels

The plasma was obtained in first day and in 7^{th} day post-stress induction after whole blood centrifugation at 3000 xg at room temperature for 10 minutes and stored at -80 ^{o}C until analysis. The corticosterone circulating level was measured employing an ELISA Kit (Enzo Life Sciences, Farmingdale, New York, USA) following the product manual. Briefly, the samples were diluted according to protocol. Then, 100 µl of the sample or standard solutions were incubated at the plate with the antibody provided by the kit at room temperature on a plate shaker for 2 hours. After, the wells were empty and washed and added 200 µl of pNpp Substrate Solution incubating for 1 hour without shaking. Finally, the plate was read at 405 nm using a microplate reader (SpectraMax I3; Molecular Devices, San Jose, California, USA).

CGRP assay

Firstly, affinity sorbent (A19482, SPI Bio, Bertin Pharma) was used with a pool of different sources of plasma to prepare CGRP-free plasma. Then, the samples were analyzed using a commercially available kit by following the manufacturer's procedures: CGRP (A05482, SPI Bio, Bertin Pharma). The concentration of CGRP is measured using Ellman's Reagent to detect the enzymatic activity of the AChE. The intensity of the yellow color formed is proportional to the amount of CGRP present in that sample [11]. The absorbance was read at 405 nm on SpectraMax I3 (Molecular Devices, San Jose, California, USA). The absorbance values of the standards were used to plot a standard curve, from which absorbance values of experimental samples were interpolated to determine their concentrations. The sensitivities of the assay were 2 pg/ml using quality control. This test was performed in mice plasma of 7th-day post-stress induction.

Cytokines level determination in plasma samples

Cytokines in plasma samples were measured on 7th-day post-stress induction with BD CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Bioscience, San Jose, CA, USA). The kit was used for the simultaneous detection of mouse interferon-γ (IFN-γ), IL-2, IL-4, IL-6, IL-10, IL-17, and TNF-α in a single sample. The operations were performed according to the manufacturer's instructions. Briefly, beads coated with

seven specific capture antibodies were mixed. Subsequently, 50 µL of the mixed captured beads, 50 µL of the unknown serum sample or standard dilutions, and 50 µL of phycoerythrin (PE) detection reagent were added consecutively to each assay tube and incubated for 2 hours at room temperature in the dark. The samples were washed with 1 mL of wash buffer for 5 minutes and centrifuged (200 g). The bead pellet was resuspended in a 300 µL buffer after discarding the supernatant. Samples were measured on the BD Accuri C6 flow cytometer and data analysis was performed with FlowJo software. Individual cytokine concentrations were indicated by their fluorescent intensities. Cytokine standards were serially diluted to facilitate the construction of calibration curves, which were necessary for determining the protein concentrations (pg/mL) of the test samples.

Statistical analysis

Data were presented as mean ± standard error of mean (S.E.M.) and statistically analyzed by parametric and nonparametric Student's t-test, one-way ANOVA, repeated measures, and two-way mixed model analysis of Variance (ANOVA) according to the experimental protocol. First to determine the presence of an interaction effect, and then to compare the NST or vehicle control and treated groups in both sex of mice at each time point tested. The post hoc comparisons employed the Bonferroni criterion. p values less than 0.05 were considered significant. The Imax was calculated using the following formula: 100 x (h post-treatment – mean of basal post-induction)/(basal post-induction mean – basal post-induction mean). Statistical analyses were performed using Graph Pad Prism 9.0 software.

RESULTS

Unpredictable sound stress evokes PMA, hind paw mechanical allodynia, and grimacing pain behavior

We first determine the development of nociception post stress in the 1, 3, 7, 10, and 14 days and address whether there would be a sexually dimorphic effect. We observed in this stress model, that on day 1 after stress induction there was an enhancement in the plasma corticosterone level, but not after 7 days of stress exposure (data not shown), as described before for this model using rats (Khasar et al., 2008).

Male and female mice showed hind paw mechanical allodynia in the 3-, 7-, and 10-days post stress induction. Mice returned to baseline withdrawal thresholds in 14 days post stress induction (Fig. 2 A and B). Similarly, when verifying PMA, in both sexes, the allodynic effect of stress started on the first-day post-stress and was maintained until the 10^{th} day (Fig 2. D and E). To measure spontaneous non evoked pain in these animals, we assessed grimace. We observed that both males and females ST, compared to NST mice had grimacing pain behavior on the first and 7th-day post stress (Fig 2. G and H).

Thus, we verified that the nociceptive peak was on the 7th-day post-stress for PMA, hind paw mechanical allodynia, and grimacing pain behavior. Interestingly, on the nociceptive peak, it was observed a sexual dimorphism for hind paw mechanical allodynia, PMA, and grimacing pain behavior. These data showed that among the ST animals, females had higher nociception compared to males in the 7th-day post-stress induction (Fig. 2 C, F, I). However, male and female mice had similar development of nociception at 1-, 3-, 10-, and 14-days post-stress induction (data not shown).

Anxiety-like symptoms induction and decrease exploratory activity after unpredictable sound stress

The open field test was evaluated in 1st, 7th, and 14th-day post-stress induction or in NST group. In this apparatus it was measured grooming (s), rearing (s), sniffing (s), time into the peripheral zone, and number of crossings. The ST group spent more time grooming compared with the NST group, on both the 1st and the 7th post-stress, but not on the 14th day (Fig. 3A). On day 7, the ST females spent more time in grooming behavior compared to male ST mice (Fig. 3A). Regarding sniffing time, in the ST group it was significantly less both in 1 and 7 days (Fig. 3B). At day 7^{th} after stress induction female mice had a higher reduction in the sniffing time compared to male ST mice (Fig. 3B). For the time of rearing male and female ST mice spent less time in this behavior at day 7^{th} after stress when compared to NST group (Fig. 3C). Also, female

ST mice showed a difference when compared to male ST mice presenting a lower time for rearing at day 7th post stress (Fig. 3C). When assessing the number of crossings, we perceived a reduction in this behavior on the 7th day only in ST female compared to NST female group and ST male mice (Fig. 3D and 3E). Also, we demonstrated that ST mice stayed longer in the peripheral zone compared to the NST group at the 7th-day post-stress induction, but not on days 1 and 14 (Fig. 3F and 3G). Also, when comparing the sex effect in the ST group, we evidenced that ST females spent more time in the peripheral zone compared to ST males at day 7th after stress (Fig. 3F and 3G). No significant difference was seen between groups in locomotor activity assessed by distance traveled (data not shown). Thus, the anxiety-like symptoms induction and decrease exploratory activity was mainly detected at 7th-day post-stress induction. Therefore, this day after stress induction was chosen to measure the antinociceptive effect of CGRP antagonist treatment and the other analysis (plasmatic levels of pro-inflammatory cytokines and CGRP, and the mRNA level of some migraine-related markers in the trigeminal ganglion).

Υνπρεδισταβλε σουνδ στρεσς ινδυσες αν ινςρεασε ιν ΙΛ-6, ΤΝΦ-α ανδ ˜ΓΡΠ λεελς ιν πλασμα

Unpredictable sound-stress induced high levels of circulating pro-inflammatory cytokine, such as IL-6 and TNF-α in plasma compared to NST animals at 7 days after induction (Fig. 4A and C). Moreover, in the same way, the levels of CGRP in plasma were higher in the ST group compared to NST (Fig. 4E). Furthermore, when comparing the effect of dimorphism sexual in the ST group, the females presented higher levels of both TNF-α, IL-6, and CGRP levels than compared to male counterparts (Fig. 4B, 4D and 4F). Nevertheless, there was no difference in the plasmatic levels of IFN-γ, IL-2, IL-4, IL-10 and IL-17 between groups (data not shown).

CGRP antagonist reduced migraine-like behaviors induced by unpredictable stress model in mice

To evaluate the CGRP role in migraine-like induced by sound stress, we administrated CGRP antagonist (BIBN4096BS or olcegepant) by i.p. route (100 mg/kg) at the 7th day after stress-induction. In this way, we observed that this compound had a robust effect to reduce the hind paw mechanical allodynia induced by stress at 1 to 3 hours post-dose in male and female ST group (Fig. 5A and B). The maximal inhibition calculated (Imax) was 87 ± 6% or 88 ± 5%, for male and female ST group 1 hour after BIBN4096BS injection for hind paw mechanical allodynia (Fig. 5C). Besides, this compound also showed a periorbital antiallodynic effect at 1 to 3 hours after treatment (Fig. 5D and F). The Imax was 92 ± 5% or 93 ± 5%, for male and female ST group 1 hour after BIBN4096BS treatment for PMA (Fig. 5F). In an analogous way, analyzing the grimacing pain behavior, we demonstrate that BIBN4096BS significantly reduced the grimacing pain behavior at 1 hour after treatment compared to the vehicle-treated group in males and females ST mice (Fig. 5G and H). Therefore, BIBN4096BS showed similar antinociceptive effects in female and male ST mice at 1 hour after treatment (Fig. 5C, F, and I). Also, at 2 and 3 hours for hind paw mechanical allodynia and PMA BIBN4096BS showed similar antinociceptive effects in female and male ST mice (data not shown).

CGRP antagonist revert the anxiety-like symptoms and increased exploratory activity after unpredictable sound stress

The effect of BIBN4096BS treatment in the open field test was evaluating 1 hour after drug administration. Thus, we perceived that this drug was able to reversion of alterations observed by stress exposition. In reference to grooming, the treatment reverted this behavior parameter compared to the vehicle-treated group in males and females ST mice (Fig. 6A). In the same, the treatment reverted the less exploratory behavior expressed as the time of sniffing in ST vehicle-treated group (Fig. 6B). Also, BIBN4096BS was able to increase the rearing (Fig. 6C) compared the stress/vehicle group (Fig. 6D). Regarding the number of crossings, the treatment reverted the stress affect in female (Fig. 6D and 6E) when compared to the female of vehicle group. Finally, the treatment reduced the time spent in the peripheral zone in the ST group when compared to the vehicle group (Fig. 6F and 6G). The treatment did not affect the distance traveled (not shown). Therefore, the treatment did not have a significant difference in their action in male or female ST

mice.

DISCUSSION

Migraine has a higher prevalence in female and negatively affects the quality of life of patients [21]. Stress is a common trigger reported in migraine patients, but the underlying mechanisms are poorly understood [30,53]. Indeed, different types of stress, such as repetitive restrain stress paradigm, social defeat stress, chronic variable stress, and early life stress have been used as models of migraine-like behavior in animals [8,20]. Besides, previous studies described that unpredictable sound stress model causes plantar allodynia and chemical hyperalgesia in male rats [17,32]. In this sense, the unpredictable sound stress was used as a model to induce painful hypersensitivity in rats [17,32]. Sound stress also represents a type of stress that normally occurs in humans [26,55]. However, nociceptive behaviors had not been evaluated in mice in this model of stress, and it had not yet been characterized as a model of migraine. Thus, here we characterize the migraine-like behaviors in a model of stress mediated by unpredictable sound in male and female mice.

In this sense, previous studies have shown that a significant proportion of individuals with migraine had experienced cutaneous allodynia and spontaneous pain during episodes of headache [28]. Thus, different models of migraine evaluate the development of periorbital and hind paw allodynia, and grimacing pain behavior [8,29]. Here, we detected these nociceptive parameters after unpredictable sound stress induction, with a nociceptive peak at 7 days after stress induction. Recently, using a model of repeated restrain stress in mice, Avona and colleagues [8] showed periorbital mechanical allodynia and grimacing pain behavior in mice. Similar, to this previous study, we also observed that nociception was not detected after 14 days of stress induction, either for mechanical allodynia or grimacing pain behavior. Thus, we characterize a new model of migraine-like pain induced by unpredictable sound stress induction. This is an interesting model, because migraine patients are more sensible to sound, and studies described that sound could be a stress factor to induce migraine pain attacks [26,55].

Moreover, migraine is also associated to psychiatric disorders, such as anxiety [27]. Some previous studies described that nociception in migraine-like models could be accompanied by exploratory behavior alteration and anxiety-like symptoms [5,50]. In this view, we also detected that stress induction decreased the time of different exploratory behaviors, such as rearing and sniffing, and the number of crossings. Besides, stress was correlated with an increased time of grooming and the permanence at the peripheral zone. In previous studies using models of migraine, the reduction in rearing behavior, was accepted as an indicative of spontaneous pain. Decrease in feeding and exploratory behavior [19], an increase in facial grooming [22] were also correlated to migraine-like pain. Besides, after exposure to repeated stress mice stayed more time in the peripheral zone than controls [25,29], and it is a behavior parameter widely used for assessing anxiety [46]. Additionally, in migraine-like model induced by nitroglycerin authors verified increased anxiety-like symptoms in the open field test in rats [50]. Thus, our study also presented other aspects related to migraine-like pain, such as anxiety-like symptoms and decrease of exploratory behavior, these are relevant aspects of a model of migraine [8,25,29].

Migraine affects three times more women than men, but the mechanisms underlying this dimorphism are not known [8,52]. Some studies showed a sexual dimorphism in migraine-like models, with female rodents showing higher nociceptive and anxiety-like responses [5]. Recently, using the migraine model induced by nitroglycerin i.p. injection, a sexual dimorphic effect was detected in which females had higher nociception than males [2]. However, most of the studies using models of migraine did not evaluate the sexual differences, and male mice or rats are normally tested [32,50]. Previous studies, utilizing sound stress demonstrated hind paw nociception, but have been performed only in male rats [15]. Only one study evaluated sexual differences in mice after stress induction (repeated restrain stress) but did not find any difference for periorbital mechanical allodynia and grimacing pain behavior [8]. Nevertheless, here we demonstrated that unpredictable sound stress induces higher nociceptive and anxiety-like parameters in females than ST males. Probably, the differences detected could be induced by the stress model used.

In migraine patients, it was already demonstrated increased TNF-α and IL-6 plasmatic levels compared to

healthy controls [34,40]. Similarly, in rodents, pro-inflammatory cytokines were described as inductors of primary afferent nociceptors sensitization [33,34]. Also, high plasmatic levels of CGRP have already been demonstrated in external jugular venous blood, saliva, and cerebrospinal fluid of migraine patients during an attack [3]. Besides, migraine animal model induce an increase in circulating CGRP levels in plasma [13,49], cerebrospinal fluid of the nestin/hRAMP1 transgenic mice [45]. Additionally, CGRP may trigger the release of pro-inflammatory cytokines [44], particularly IL-6 and TNF-α, which are also increased in the plasm of migraine patients [14,56].

In accordance with these findings, we had demonstrated that unpredictable sound-stress induced elevated the levels of circulating of IL-6, TNF-α, and CGRP in male and female mice. Acute stress models, including restrain and social isolation, caused high plasmatic levels of IL-6 [43,47]. However, no model of stress inducing migraine-like behavior had done this measure before [8,20,29]. The injection of IL-6 (dura mater and cisterna magna) and CGRP (dura mater and intraganglionar injection) induces nociception [5,7]. Dural meningeal nociceptors were also sensitized by TNF-α application [57]. Besides, unpredictable sound stress model induced muscle nociception could be reduced by antisense injection to IL-6 or TNF-α receptors [17]. Seeking to elucidate the sexual dimorphism presented in nociceptive parameters, the ST females presented higher levels of both TNF-α, IL-6, and CGRP levels than compared to male ST mice. Our results corroborate the study of Avona and colleagues, in which the sexually dimorphic effect of CGRP female-specific hypersensitivity responses was seen in mice, where increased grimace responses were also observed [6]. In fact, estrogen has been shown that was able to regulate the release of CGRP [42,48]. Therefore, data are innovative and to our knowledge already have not been assessed in migraine models. Thus, it can be explored in future pre-clinical studies and in research with migraine patients.

Recent reports indicate that mechanisms of pain may differ between the sexes, and a potential role for spinal prolactin has been implicated in the production of IL-6 induced hind paw allodynia [1,38]. Similarly, co-injection of prolactin with IL-6 increases hind paw hypersensitivity in female mice [41]. Furthermore, several studies indicate that fluctuations of ovarian steroid hormone (mainly estrogen) levels modulate CGRP in the trigeminovascular system during different reproductive milestones migraine and suggest that female-specific mechanisms downstream of CGRP receptor activation contribute to the higher prevalence of migraine in women [35].

Recent clinical studies have further confirmed a protagonist of CGRP in migraine due to the use of inhibitors of CGRP signaling, such as antagonists and monoclonal antibodies to CGRP receptor [24]. Here, we demonstrated that a CGRP receptor antagonist had antinociceptive and anxiolytic-like effect and revert the reduced exploratory behavior alterations, showing similar efficacy in male and female ST mice. Olcegepant (BIBN4096BS) was also used to reduce nociception observed in other models of migraine [16]. Besides, using a model of repetitive retrain stress authors showed that CGRP signaling may be involved in migraine-like behaviors detected in this model of stress. In this study calcitonin gene-related peptide monoclonal antibody ALD405 reduced the nociception mainly in female mice, but authors used a priming effect of a nitric oxide donor [8]. In migraine patients, olcegepant can reduce migraine pain in both male and female patients [54]. Intracerebroventricular CGRP infusions is reported to be involved in various behaviors suggestive of anxiety [12]. In this sense, CGRP antagonist showed antidepressant-like effects in stressed mice, and in rats, CGRP antagonism has been shown to suppress anxiety-like behaviors [22].

Our data showed that unpredictable sound stress model in mice causes periorbital/hind paw mechanical allodynia, and grimacing pain behavior. Besides, we detected in this model anxiety-like symptoms and decrease exploratory activity. The nociception, anxiety-like behavior, and exploratory activity alterations detected in this model showed sexual dimorphism. Thus, female mice after stress induction presented higher levels of hind paw mechanical allodynia, PMA, grimacing pain behavior, and anxiety-like symptoms. In addition, we demonstrated that ST female presented lower levels of exploratory behavior than compared with ST male group. Besides, the plasmatic levels of inflammatory cytokines (IL-6 and TNF-α) and CGRP were increased in ST mice, particularly in female ST mice. The CGRP antagonist also caused an antinociceptive effect in male and female ST mice and reduced the anxiety-like behavior and associated exploratory alterations

related to this model. Therefore, these data support the use of this stress priming model to the study of the mechanisms by which stress contributes to migraine-related pain and anxiety-like symptoms.

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Figure legends

Figure 1. Schematic representation of experimental design**.** Exposure to unpredictable sound stress occurred over 3 days. Animals were placed 4-5 per cage and the cage placed 25 cm from a speaker that emitted 4 pure tones (5, 11, 15, and 19 kHz), whose amplitudes varied through time independently from 20-110 dB sound pressure level at random times each minute, lasting 5 or 10 seconds. Non-stressed (NST) animals were placed in the sound chamber for 30 minutes but without exposure to the sound stimulus. Following sound or non-stress, mice were returned to their home cages in the animal care facility. Firstly, baseline grimace scale, periorbital and hind paw thresholds to von Frey filaments were recorded for each animal. After post-stress induction the behavioral experiments were evaluated on days 1, 3, 7, 10, and 14 days. Subsequently, on the nociceptive peak after stress induction (7 days) the treatment protocol with CGRP antagonist or the different plasma or trigeminal ganglion analyses were performed.

Figure 2. Unpredictable sound stress evokes periorbital mechanical allodynia (PMA), hind paw mechanical allodynia and grimacing pain behavior in mice. Hind paw mechanical allodynia was measured in (A) male and in (B) female mice at different time points after stress (ST group) or control (non-stressed group, NST).

(C) Comparison of hind paw mechanical allodynia between male and female mice at day 7 after stress or in NST group. PMA was measured in (D) male and in (E) female mice. (F) Comparison of PMA between male and female mice at day 7 after stress or in NST group. Grimacing pain behavior were evaluated in (G) male and in (H) female at different time points after stress (ST group) or control (non-stressed group, NST). (I) Comparison of grimacing pain behavior male and female mice at day 7 after stress or in NST group. Baseline measurements (described as B in the graph) were taken before induction. Spontaneous nociception was analyzed by mouse grimace scale (MGS). Data are expressed as mean +- S.E.M. (n = 8). $^{\#}P < 0.05$ when compared to NST group, and between days (1, 7, and 14 post-stress) comparing the ST group and NST group. $^{*}P < 0.05$, when compared between the sexes within ST group [Repeated measures two-away ANOVA followed by Bonferroni's post hoc test].

Figure 3. Anxiety-like symptoms induction and decrease exploratory activity after unpredictable sound stress induction. Time spent (A) grooming, (B) sniffing, (C) rearing, (D) number of crossings, (F) time in the peripheral zone were observed in the open field test on days 1, 7, and 14 post-stress (ST group) or in non-stressed group (NST). (G) Representative image of crossings and time in the peripheral zone represented by heat map. Data are expressed as mean +- S.E.M. (n = 8).* $P < 0.05$, when compared between the sexes within the ST group. $^{\#}$ $P < 0.05$ when compared to the NST group. [Repeated measures two-away ANOVA followed by Bonferroni's post hoc test].

Figure 4. Unpredictable sound stress induces an increase in IL-6 (Interleukin-6), TNF-α TNF-α (Tumor necrosis factor-alpha) and CGRP calcitonin gene-related peptide levels in plasma. Plasmatic levels of (A and B) TNF-α, (C and D) IL-6 (Interleukin-6), and (E and F) CGRP detected at 7 days after stress (ST group) or in non-stressed group (NST). Data are expressed as mean ± S.E.M. (n = 12) in the graphs (A,C and D) and n = 6 in the graphs (B, D and F) $^{\#}P < 0.05$, when compared to NST group in (A, C, and E) [nonparametric Student's t-test], and (B, D, and F) [Two-way ANOVA followed by Bonferroni's post hoc test]. * $P < 0.05$ when compared between the sexes within the ST group.

Figure 5. CGRP antagonist reduced migraine-like behaviors induced by unpredictable stress model in mice. BIBN4096BS (olcegepant, 100 mg/kg) intraperitoneal (i.p.) administration reduced the (A and B) hind paw mechanical allodynia, (D and E) periorbital mechanical allodynia (PMA), and (G and H) grimacing pain behavior in male and female stressed (ST) mice when compared to vehicle (Veh) treated group. Antinociceptive effect of BIBN4096BS at 1 hour after treatment for (C) hind paw mechanical allodynia, (F) PMA, and (I) grimacing pain behavior. Baseline measurements (described as B in the graph) were observed before induction. Time 0 represents the measures taken 7 days after ST or in NST group. Spontaneous nociception was analyzed by mouse grimace scale (MGS). Data are expressed as mean ± S.E.M. (n = 8). * $P<$ 0.05, when compared vehicle/stressed group (ST) or between the sexes within the ST group. $^{\#}$ $P < 0.05$ when compared to the NST group § $P< 0.05$ when compared to the vehicle (Veh) treated group [Repeated measures two-away ANOVA followed by Bonferroni's post hoc test].

Figure 6. CGRP antagonist revert the anxiety-like symptoms and increased exploratory activity after unpredictable sound stress. BIBN4096BS (olcegepant, 100 mg/kg) intraperitoneal (i.p.) administration reverted the alterations of different behavioral parameters: Time spent (A) grooming, (B) sniffing, (C) rearing, (D) number of crossings, (F) time in the peripheral zone were observed in the open field test at day 7 post-stress (ST group) or in non-stressed group (NST) 1 hour- post BIBN4096BS administration. (G) Representative image of crossings and time in the peripheral zone represented by heat map. Data are expressed as mean ± S.E.M. (n = 8). * $P < 0.05$, when compared between the sexes within the ST group. $^{\#}$ $P< 0.05$ when compared to the NST group. $^{\S}P < 0.05$ when compared to the vehicle (Veh) treated group [Repeated measures two-away ANOVA followed by Bonferroni's post hoc test].