Neuroendocrine Mechanisms Governing Sex Differences in Hyperalgesic Priming Involve Prolactin Receptor Sensory Neuron Signaling

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Many clinical and preclinical studies report higher prevalence and severity of chronic pain in females. We used hyperalgesic priming with interleukin 6 (IL-6) priming and PGE₂ as a second stimulus as a model for pain chronicity. Intraplantar IL-6 induced hypersensitivity was similar in magnitude and duration in both males and females, while both paw and intrathecal PGE₂ hypersensitivity was more persistent in females. This difference in PGE₂ response was dependent on both circulating estrogen and translation regulation signaling in the spinal cord. In males, the duration of hypersensitivity was regulated by testosterone. Since the prolactin receptor (Prlr) is regulated by reproductive hormones and is female-selectively activated in sensory neurons, we evaluated whether Prlr signaling contributes to hyperalgesic priming. Using ΔPRL, a competitive Prlr antagonist, and a mouse line with ablated *Prlr* in the Nav1.8 sensory neuronal population, we show that Prlr in sensory neurons is necessary for the development of hyperalgesic priming in female, but not male, mice. Overall, sex-specific mechanisms in the initiation and maintenance of chronic pain are regulated by the neuroendocrine system and, specifically, sensory neuronal Prlr signaling.

Key words: estrogen; nociceptor; prolactin; sex dimorphism; testosterone; translation regulation

Significance Statement

Females are more likely to experience chronic pain than males, but the mechanisms that underlie this sex difference are not completely understood. Here, we demonstrate that the duration of mechanical hypersensitivity is dependent on circulating sex hormones in mice, where estrogen caused an extension of sensitivity and testosterone was responsible for a decrease in the duration of the hyperalgesic priming model of chronic pain. Additionally, we demonstrated that prolactin receptor expression in Nav1.8⁺ neurons was necessary for hyperalgesic priming in female, but not male, mice. Our work demonstrates a female-specific mechanism for the promotion of chronic pain involving the neuroendocrine system and mediated by sensory neuronal prolactin receptor.

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Introduction

Many chronic pain conditions, such as migraine, fibromyalgia, temporomandibular joint disorders, irritable bowel syndrome, and rheumatoid arthritis, have a 2- to 6-fold greater prevalence or symptom severity in women compared with men (Unruh, 1996; Berkley, 1997; Fillingim et al., 2009; Traub and Ji, 2013). Pain symptoms in women with these chronic pain conditions can change during the menstrual cycle as gonadal hormone concentrations fluctuate; and some, but not all, pain conditions decrease in frequency or intensity after menopause (Houghton et al., 2002; LeResche et al., 2003; Slade et al., 2011; Mathew et al., 2013). There is a general consensus that plasticity mechanisms in peripheral and central nociceptive pathways are critical for

Figure 1. Persistence of hyperalgesic priming is greater in female mice. **A**, Schematic of the IL-6-induced hyperalgesic priming model. BL, Baseline measurements. Brown arrows indicate injection time points. Blue arrows indicate post-PGE₂ mechanical nociception measurement time points. **B**, Hyperalgesic priming model: IL-6 priming into paw and PGE₂ injection into paw of female and male C57BL/6 mice. **C**, Hyperalgesic priming model: IL-6 priming into paw and PGE₂ injection into SC of female and male C57BL/6 mice. **D**, The same model as in **C** in female and male Institute of Cancer Research (ICR) mice. Arrows indicate injection time points for IL-6/Veh and PGE₂. Repeated-measures ANOVA with Bonferroni *post hoc* test: **C**, IL-6 male compared with Veh-female: **** $p < 0.0001$; IL-6 female compared with Veh-female: ★★★★★ $p < 0.0001$; ★★★★★ $p < 0.001$; all others: * $p < 0.05$; **** $p < 0.0001$. $n = 5-8$.

chronic pain development in males and females, but the precise mechanisms governing this plasticity are increasingly recognized as sex dimorphic and are still largely unknown. Nevertheless, recent progress was made in understanding underlying mechanisms for sex-dependent mechanisms of nociceptive plasticity (Mogil et al., 2011; Sorge et al., 2011, 2015; Rosen et al., 2017; Martin et al., 2019). These findings on sex differences in nociceptive plasticity mechanisms, combined with abundant clinical and rodent data on the effects of gonadal hormones on pain, indicate a critical role for gonadal hormones in regulation of pain chronicity (Fillingim et al., 2009; Traub and Ji, 2013).

Clearly, there are gonadal hormone-regulated mechanisms that promote chronic pain in females, but these mechanisms have not been thoroughly characterized. Prolactin (PRL) and its receptor (Prlr) are prime candidates for this potential mechanism, since responsiveness to PRL in a variety of cells, including sensory neurons, is closely regulated by estrogen (Childs et al., 1999; Pi and Voogt, 2002; Diogenes et al., 2006; Belugin et al., 2013). Thus, Prlr signaling sensitizes pain-related ion channels and causes increased excitability in nociceptors, specifically in females (Diogenes et al., 2006; Patil et al., 2013b, 2019a,b; Liu et al., 2016). Moreover, Prlr function in female nociceptors is governed by estrogen signaling via a nongenomic pathway that involves sex-specific translation of Prlr mRNA (Patil et al., 2019b). Clinically, high PRL levels are linked to migraine, and blocking PRL actions in women with hyperprolactinemia resolves headache (Silberstein, 1992; Cavestro et al., 2006; Oliveira et al., 2020). Based on these previous studies, we hypothesized that Prlr signaling might differentially contribute to female-specific regulation of chronic pain that can be assessed by use of the hyperalgesic priming model (Aley et al., 2000). Our work reveals that initiation, maintenance, and magnitude of hyperalgesic priming are governed by estrogen-dependent regulation of Prlr signaling in sensory neurons. Therefore, PRL signaling to Prlr is a gonadal hormone-

dependent mechanism that promotes plasticity in the nociceptive pathway, supporting development of chronic pain specifically in females.

Materials and Methods

Animals. All animal experiments were approved by the University Texas Health Science Center at San Antonio and University of Texas at Dallas Institutional Animal Care and Use Committee. We followed guidelines issued by the National Institutes of Health and the Society for Neuroscience to minimize suffering and the number of animals used.

Key reagents and mouse lines. Eight- to 12-week-old female and male mice were purchased from The Jackson Laboratory. Ovariectomized (OVX) and gonadectomized (GdX) mice were purchased from The Jackson Laboratory. The estrous phases in adult females were determined by vaginal gavage as described by Caligioni (2009). Estrogen and testosterone replacement procedures to generate OVX-E-2 and GdX-T mice were performed as previously described (Diogenes et al., 2006; Nettleship et al., 2007). 17 β -Estradiol (E-2; 300 μ g per injection) or testosterone (T; 300 μ g per injection) was injected intraperitoneally 2 times a week for 3 weeks into OVX and GdX mice, respectively.

The *Prlr*^{fl/fl} line was generated as previously described (Brown et al., 2016). *Prlr*^{fl/fl} line has inverse lox sites; hence, *Cre* recombination ablates the *Prlr* gene and activates GFP in targeted cells.

Estrogen was purchased from Sigma Millipore (catalog #PHR1353-1G). Testosterone was purchased from Sigma Millipore (catalog #T1875-1G). 4EGI was purchased from Tocris Bioscience. Vehicle for IL-6, PGE₂, PRL, and Δ 1-9-G129R-hPRL (Δ PRL) was 0.9% saline or PBS. Vehicle for 4EGI-1 was 0.1% DMSO in 0.9% saline.

Human PRL was generated in an *Escherichia coli* expression system containing plasmid with human PRL (Goffin; Institut National de la Santé et de la Recherche Médicale). Thus, PRL is fully processed, unmodified (i.e., no glycosylation and phosphorylation), and has molecular weight of ~23 kDa. The Prlr antagonist Δ PRL (Rouet et al., 2010), which is a modified PRL that binds to and blocks the function of Prlr in rat, mouse, and human (Bernichtein et al., 2003), was also synthesized by Goffin (Institut National de la Santé et de la Recherche Médicale).

We and others thoroughly confirmed the specificity of Δ PRL using *in vitro* (Bernichtein et al., 2003; Scotland et al., 2011), and *in vivo* studies (Rouet et al., 2010), including using Prlr KO mice (Belugin et al., 2013).

RT-PCR. RT-PCR was performed on hindpaw, L3-L5 DRG, and spinal cord (SC) total RNA. Dissected tissue was stored in RNA. Later at -20°C (QIAGEN). RNA extraction was done using the QIAzol lysis reagent and the RNeasy Mini Kit (QIAGEN), and manufacturer's instructions were followed. cDNA was synthesized using Superscript III First Strand Synthesis kit (Invitrogen). Primers were as follows: Prlr-F (5'-CCATTACCTGCTGGTGAATCCT-3'), GFP-F (5'-AAGGCTACGTCCAGGAGCGCACCA-3'), GFP-R1 (5'-CGTCCTCGATGTTGTGGCGGATC-3'), and GFP-R2 (5'-TGGTGCCTCCTGGACGTAGCCTT-3'). Amplification of target sequences was detected on 1 or 1.5% agarose gel depending on band size.

Behavior experiments. Hyperalgesic priming was established using a previously described model (Aley et al., 2000; Kim et al., 2016). IL-6 was injected intraplantarly, which created a transient mechanical hypersensitivity and initiated hyperalgesic priming. After IL-6-induced mechanical hypersensitivity resolved and thresholds returned to a baseline level, PGE₂ was administered either intraplantarly or intrathecally to precipitate the primed state and induce mechanical hypersensitivity. PRL, Δ PRL, or 4EGI-1 was administered immediately before IL-6 or PGE₂ administration. To evaluate mechanical hypersensitivity following the intraplantar or intrathecal injections, animals were habituated for 45–60 min in elevated behavior racks, and then paw withdrawal threshold was determined using the up-down von Frey method (Chaplan et al., 1994). Both the experimenters performing the behavior and data analysis were done blinded.

Experimental design and statistical analysis. GraphPad Prism 7.0 (GraphPad) was used for all statistical analyses of data. Data are presented as mean $\pm$ SEM, with *n* indicating the number of independent animals per group in behavioral experiments. The sex of the animals used in each experiment is described in the text. Differences between groups were assessed by either mixed-effects or repeated-measures ANOVA with Bonferroni *post hoc* tests (noted for each figure). Statistical significance was determined as $p < 0.05$. Interaction *F* ratios and the associated *p* values are reported in the text.

Results

Sex differences in hyperalgesic priming in mice are regulated by gonadal hormones and translation regulation

Chronic pain occurs more frequently in females (Unruh, 1996; Berkley, 1997; Fillingim et al., 2009; Traub and Ji, 2013). This difference could be mediated by sex-dependent mechanisms controlling the transition from acute to chronic pain. We used the

Figure 2. Contribution of gonadal hormones profoundly influences hyperalgesic priming in female and male mice. **A**, Hyperalgesic priming model with spinal PGE₂ injection in WT female, OVX, and OVX + E (**B**) GdX, and (**C**) WT male, GdX, and GdX-T C57BL mice. **B**, The disparity in timelines between GdX animals and those animals that are naive or GdX-T. Arrows indicate injection time points for IL-6 and PGE₂. Repeated-measures ANOVA with Bonferroni *post hoc* test: **A**, *** $p < 0.001$; **** $p < 0.0001$, for OVX-E compared with female; ★★ $p < 0.01$ for OVX compared with female; **C**, *** $p < 0.001$; ★★ $p < 0.01$ for GdX-T compared with male; $n = 5$ or 6 . ★★★★★ $p < 0.0001$.

hyperalgesic priming paradigm (Aley et al., 2000) to gain insight into female-specific mechanisms involved in the acute to chronic pain transition. In our experiments, hyperalgesic priming was initiated with an intraplantar injection of IL-6 (0.5 ng). When the initial hypersensitivity from this IL-6 injection had resolved, the presence of priming was assessed with either an intraplantar or intrathecal injection of PGE₂ (0.1 μg ; Fig. 1A). Female C57BL/6 mice that were primed with IL-6 and then subsequently received an intraplantar injection of PGE₂ had a slightly longer persistence of mechanical hypersensitivity compared with males (repeated-measures ANOVA; $F_{(11,88)} = 1.437$; $p = 0.1708$; $n = 5$; Fig. 1B). Intrathecal injection of PGE₂ in females had a significantly longer duration of response to PGE₂ compared with males (repeated-measures ANOVA; $F_{(14,91)} = 8.975$ $p < 0.0001$ $n = 5$; Fig. 1C). To

Figure 3. Spinal local translation only contributes to hyperalgesic priming in intact female mice. **A**, Hyperalgesic priming model with spinal PGE₂ injection in WT male, **B** WT female, and **C** OVX mice. 4EGI-1 (10 μg) or vehicle was administered spinally at 30 min before PGE₂ injection. Arrows indicate injection time points for IL-6 and 4EGI-1/PGE₂. Repeated-measures ANOVA with Bonferroni *post hoc* test: *****p* < 0.0001. *n* = 5 or 6.

rule out a possible strain-dependent sex effect, the experiment with spinal administration of PGE₂ following intraplantar IL-6 was repeated in male and female Swiss Webster mice. The difference in the length of the PGE₂ response following intrathecal administration was even longer lasting in this outbred strain (repeated-measures ANOVA; $F_{(10,140)} = 8.409$; $p < 0.0001$; $n = 8$; Fig. 1D). To gain insight into the mechanistic underpinnings of the sex difference we identified, we did the remaining experiments with intraplantar injection of IL-6 and intrathecal administration of PGE₂ in C57BL/6 mice.

In the above and following experiments, the female animals were all in the estrous phase of the estrous cycle at the time of IL-6 injections. The hyperalgesic priming model lasts at least a week; and despite controlling for the estrous phase at the time of IL-6 injection, female mice cycle quickly (4–5 d) through other

phases of the cycle, and circulating blood estrogen (E-2) levels will vary on a day-to-day basis. Hence, to control for E-2 levels, we used OVX and OVX with E-2 supplementation (OVX-E-2) female mice. The substantial reduction of circulating E-2 in OVX mice (Green et al., 2016) did not change the initiation phase of priming, but the persistence of the response following the PGE₂ injection was significantly shorter (mixed-measures ANOVA; $F_{(34,220)} = 8.710$; $p = 0.0011$; $n = 5$ or 6; Fig. 2A). Administering E-2 to keep circulating E-2 at approximately proestrus phase levels in OVX-E-2 mice (Green et al., 2016) resulted in a significant extension of both the initiation (IL-6) and priming (PGE₂) phases compared with intact females in the hyperalgesic priming model. Testosterone mediates male-specific nociceptive responses (Sorge et al., 2011). GdX mice have almost no circulating testosterone (Green et al., 2016). These GdX mice had a substantially longer response to IL-6 injection than intact male mice. Males with IL-6-induced mechanical hypersensitivity usually return to baseline mechanical sensitivity within 4–5 d (Kim et al., 2016) (Fig. 1B–D). In contrast, the initiation phase of hyperalgesic priming lasted >31 d in GdX males, and only partially recovered to baseline levels, which we displayed on a separate graph (Fig. 2B). The PGE₂ phase was also lengthened in GdX compared with intact male mice (repeated-measures ANOVA; $F_{(12,84)} = 5.016$; $p < 0.0001$; $n = 5$ or 6; Fig. 2C). Testosterone rescue in GdX-T animals returned the initiation and priming phases to the same timeline as intact males (Fig. 2C). These results show that the time course of hyperalgesic priming is more pronounced in female mice compared with male mice, and is closely regulated by circulating gonadal hormones in both sexes.

Translation regulation plays a key role in the development of hyperalgesic priming (Price and Inyang, 2015; Khoutorsky and Price, 2018). In previous experiments that were mostly done in male animals, we have shown that inhibition of cap-dependent translation at the time of initiation blocks the development of hyperalgesic priming (Melemedjian et al., 2010, 2014; Asiedu et al., 2011; Moy et al., 2017). Inhibition of cap-dependent translation during the maintenance phase fails to reverse established priming (Asiedu et al., 2011). In concordance with previous experiments (Melemedjian et al., 2010, 2014; Asiedu et al., 2011), the cap-dependent translation inhibitor 4EGI-1 (10 μg) given intrathecally immediately before PGE₂ stimulation did not affect PGE₂-induced mechanical hypersensitivity in males (repeated-measures ANOVA; $F_{(8,64)} = 0.3153$; $p = 0.9575$; $n = 5$; Fig. 3A). In stark contrast, 4EGI-1 dramatically reduced the persistence of PGE₂ precipitated mechanical hypersensitivity in females (repeated-measures ANOVA; $F_{(8,64)} = 10.60$; $p < 0.0001$; $n = 5$; Fig. 3B). We next evaluated whether the

difference between males and females in the regulation of maintenance of hyperalgesic priming was defined by gonadal hormone status. Removal of circulating E-2 using OVX females abolished the influence of 4EGI-1 on the hyperalgesic priming (repeated-measures ANOVA; $F_{(9,90)} = 0.7394$; $p = 0.6719$; $n = 6$; Fig. 3C). These results indicate that the magnitude and maintenance of chronic pain are regulated by gonadal hormones in male and female mice and that the enhanced priming effect seen in intact female mice is dependent on translation regulation at the level of the DRG and/or SC. Interestingly, previous work done entirely in male rodents suggested that translation regulation events at the level of the DRG and/or SC were not involved in maintenance of hyperalgesic priming (Asiedu et al., 2011; Ferrari et al., 2015).

A female-specific role for sensory neuronal *Prlr* in hyperalgesic priming
Responsiveness to PRL in sensory neurons is substantially higher in females (>40 fold) than in males (Patil et al., 2013b, 2019a,b), and strictly controlled by E-2 (Diogenes et al., 2006; Patil et al., 2019b). Endogenous and extrapituitary PRL is elevated in paw and SC after inflammation and surgical injury (Scotland et al., 2011; Patil et al., 2013a). Accordingly, we examined whether mimicking the presence of endogenous PRL after injury could prolong mechanical hypersensitivity induced by spinal PGE₂ in both females and males. To do this, we primed the nociceptive pathway with a single injection of exogenous PRL (1 μ g) intraplantarly (this PRL dosage is active in females but not males) (Patil et al., 2019b) and precipitated hyperalgesic priming with intrathecal PGE₂ at 1 d after PRL treatment (Fig. 4A). We were able to administer PGE₂ at 24 h after intraplantar PRL because 1 μ g PRL produces mechanical hypersensitivity for only ~3–4 h in females (Patil et al., 2019b). PGE₂ evoked mechanical hypersensitivity was substantially longer lasting in PRL-treated female mice compared with vehicle-primed females (repeated-measures ANOVA; $F_{(6,60)} = 6.398$; $p < 0.0001$; $n = 6$; Fig. 4C), but this was not the case in male mice (repeated-measures ANOVA; $F_{(4,40)} = 0.2318$; $p = 0.9189$; $n = 5$ –7; Fig. 4B).

Our present findings suggest that a translation regulation event at the spinal level is critical for enhanced pain chronification in female mice. Our previous work demonstrated that Prlr signaling in the central terminals of nociceptors is important for acute pain models, specifically mechanical hypersensitivity in response to inflammation and injury in females (Patil et al., 2019b). This sex difference can be accounted for by increased translation of *Prlr* mRNA in central terminals of female mice (Patil et al., 2019b), suggesting transport of this mRNA to central terminals of nociceptors. To gain better insight into regulation of *Prlr* mRNA

Figure 4. Peripheral PRL only induces hyperalgesic priming in female mice. **A**, Schematic of the PRL-induced hyperalgesic priming model. BL, Baseline measurements after PRL-induced hypersensitivity is fully resolved. Brown arrows indicate injection time points. Blue arrows indicate post-PGE₂ treatment mechanical nociception measurement time points. **B**, **C**, Hyperalgesic priming model; PRL or vehicle priming into paw and PGE₂ injection into SC of male (**B**) or female (**C**) C57BL mice. Arrows indicate injection time points for PGE₂. Repeated-measures ANOVA with Bonferroni *post hoc* test: *** $p < 0.001$; **** $p < 0.0001$. $n = 5$ –7.

localization, we used *Prlr*^{fl/fl} mice and crossed them with Nav1.8^{cre/-} animals to generate a Nav1.8^{cre/-}/*Prlr*^{fl/fl} (*Prlr* CKO) in a set of sensory neurons (Fig. 5A). The *Prlr*^{fl/fl} line has inverse lox sites; hence, *Cre* recombination ablates the *Prlr* gene and activates GFP expression in targeted cells driven by the *Prlr* promoter (Fig. 5A). Analysis of the *Prlr* CKO showed that the truncated transgene *Prlr* mRNA contains the entire 5'-UTR; four exons and an intron between exons 1 and 4 (EI-EIV) and GFP, but it does not have the remaining *Prlr* exons or the 3'-UTR (Fig. 5A). Accordingly, we observed that RT-PCR with *Prlr*-F and GFP-R1 primers (Fig. 5A, red arrows) produced a 2500 bp band containing an intron sequence from total RNA of female *Prlr* CKO, but not *Prlr*^{fl/fl} (Fig. 5C).

The unique splicing of EI-EIV and GFP for *Prlr* mRNA in *Prlr* CKO mice allowed us to detect localization of this hybrid *Prlr* mRNA synthesized from *Prlr* gene promoter in sensory neuronal cell bodies, as well as peripheral and central terminals. For these experiments, we also used a positive control, PGP9.5 mRNA (*Uchl1* gene), which undergoes axonal transport in DRG

Figure 5. Evidence for translocation of *Prlr* mRNA to sensory neuronal peripheral and central terminals in female mice. **A**, Schematic of *Prlr* fl/fl and Nav1.8^{cre}/Prlr^{fl/fl} (*Prlr* CKO) genes and corresponding transcribed mRNA from these genes. Location of *Prlr*-F and GFP-R1 (red arrows) and GFP-F and GFP-R2 (blue arrow). **B**, Representative panel for PCR of PGP9.5 mRNA (*Uchl1* gene) from total RNA isolated from DRG and hindpaws (HP) of *Prlr* fl/fl (Con) and *Prlr* CKO female mice. **B'**, Quantification of data shown in **B** for *Prlr* CKO female mice (** $p < 0.01$; $n = 3$). **C**, Representative panel for PCR of hybrid 2500 bp *Prlr* mRNA using *Prlr*-F (exon 4) and GFP-R1 primers (see **A**) for DRG and SC total RNA isolated from *Prlr* fl/fl (Con) and *Prlr* CKO of female mice. **C'**, Quantification of data shown in **C** for *Prlr* CKO female mice (** $p < 0.01$; $n = 3$). **D**, Representative panel for PCR of GFP mRNA using GFP-F and GFP-R2 primers (see **A**) for DRG and SC total RNA isolated from *Prlr* fl/fl (Con) and *Prlr* CKO of female mice. **D'**, Quantification of data shown in **D** for *Prlr* CKO female mice (* $p < 0.05$; $n = 3$). **E**, Representative panel for PCR of GFP mRNA using GFP-F and GFP-R2 primers (see **A**) for DRG and HP total RNA isolated from *Prlr* fl/fl (Con) and *Prlr* CKO of female mice. **E'**, Quantification of data shown in **E** for *Prlr* CKO female mice (** $p < 0.01$; $n = 3$).

neurons (Willis et al., 2005, 2007). *Uchl1* mRNA was found in the hindpaw, but at an apparently lower level than observed in the DRG (unpaired two-tailed t test; $t = 6.521$ df = 4; Figs. 5B,B'). The hybrid *Prlr* mRNA in sensory neuronal cell bodies (DRG) and sensory neuronal central terminals (SC) was detected by amplification of the 2500 bp band from total RNA isolated from DRG and SC tissues of *Prlr* CKO (Fig. 5C). This 2500 bp band was

not noticed in the *Prlr*^{fl/fl} mouse line (Fig. 5C). Interestingly, the 2500 bp PCR product was detected in SC mRNA, suggesting constitutive axonal transport of *Prlr* mRNA (Fig. 5C). Moreover, hybrid *Prlr* mRNA was at significantly higher levels in SC compared with DRG (unpaired two-tailed t test; $t = 4.797$ df = 4; Fig. 5C'). To confirm this result with different primers, we PCR-amplified GFP (253 bp band) with GFP-F and GFP-R2 primers (Fig. 5A, blue arrows), using DRG and SC total RNA from *Prlr*^{fl/fl} and *Prlr* CKO mice. GFP mRNA was detected not only in DRG of *Prlr* CKO females, but also in SC (Fig. 5D), and it was at higher density in SC compared with DRG (unpaired two-tailed t test; $t = 3.608$ df = 4; Fig. 5D'). To examine translocation of *Prlr* mRNA to peripheral terminals, we examined the presence of GFP mRNA in hindpaw tissues of *Prlr* CKO mice (Fig. 5E). Again, GFP mRNA in *Prlr* CKO was at higher levels in hindpaw compared with DRG tissues (unpaired two-tailed t test; $t = 5.036$ df = 4; Fig. 5E'). These findings suggest that female *Prlr* mRNA is extensively translocated to sensory neuron peripheral and central terminals, likely via an element in the 5' UTR of the mRNA that is preserved in the transgene.

Next, we used *Prlr* CKO mice to examine the contribution of sensory neuronal *Prlr* to the regulation of the development of chronic pain in females and males. *Prlr* ablation in male sensory neurons did not affect either the initiation or persistent phase in the hyperalgesic priming model (repeated-measures ANOVA; $F_{(12,66)} = 9.445$; $p < 0.001$; $n = 4-6$; Fig. 6A). In contrast, *Prlr* CKO female mice showed both a significant reduction in mechanical hypersensitivity in response to IL-6 injection and these mice also showed a greatly reduced response to PGE₂ injection (persistent phase) compared with *Prlr*^{fl/fl} mice (repeated-measures ANOVA; $F_{(12,120)} = 1.309$, $p = 0.2219$; $n = 5-7$; Fig. 6B). We conclude from this experiment that *Prlr* in sensory neurons plays a key role in initiation and maintenance of chronic pain in female, but not male, mice.

Prlr signaling and the initiation and maintenance of hyperalgesic priming in female mice

Ablation of *Prlr* in sensory neurons does not allow for identification of peripheral or central sites driving hyperalgesic priming or the time course of when *Prlr* signaling occurs during hyperalgesic priming. To explore this in detail, the *Prlr* antagonist ΔPRL (Rouet et al., 2010; Patil et al., 2019b), was delivered at different locations and time points. A single injection of the antagonist ΔPRL (5 μg) into the

paw immediately before the intraplantar IL-6 priming injection did not affect mechanical hypersensitivity in response to the IL-6 injection (Fig. 7A). However, there was a significant reduction in mechanical hypersensitivity during the persistent, post-PGE₂ phase in animals that received Δ PRL compared with those that received the vehicle. These Δ PRL-treated female mice returned to baseline levels of mechanical sensitivity within 2 d of the PGE₂ injection (repeated-measures ANOVA; $F_{(30,195)} = 6.043$; $p < 0.0001$; $n = 5$ or 6; Fig. 7A). Intrathecal administration of Δ PRL (5 μ g) immediately before intraplantar IL-6 led to a transient reduction in mechanical hypersensitivity for <3 h following intraplantar IL-6 injection (Fig. 7A). The degree of mechanical hypersensitivity following PGE₂ injection was almost identical to that observed with intraplantar administration of Δ PRL. We then evaluated the role of Prlr signaling in the maintenance of hyperalgesic priming with intraplantar or intrathecal administration of Δ PRL (5 μ g) before PGE₂ administration. Blockage of Prlr signaling in the paw by intraplantar injection of Δ PRL before the injection of PGE₂ did not influence mechanical hypersensitivity magnitude or duration. In contrast, intrathecal administration of Δ PRL coupled with intrathecal PGE₂ administration led to inhibition of mechanical hypersensitivity and a faster return to baseline (repeated-measures ANOVA; $F_{(30,210)} = 7.192$; $p < 0.0001$; $n = 5$ or 6; Fig. 7B).

The conventional view is that endogenous PRL comes almost exclusively from the pituitary gland (Ben-Jonathan et al., 2008). However, extrapituitary sources for PRL have been reported, and these PRL sources are especially abundant in humans (Ben-Jonathan et al., 1996). In rodents, inflammation and tissue injury cause an increase in PRL in the paw and SC (Scotland et al., 2011; Patil et al., 2013a). We examined whether endogenous pituitary PRL is involved in the regulation of pain chronicity in male and female mice. Circulating PRL that has originated in the pituitary can be removed by either hypophysectomy (Green et al., 2016) or systemic treatment with bromocriptine (Grattan, 2015). Both approaches have downsides, but we opted to use the systemic bromocriptine approach because bromocriptine is used in clinical studies and hypophysectomy drastically affects gonadal hormone production. We systemically treated both male and female mice with bromocriptine as previously described (Yip et al., 2012). Removal of endogenous pituitary PRL did not influence mechanical hypersensitivity during the IL-6 phase of hyperalgesic priming initiation in male mice but did slightly prolong the PGE₂ precipitated hypersensitivity (repeated-measures ANOVA; $F_{(10,90)} = 2.980$; $p = 0.0028$; $n = 5$ or 6; Fig. 8A). In female mice, the IL-6 response was enhanced in the bromocriptine-treated females, but the priming response to PGE₂ was equivalent in vehicle and bromocriptine-treated mice (repeated-measures ANOVA; $F_{(14,126)} = 3.127$; $p = 0.0003$; $n = 5$ or 6; Fig. 8B).

Figure 6. Regulation of hyperalgesic priming by sensory neuronal Prlr selectively in female mice. **A, B.** Hyperalgesic priming model with peripheral IL-6/Veh and spinal PGE₂ in *Prlr^{fl/fl}* (*Prlr-lox*; control) and *Nav1.8^{cre/+}/Prlr^{fl/fl}* (*Prlr CKO*) in male (**A**) and female (**B**) mice. Arrows indicate injection time points for IL-6/Veh and PGE₂. Repeated-measures ANOVA with Bonferroni *post hoc* test: **A**, **** $p < 0.001$, for *Prlr-lox* compared with Veh-*Prlr-lox*; and ★★★★★ $p < 0.0001$; ★★★★★ $p < 0.001$, for *Prlr CKO* compared with Veh-*Prlr-lox*. **B**, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. $n = 4–7$.

Collectively, these results suggest that endogenous extrapituitary PRL signaling plays a key role in hyperalgesic priming in female mice. During initiation of chronic pain, this source can be peripheral or central, but the crucial source of PRL during the maintenance of pain chronicity is likely central.

Discussion

Studies in both animals and humans demonstrate sexually dimorphic mechanisms controlling the development and resolution of chronic pain (Joseph et al., 2003; Sorge et al., 2015; Nasir et al., 2016; Taves et al., 2016; Lopes et al., 2017; Rosen et al., 2017, 2019; Mapplebeck et al., 2018; Paige et al., 2018; Dance, 2019; North et al., 2019; Patil et al., 2019b; Ray et al., 2019). Among these sex differences, several factors have been discovered that drive chronic pain specifically in males (Sorge et al., 2015; Taves et al., 2016; Mapplebeck et al., 2018; Megat et al., 2018; Paige et al., 2018; Shiers et al., 2018; Martin et al., 2019), but relatively little is known about such chronic pain mechanisms in females. There is evidence that these mechanisms are closely regulated by gonadal hormones (Traub and Ji, 2013). For instance, the apparent male-specific effect of microglia-driven P2X4 signaling in neuropathic pain can be conferred to females

Figure 7. Regulation of the initiation and maintenance of hyperalgesic priming by peripheral and spinal Prlr in female mice. Hyperalgesic priming model in female mice with peripheral IL-6 and spinal PGE₂. **A**, Schematic of injection locations and timing. **B**, Vehicle (no ΔPRL), Prlr antagonist (ΔPRL; 5 μg) was coadministered with IL-6 in paw or SC. **C**, Schematic of injection locations and timing. **D**, Vehicle (no ΔPRL) was injected into paw. ΔPRL (5 μg) was given into the paw or SC 30 min before spinal PGE₂. Repeated-measures ANOVA with Bonferroni *post hoc* test: **p* < 0.05; *****p* < 0.0001, for ΔPRL intrathecally compared with no ΔPRL; and ★★★★★*p* < 0.0001; ★*p* < 0.05, for ΔPRL intraplantarly compared with no ΔPRL; *n* = 5–7).

with testosterone treatment (Sorge et al., 2015). In humans, sex differences in tibial nerve transcriptomes also demonstrate a signature for gonadal hormone influence on sensory neuronal transcriptomes across the lifespan in females (Ray et al., 2019). Experiments described here clearly demonstrate differential roles of gonadal hormones in development of chronicity in painful conditions with estrogen exacerbating priming effects and testosterone playing a protective role.

Additionally, we demonstrated that translation regulation plays a sex-specific role in the maintenance of chronic pain in female mice. This is especially relevant considering that translation control mechanisms are known contributors to the sensitization of nociceptors (Khoutorsky and Price, 2018; Megat and Price, 2018). Our previous work demonstrated that disruption

of translation regulation signaling in the periphery or SC was only capable of interfering with hyperalgesic priming if these treatments were given at the time of the priming event (Melemedjian et al., 2010, 2014; Asiedu et al., 2011). However, these previously published experiments were done entirely in male mice. Our data suggest that targeting these translation regulation mechanisms for the treatment of pain may have additional therapeutic benefits in women. A potential explanation for this differential effect on translation machinery in females is an effect of estrogen on translation machinery (Bronson et al., 2010; Ochnik et al., 2016). E-2-dependent connections between translational control of the suppressor of cytokine signaling and mTOR phosphorylation (Augusto et al., 2010) or regulation of Rheb signaling (Pochynuk et al., 2006) have been proposed (Matthews et al., 2005; Arbocco et al., 2016). These signaling pathways also play key roles in the excitability of nociceptors (Moy et al., 2017; Khoutorsky and Price, 2018; Megat et al., 2019a,b) and may play a more prominent role in the maintenance of persistent nociceptor plasticity in females than males.

Previous work indicates that sex-dependent mechanisms regulating hypersensitivity in inflammatory and neuropathic pain conditions can be attributed to distinct immune cell types: microglia in males (Sorge et al., 2011, 2015; Taves et al., 2016; Paige et al., 2018) and T cells in females (Sorge et al., 2015). Importantly, there is an opinion that these cells are regulated by gonadal hormones. Together, key molecules involved in sex-dependent regulation of the initiation, maintenance, and resolution would need to (1) be controlled by gonadal hormones, (2) induced by injury, (3) regulate immune cells, (4) undergo local translation control, and (5) be capable of regulating many other genes. The neuroendocrine hormone PRL and its receptor Prlr fit all these requirements. First, Prlr-mediated PRL effects are sex- and gonadal hormone-dependent in many tissues and cell types, including sensory neurons (Torner et al., 2001; Ben-Jonathan et al., 2008; Belugin et al., 2013; Patil et al., 2013a, 2019a,b). Second, many clinical and pre-clinical studies show that endogenous release of PRL from both pituitary and extrapituitary origins is induced by inflammation and tissue injury (Chernow et al., 1987; Noreng et al., 1987; Ben-Jonathan et al., 1996; Yardeni et al., 2007; Scotland et al., 2011; Patil et al., 2013a). Third, PRL is an effective direct and/or indirect activator of immune cells, especially macrophages and T cells (Matera et al., 2001; Savino et al., 2016; Tang et al., 2017). Moreover, many chronic autoimmune diseases affect females more frequently than males, and a potential role for PRL may in part explain this phenomenon for certain autoimmune diseases, such as lupus (Tang et al., 2017; Rizzetto et al., 2018). Fourth,

translational regulation of *Prlr* in sensory neurons has been suggested in our previous work (Patil et al., 2019b), and herein we show evidence in support of the translocation of *Prlr* mRNA to peripheral and central terminals of female or male sensory neurons where it could be translated in a female sex hormone-specific fashion (Patil et al., 2019b). Finally, *Prlr* activation leads to epigenetic changes and transcription regulation of many genes via the STAT5 pathway (Bole-Feyssot et al., 1998; Ben-Jonathan et al., 2008). The data presented here are one of the first demonstrations of a female-specific chronic pain initiation and maintenance mechanism acting directly on sensory neurons. Another is calcitonin gene-related peptide, which is released from sensory neurons, but its site of action to produce pain specifically in female mice is not known (Avona et al., 2019). Our findings using a sensory neuron-specific KO of *Prlr* combined with pharmacological antagonism of *Prlr* at specific sites suggests that *Prlr* signaling in sensory neuronal terminals of the SC controls initiation and maintenance of a chronic pain state in female mice. Having said this, we cannot rule out the possible influence of immune cells in our observations, including immune cells as a possible source of PRL that acts on *Prlr* in the setting of hyperalgesic priming in female mice.

A previous study in rats demonstrated that hyperalgesic priming to carrageenan does not occur in females (Joseph et al., 2003). Subsequent studies in mice and rats have shown additional sexual dimorphisms (Megat et al., 2018; Paige et al., 2018; Inyang et al., 2019), across the lifespan (Moriarty et al., 2019), but none of them has observed a similar absence of priming in female rodents. Indeed, our work shows, at least with IL-6 as the priming stimulus, that the magnitude and duration of the response to PGE₂ given peripherally or intrathecally are longer in female mice than in male mice.

Previous work from our laboratory (Patil et al., 2013a, 2019a, b) and other groups (Chen et al., 2020b) has established that PRL signaling to nociceptor PRL receptors plays an important role in promoting pain in response to injury, specifically in female rodents (Chen et al., 2020a). Our findings presented here extend this work to show that PRL signaling to nociceptors promotes development of chronic pain, also specifically in females. Interestingly, there is literature linking PRL to migraine (Silberstein, 1992; Cavestro et al., 2006), and a recent clinical study demonstrates that reducing PRL levels leads to resolution of headache, suggesting a causative role (Oliveira et al., 2020). These preclinical and clinical results suggest that targeting PRL signaling may be a treatment avenue for certain types of chronic pain, but likely only in women.

In conclusion, our findings demonstrate that sensory neuronal *Prlr* signaling relies on gonadal hormones and translation

Figure 8. Lack of effect of systemic bromocriptine on hyperalgesic priming in female and male mice. **A, B**, Hyperalgesic priming model with peripheral IL-6 and spinal PGE₂ in vehicle and bromocriptine (i.p.; BrCre) treatments of male (**A**) and female (**B**) mice. Arrows indicate injection time points for IL-6 and PGE₂. Repeated-measures ANOVA with Bonferroni *post hoc* test: ****p* < 0.001; *****p* < 0.0001. *n* = 5 or 6.

mechanisms to contribute to a female-specific regulation of the initiation and maintenance of pain chronicity. These results add a new depth to our understanding of sexually dimorphic signaling pathways involved in chronic pain development. Additionally, our data further substantiate the critical role that the neuroendocrine system and translation regulation play in nociceptor and nociceptive circuit excitability in response to a broad variety of important physiological stimuli.

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