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Prolactin signaling modulates stress-induced behavioral responses in a preclinical mouse model of migraine

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Abstract

Objective: The aim of this study was to determine if prolactin signaling modulates stress-induced behavioral responses in a preclinical migraine model.

Background: Migraine is one of the most complex and prevalent disorders. The involvement of sex-selective hormones in migraine pathology is highly likely as migraine is more common in women and its frequency correlates with reproductive stages. Prolactin has been shown to be a worsening factor for migraine. Normally prolactin levels are low, however levels can surge during stress. Dopamine receptor agonists, which suppress pituitary prolactin release, are an effective migraine treatment in a subset of patients. Previously, we showed that administration of prolactin onto the dura mater induces female-specific behavioral responses, suggesting that prolactin may play a sex-specific role migraine.

Methods: The effects of prolactin signaling were assessed using a preclinical migraine model we published recently where behavioral sensitization is induced by repeated stress. Plasma prolactin levels were assessed in naïve and stressed CD1 mice (n=3–5/group) and transgenic mice with conditional deletion of the Prlr in Nav1.8-positive sensory neurons (Prlr CKO; n=3/group). To assess the contribution of prolactin release during stress, naïve or stressed male and female CD1 mice were treated with the prolactin-release inhibitor bromocriptine (2mg/kg; n=7–12/group) or vehicle for 5 days (8–12/group) and tested for facial hypersensitivity following stress. Additionally, the contribution of ovarian hormones in regulating the prolactin-induced responses was assessed in ovariectomized female CD1 mice (n=6–10/group). Furthermore, the contribution of Prlr activation on Nav1.8-positive sensory neurons was assessed. Naïve or stressed male and female Prlr CKO mice and their control littermates were tested for facial hypersensitivity (n=8–9/group). Immunohistochemistry was used to confirm loss of Prlr in Nav1.8-positive neurons in Prlr CKO mice. The total sample size is n=245, the full analysis sample size is n=221.

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Conflict of Interest:
The authors report no relevant conflicts of interest

Results: Stress significantly increased prolactin levels in vehicle treated female mice (+39.70 ± 2.77; $p<0.0001$). Bromocriptine significantly reduced serum prolactin levels in stressed female mice compared to vehicle-treated mice (−44.85 ± 3.1; $p<0.0001$). Additionally, no difference was detected between female stressed mice that received bromocriptine compared to naïve mice treated with bromocriptine (−0.70 ± 2.9; $p=0.995$). Stress also significantly increased serum prolactin levels in male mice, although to a much smaller extent than in females (+0.61 ± 0.08; $p<0.001$). Bromocriptine significantly reduced serum prolactin levels in stressed males compared to those treated with vehicle (−0.49 ± 0.08; p=0.002). Furthermore, bromocriptine attenuated stress-induced behavioral responses in female mice compared to those treated with vehicle (maximum effect observed on day 4 post stress (+0.21 ± 0.08; $p=0.03$)). Bromocriptine did not attenuate stress-induced behavior in males at any timepoint compared to those treated with vehicle. Moreover, loss of ovarian hormones did not affect the ability of bromocriptine to attenuate stress responses compared to vehicle-treated OVX mice that were stressed (maximum effect observed on day 4 post stress (+0.29 ± 0.078; $p=0.013$)). Similar to CD1 mice, stress increased serum prolactin levels in both Prlr CKO female mice (+27.74 ± 9.96; $p=0.047$) and control littermates (+28.68 ± 9.9; $p=0.041$) compared to their naïve counterparts. There was no significant increase in serum prolactin levels detected in male Prlr CKO mice or control littermates. Finally, conditional deletion of Prlr from Nav1.8-positive sensory neurons led to a female-specific attenuation of stress-induced behavioral responses (maximum effect observed on day 7 post stress (+0.32 ± 0.08; p=0.007)) compared to control littermates.

Conclusion: These data demonstrate that prolactin plays a female-specific role in stress-induced behavioral responses in this preclinical migraine model through activation of Prlr on sensory neurons. They also support a role for prolactin in migraine mechanisms in females and suggest that modulation of prolactin signaling may be an effective therapeutic strategy in some cases.

Keywords

Migraine; Stress; Prolactin; Sex Differences; Hypersensitivity

Introduction

Migraine is a complex, highly prevalent neurological disorder that is the 2nd-leading cause of disability worldwide and is also 2–3 times more common in women[1, 2]. Approximately 40% of women will have experienced at least one migraine by the end of their reproductive years. Mechanisms contributing to this increased susceptibility to migraine in women are poorly understood. It is thought that the sex differences observed are connected to changes in hormonal regulation in women[3]. In fact, the prevalence of migraine between males and females are similar prior to puberty[4, 5]. However, after the onset of puberty migraine prevalence drastically increases in females suggesting a role for female-selective hormones that influence migraine pathology[5].

While the mechanisms contributing to the sex differences in migraine are considerably complex and not mediated by a single pathway, gonadal hormones are proposed as key contributors to these differences[6–8]. Previous studies have mainly focused on the hormones estrogen and progesterone, however so far there has only been indirect evidence linking migraine occurrence to gonadal hormones. Clinical studies have also shown that elevated

levels of the cytokine-like, pituitary-derived hormone prolactin is a worsening factor for migraine occurrence[9, 10]. In a subset of patients with microprolactinomas, serum prolactin levels can surge at the onset of an attack[11]. Moreover, the involvement of prolactin in migraine is also supported by the ability of dopaminergic agonists like bromocriptine, which suppress pituitary prolactin release by preferentially binding to D2 receptors, to be effective in treating migraine in these patients[10, 12]. Although most studies are from subjects with prolactinomas, the presence or intensity of the headache does not always correlate with tumor size suggesting a role for the biochemical activity of prolactin[13, 14]. This supports the idea that higher prolactin levels increase susceptibility of migraine but validation with controlled studies are needed.

Prolactin contributes to hundreds of physiological functions in the body, but excess prolactin can result in malignant disorders and many pain conditions. One major contributor to elevated prolactin release is stress. Stress is consistently reported as a major trigger of migraine for both men and women, however stress-triggered attacks tends to be more common in females[15, 16]. Attacks do not always occur during the stressful event and the greatest susceptibility to a migraine attack often occurs during the first 6–18 hours after resolution of the stressful event[17–19]. Susceptibility is also greatest after multiple days of intense stress rather than a single incident[17]. We have previously reported that repeated restraint stress can cause long-lasting hypersensitivity and priming to a subthreshold dose of an NO donor in rodents, mimicking conditions present in migraine[20]. Clinical studies report that stressful events significantly elevate serum prolactin levels[21, 22] and we have shown that dural application of prolactin can cause facial hypersensitivity in female rodents[23]. Thus, we hypothesize that prolactin signaling modulates stress-induced migraine-like behavior in mice. The purpose of these studies was to test the relationship of prolactin, stress, and migraine-like behavioral responses, using a preclinical mouse model.

Methods

Animals

Two strains of mice were used. CD-1 mice that were pathogen-free were obtained from Charles River (R09; Raleigh, NC). These mice were used because they are outbred and provide increased genetic diversity between animals over standard inbred mouse strains. Transgenic prolactin receptor conditional knockout (CKO) mice (Nav1.8/Prlr-lox) and their littermates were bred in the animal facility at the University of Texas at Dallas. The transgenic CKO mice have been previously described[23–26]. In brief, double transgenic progeny from crosses of the PRL$^{lox/lox}$ parental line with a Scn10a$^{+/-}$ (Nav1.8)-cre line were used in this study. For the ovariectomized (OVX) female mice, Charles River performed ovariectomy on the mice at 4 weeks. Following one week of recovery, the mice were shipped to the animal facility at the University of Texas at Dallas. All mice that were delivered to the animal facility at the University of Texas at Dallas were allowed to acclimate to the facility for approximately 1 week before any experiments were done. Both male and female mice were used in all experiments unless otherwise noted, aged 6–8 weeks. Animals weights varied from 25 grams up to a maximum of 35 grams. Mice were housed in groups of 2–4 per cage on a 12-hour light-dark cycle and had access to food and

water *ad libitum*. Prior to handling, CD-1 mice animals were a allowed a minimum of 72 hours to acclimate to the animal facility. For all experiments, investigators were blinded to treatment groups. Animals were randomly assigned to treatment groups. At the end of all experiments, mice are humanely euthanized as they are not able to be used in other experiments without introducing a confounding factor in data interpretation. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas at Dallas.

Intraperitoneal drug administration

The experiments in this study used either corn oil (Sigma-Aldrich) or 1X PBS (Thermo Fisher Scientific) as vehicle. The amounts injected were as follows: 2 mg/kg bromocriptine (Sigma-Aldrich), 0.1 mg/kg sodium nitroprusside (SNP; Sigma Aldrich). Bromocriptine was administered at 10 μl/g bodyweight with a 30 g x 0.5 in. needle for 5 days (2 days prior to stress and on the 3 days the mice were stressed). SNP was administered with a total injection volume of 150 μl. All injections were performed by BNM or RK. Animals were held gently during injection. After SNP injection, mice were allowed to recover for 1 hour prior to testing.

Enzyme linked immunosorbent assay (ELISA)

Mouse serum prolactin was obtained by acquiring blood from a cardiac puncture from mice under deep terminal anesthesia. Approximately 1 ml of blood was obtained from each animal. Blood was allowed to clot at room temperature for approximately 30 min. After 30 min elapsed, samples were centrifuged at 3,000 rpm at 4°C for 10 min. The supernatant was placed in fresh tubes and samples were either assayed immediately or frozen at −80 until assayed with mouse prolactin ELISA (Boster Bio).

Repetitive restraint stress paradigm

Animals were subjected to restraint stress 24 hours following baseline according to our previously published protocol[20]. 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 12:00 pm 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 [27]. 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.

Facial Mechanical Hypersensitivity

Twenty-four hours prior to habituation, all animals were handled for a minimum of 5 minutes. During each session of habituation, mice were placed in paraffin wax-free paper cups (Choice) for 3 consecutive days and 2 hours/day as previously described[28]. 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 was used to establish baseline values following habituation prior to bromocriptine treatment and stress. von Frey was also used to assess hypersensitivity that resulted from either stress or drug treatment during the experiments. On the days the mice were subjected to stress, facial von Frey thresholds were not measured. Testing commenced 24 hours after the third day of restraint stress. Mechanical thresholds were determined by using the Dixon “up-and-down” method. Von Frey filaments were applied to the periorbital region of the face for at least 3 seconds but no more than 5 seconds or until the mouse withdraws its head and swipes at the filament with their paw. Testing began with the 0.07 g filament and progressed with a maximum of 0.6 g or a minimum of 0.008 g. Following baseline testing, mice were randomly allocated to an experimental group.

Bromocriptine and Stress Testing Paradigm

Baseline withdrawal threshold was established for male and female CD1 mice. Then approximately 24 hours after the mice reached their target baseline, 2 mg/kg bromocriptine or vehicle was administered intraperitoneally for 2 days prior to undergoing restraint stress and for 3 days during the days the mice were subjected to restraint stress (Fig. 1A). This dose was modified from previous studies that showed suppression of serum prolactin levels in rats[29].

Immunohistochemistry

Trigeminal ganglion (TG) from perfusion-fixed female Prlr CKO mice and their control littermates were embedded in optimal cutting temperature compound (OCT) and frozen. Embedded TG were sliced into 15 μm sections. Antibodies used in this study: anti-Nav1.8 sodium channel mouse monoclonal N134/12 (NeuroMab; Davis, CA; 1:500; RRID: AB_2183861), anti-Prlr rabbit polyclonal R31199 (NSJ Bioreagents; San Diego, CA;

1:500). Sections were incubated with species appropriate Alexa Fluor secondary antibodies (Invitrogen; Waltham, MA; 1:1000). Images were acquired using an Olympus FV3000RS confocal laser scanning microscope. Control IHC was performed on tissue sections lacking primary antibody.

Statistical Analysis

All hypothesis testing was two-tailed. Each experiment was conducted separately with a new cohort of mice. In most experiments a two-way, repeated measures ANOVA (factors: time, treatment) were used. Tukey multiple-comparisons test was used as the post hoc analysis. In each graph, each group was compared against every other group. All von Frey experiments were analyzed separately for the acute phase (BL-D10) and the priming phase (D14–24 hr) but represented on the same graph. For serum analysis represented on the graphs in Figure 1B and 4B, male and female data were analyzed separately using a One-way ANOVA with Tukey multiple-comparisons test as the post hoc analysis but represented on the same graph. Additional analysis was also done comparing serum levels between male and female mice using a One-way ANOVA. Data were analyzed using Graphpad Prism 9, version 9.0.0 software. Significance was set at $p<0.05$ for all analyses. G power was used to generate a power analysis. Expected effected size was based on (Avona *et al.*, 2020)[20]. All sample sizes for periorbital von Frey were greater than or equal to the suggested sample size of 5 calculated in G power for a 0.8 desired power. The family of tests used in G power was F tests and statistical test was repeated measures ANOVA (between factors). The type of power analysis was A priori with an effect size of 12.12, alpha of 0.05, and the number of measurements used was 7. For all analyses using both a one-way and two-way ANOVA we have verified the assumptions by generating a Q-Q plot to ensure normal distribution of the data in GraphPad Prism. All points plotted on the Q-Q graph roughly lie in a straight line with almost no scattering at the ends of the line. We also performed a goodness of fit line test for the Q-Q plots: fig. 2A (R^2=0.98); fig. 2B (R^2=0.97); fig 3 (R^2=0.99); fig. 5A (R^2= 0.94); fig. 5B (R^2=0.98) In addition, we also performed a Shapiro-Wilk test where the data passed the normality test. Additionally, for the one-way ANOVA we used a Brown-Forsythe test to test for equal variances. Both figure 1B ($F_{(7,19)}$=1.532; p=0.216) and 4B ($F_{(7,16)}$=2.095; p=0.445) showed no significance and thus assumed to have equal variances. The total sample size analyzed in this study is 221: 132 female mice and 89 male mice. Animals were not included in the study if they did not reach the target baseline at the beginning of each experiment. The total sample size for each experiment prior to exclusions is n=245: Figure 1A (female n=15, male n=12); Figure 2 (female n=44, male n=39); Figure 3 (n=36); Figure 4 (female n=12, male n=12); Figure 5 (female n=37, male n=38). The following were excluded because they failed to reach target baseline: Figure 1 (n=4 female, n=9 male); Figure 3 (n=5); Figure 5 (n=3 female, n=3 male). The total number of mice excluded from this study is 24. No mice were excluded after baseline exclusion. No data points were excluded in this study. For complete statistical analysis see Table 1. All Data in this study are reported as mean ± SD.

Results

Bromocriptine lowers serum prolactin levels and attenuates stress-induced facial hypersensitivity in female mice

We have previously reported that dural administration of prolactin induces female-specific pain responses in mice[23, 25, 26, 30]. Stress is well known to cause prolactin release from the pituitary and we have shown that repeated restraint stress causes facial hypersensitivity and priming to the NO donor SNP in mice[20, 21]. Here we investigated whether our previously published stress protocol caused elevated serum prolactin levels in CD1 mice and whether changes in these levels may play a role in facial hypersensitivity triggered by stress.

First, we tested whether stress caused elevated levels of prolactin. Female control mice that did not undergo restraint stress had an average of 6 ng/ml serum prolactin detected in their blood whereas males averaged approximately 0.1 ng/ml (Fig. 1B; p=0.148; mean difference of 5.95 ng/ml). Twenty-four hours after the third day of stress, female mice had a significant increase of serum prolactin to ~46 ng/ml compared to control female mice that received vehicle (p<0.0001; mean difference of 39.70 ng/ml; 86.3% increase from control). Males also had an increase in serum prolactin 24 hours after stress compared to control male mice (0.7 ng/ml) (Fig. 1B; p=0.0004; mean difference of 0.61 ng/ml; 87.14% increase from control). We further analyzed serum prolactin levels between male and females who were stressed. Vehicle-treated females that were stressed had significantly elevated prolactin levels compared to males that were stressed (Fig. 1B; p<0.0001; mean difference of 45.65 ng/ml). Next, we determined whether bromocriptine treatment lowers serum prolactin levels. Female (~0.2 ng/ml) and male (0.1 ng/ml) control mice that were treated with bromocriptine had similar levels of prolactin (p>0.999; mean difference of 0.004 ng/ml). Although prolactin levels still increased in female mice that were stressed and treated with bromocriptine (~0.9 ng/ml), this was not significant from female control mice treated with bromocriptine (p=0.995; mean difference of 0.70 ng/ml). Additionally, bromocriptine had no effect on prolactin release in male mice that were subjected to stress (~0.2 ng/ml) compared to control conditions (Fig. 1B; p=0.995, mean difference of 0.02 ng/ml). Lastly, there was no significant difference between bromocriptine-treated stressed males compared to their female counterparts (p=0.412; mean difference of 0.69 ng/ml). These findings establish that the specific stress protocol used in our studies causes elevated serum prolactin levels, bromocriptine lowers serum prolactin levels, and females overall had higher serum prolactin levels compared to males ($F_{(7,19)}$=91.33; p<0.0001).

Based on the ability of 2 mg/kg bromocriptine to inhibit serum prolactin release, we determined whether bromocriptine treatment had any effect on stress-induced hypersensitivity. Again, mice were treated for 5 days with bromocriptine (Fig. 1A). They were subjected to the stress protocol then tested for facial hypersensitivity until they returned to baseline levels, which is approximately 14 days after the last day of restraint stress. Once the mice have returned to baseline, we next wanted to determine whether suppression of prolactin signaling also modulates priming to the SNP response observed in this stress model[20]. Twenty-four hours after stress had concluded, both bromocriptine-treated (p=0.045; mean withdrawal threshold difference of 0.24 g; 57.62% decrease from

bromocriptine-treated controls) and vehicle-treated (p=0.002; mean withdrawal threshold difference of 0.38 g; 75.23% decrease from vehicle-treated controls) female mice exhibited a reduction in facial withdrawal thresholds compared to their respective controls (Fig. 2A). However, stressed mice treated with bromocriptine started returning to baseline levels faster than their vehicle-treated counterparts. This was only statistically significantly at day 4 following the conclusion of stress (p=0.03; mean withdrawal threshold difference of 0.21 g). Once all the mice had returned to their baseline withdrawal thresholds, we injected the mice with 0.1 mg/kg SNP. Stressed mice that received bromocriptine treatment exhibited no response to SNP at all timepoints, whereas stressed mice treated with vehicle were hypersensitive at 1, 3, and 24 (p=0.032, p=0.004, and p=0.018; mean withdrawal threshold differences of 0.25 g, 0.36 g, and 0.28 g, respectively) hours post-injection. On the contrary, male mice that were stressed and treated with bromocriptine exhibited withdrawal thresholds that were indistinguishable from their stress counterparts that received vehicle (Fig. 2B). Likewise, there was no difference in their response to SNP. Stressed mice treated with bromocriptine were significantly different from the control mice treated with bromocriptine at all timepoints following SNP injection (1h (p=0.041; mean withdrawal threshold difference of 0.27 g; 45.57% decrease), 3h (p<0.0001; mean withdrawal threshold difference of 0.53 g; 87.57% decrease), 24h (p<0.001; mean withdrawal threshold difference of 0.45 g; 74.85% decrease). Stressed mice treated with vehicle were significant at 3 (p=0.037; mean withdrawal threshold difference of 0.35 g; 71.39% decrease) and 24 hours (p<0.0001; mean difference withdrawal threshold of 0.41 g; 68.22% decrease) after administration of SNP compared to their controls (Fig. 2B). These data show that lowering serum prolactin levels with bromocriptine treatment selectively attenuates the stress response and priming to SNP in females ($F_{(3,36)}$=18.83; p<0.0001) but not males ($F_{(3,26)}$=63.64; p<0.0001).

Several studies report that in rodents, plasma levels of prolactin markedly rise in response to estrogen and progesterone[31, 32]. Additionally, ovariectomy (OVX) decreases circulating prolactin levels in rodents[33]. Given the cross-talk between pituitary-derived and ovarian-derived hormones, we determined whether disruption of this circuit would alter the stress response in female mice. Interestingly, loss of ovarian hormones did not alter the facial withdrawal thresholds following stress in mice treated with vehicle (Fig. 3). Similar to intact females, bromocriptine significantly attenuated the withdrawal threshold starting approximately 2 days after the last day of stress ($F_{(3,27)}$=13.77; p<0.0001) compared to stressed mice treated with vehicle. This was significant on day 4 (p=0.013; mean withdrawal threshold difference of 0.29 g). Likewise, the response to SNP was also diminished in the OVX mice treated with bromocriptine compared to those treated with vehicle at all timepoints. These data suggest that prolactin release during stress plays a significant role in facial hypersensitivity regardless of ovarian status.

Loss of prolactin receptors on Nav1.8-positive sensory neurons attenuates stress-induced facial hypersensitivity in female mice

We have previously shown that Prlr expression on neuronal fibers in the meninges was more prominent in female mice compared to males and also that application of PRL to cultured trigeminal ganglion neurons only causes sensitization of action potential firing in

female neurons[23]. Given that bromocriptine was able to attenuate stress-induced behavior, we asked whether Prlr activation on sensory neurons played a significant role in stress-induced behavior. Here, we took advantage of a cre-mediated conditional knockout mouse where Prlr is selectively excised from Nav1.8-positive sensory neurons. We have previously characterized the loss of Prlr expression in the dorsal root ganglion of this mouse line[24, 26]. To begin to address this, we first confirmed the loss of Prlr in trigeminal sensory neurons, the source of sensory innervation of cranial tissues including the meninges. Trigeminal ganglion from 8-week-old female mice were extracted and used for IHC. We confirmed that neurons immunoreactive for Nav1.8 did not exhibit colocalization with Prlr in the trigeminal ganglion (Fig. 4A). On the contrary, we observed colocalization of Prlr and Nav1.8 expression in the trigeminal ganglion of control littermates. To ensure that Prlr deletion in sensory neurons has no effect on serum prolactin levels, serum PRL was measured in male and female Prlr CKO mice and their control littermates following stress or control conditions. Under basal conditions serum prolactin levels were not statistically significant between the female control littermates (~17 ng/ml) and Prlr CKO mice (10 ng/ml) (Fig. 4B; p=0.904; mean difference of 6.72 ng/ml). Similarly, when females were stressed, prolactin levels significantly surged in both Prlr CKO mice (39 ng/ml) and their control littermates (44 ng/ml) compared to their respective controls (p=0.041 and p=0.047; mean difference of 27.74 ng/ml (129% increase) and 28.68 ng/ml (186% increase), respectively; $F_{(3,8)}$=5.606; p=0.023). Likewise, there was no statistical difference detected in serum prolactin between male Prlr CKO mice and their control littermates in both control and stress conditions (Fig. 4B; $F_{(3,8)}$=1.673; p=0.249). Although females had overall higher levels of prolactin under control conditions, no statistical significance was detected when compared to males (p=0.299; mean difference of 17.01 ng/ml). In contrast, stressed female Prlr CKO mice had significantly higher serum prolactin levels compared to stressed male Prlr CKO mice (p=0.001; mean difference of 38.24 ng/ml).

We next investigated whether the lack of Prlr on sensory neurons altered the behavior caused by stress in Prlr CKO mice. Both female and male Prlr CKO mice and their control littermates were stressed for 3 days and facial hypersensitivity was measured (Fig. 5). The reduction in facial withdrawal thresholds of female Prlr CKO mice started to attenuate 2 days after stress compared to stressed littermates (Fig. 5A). This was only statistically significant on day 7 (p=0.007; mean withdrawal threshold difference of 0.395 g). Furthermore, Prlr CKO mice that were stressed and received SNP treatment did not exhibit a hypersensitive response compared to control Prlr CKO mice (Fig. 5A). Additionally, stressed Prlr CKO mice exhibited significantly higher withdrawal thresholds compared to stressed control littermates at 1 h (p=0.005; mean withdrawal threshold difference of 0.27 g) and 3 h (p=0.019; mean withdrawal threshold difference of 0.31 g) after administration of SNP ($F_{(3,30)}$=58.08; p<0.0001). Contrary to females, both male Prlr CKO and their control littermates were hypersensitive until day 10 after the last day of stress (Fig. 5B). Moreover, Prlr CKO mice exhibited similar SNP responses as their control littermates ($F_{(3,31)}$=42.71;p<0.0001). These data show a requirement for Prlr activation in sensory neurons selectively in females to cause facial hypersensitivity.

Discussion

While migraine is a very complex condition that has a multitude of causative factors, it is likely that female-selective hormones play a significant role in the increased susceptibility of migraine in women. Among hormonal influences, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is also implicated in primary headache disorders[34, 35]. Furthermore, during stress, maladaptive physiological responses can occur which include hyperactivity of the HPA axis[36]. Since stress impacts males and females differently, there may be different mechanisms between the sexes for how stress contributes to migraine. Stress in general has a negative impact on mental health and to mimic the effects of stress using a rodent model, we used repeated restraint as a surrogate[37]. We have previously reported that repetitive restraint stress is sufficient to induce migraine-like behaviors in mice[20]. Stress alone did not cause any detectable difference in the hypersensitive behaviors between male and female mice. In this study, we show that suppression of pituitary prolactin release is sufficient to attenuate stress-induced behaviors in female, but not male mice. Moreover, we show that Prlr signaling in sensory neurons plays an important role in this hypersensitive behavior given that loss of Prlr in Nav1.8-positive neurons also attenuated the pain responses only in females. Lastly, we show that along with the attenuation of this long-lasting hypersensitivity in females, there is also a diminished hyperalgesic priming response to the NO donor SNP. Taken together, our present findings support an emerging role for prolactin in female-specific migraine mechanisms where prolactin signaling may play a role in sensitizing the nociceptive system during stressful events to trigger migraine attacks. Further, these findings support that modulation of the prolactin system may be a treatment strategy for migraine in a subset of humans with the disorder.

Prolactin secretion is controlled by hypothalamic endocrine factors that either inhibit or stimulate its release and tuberoinfundibular neurons are well known to regulate prolactin secretion from the pituitary[38]. Males and female mice have been reported to have the same density of tuberoinfundibular nerve terminals but have drastic differences in how they respond to psychological and pharmacological stimuli. In fact, tuberoinfundibular neuronal activity is higher in females and also restraint stress reduces tuberoinfundibular neuronal activity in females compared to males[38, 39]. However, low testosterone levels and orchidectomy increases tuberoinfundibular activity in males[40]. The ability of bromocriptine to suppress prolactin and attenuate stress-induced hypersensitivity only in female mice is consistent with these reports. These responses in females may be attributed to higher prolactin responsiveness detected in sensory neurons/fibers found in their trigeminal ganglion, dorsal root ganglion, and dura mater[23, 41, 42]. Interestingly, the initial stress response 24 hours after stress was not affected in females that received bromocriptine although behavior at later time points was attenuated. These data combined with our prior reports on acute dural prolactin suggest a mechanism whereby serum prolactin contributes to the development and maintenance of neuronal sensitization and promotes the chronicity of hypersensitivity in this model. In addition to migraine, these findings may also be relevant for sex differences in other pain states where stress is a contributing factor.

Although previous studies report that pituitary prolactin is strictly governed by gonadal estrogen regulation, there are reports that indicate basal serum prolactin is only transiently

diminished in rats after ovariectomy and levels are back at normal ranges approximately 7–10 days after the procedure[43]. Consistent with preclinical studies, women who underwent a bilateral oophorectomy had prolactin levels return to levels prior to surgery by approximately 6 weeks after surgery. In this study, mice are tested approximately 10–14 days after ovariectomy has been performed. This may explain the finding that ovariectomy did not decrease the stress-induced hypersensitivity nor did it alter the response to bromocriptine, suggesting plasticity within the prolactin system in the absence of gonadal hormones. Serum prolactin was unchanged in male mice that received bromocriptine treatment following both control conditions and after stress. This is in contrast with several human studies that show bromocriptine can modulate serum prolactin in men[44, 45]. However, these changes in serum prolactin occur after long-term treatment with bromocriptine. Similarly, in male rats, bromocriptine decreased prolactin concentrations after approximately 28 days after treatment[46]. Regardless, the 5-day bromocriptine treatment used here had no effect on stress-induced behavioral responses, suggesting that other mechanisms contribute to these behaviors in males.

Disruption of prolactin signaling in sensory neurons revealed a possible cellular site of prolactin action in the development and maintenance of stress-induced hypersensitivity. Surprisingly, bromocriptine treatment and loss of Prlr in peripheral sensory neurons exhibit a very similar phenotype suggesting a convergence of mechanisms where the prolactin released from the pituitary acts on Prlr in sensory neurons. However, there are reports that bromocriptine can have actions on dopamine receptors in peripheral tissues[47]. For example, a study using peripheral human adipocytes showed that administration of either dopamine or bromocriptine showed inhibition of prolactin release in these cells[48]. Whether bromocriptine is acting to inhibit both pituitary and extra-pituitary (ePit) prolactin release in the model used here is not known. However, this leaves the possibility of a role for ePit prolactin release following stress in the behavioral responses shown in this study. It would thus be interesting to evaluate the effects of selective modulation of pituitary vs ePit prolactin release on stress-induced hypersensitivity. Prolactin and Prlr are both expressed in an abundance of cell types. Although pituitary-derived prolactin can diffuse into peripheral tissues, we cannot rule out the possibility that ePit prolactin modulates sensory neurons/fibers in a local paracrine manner. Additionally, we have recently reported a mechanism of Prlr and calcitonin gene-related peptide (CGRP) crosstalk in the meninges and that CGRP also plays a role in stress-induced migraine behaviors[20, 23]. While we cannot address what role CGRP plays in the effects shown here, we do expect the conditional knockout of Prlr to impact CGRP-expressing neurons. The Nav1.8-expressing population of sensory neurons includes both peptidergic (containing CGRP and substance P) and non-peptidergic neurons, with reports showing that this population is ~75% of dorsal root ganglion neurons[49]. If TG neurons are similar, this is clearly a majority of the neurons in the trigeminal ganglion and deleting Prlr from this large population should influence CGRP-expressing and neurons. It remains to be explored whether dual antagonism of Prlr and CGRP receptors would be more effective than either alone. Nonetheless, the present work adds to the building literature supporting a role for prolactin in migraine-relevant mechanisms and provides additional support for targeting the prolactin system in women as a sex-specific migraine therapeutic approach.

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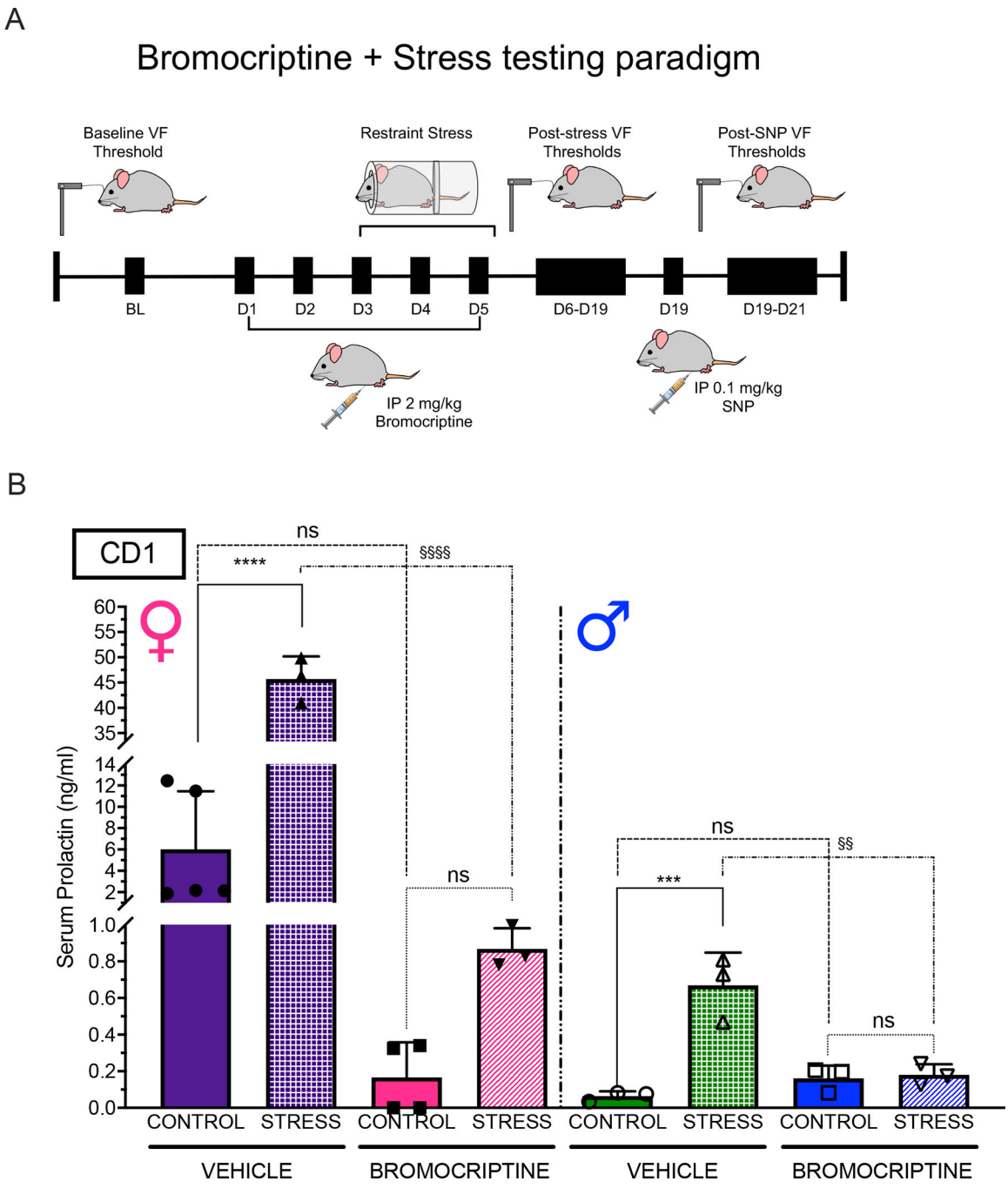

Figure 1. Stress elevates and bromocriptine decreases serum prolactin levels in female mice.

A. Paradigm of bromocriptine treatment and testing of facial hypersensitivity in male and female wild-type CD1 mice. Baseline withdrawal thresholds were determined prior to administration of 2 mg/kg bromocriptine for 5 days. Twenty-four hours after 3 days of stress, facial withdrawal thresholds were tested until mice return to baseline. Following return to baseline, mice were administered 0.1 mg/kg SNP and tested for 1 h, 3 h, and 24 h after injection. B. Serum prolactin was determined 24 hours after the last day of stress after vehicle or bromocriptine administration for 5 days in male and female mice. Female

mice that were stressed and treated with vehicle (n=3) exhibited a significant increase in serum prolactin compared to mice treated with vehicle under control conditions (n=5) and stressed mice treated with bromcriptine (n=4). No apparent difference in prolactin secretion in female mice that were stressed and treated with bromocriptine (n=3) and bromocriptine-treated control mice. There was no significance detected in between male control mice that were treated with vehicle (n=3) or bromocriptine (n=3) and stressed mice that were either treated with vehicle (n=3) or bromocriptine (n=3). §§ $p<0.01$, *** $p<0.001$, §§§§,**** $p<0.0001$. "n" refers to 1 serum sample/mouse. Data are represented as mean ± SD.

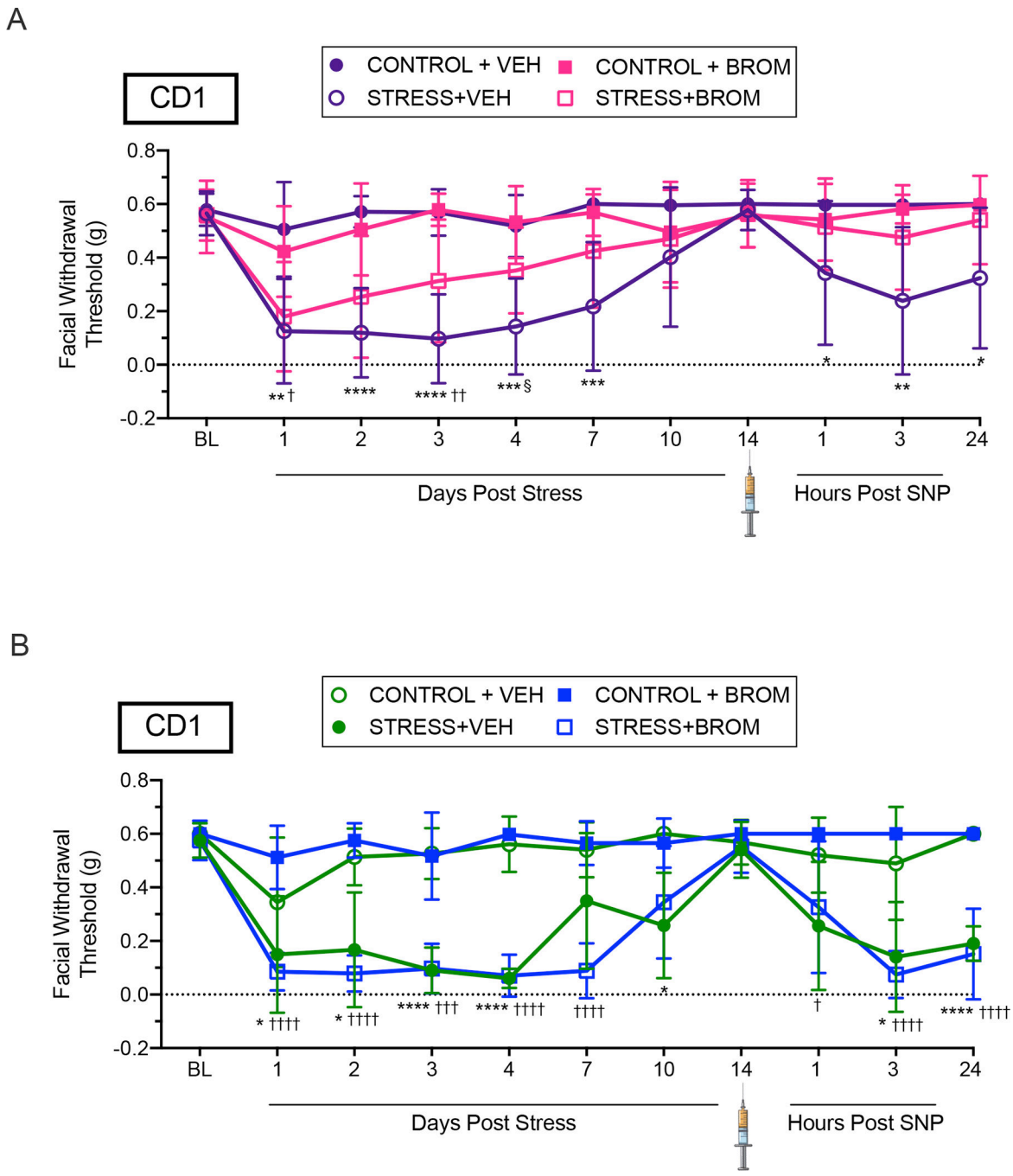

Figure 2. Bromocriptine attenuates stress-induced facial hypersensitivity in female, but not male mice.

A. Facial withdrawal thresholds in female mice following control conditions or stress and treatment with either bromocriptine or vehicle. Female mice that underwent stress and were treated with vehicle (n=12) exhibited significantly decreased withdrawal thresholds compared to control mice that were treated with vehicle (n=8). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Mice that underwent stress and were treated with bromocriptine (n=12) were significantly different from control mice treated with bromocriptine (n=8). $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01. Stressed mice treated with bromocriptine had significantly higher

withdrawal thresholds from stressed mice treated with vehicle at 4 days after stress. $^{\S}p<0.05$. B. Male mice that underwent stress and were treated with vehicle (n=7) exhibited significantly decreased withdrawal thresholds compared to control mice that were treated with vehicle (n=7). $^{*}p<0.05$, $^{****}p<0.0001$. Mice that underwent stress and treated with bromocriptine (n=9) had significantly lower withdrawal thresholds compared to control mice treated with bromocriptine (n=7). $^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$, $^{\dagger\dagger\dagger\dagger}p<0.0001$. There was no significant difference detected between stressed mice treated with bromocriptine and stressed mice treated with vehicle. “n” refers to number of mice tested. Data are represented as mean ± SD.

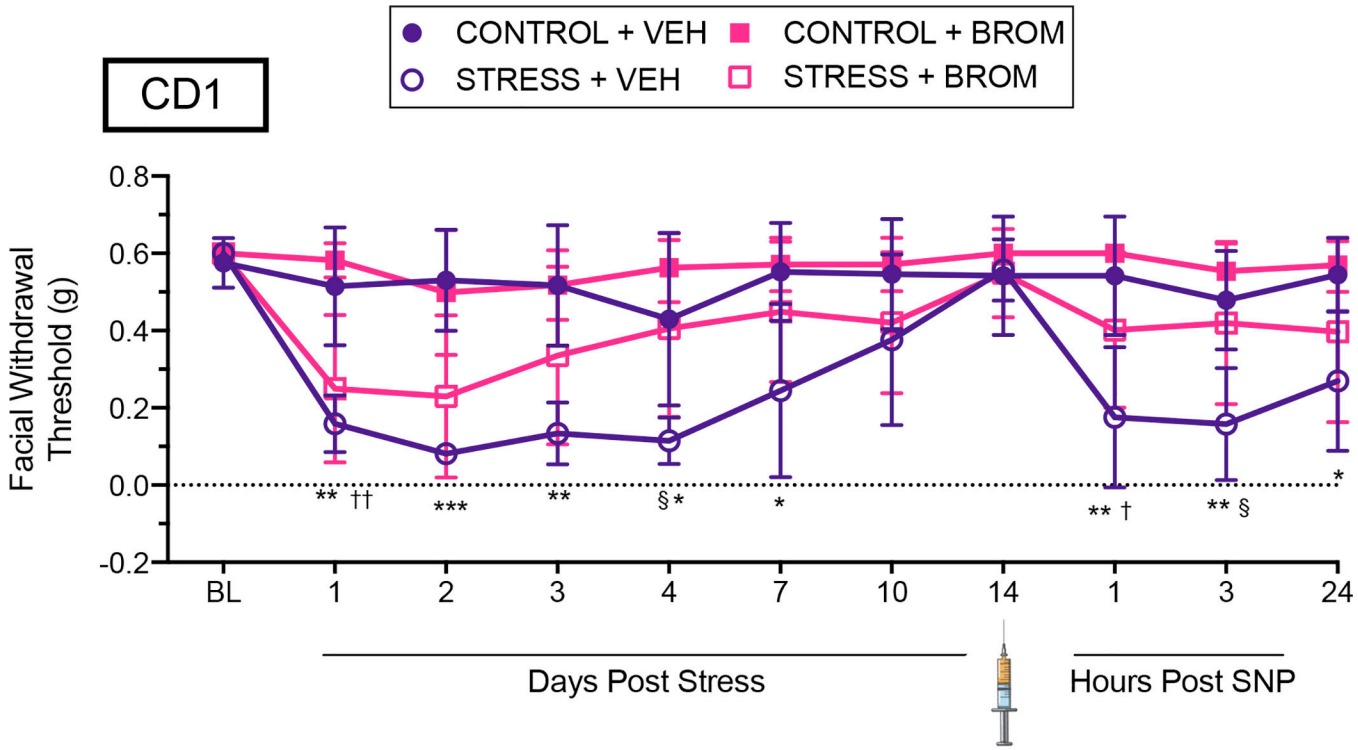

Figure 3. Ovariectomy has no effect on either stress or bromocriptine responses in female mice.

Facial withdrawal thresholds in ovariectomized female mice following control conditions or stress and treatment with either bromocriptine or vehicle. Ovariectomized mice that underwent stress and were treated with vehicle (n=8) exhibited significantly decreased withdrawal thresholds compared to control mice that were treated with vehicle (n=7). *p<0.05, **p<0.01, ***p<0.001. Mice that underwent stress and treated with bromocriptine (n=10) had significantly lower thresholds at day 1 and 1 hr after SNP compared to control mice treated with bromocriptine (n=6). $^{\dagger}p$<0.05, $^{\dagger\dagger}p$<0.01. Stressed mice treated with bromocriptine were significantly different from stressed mice treated with vehicle 4 days after stress. $^{\S}p$<0.05. “n” refers to number of mice tested. Data are represented as mean ± SD.

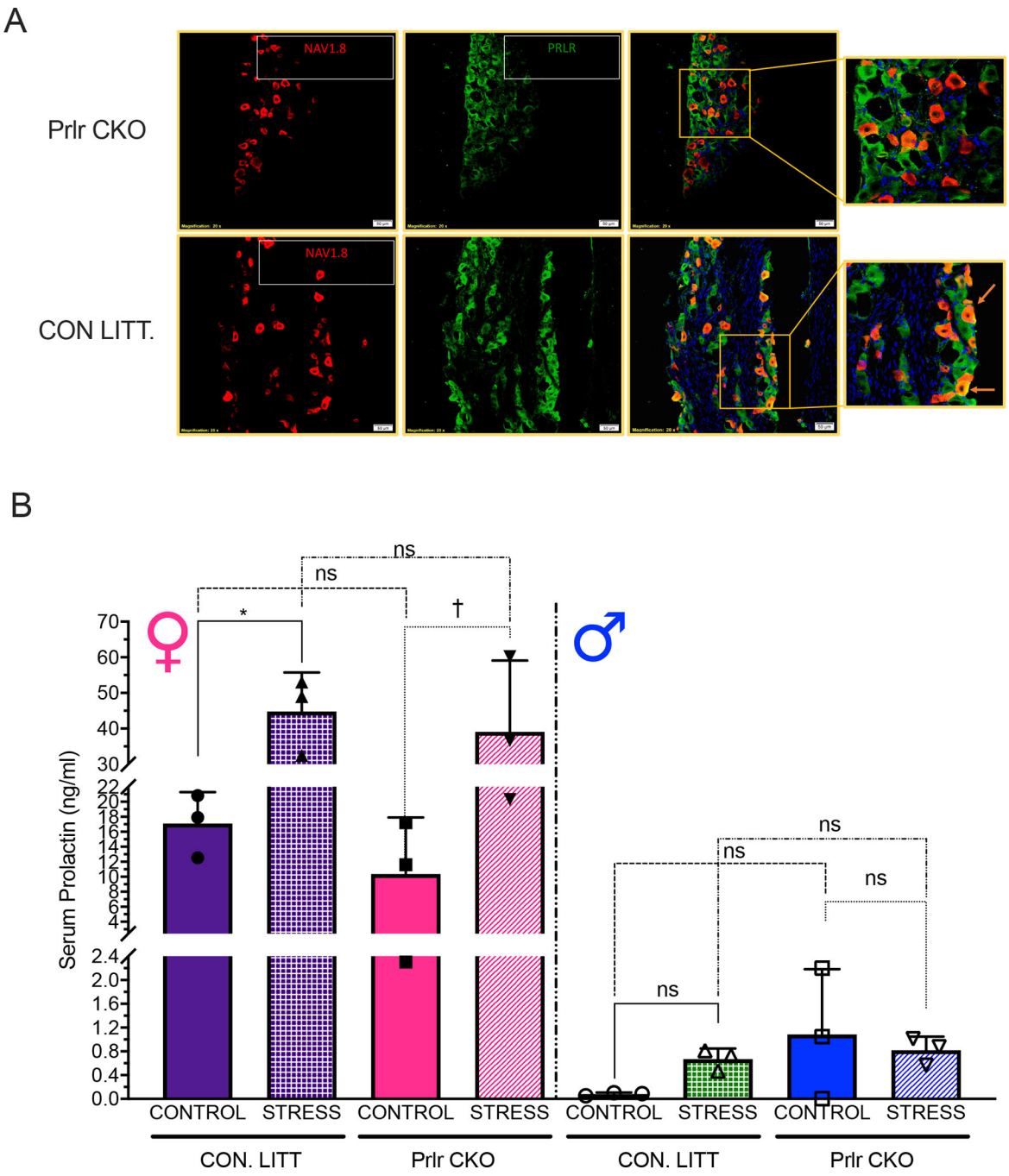

Figure 4. Characterization of sensory neuron Prlr expression and serum prolactin levels in Prlr CKO mice.

A. Trigeminal ganglion of Prlr CKO mice exhibit loss of Prlr in Nav1.8-positive sensory neurons whereas colocalization of Prlr and Nav1.8 (orange arrows) are seen in control littermates. B. Serum prolactin was determined 24 hours after the last day of stress in male and female Prlr CKO mice and their control littermates. Female Prlr CKO mice that were stressed (n=3) exhibited a significant increase in serum prolactin compared to Prlr CKO mice under control conditions (n=3) and stressed control littermates (n=3). Elevated prolactin levels were detected in control littermates that were stressed compared

to control littermates that underwent control conditions(n=3). There was no significant difference detected between male Prlr CKO mice that were stressed and Prlr CKO mice under control conditions (n=3) or control littermates that were stressed (n=3) or underwent control conditions (n=3) *p<0.01, $^{\dagger}p$<0.05. “n” refers to 1 serum sample/mouse. Data are represented as mean ± SD.

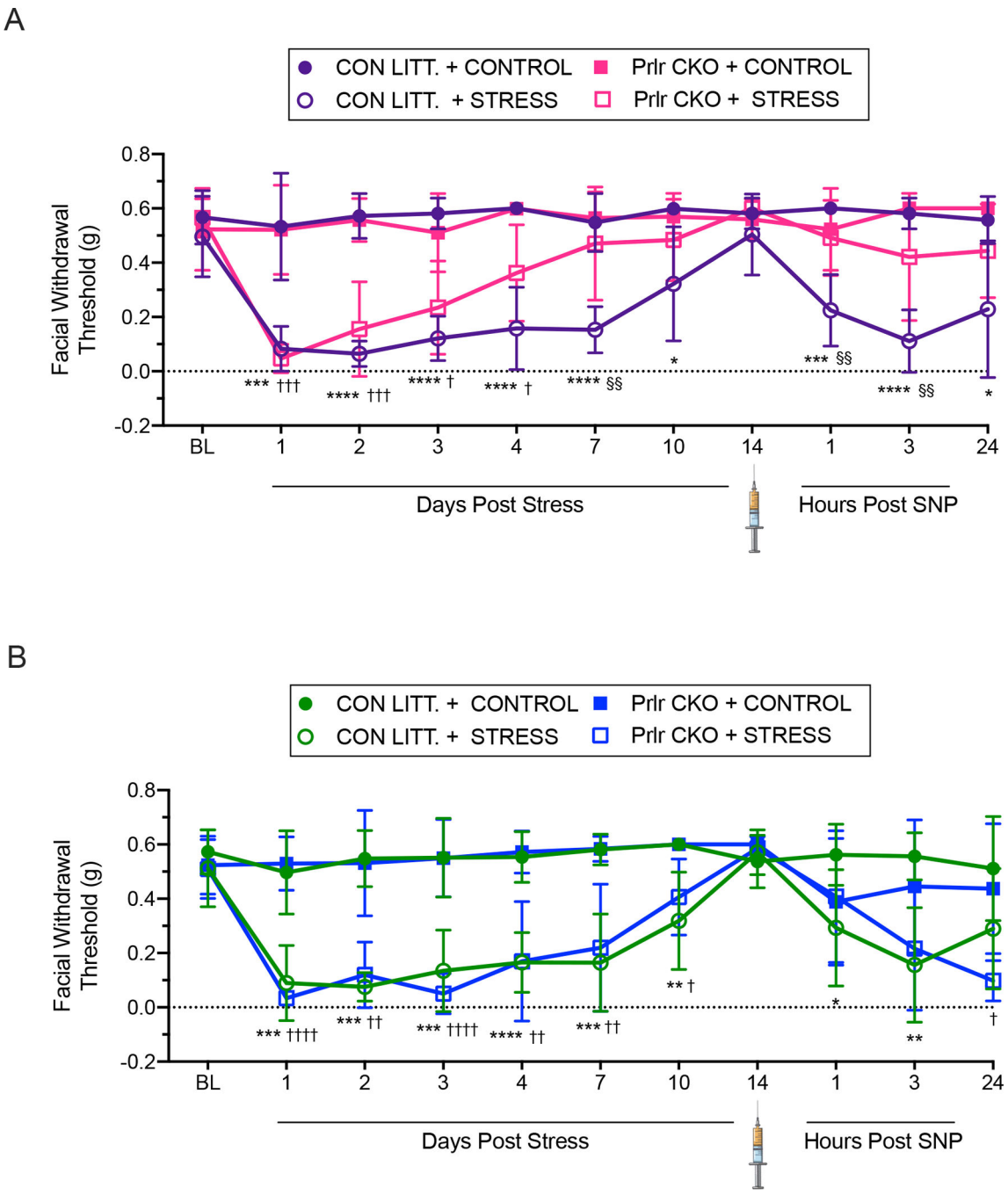

Figure 5. Female-specific attenuation of stress-induced behavioral responses in mice with loss of Prlr in Nav1.8-positive sensory neurons.

Facial withdrawal thresholds in Prlr CKO female mice following control conditions or stress. Prlr CKO mice that underwent stress (n=9) exhibited significantly decreased withdrawal thresholds compared to control Prlr CKO mice (n=8). $^{\dagger}p<0.05$, $^{\dagger\dagger\dagger}p<0.001$. Control littermates that underwent stress (n=8) had significantly lower withdrawal thresholds compared to control littermates under control conditions (n=9). $^{*}p<0.05$, $^{***}p<0.001$, $^{****}p<0.0001$. Prlr CKO mice that were stressed had significantly higher withdrawal thresholds compared to stressed control littermates on day 7 after stress.

§§$p<0.05$. B. Male Prlr CKO mice that underwent stress (n=9) exhibited significant decreased withdrawal thresholds compared to Prlr CKO mice under control conditions (n=8). †$p<0.05$, ††$p<0.01$, ††††$p<0.0001$. Control littermates that underwent stress (n=9) exhibited significantly decreased withdrawal thresholds compared to control littermates under control conditions. (n=9). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. There no was significant difference detected between Prlr CKO mice that were stressed and stressed control littermates. “n” refers to number of mice tested. Data are represented as mean ± SD.

Table 1.

Figure #	Analysis	Statistics
Figure 1B	One-way ANOVA for treatment	Female: $F_{(3,11)}$=104.4; p<0.0001 Male: $F_{(3,8)}$=21.89; p<0.001
	Female	
	Veh Control vs Veh Stress	p<0.0001 (****)
	Brom Control vs Brom Stress	p=0.995
	Veh Stress vs Brom Stress	p<0.0001 (§§§§)
	Male	p<0.001(***)
	Veh Control vs Veh Stress	p=0.995
	Brom Control vs Brom Stress	p=0.002 (§§)
	Veh Stress vs Brom Stress	
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 2A Acute Phase	Two-Way repeated measures ANOVA	
	Interaction Factor	$F_{(18,216)}$=3.78; p<0.0001
	Treatment Factor	$F_{(3,36}$=20.45; p<0.0001
	Time Factor	$F_{(4.824,\ 173.6)}$=13.50; p<0.0001
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 2A Priming Phase	Two-Way repeated measures ANOVA	
	Interaction Factor	$F_{(9,108)}$=2.865; p=0.005
	Treatment Factor	$F_{(3,36)}$=8.46; p<0.001
	Time Factor	$F_{(2.528,\ 90.99)}$=3.71; p=0.019
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 2B Acute Phase	Two-Way repeated measures ANOVA	
	Interaction Factor	$F_{(18,156)}$=6.39; *p<0.0001*
	Treatment Factor	$F_{(3,26)}$=59.76; *p<0.0001*
	Time Factor	$F_{(4.307,\ 112)}$=26.18; *p<0.0001*
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 2B Priming Phase	Two-Way repeated measures ANOVA	
	Interaction Factor	$F_{(9,78)}$=6.67; *p<0.0001*
	Treatment Factor	$F_{(3,26)}$=27.16; *p<0.0001*
	Time Factor	$F_{(2.012,\ 52.32)}$=21.12; *p<0.0001*
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 3 Acute Phase	Two-Way repeated measures ANOVA	
	Interaction Factor	$F_{(18,\ 162)}$=3.926; p<0.0001
	Treatment Factor	$F_{(3,27)}$=14.60; p<0.0001
	Time Factor	$F_{(5.083,\ 137.3)}$=16.23; p<0.0001
	Tukey's multiple comparisons between treatments *p<0.05	

Figure #	Analysis	Statistics
Figure 3 Priming Phase	Two-Way repeated measures ANOVA	
	Interaction Factor	$F_{(9,81)}$=3.76; p=0.001
	Treatment Factor	$F_{(3,27)}$=9.18; p<0.001
	Time Factor	$F_{(2.779, 75.04)}$=10.52; p<0.0001
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 4B	One-way ANOVA for treatment	Female: $F_{(3,8)}$=5.60; p=0.023 Male: $F_{(3,8)}$=1.63; p=0.25
	Female	
	Control Con. Lit. vs Stress Con. Lit.	p=0.047 (*)
	Control Prlr CKO vs Stress Prlr CKO	p=0.041 (†)
	Control Con. Lit. vs Control Prlr CKO	p=0.904
	Stress Con. Lit. vs Stress Prlr CKO	p=0.936
	Male	
	Control Con. Lit. vs Stress Con. Lit.	p=0.61
	Control Prlr CKO vs Stress Prlr CKO	p=0.808
	Control Con. Lit. vs Control Prlr CKO	p=0.213
	Stress Con. Lit. vs Stress Prlr CKO	p=0.988
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 5A Acute Phase	Two-Way repeated measures ANOVA	
	Interaction Factor	$F_{(18,180)}$=7.327; p<0.0001
	Treatment Factor	$F_{(3,30)}$=53.05; p<0.0001
	Time Factor	$F_{(5.13, 153.9)}$=20.01; p<0.0001
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 5A Priming Phase	Two-Way repeated measures ANOVA	
	Interaction Factor	$F_{(9, 90)}$=4.673; p<0.0001
	Treatment Factor	$F_{(3, 30)}$=26.42; p<0.0001
	Time Factor	$F_{(2.419, 72.56)}$=8.756; p<0.001
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 5B Acute Ph	Two-Way repeated measures ANOVA	
	Interaction Factor	$F_{(18, 186)}$=5.76; p<0.0001
	Treatment Factor	$F_{(3,31)}$=67.59; p<0.0001
	Time Factor	$F_{(4.299, 133.3)}$=21.33; p<0.0001
	Tukey's multiple comparisons between treatments *p<0.05	
Figure 5B Priming Phase	Two-Way repeated measures ANOVA	$F_{(9, 93)}$=4.70; p<0.0001
	Interaction Factor	$F_{(3, 31)}$=7.37; p=0.001
	Treatment Factor	$F_{(2.801, 86.83)}$=18.62; p<0.0001
	Time Factor	
	Tukey's multiple comparisons between treatments *p<0.05	