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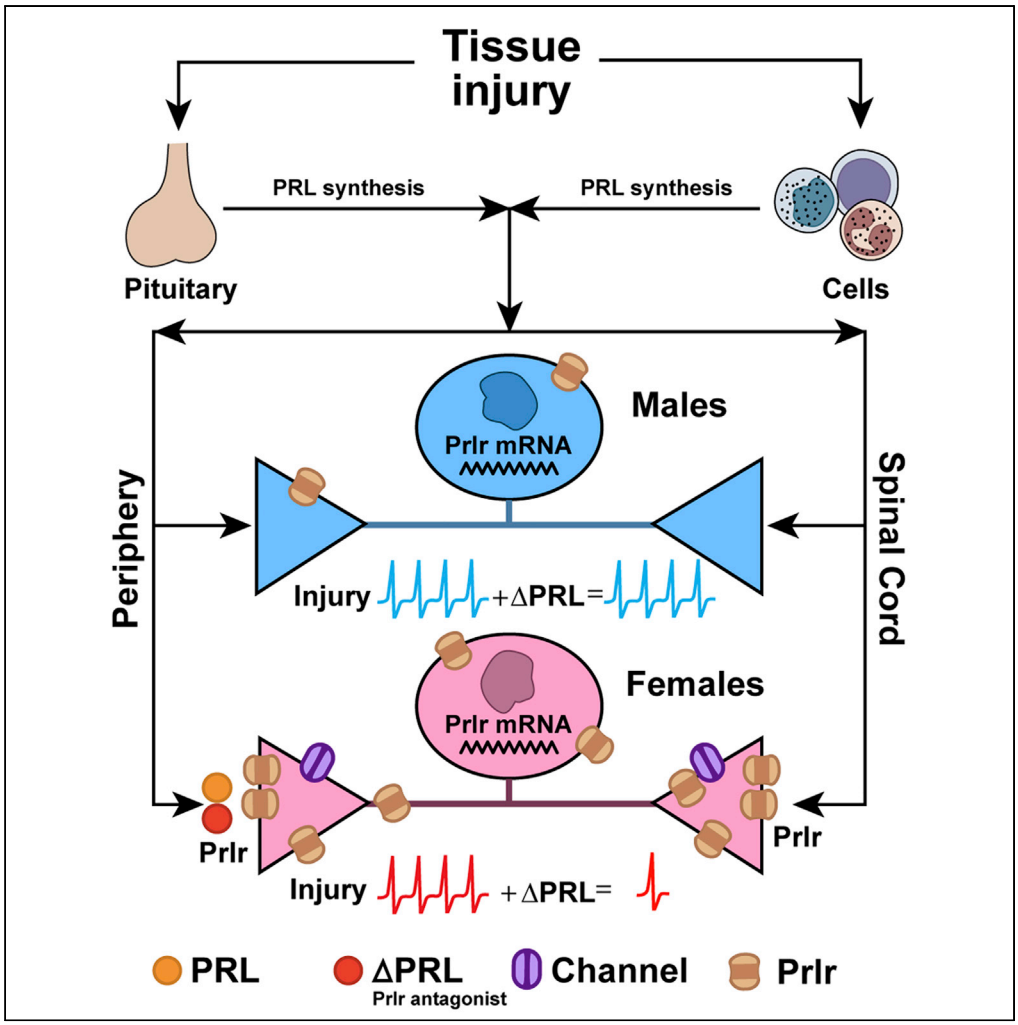

Mayur Patil, Sergei Belugin, Jennifer Mecklenburg, ..., Gregory Dussor, Theodore J. Price, Armen N. Akopian

theodore.price@utdallas.edu (T.J.P.)
akopian@uthscsa.edu (A.N.A.)

HIGHLIGHTS

Local or spinal PRL injection induces hyperalgesia in a female-selective manner

Sensory neuron Prlr regulates tissue injury-induced pain only in females

PRL regulates excitability in Prlr^{+} neurons depending on sex and estrogen

Regulation of Prlr translation defines female-selective neuronal excitability

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Prolactin Regulates Pain Responses via a Female-Selective Nociceptor-Specific Mechanism

Mayur Patil,[1,7] Sergei Belugin,[1] Jennifer Mecklenburg,[1] Andi Wangzhou,[3] Candler Paige,[3] Priscilla A. Barba-Escobedo,[1] Jacob T. Boyd,[1,2] Vincent Goffin,[4] David Grattan,[5] Ulrich Boehm,[6] Gregory Dussor,[3] Theodore J. Price,[3,*] and Armen N. Akopian[1,2,8,**]

SUMMARY

Many clinical and preclinical studies report an increased prevalence and severity of chronic pain among females. Here, we identify a sex-hormone-controlled target and mechanism that regulates dimorphic pain responses. Prolactin (PRL), which is involved in many physiologic functions, induces female-specific hyperalgesia. A PRL receptor (Prlr) antagonist in the hind paw or spinal cord substantially reduced hyperalgesia in inflammatory models. This effect was mimicked by sensory neuronal ablation of Prlr. Although *Prlr* mRNA is expressed equally in female and male peptidergic nociceptors and central terminals, Prlr protein was found only in females and PRL-induced excitability was detected only in female DRG neurons. PRL-induced excitability was reproduced in male *Prlr*$^{+}$ neurons after prolonged treatment with estradiol but was prevented with addition of a translation inhibitor. We propose a novel mechanism for female-selective regulation of pain responses, which is mediated by Prlr signaling in sensory neurons via sex-dependent control of Prlr mRNA translation.

INTRODUCTION

In recent years a renewed focus on sexual dimorphic mechanisms of pain has emerged. It is now widely recognized that many key mechanisms driving persistent pain differ between males and females in both animals and humans (Martin et al., 2019; Mogil et al., 2011; North et al., 2019; Sorge et al., 2011). Although time course and magnitudes of nociceptive hypersensitivity for a variety of pain conditions are often similar in females and males, the mechanisms responsible for this hypersensitivity and degree of chronicity are sex dependent (Martin et al., 2019; Mogil et al., 2011; Rosen et al., 2017; Sorge et al., 2011, 2015). Gonadal hormones, for instance, are known to be key contributors to sex differences in a variety of physiological and pathophysiological processes (Karp et al., 2017; Morselli et al., 2017). Human and animal studies of pain symptoms and severity have established correlations with the menstrual cycle, menopause, and alterations in gonadal hormone concentrations (Aloisi and Sorda, 2011; Houghton et al., 2002; LeResche et al., 2003; Slade et al., 2011; Traub and Ji, 2013).

Recent findings on sexual dimorphisms have demonstrated a role for spinal microglia in male-specific pain mechanisms (Sorge et al., 2011) and a T cell selective contribution to nociceptive transmission in females (Rosen et al., 2019; Sorge et al., 2015), although other investigators have described T cells to be involved in protection and resolution of pain (Krukowski et al., 2016; Laumet et al., 2019). It is possible that a neuron-specific, sexually dimorphic pain mechanism also could be involved and mediated by gonadal hormone controlled signaling. A prime candidate for this potential mechanism is prolactin (PRL) and its receptor (Prlr), since responsiveness to PRL in a variety of cell types depends on sex, menstrual cycle phase, pregnancy status, and lactation (Belugin et al., 2013; Childs et al., 1999; Diogenes et al., 2006; Pi and Voogt, 2002). PRL is involved in female-specific regulation of transient receptor potential (TRP) and other ligand-gated channels in sensory neurons (Diogenes et al., 2006; Liu et al., 2016; Patil et al., 2013b). Global ablation of PRL and Prlr leads to a substantial and female-selective reduction in postoperative and inflammatory heat hypersensitivity (Patil et al., 2013a, 2013b) and mechanical hypersensitivity, but the latter effect is observed in male and female mice (Patil et al., 2013a, 2013b). These studies demonstrate a clear role for PRL-Prlr signaling in pain hypersensitivity after injury, but the cells mediating these effects and the mechanisms generating female-specific nociceptive responses remain unknown.

[1]Department of Endodontics, The School of Dentistry, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA

[2]Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, TX 78229, USA

[3]School of Behavioral and Brain Sciences and Center for Advanced Pain Studies, University of Texas at Dallas, 800 W Campbell Road, Richardson, TX 75080, USA

[4]Inserm U1151, Université Paris Descartes, Paris, France

[5]Centre for Neuroendocrinology and Department of Anatomy, University of Otago School of Biomedical Sciences, Dunedin, New Zealand

[6]Department of Pharmacology and Toxicology, Center for Molecular Signaling (PZMS), Saarland University School of Medicine, Homburg, Germany

[7]Department of Molecular Pharmacology and Physiology, University South Florida (USF), Tampa, FL 33612, USA

[8]Lead Contact

*Correspondence: theodore.price@utdallas.edu (T.J.P.)

**Correspondence: akopian@uthscsa.edu (A.N.A.)

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The central goals of the work described here were to gain insight into whether Prlr expression in sensory neurons drives female-specific nociceptive responses to PRL and to understand how these female-specific effects emerge. We show that PRL signaling to Prlr expressed in sensory neurons at the level of peripheral and central terminals regulates female-specific hyperalgesia in several pain models. We also elucidate mechanisms responsible for PRL's female-selective actions in the regulation of pain. Gonadal hormones regulate cellular phenotypes via classic genomic and transient non-genomic signaling pathways (Amandusson and Blomqvist, 2013; Kelly et al., 1976; Revankar et al., 2005). However, surprisingly, our work points to a novel mechanism for sex-specific regulation of nociceptor plasticity that is dependent on selective and estrogen-dependent translation of *Prlr* mRNA in female DRG neurons. Overall, our work establishes sensory neuron participation of a major neuroendocrine hormone PRL in female-selective regulation of pain as well as a novel paradigm connecting sex- and gonadal hormone-dependent translational control that could be critical to understanding sexual dimorphism in many biological processes.

RESULTS

Exogenous PRL Induces Thermal and Mechanical Hypersensitivity in Females but Not in Males

Exogenous fully processed and non-modified human PRL (PRL) generated in an expression system sensitizes a subset of mouse female sensory neurons (Belugin et al., 2013; Patil et al., 2013b). Statistically significant sensitization in male sensory neurons is achieved with approximately a 40-fold higher concentration of PRL (Patil et al., 2013b). To establish if this major difference is also found *in vivo*, we evaluated whether PRL produces hyperalgesia in female and/or male mice. PRL injected into the hind paw (*ipl*) generated profound heat (Figure 1A) and mechanical hyperalgesia (Figure 1B) in a dose-dependent manner in estrus female mice (two-way ANOVA; heat - $F(3, 40) = 13.4$; $P < 0.0001$; mechanical - $F(3, 37) = 10.9$; $P < 0.0001$). As low as 0.1 μg PRL generated thermal and mechanical hyperalgesia in females, whereas 1 μg PRL injected in the contralateral paw did not produce an effect ipsilaterally (bars "*Cont*" on Figures 1A and 1B). This indicates that PRL-induced hyperalgesia involves peripheral (i.e., local) mechanisms. In contrast, for male mice, higher amounts of PRL (10 μg) produced heat (two-way ANOVA; $P < 0.0001$; Figure 1A) but not mechanical hypersensitivity $P = 0.1$; Figure 1B).

Administration of PRL into the spinal cord via intrathecal injection (*it*) also produced substantial heat (Figure 1C) and mechanical hypersensitivity (Figure 1D) predominantly in females (two-way ANOVA; heat - $F(3, 51) = 14$; $P < 0.0001$; mechanical - $F(3, 45) = 20.6$; $P < 0.0001$). Spinal PRL-induced hypersensitivity was not significant in male mice (two-way ANOVA; heat $P = 0.1$; mechanical $P = 0.2$; Figures 1C and 1D). Unbound PRL protein undergoes relatively fast degradation (Freeman et al., 2000). Consistent with this pharmacokinetic property, PRL (1 and 10 μg) injected in the hind paw (Figures S1A and S1B) or spinal cord (Figures S1C and S1D) of female mice produced significant heat and mechanical hypersensitivity for up to ~4 h (especially for high doses) and peaked at 1–2 h post administration.

Prolonged withdrawal of estrogen and progesterone in ovariectomized (OVX) females totally ablates PRL responsiveness in rats (Diogenes et al., 2006). We evaluated whether PRL responsiveness depends on the estrous phase of mice. Intraplantar (*ipl*) or spinal cord (*it*) injection of PRL (1 μg) induced mechanical hypersensitivity in females but not in males (Figures 1E and 1F two-way ANOVA; peripheral - $F(6, 48) = 9.7$; $P < 0.0001$; $n = 5$; Figure 1E; and spinal - $F(6, 48) = 4.5$; $P = 0.0011$; $n = 5$). PRL sensitivity was not affected by female estrous phases (Figure 1F). These results show that exogenous PRL delivered locally into the hind paw or spinal cord triggers 4-h-long-lasting pain hypersensitivity in a female-selective manner, but independent of female estrous phases.

Female-Selective Suppression of Postoperative Pain by Prlr Antagonist

Incision surgery and inflammation up-regulates PRL in a sex-dependent fashion in the hind paw and especially spinal cord, where the larger magnitude of upregulation is found (Patil et al., 2013a; Scotland et al., 2011). We used the specific Prlr antagonist, Δ1-9-G129R-hPRL (ΔPRL) (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), to evaluate the role of Prlr in the regulation of postoperative pain in female and male mice and rats. In estrus female mice at 1 day post incision, ΔPRL (5 μg) applied into the spinal cord by intrathecal injection (*it*) significantly reversed heat (two-way ANOVA; $F(6, 44) = 8.2$; $P < 0.0001$; $n = 4–5$; Figure S2A) and mechanical hypersensitivity ($P = 0.014$ at 60 min; $P = 0.03$ at 120 min; $n = 5–6$; Figure S2B). In contrast, ΔPRL (5 μg) did not

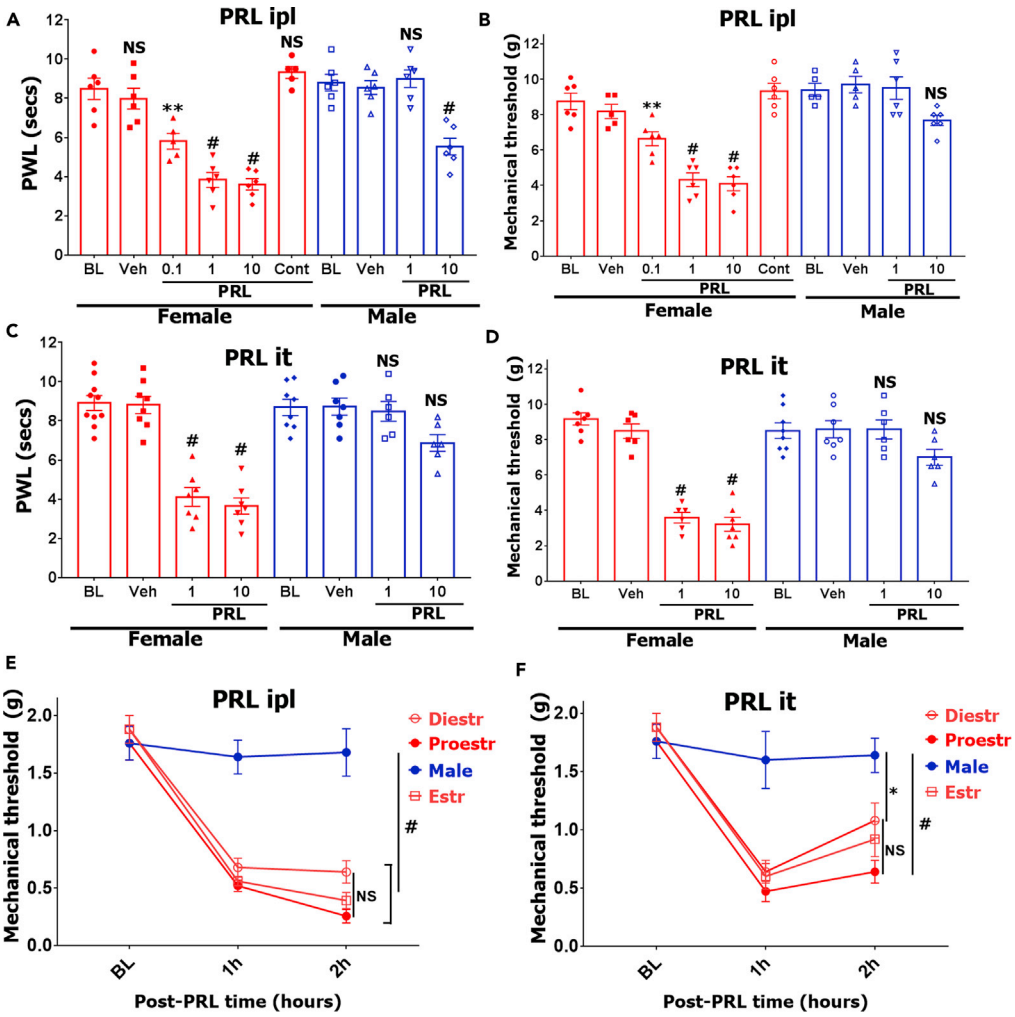

Figure 1. Exogenous PRL-Induced Hypersensitivity in Female and Male Mice

(A–D) PRL-induced heat (A and C) and mechanical (B and D) hypersensitivity was assessed at 1 h post-PRL-administration time point in male and estrous female mice. PRL was administrated into the hind paw (ipl; A and B) or intrathecal space of spinal cord (SC; C and D). PRL dosages (0.1, 1, or 10 μg) and sex of mice are indicated. Mechanical threshold was measured with the Dynamic Plantar Aesthesiometer. "*Cont*" indicates contralateral injection of 1 μg PRL and measurements of hyperalgesia in the ipsilateral hind paw.

(E and F) PRL (1 μg) was injected *ipl* (intra-plantar; panel E) or *it* (intrathecal; panel F), and mechanical hypersensitivity was measured in males and females at different estrous phases (diestrus [*Diestr*], estrous [*Estr*], and proestrus [*Proestr*]). BL is baseline reading before PRL administration.

Data are represented as mean ± SEM. Statistical test is regular two-way ANOVA with Tukey's post hoc test (n = 5–10; NS, non-significant; *p < 0.05; **p < 0.01; #p < 0.0001). See also Figure S1.

show antagonism of incision-induced heat and mechanical hypersensitivity in male mice (Figures S2C and S2D). We did not escalate the dosage of ΔPRL, since at dosages >25 μg, it could show agonistic properties (Scotland et al., 2011). However, 5 μg of ΔPRL did not exhibit agonistic or antagonistic properties on mice that underwent sham procedures (Figures S2A–S2D).

Since peak effects were observed at 60 min post ΔPRL, we recorded vehicle and ΔPRL actions at 60 min post injection. Male and estrous females were injected with vehicle or ΔPRL into hind paws (*ipl*) at 1 day post incision. Heat hypersensitivity in females, but not in males, was significantly reversed with ΔPRL (two-way ANOVA; $F(4, 31) = 9.4$; $P < 0.0001$; n = 4–5; Figure 2A). Mechanical hyperalgesia in males as well as females was not significantly affected by hind paw administration of ΔPRL (two-way ANOVA; $F(4, 50) = 0.2$; $P = 0.9$; Figure 2B). Spinal injection of ΔPRL substantially reversed both heat (two-way ANOVA; $F(4, 41) = 12.6$;

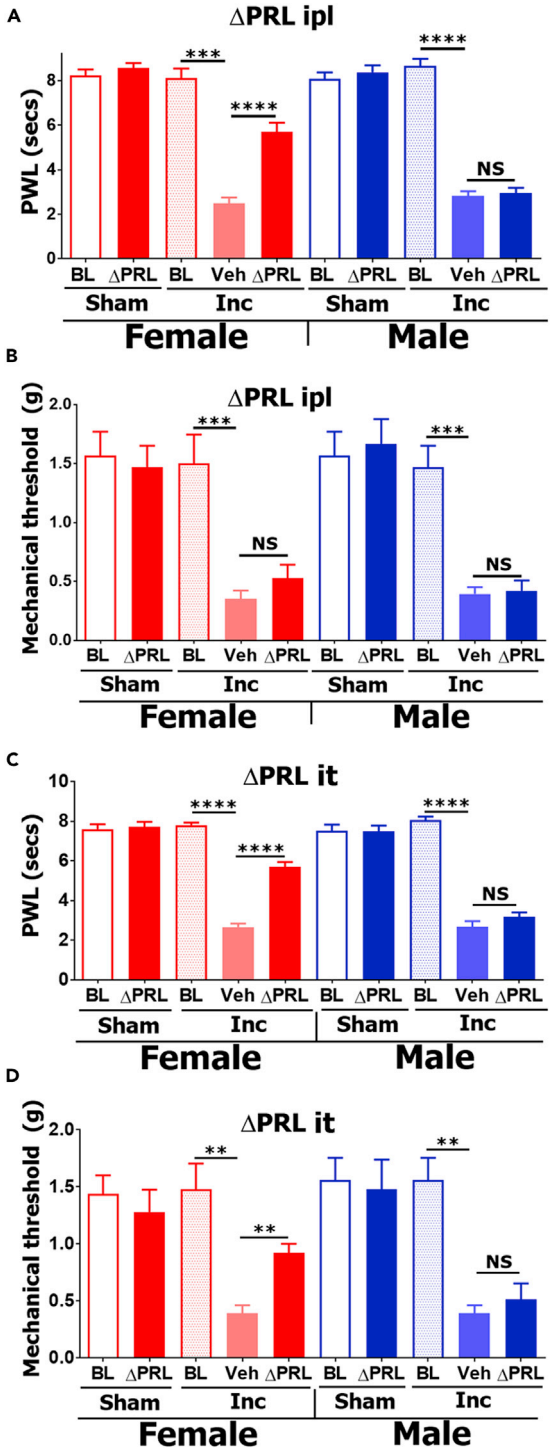

Figure 2. Suppression of Postoperative Pain by *Prlr* Antagonist in Female and Male Mice

Vehicle (Veh) or Prlr antagonist (5 μg; ΔPRL) was injected into hind paw (*ipl*) of male and estrous female mice at 1 day post incision (Inc) or sham procedures. Heat (A) and mechanical (B) hypersensitivity was assessed at 1 h post Veh/ΔPRL injection. Vehicle or ΔPRL (5 μg) was injected intrathecally (*it*) into spinal cord of male and estrous female mice at 1 day post incision or sham procedures. Heat (C) and mechanical (D) hypersensitivity was assessed at 1 h post Veh/ΔPRL injection. BL are baseline values before incision procedures. Procedures and animal sex are indicated below the x axis. Data are represented as mean ± SEM. Statistical test is regular two-way ANOVA with Tukey's post hoc test (NS, $p > 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; n = 5–7). See also Figure S2.

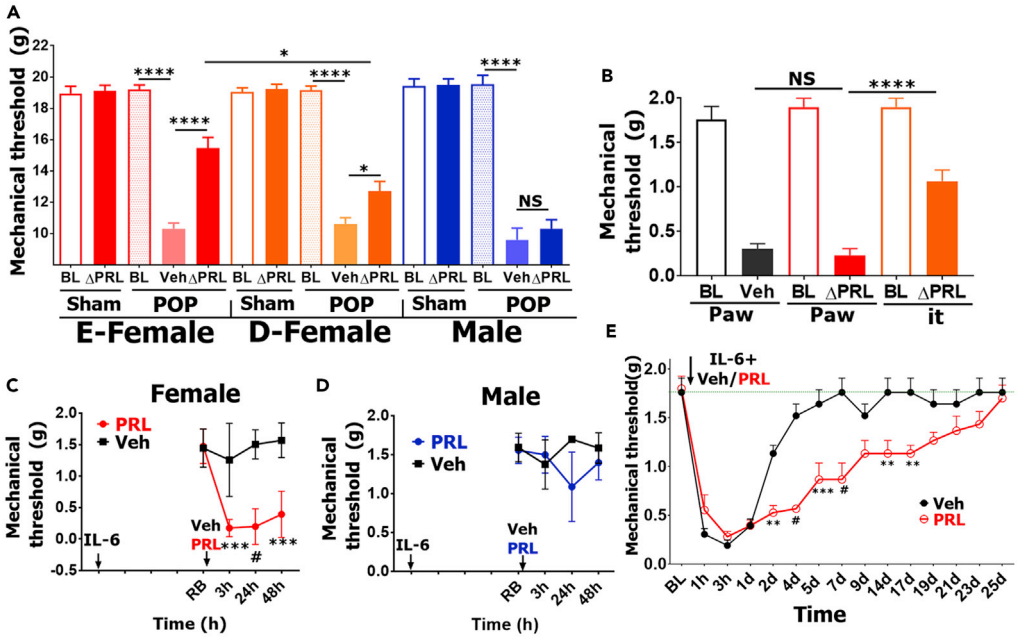

Figure 3. Effects of *Prlr* Antagonist and Agonist in Different Inflammatory Pain Models in Female and Male Rats and Mice

(A) Vehicle (Veh) or ΔPRL (5 μg) was injected into spinal cord of male, diestrus female (D-female), or estrous female (E-female) rats at 1 day post incision surgery (POP) or sham procedures. Mechanical hyperalgesia was assessed with Dynamic Plantar Aesthesiometer at 1 h post Veh/ΔPRL injection. BL are baseline values. Statistical test is two-way ANOVA with Tukey's post hoc test (NS, $p > 0.05$; *$p < 0.05$; ****$p < 0.0001$; n = 5–6).

(B) IL-6 (1 ng) was injected into hind paw, and vehicle or ΔPRL (5 μg) was injected approximately simultaneously into hind paw (*paw*) or spinal cord (*it*) of estrous female mice. Mechanical hyperalgesia was assessed at 1 h post IL-6/Veh or IL-6/ΔPRL co-injections. BL is baseline value. Data are represented as mean ± SEM. Statistical test is regular two-way ANOVA Bonferroni's post hoc test (NS, $p > 0.05$; ****$p < 0.0001$; n = 6).

(C and D) In the model of hyperalgesic priming, 0.5 ng PRL produces mechanical hypersensitivity in IL-6 (0.1 ng)-primed females (panel C) but not males (panel D). RB is baseline. Statistical test is regular two-way ANOVA Bonferroni's post hoc test ***$p < 0.001$; $^{\#}p < 0.0001$; n = 5).

(E) IL-6 (1 ng) and vehicle or PRL (1 μg) were co-injected into the paw in estrous-phase female mice. Mechanical hyperalgesia was assessed at indicated time points. BL is baseline value. Data are represented as mean ± SEM. Statistical test is regular two-way ANOVA Bonferroni's post hoc test (**$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.0001$; n = 6).

$P < 0.0001$; n = 4–6; Figure 2C) and mechanical hypersensitivity ($F(4, 40) = 18.2$; $P < 0.0001$; n = 5–6; Figure 2D) in a female-selective fashion.

PRL actions could vary between species and PRL release from the pituitary depends on the estrous phase (Freeman et al., 2000), but exogenous PRL responsiveness at periphery and spinal cord of naive female mice is not dependent on the estrous phase (Figures 1E and 1F). Accordingly, we evaluated whether ΔPRL effects differ in diestrus (diestrus 1 and 2 combined) versus estrus female rats. Spinal (*it*) injection of ΔPRL (5 μg) at 1 day post incision significantly reversed postoperative mechanical hypersensitivity in diestrus and estrus female (two-way ANOVA; $F(8, 60) = 6.5$; $P < 0.0001$; n = 5) but not in male rats (Figure 3A). The inhibition of mechanical hypersensitivity was more pronounced in estrus compared with diestrus female rats (two-way ANOVA; $P = 0.011$; Figure 3A). Our findings indicate that blockage of Prlr leads to female-selective inhibition of postoperative hypersensitivity, which depends on the site of Prlr antagonist action (Figures 1B versus 1D) and partially on the estrous phase (Figure 3A) but not the rodent species (Figures 2 and 3A).

Prlr Modulates Inflammatory Hypersensitivity in Female Mice

Inflammatory hypersensitivity was induced by hind paw injection of interleukin (IL)-6 (Melemedjian et al., 2010). Co-injection of IL-6 (1 ng; into the hind paw, *ipl*) and ΔPRL (5 μg; into the spinal cord, *it*) effectively

inhibited mechanical hypersensitivity in female mice, whereas IL-6 and vehicle or ΔPRL co-administration into the hind paw did not produce anti-mechanical hypersensitivity effects (two-way ANOVA; $F(2, 28) = 13.3$; $P < 0.0001$; $n = 5–6$; Figure 3B).

The concentration of endogenous PRL in serum of rodents could be increased up to 100 ng/mL during inflammatory conditions (Patil et al., 2013a; Scotland et al., 2011). It could be presumed that inflammation will sensitize Prlr signaling. To evaluate this possibility, we used the hyperalgesic priming model (Melemedjian et al., 2010) and examined whether hind paw injection of PRL could precipitate hyperalgesic priming in mice primed with IL-6. IL-6 (0.1 ng) was injected into the hind paw and following hypersensitivity resolution (5 days post IL-6), PRL was injected into the same hind paw. Priming with IL-6 dramatically (>100-fold) sensitized Prlr signaling wherein estrus female, but not male mice, showed hypersensitivity to as low as 0.5 ng PRL (two-way ANOVA; for females; $F(3, 24) = 7$; $P = 0.0015$; $n = 5$; Figure 3C and for males; $F(3, 20) = 2.5$; $P = 0.085$; $n = 5$; Figure 3D).

It is well documented that many inflammatory and idiopathic chronic pain conditions have 2- to 6-fold greater prevalence, chronicity, and symptom severity in women as compared with men (Berkley, 1997; Fillingim et al., 2009; Traub and Ji, 2013; Unruh, 1996). Hence, we examined whether addition of exogenous PRL to IL-6 could alter chronicity and/or severity of mechanical hypersensitivity. Single co-administration of PRL (1 μg) and IL-6 (1 ng) into the hind paw resulted in a substantial increase in the duration of mechanical hypersensitivity compared with co-injection of vehicle and IL-6 into females (two-way ANOVA; $F(14, 136) = 4.6$; $P < 0.0001$; $n = 5–6$; Figure 3E). However, PRL did not increase the severity (i.e., magnitude) of inflammatory hypersensitivity (Figure 3E). In summary, these results indicate that peripheral and spinal Prlr signaling is involved in modulation of inflammatory pain in females.

Sensory Neuronal Prlr Regulates Inflammatory Pain in a Female-Selective Fashion

Prlr is expressed not only in sensory neurons but also in DRG fibroblasts and satellite glial cells, some immune cells, and possibly by intrinsic spinal cord neurons (Ben-Jonathan et al., 2008; Haring et al., 2018; Patil et al., 2014, 2019). Here, we evaluated whether sensory neuronal Prlr is essential in female-selective regulation of chemical-induced, inflammatory, and neuropathic pain. To do so, we ablated the *Prlr* gene in the Nav1.8^{+} subset of sensory neurons (*Prlr* CKO). The *Prlr*$^{fl/fl}$ line was generated by insertion of inverse lox sites around exon 5 (Brown et al., 2016). Hence, cre-recombination ablates the gene and activates GFP in Nav1.8^{+} neurons (Figures S3A and S3B). C*re*-recombination was verified by GFP mRNA expression that can be amplified from DRG RNA of *Prlr* CKO but not *Prlr*$^{fl/fl}$ female mice (Figure S3C). To show conditional ablation of Prlr protein in sensory neurons, we performed immunohistochemistry (IHC) on spinal cord sections with CGRP and Prlr antibodies. Figure S3D shows that Prlr protein is eliminated in central terminals of the dorsal horn of spinal cord but not in other Prlr^{+} cells of Nav1.8$^{cre/-}$/Prlr$^{fl/fl}$ female mice. IHC was performed only in female mice, since Prlr antibodies do not reliably label DRG sensory neurons and central terminals in spinal cord of male mice, probably owing to low Prlr expression in males and/or low sensitivity of Prlr antibodies. As an additional test of the validity of our conditional deletion approach, we also tested sensitization of TRPV1 by exogenous PRL in female mice and found that 1 μg/mL PRL sensitizes capsaicin (CAP)-evoked CGRP release in *Prlr*$^{fl/fl}$ but not Nav1.8$^{cre/-}$/Prlr$^{fl/fl}$ (Prlr CKO) spinal cord slices (Figure S3E).

Ablation of *Prlr* gene in sensory neurons substantially and female-selectively reduced postoperative heat (two-way ANOVA; $F(3, 40) = 5.2$; $P = 0.004$; $n = 5–8$; Figure 4A) and mechanical hypersensitivity ($F(3, 48) = 3.5$; $P = 0.021$; $n = 5–8$; Figure 4B) at the 1-day post-incision time point. In *Prlr* CKO animals, IL-6-induced mechanical hypersensitivity was also significantly reversed in females (two-way ANOVA; $F(3, 28) = 13.3$; $P < 0.0001$; $n = 5$) but in not males ($P = 0.99$; $n = 5$) at 3 h post-IL6 time point (Figure 4C). Examination of the time course of IL-6 hypersensitivity development showed that IL-6-induced heat (two-way ANOVA; $F(4, 40) = 0.74$; $P = 0.57$; $n = 5$) and mechanical hypersensitivity ($F(4, 40) = 0.09$; $P = 0.99$; $n = 5$) were equally well developed in *Prlr*$^{fl/fl}$ and *Prlr* CKO male mice (Figures 4D and 4E). In contrast, IL-6-induced heat (two-way ANOVA; $F(4, 30) = 3.8$; $P = 0.012$; $n = 5$) and mechanical hypersensitivity ($P = 0.011$ at 1 h post IL-6; $P = 0.004$ at 3 h post IL-6; $n = 5$) were substantially lesser in *Prlr* CKO compared with *Prlr*$^{fl/fl}$ females at all time points (Figures 4F and 4G).

In a neuropathic model of chronic constriction injury (CCI), heat and mechanical hyperalgesia were similarly developed in *Prlr*$^{fl/fl}$ and *Prlr* CKO males (Figures S4A and S4B). CCI-induced hypersensitivity was slightly

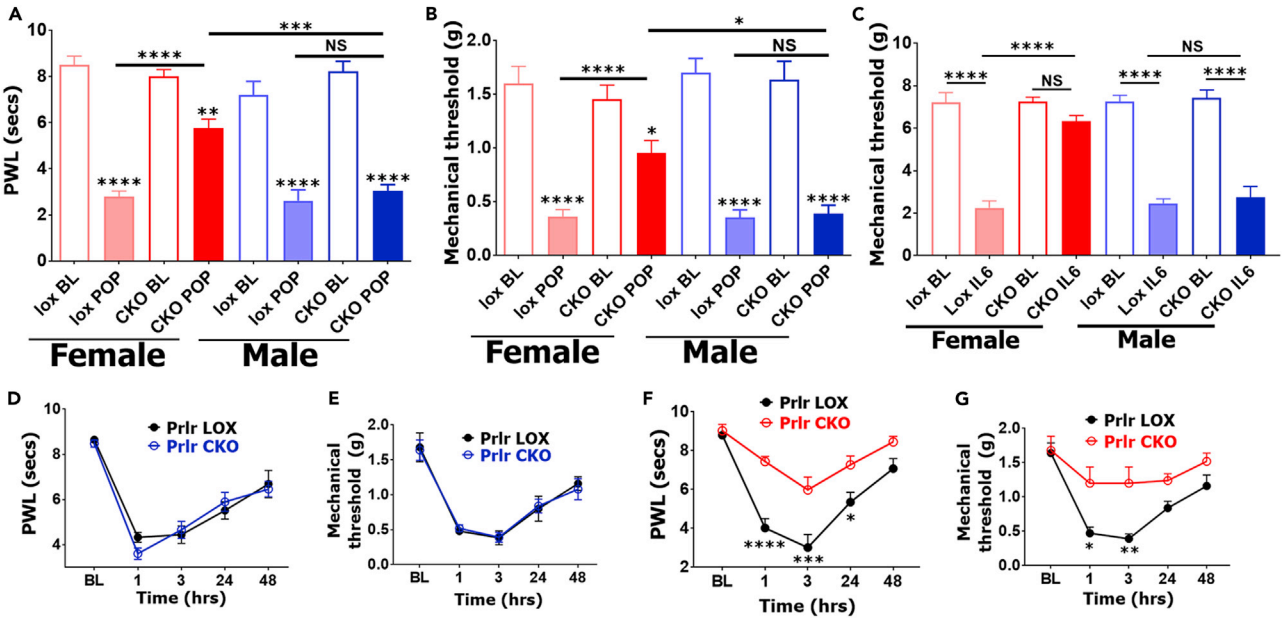

Figure 4. Hypersensitivity in Inflammatory Pain Models in Sensory Neuronal Prlr CKO Male and Female Mice

(A) Postoperative (POP) heat hypersensitivity was measured 1 day post incision in $Prlr^{fl/fl}$ (lox; control) and $Nav1.8^{cre/-}/Prlr^{fl/fl}$ (CKO) female and male mice.

(B) POP mechanical hypersensitivity was measured 1 day post incision in lox and CKO female and male mice.

(C) IL-6 (1 ng)-induced mechanical hyperalgesia was measured 3 h post IL-6 (ipl) in lox and CKO female and male mice.

For (A)–(C), Lox BL and CKO BL are baseline measurements in indicated mouse lines. For (A)–(C), data are represented as mean ± SEM and the statistical test is regular two-way ANOVA with Tukey's post hoc test (NS, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$; n = 5–7).

(D and E) Development of IL-6-induced heat (D) and mechanical (E) hyperalgesia in Prlr LOX (control) and Prlr CKO male mice.

(F and G) Development of IL-6-induced heat (F) and mechanical (G) hyperalgesia in Prlr LOX and Prlr CKO female mice. BL are baseline measurements in indicated mouse lines.

For (D)–(G), data are represented as mean ± SEM and the statistical test is regular two-way ANOVA with Bonferroni's post hoc test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; n = 5–6). See also Figures S3 and S4.

less pronounced in *Prlr* CKO compared with $Prlr^{fl/fl}$ females (Figures S4C and S4D). In a test of chemical nociception, mustard oil-induced hypersensitivity was quickly resolved (within 30 min) and identical in $Prlr^{fl/fl}$ and *Prlr* CKO females (Figures S4E and S4F). Overall, these data show that sensory neuronal Prlr contributes to female-selective regulation of hypersensitivity in inflammatory pain models but may play a lesser role in neuropathic and chemical-induced pain models.

Prlr Isoform mRNA Expression by DRG Sensory Neuronal Subtypes in Females and Males

Sensory neuronal Prlr signaling appears to be female-selective for *in vivo* (Figure 4) and *in vitro* models (Diogenes et al., 2006; Patil et al., 2013b). This suggests that *Prlr* mRNA should have predominant expression in female compared with male sensory neurons. *Prlr* mRNA is mainly expressed in a subset of medium- and small-sized peptidergic and $CGRP^{-}/trpV1^{+}$ sensory neurons of female and male mice (Patil et al., 2019). $Prlr^{+}$ medium-sized peptidergic neurons can be divided into two subpopulations: $NPY2R^{+}$ and $NPY2R^{-}$ (Patil et al., 2019). Separate analysis of single-cell sequencing for female versus male data shows Prlr expression is at similar levels in $Prlr^{+}$ sensory neuronal groups (Patil et al., 2019; Usoskin et al., 2015). However, data on sex-dependent expressions of Prlr long (Prlr-L) and short (Prlr-S) isoforms in sensory neurons, which have distinct functions (Belugin et al., 2013; Ben-Jonathan et al., 2008; Freeman et al., 2000), are not available. Accordingly, we examined Prlr-L and Prlr-S mRNA expression in sensory neurons using single-cell quantitative PCR (qPCR). We randomly collected single small or medium-sized *Prlr-cre*$^{+}$ DRG neurons from female and male $Prlr^{cre/+}/Rosa26^{LSL\text{-}tDTomato/+}$ mice and performed real-time qPCR with Prlr-L, Prlr-S, as well as sensory neuronal marker CGRP, TRPV1, and NPY2R primer sets. Expression of GAPDH was used as a loading and normalization control. All visualized and collected *Prlr-cre*$^{+}$ neurons contained Prlr-L and Prlr-S mRNA (Figure 5A). Statistical analysis showed that Prlr-L and Prlr-S mRNA were expressed at approximately similar levels in female and male neuron subtypes: $CGRP^{+}/TrpV1^{+}$, $CGRP^{-}/TrpV1^{+}$, $CGRP^{+}/NPY2R^{-}$, and $CGRP^{+}/NPY2R^{+}$ (for Prlr-L $t = 0.1$ $df = 34$; $P = 0.92$ and for Prlr-S $t = 0.51$

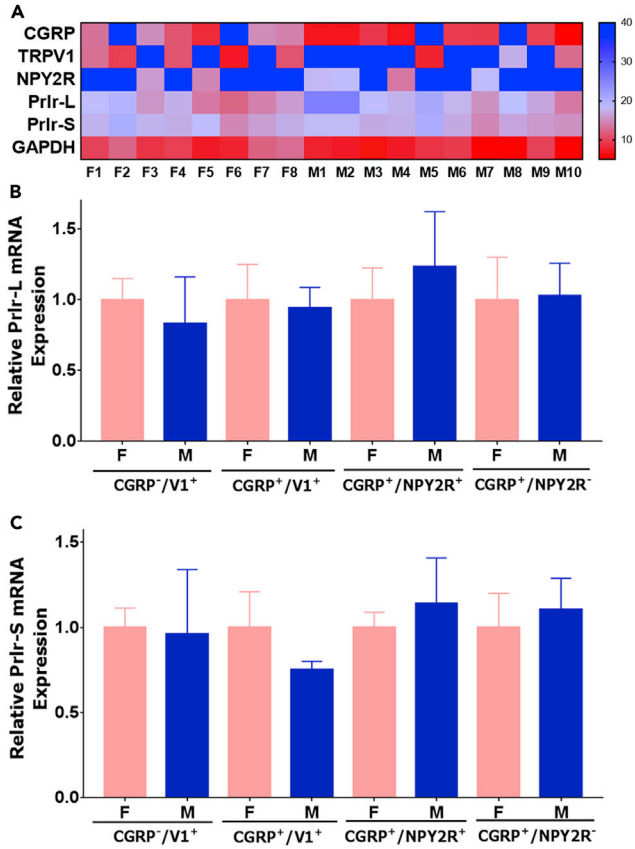

Figure 5. Real-time Single Cell Quantitative PCR for *Prlr-L* and *Prlr-S* from *Prlr-cre*$^{+}$ Female and Male DRG Neurons

(A) Representative heatmap showing Ct values generated by single-cell RT-PCR from *Prlr*-cre^{+} female (F) and male (M) DRG neurons. Y axis shows amplified set of genes. X axis marks randomly picked cells for PCR. Values of $\geq$38 on the heatmap is considered as no amplification. Normalized mRNA expression levels of *Prlr-L* (B) and *Prlr-S* (C) isoforms in sensory neuronal groups. Groups for single *Prlr-cre*$^{+}$ neurons from female and male mouse DRG are indicated on the x axis. Data are represented as mean $\pm$ SEM. Statistical test is unpaired t test (non-significant $p > 0.05$; n = 6–32 depending on *Prlr-cre*$^{+}$ neuronal group) for each sensory neuronal group.

df = 36; P = 0.62; Figures 5B and 5C). These findings lead to the unexpected conclusion that there are not clear differences for Prlr-L and Prlr-S mRNA expression levels between sexes in a variety of *Prlr-cre*$^{+}$ sensory neuronal subtypes.

Sex- and Estrogen-Dependent Regulation of Neuronal Excitability in Prlr-cre^{+} Nociceptors

We have shown that exogenous, unmodified 23 kDa-PRL is able to produce hypersensitivity in a female-selective manner (Figures 1 and S1) and enhance excitability in female DRG neurons (Patil et al., 2014). Hence, regulation of excitability is a valid, dependable measure for Prlr activity in DRG neurons. We investigated whether PRL could regulate neuronal excitability in *Prlr-cre*$^{+}$ sensory neurons isolated from Prlr$^{cre/+}$/Rosa26$^{LSL\text{-}tDTomato/+}$ female and male mouse DRG and whether this regulation is reliant on estrogen (E2). For these experiments, only small-sized *Prlr-cre*$^{+}$ neurons belonging to CGRP^{+}/TrpV1^{+} or CGRP^{-}/TrpV1^{+} groups were selected (Patil et al., 2019), since it is challenging to reliably produce an action potential (AP) train in medium- to large-diameter DRG neurons using whole-cell current-clamp patch recordings. Initially, a single depolarizing pulse was applied to identify the *Prlr-cre*$^{+}$ neuronal group on the basis of AP parameters that clearly distinguish CGRP^{-}/TrpV1^{+} from CGRP^{+}/TrpV1^{+} as well as from medium-sized CGRP^{+}/TrpV1^{-}/NPY2R^{-} and CGRP^{+}/TrpV1^{-}/NPY2R^{+} neurons (Patil et al., 2018, 2019). CGRP^{+}/TrpV1^{+} neurons could be further sub-grouped (Patil et al., 2018; Usoskin et al., 2015). However, data from different CGRP^{+}/TrpV1^{+} sub-types were grouped together, because additional recording are required to discriminate these CGRP^{+}/TrpV1^{+} neuronal sub-types. As soon as the *Prlr-cre*$^{+}$ neuronal subtype was defined, a

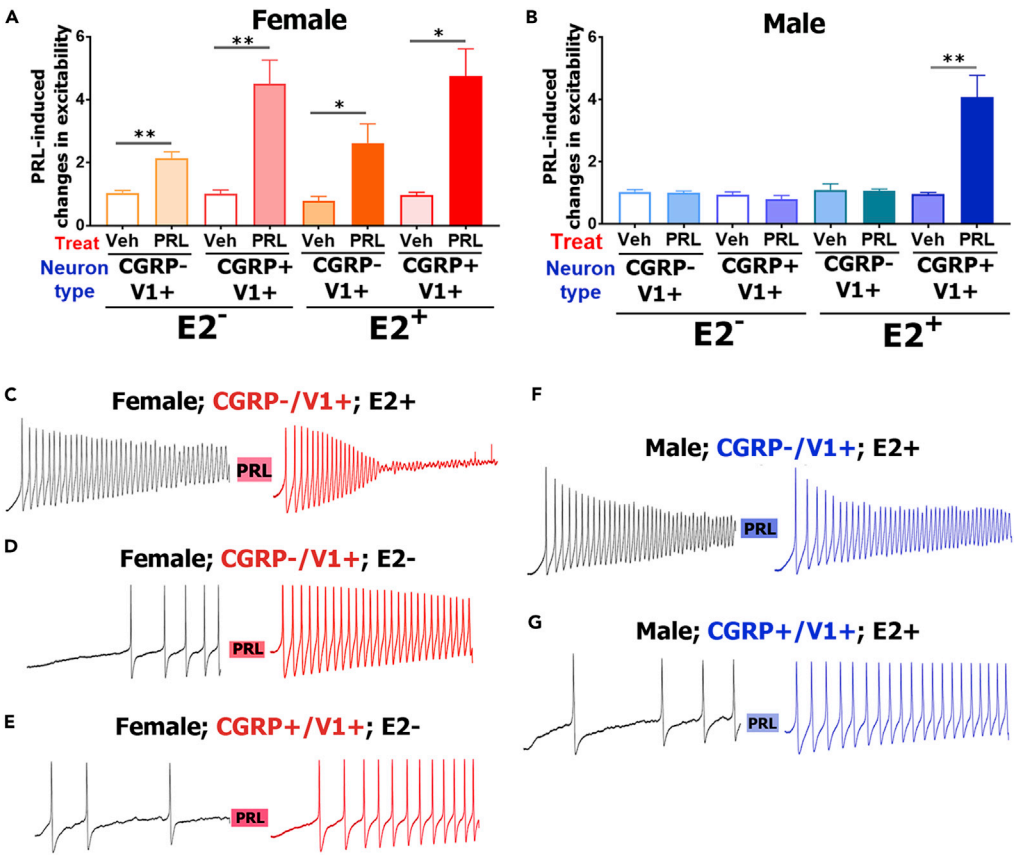

Figure 6. Sex and Estrogen-dependent Regulation of PRL-induced Excitability of *Prlr*-cre$^+$ DRG Neurons

Exogenous PRL induces regulation of excitability in female (A) and male (B) DRG neurons. DRG neurons in culture were treated with vehicle (E2-) or 17β-estradiol (E2+; 1 μg/mL) for 6–36 h in culture. Y axis is change in action potential (AP) frequency (i.e., excitability) after treatment with vehicle or PRL. Data are represented as mean ± SEM. The statistical test is one-way ANOVA with Tukey's post hoc test separately for females or males and for vehicle- or E2-treated groups (*$p < 0.05$; **$p < 0.01$; $n = 4–12$). Examples of AP trains before and after treatment with PRL for 2–3 min are shown in female CGRP$^-$/trpV1$^+$ E2-treated DRG neurons (C); female CGRP$^-$/trpV1$^+$ vehicle-treated DRG neurons (D); female CGRP$^+$/trpV1$^+$ vehicle-treated DRG neurons (E); male CGRP$^-$/trpV1$^+$ E2-treated DRG neurons (F); and male CGRP$^+$/trpV1$^+$ E2-treated DRG neurons (G).

ramp protocol was applied to evaluate how PRL (0.2 μg/mL) treatment (2–3 min) regulates excitability in *Prlr-cre*$^+$ neurons.

PRL triggered an increase in excitability in vehicle-treated CGRP$^+$/TrpV1$^+$ and CGRP$^-$/TrpV1$^+$ *Prlr-cre*$^+$ estrus female (one-way ANOVA; $F(3, 26) = 7.9$; $P = 0.0007$; Figures 6A, 6D, and 6E) but not male DRG neurons (one-way ANOVA; $F(3, 16) = 1.3$; $P = 0.3$; Figure 6B). Mean value of PRL-induced excitability was larger in *Prlr*-cre$^+$ CGRP$^+$/TrpV1$^+$ compared with CGRP$^-$/TrpV1$^+$ DRG female neurons (Figure 6A). Some female CGRP$^-$/TrpV1$^+$ *Prlr-cre*$^+$ neurons had a higher firing frequency upon first ramp application and showed only a short AP train "burst" after PRL treatment (Figure 6C). We did not analyze these neurons. This burst firing pattern is typical for sensitized C-nociceptors (or naive A-LTMR neurons) (Koltzenburg et al., 1997). These data indicate that despite equal *Prlr-L* and *Prlr-S* mRNA expression in female and male DRG neurons, Prlr produces female-selective regulation of excitability in small-sized *Prlr-cre*$^+$ DRG nociceptors. Moreover, these data provide strong independent support for the behavioral findings that sensory neuronal Prlr signaling contributes to mediation of pain in a female-specific manner (Figures 1 and 4).

OVX females and males have very low PRL sensitivity in sensory neurons (Diogenes et al., 2006; Patil et al., 2013b). OVX females and males have substantially lower E2 serum levels than cycling females. Hence, we asked whether E2 treatment could enhance PRL sensitivity in female neurons and generate PRL

responsiveness in male neurons. To test this possibility, DRG neurons in culture were maintained in the presence of 17β-estradiol (E2; 1 μg/mL) for 6–36 h. In such conditions (i.e., $E2^+$), PRL (0.2 μg/mL) increased the excitability of male $CGRP^+/TrpV1^+$, *Prlr-cre*$^+$ neurons (one-way ANOVA; F (3, 18) = 10.3; P = 0.0004; Figures 6B and 6G). However, E2 treatment did not change PRL sensitivity of male $CGRP^-/TrpV1^+$, *Prlr-cre*$^+$ neurons (Figures 6B and 6F). Interestingly, according to single-cell sequencing data, estrogen receptor mRNA is absent in $CGRP^-/TrpV1^+$ (i.e., NP-3 group) male neurons (Usoskin et al., 2015). E2 treatment for less than 6 h did not have any effect on male DRG neurons (Veh 1 ± 0.1 versus PRL 0.9 ± 0.2, n = 6, P = 0.9). E2 treatment of female DRG neurons did not substantially enhance the already existing PRL sensitivity of $CGRP^+/TrpV1^+$ and $CGRP^-/TrpV1^+$ *Prlr-cre*$^+$ neurons (two-way ANOVA; variables are sensory neuronal groups and treatment with E2; F (3, 41) = 0.09; P = 0.99 [for $CGRP^+/TrpV1^+$]; P = 0.98 [for $CGRP^-/TrpV1^+$]; Figure 6A). These data show that Prlr activity in female DRG neurons does not undergo additional sensitization by E2 treatment and may explain the independence of PRL responsiveness from the estrous phase (Figures 1E and 1F). In summary, PRL increases excitability only in female DRG neurons and prolonged (≥6 h) E2 treatment establishes PRL responsiveness in male $CGRP^+/TrpV1^+$ but not $CGRP^-/TrpV1^+$ *Prlr-cre*$^+$ neurons.

PRL Responsiveness in Prlr-cre$^+$ Neurons Are Regulated by a Prlr mRNA Translation Mechanism

Generation of PRL sensitivity in male DRG neurons by E2 treatment implies that E2 is responsible for the production of functional Prlr. In the classical pathway for nuclear receptors, E2 would increase male *Prlr* mRNA and in doing so generate functional Prlr in sensory neurons. To evaluate regulation of *Prlr* mRNA expression by E2, we performed *in vivo* E2 treatment/replacement of male as well as female mice according to a standard protocol (Diogenes et al., 2006; Patil et al., 2014). Then, *Prlr-cre*$^+$ neurons were counted in single-cell suspension or Prlr-L and Prlr-S mRNA expressions were quantified. E2 treatment did not significantly alter the percentage of *Prlr-cre*$^+$ male DRG neurons (9.71 ± 0.34% for vehicle ($E2^-$) versus 10.15 ± 0.68% for $E2^+$, n = 3 independent mice for single-cell suspensions) suggesting that E2 has little impact on *Prlr* transcription. Similarly, E2 did not affect the percentage of *Prlr-cre*$^+$ female neurons (10.2 ± 0.4% for $E2^-$ versus 12.0 ± 0.5% for $E2^+$, n = 3). Quantitative RT-PCR on DRG tissue from wild-type males showed that neither *Prlr-L* nor *Prlr-S* mRNA expression in DRG was substantially altered by *in vivo* E2 treatment (unpaired t test for Prlr-S: t = 0.1; df = 6; P = 0.9; for Prlr-L: t = 0.8; df = 6; P = 0.4; Figure 7A). Female DRG *Prlr-L* and *Prlr-S* mRNA expression levels were also not affected by E2 treatment (unpaired t test for Prlr-S: t = 1.4 df = 4; P = 0.2; for Prlr-L: t = 1.2 df = 4; P = 0.3; Figure 7B). Therefore, we find no evidence for E2 control of transcription of either Prlr isoform.

To this point our results reveal that: (1) sensory neuronal expressed Prlr regulates inflammatory pain in a female-selective manner, (2) prolonged (>6 h) E2 treatment is required to confer PRL sensitivity in male DRG neurons without affecting *Prlr* mRNA expression levels, (3) Prlr mRNA level is similar between *Prlr-cre*$^+$ neuronal groups in male and female mice, and (4) E2 treatment does not induce changes in *Prlr-L* or *Prlr-S* mRNA expression. These results lead us to conclude that female-selective regulation of inflammatory pain via Prlr signaling pathway and female-selective and E2-mediated control of PRL sensitivity are not due to transcriptional or post-translational regulation of Prlr function in DRG neurons. Accordingly, we examined whether translation regulation of *Prlr* mRNA could be the mechanism underlying female-selective and E2-controlled PRL responsiveness. To test this, we first estimated the half-life for Prlr protein function. Cap-dependent translation in the DRG and spinal cord was blocked by intrathecal injection of 4EGI-1 (10 μg). Intrathecal PRL (1 μg)-induced mechanical hypersensitivity was measured at 1–72 h post 4EGI-1 time points in female mice. PRL-induced hypersensitivity was assessed 1 h post PRL. PRL-triggered mechanical hypersensitivity became insignificant at >24 h post 4EGI-1 (one-way ANOVA; F (6, 28) = 5; P = 0.0013; Figure 7C). From this we conclude that the half-life for Prlr functional protein is likely 16–20 h and that new functional Prlr protein is not readily produced when cap-dependent translation is blocked (Figure 7C).

To test this finding in an independent system, we evaluated the effects of 4EGI-1 on PRL sensitivity of female DRG neurons in culture. To do so, we first evaluated whether 12- to 24-h-long 4EGI-1 treatment affects AP properties and/or excitability. CGRP-cre^+/TRPV1-GFP^+ DRG neurons, which represent the $CGRP^+/TRPV1^+$ group, were selected from female reporter mice. Since transient effects of PRL in sensory neurons are mediated via PKCε-dependent mechanisms (Belugin et al., 2013), cultured DRG neurons were treated with 4EGI-1 for the indicated time (Figure S5A) and then the increase in excitability by PMA (a PKC activator) was evaluated. 4EGI-1 had no adverse effect on AP properties and did not block PMA-induced

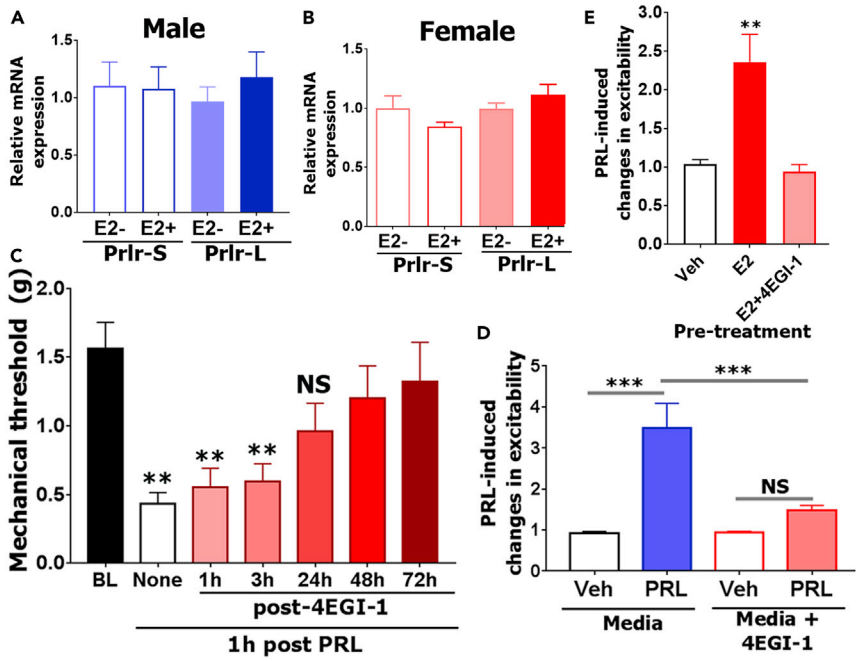

Figure 7. E2-controlled *Prlr* mRNA Transcription and Translation in Male and Female DRG Neurons

(A and B) Expression of *Prlr-L* and *Prlr-S* mRNA in male (A) and female (B) DRG tissues that was isolated from mice *in vivo* treated with vehicle ($E2^-$) or E2 ($E2^+$) for 7 days. mRNA levels were assessed by quantitative RT-PCR. Data were analyzed by one-way ANOVA (n = 3–4).

(C) Inhibition of PRL (1 μg)-induced mechanical hypersensitivity in female mice by spinal treatment for 1–72 h with translation inhibitor 4EGI-1 (10 μg). BL is baseline read before PRL and 4EGI-1 treatment. "None" is no treatment with 4EGI-1. Data were analyzed by one-way ANOVA with Tukey's post hoc test (NS, $p > 0.05$; $^{**}p < 0.01$; n = 5).

(D) PRL (0.2 μg/mL)-induced increase in excitability in female *Prlr-cre*$^+$ cultured DRG neurons pre-treated for 16–20 h with 4EGI-1 (1 μg/mL). Data are represented as mean ± SEM. Statistical test is regular two-way ANOVA with Tukey's post hoc test (variables are treatments with Veh/PRL and Media/4EGI-1; $^{***}p < 0.001$; n = 4–8).

(E) PRL (0.2 μg/mL)-induced excitability of male *Prlr-cre*$^+$ DRG neurons pre-treated for 16–20 h with mixtures of indicated drugs. Data are represented as mean ± SEM. Statistical test is one-way ANOVA with Tukey's post hoc test ($^{**}p < 0.01$; n = 6–13).

See also Figure S5.

hyperexcitability over a 16- to 20-h-long 4EGI-1 treatment (one-way ANOVA; F (3, 19) = 7.5; P = 0.0017; Figures S5A and S5B). Longer treatment led to an inhibition of PMA-induced excitability and distortion in AP shapes (Figure S5A). The findings in Figures 7C and S5 indicate that PRL-induced increase in *Prlr-cre*$^+$ neuron excitability should be evaluated only during the 16- to 20-h pre-treatment window with 4EGI-1.

Figure 7D shows that 16- to 20-h-long 4EGI-1 pre-treatment resulted in a substantial inhibition of PRL responsiveness in female *Prlr-cre*$^+$ neurons (two-way ANOVA; F (3, 19) = 13.4; P < 0.0001). E2 conferred PRL sensitivity in male DRG neurons (Figures 6A and 7E). We examined whether this E2 action depends on translation mechanisms in male neurons. A 16- to 20-h-long pre-treatment of male *Prlr-cre*$^+$ DRG neurons with a mix of E2 and 4EGI-1 (1 μg/mL each) totally blocked E2-dependent establishment of PRL sensitivity in these neurons (Figure 7E). These findings support the hypothesis that sex dimorphism in Prlr functional expression and consequently PRL responsiveness involve translation control mechanisms in DRG neurons wherein *Prlr* mRNA in male neurons is not effectively translated into functional protein owing to E2-driven control of a translational machinery.

To provide additional *in vivo* evidence for this hypothesis, we examined the relative presence of *Prlr* mRNA and protein in DRG neuron central terminals of males and females. We focused on central terminals because our behavioral findings had the largest magnitudes with intrathecally delivered treatments. IHC was conducted on spinal cord sections from male and female $Prlr^{cre/+}$/$Rosa26^{LSL\text{-}tDTomato/+}$ mice

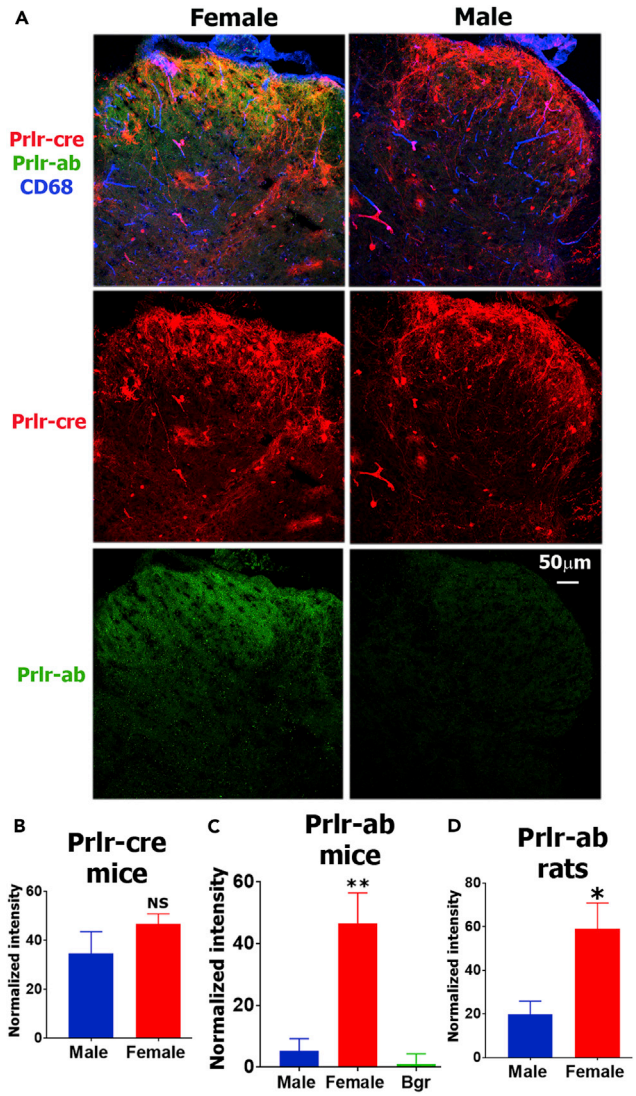

Figure 8. *Prlr* mRNA Reporter Expression and Prlr Protein Localization in Female and Male Mouse Spinal Cord

(A) IHC with Prlr antibodies (polyclonal) and CD68 (rat monoclonal) on spinal cord sections from $Prlr^{cre/+}$/$Rosa26^{LSL\text{-}tDTomato/+}$ female and male mice.

(B) Intensity of TdTomato (Prlr-cre) labeling in spinal cord of female and male $Prlr^{cre/-}$/TdTomato mice.

(C) Intensity of Prlr protein (Prlr-ab) labeling in spinal cord of female and male $Prlr^{cre/-}$/TdTomato mice. *Bgr* is normalized intensity of background.

(D) Intensity of Prlr protein (Prlr-ab) labeling in spinal cord of female and male rats. A representative scale bar of 50 μm is shown.

Data are represented as mean ± SEM. Statistical test is unpaired t test (B and D) or one-way ANOVA with Tukey's post hoc test (panel C) (NS, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; $n = 3$). See also Figure S6.

(Figure 8A). *Prlr* mRNA reporter expression in *Prlr-cre*$^{+}$ fibers (red) was very similar in females and males (un-paired t test; $t = 1.2$ df = 4; $P = 0.3$; $n = 3$; background is subtracted and data normalized per measured area; Figure 8B), whereas Prlr protein detected by polyclonal anti-Prlr antibodies was substantially greater in females than in males in the dorsal horn (one-way ANOVA; $F(2, 12) = 14.9$; $P = 0.0006$; $n = 3$; Figure 8C). Similarly, female-predominant expression of Prlr protein was observed in spinal cord of rats using mouse monoclonal anti-Prlr (U5) antibodies (un-paired t test; $t = 3$ df = 4; $P = 0.042$; $n = 3$; Figures 8D and S6). Taken together, these findings support the conclusion that the female-selective regulation of inflammatory pain by sensory neuronal Prlr and PRL sensitivity found in the DRG is due to translational regulation of Prlr function in sensory neurons.

DISCUSSION

Studies in animals and humans demonstrate clear sex dimorphisms in mechanisms that control development of chronic pain (Dance, 2019), and the existing paradigm is that these dimorphisms are directly or indirectly regulated by gonadal hormones (Traub and Ji, 2013). A growing body of research suggests that an important mechanistic difference between development of chronic pain in male and female mice is that distinct immune cells are critical drivers, microglia in males (Paige et al., 2018; Sorge et al., 2011, 2015; Taves et al., 2016) and T cells in females (Sorge et al., 2015). The male-specific microglia effects are conserved for mice and rats (Mapplebeck et al., 2018) and can be conferred to females with testosterone treatment (Sorge et al., 2015). On the other hand, the T cell findings are controversial because T cells also play a critical role in pain resolution in some pain models (Krukowski et al., 2016; Laumet et al., 2019). Several recent studies have also found sex differences in mRNA expression using RNA sequencing in whole human DRG and tibial nerve (North et al., 2019; Ray et al., 2019). The DRG transcriptomic work suggests that of the monocyte lineage in DRG and nerves may play a critical role in promoting neuropathic pain in males but to a lesser extent in females (North et al., 2019). Sex differences in the tibial nerve transcriptomes, although not directly related to chronic pain since the tissues were from organ donors and not patients, found a clear signature for gonadal hormones in regulating transcriptomes in this tissue across the lifespan in females (Ray et al., 2019). Collectively, these findings in rodents and humans support the classical viewpoint that gene regulation via gonadal hormone nuclear receptor-mediated transcription control mechanisms is a cornerstone of sex-dependent processes (Ormandy and Sutherland, 1993) including sex dimorphisms in pain (Rosen et al., 2017). Our findings suggest a new twist on this paradigm wherein gonadal hormones could also regulate sensory neuron excitability via regulation of translation machinery or transcription of proteins belonging to the translation complex. As a key example, we demonstrated this mechanism for female-selective *in vitro* and *in vivo* PRL responsiveness in sensory neurons.

Previous studies showed gonadal hormones-dependent regulation of Prlr in non-neuronal cells (Furigo et al., 2014; Hu et al., 1996, 1998; Tanaka et al., 2005), but this is the first demonstration of such an effect in sensory neurons. Since Prlr does not have classical gonadal hormone-response element (Ormandy and Sutherland, 1993), *Prlr* mRNA expression is thought to be regulated by E2 utilizing alternative transcription binding sites, such as C/EBP, Sp3, and/or Sp1A (Dong et al., 2006; Goldhar et al., 2011). Based on the literature, it could be extrapolated that *Prlr* mRNA should be predominantly expressed in female DRG neurons in an estrogen-dependent fashion. However, surprisingly, our data, which were generated by multiple, independent methods, show that *Prlr-L* and *Prlr-S* mRNA expression in sensory neuronal subtypes are not sex- or E2-dependent. Post-translational (i.e., phosphorylation, glycosylation) regulation is also unlikely, since prolonged E2 treatment (>6 h) is required for establishing PRL sensitivity in male neurons despite expression of *Prlr* mRNA. Our data support the conclusion that translation regulation of Prlr function is critical for the observed dimorphisms in nociceptive processes during inflammatory pathological pain conditions. Accordingly, inhibition of *cap*-dependent translation almost entirely ablated PRL responsiveness in *Prlr-cre*$^{+}$ female neurons and blocked the behavioral response to PRL *in vivo*. In further support of this model, we showed that blockage cap-dependent translation eliminated E2-established PRL responsiveness in male DRG neurons. Moreover, we also observed robust expression of *Prlr-cre*$^{+}$ sensory neurons and fibers in both male and female mice but found substantially higher Prlr protein expression in female rodent (rat and mice) spinal cord.

Based on these findings, we propose that sex- and E2-dependent translational regulation could be a novel mechanism for sexual dimorphism observed in many pain conditions (Fillingim et al., 2009). This is especially relevant considering that translation control mechanisms are already known to strongly contribute to modulation and sensitization of nociceptors but suggests that therapeutic targeting of these mechanisms may have additional benefits in females (Khoutorsky and Price, 2018; Megat and Price, 2018). Gonadal hormone-controlled translational regulation has been a subject of speculation but not studied in detail (Bronson et al., 2010; Ochnik et al., 2016). This mechanism could be due to an increase of efficiency of translation by gonadal hormones and/or gonadal hormone-controlled additional mRNA transcription of proteins involved in translational machinery. Thus, translation regulation factors encoded by *Eif2s3y* and *Eif2s3x* genes exhibit strong sex dependency in mRNA expression in many types of neurons (Armoskus et al., 2014; Ray et al., 2019). E2-driven translational control over the suppressor of cytokine signaling (SOCS) protein family has been proposed (Arbocco et al., 2016; Matthews et al., 2005) where E2 can affect the translational machinery via mTOR phosphorylation (Augusto et al., 2010) and regulation of Rheb

signaling (Pochynyuk et al., 2006). Translation can also be controlled by factors binding to mRNA. One of such factors is Musashi (*Msi-1* and 2 genes), which binds specific sequences in the 3′ untranslated region (UTR) of mRNA and controls translation of proteins. It was shown that leptin can control translation of proteins by regulating Msi-1 expression (Odle et al., 2018). These signaling pathways also play key roles in pain sensitization whereby they control the on-demand synthesis of new proteins that alter the excitability of nociceptors (Khoutorsky and Price, 2018; Moy et al., 2017).

A corollary of our work is that molecules involved in sex-dependent regulation of nociceptive pathways could be (1) induced by injury, (2) controlled by gonadal hormones, and (3) capable of regulating many other genes. PRL and its receptor Prlr fit these requirements. First, Prlr-mediated PRL effects are sex dependent in many tissues and cell types (Belugin et al., 2013; Ben-Jonathan et al., 2008; Patil et al., 2013a; Torner et al., 2001). It is well documented that PRL responsiveness is closely controlled by E2 and to a lesser extent progesterone. Many clinical and preclinical studies also show that stress related to injury and inflammatory conditions trigger PRL release not only from the pituitary (Chernow et al., 1987; Noreng et al., 1987; Yardeni et al., 2007), but also from extra-pituitary tissues, such as cells in skin and in the spinal cord (BenJonathan et al., 1996; Patil et al., 2013a; Scotland et al., 2011). Finally, Prlr activation could lead to epigenetic changes and transcription regulation of many genes via the STAT5 pathway (Ben-Jonathan et al., 2008; Bole-Feysot et al., 1998). This downstream transcriptional control may lead to additional diversification of nociceptor responses to injury since this pathway would not be induced in male neurons, which have very low levels of Prlr functional protein in most sensory neuronal types. The preponderance of data on sex-specific mechanisms of chronic pain has focused on male rodents. To our knowledge, this is one of the first demonstrations of a female-specific inflammatory pain mechanism acting directly on the sensory neuron. Interestingly, the discovery of a far greater potency of calcitonin gene related peptide (CGRP) in producing migraine pain-like behaviors in female mice also involves a peptide with intimate connections to nociceptor biology (Avona et al., 2019).

In conclusion, our data demonstrate that sensory neuronal Prlr contributes to female-selective regulation of hypersensitivity in inflammatory pain models via local and spinal mechanisms. Sex dependency of PRL responsiveness and the generation of hypersensitivity by this mechanism in sensory neurons is likely controlled via translation mechanisms. We favor the hypothesis that sex-specific regulation of sensory neuronal excitability by PRL is governed via E2-controlled translation regulation of *Prlr* mRNA, and this in turn explains female-selective mechanisms for regulation of PRL-induced hypersensitivity in non-injured animals and inflammatory pain by sensory neuronal Prlr. These results add a new layer to our understanding of sex dimorphisms in pain signaling and further substantiate the critical role that translation regulation plays in setting nociceptor excitability in response to a broad variety of important physiological stimuli.

Limitations of Study

PRL responsiveness in humans is influenced by the menstrual phases (Freeman et al., 2000). Unfortunately, data obtained in different estrous phases of rodent females (Figures 1E, 1F, and 3A) do not translate well to human menstrual phases. There are several reasons for this lack of direct translation. First, the full estrous cycle in rodents is only 4–5 days. Therefore, stable proteins, such as Prlr (Figure 7C), synthesized in the previous estrous phase may still be present in the subsequent phase. Thus, in the diestrus phase, some of Prlr synthesized in estrous is still functional. Second, changes in patterns and levels of estrogen and progesterone from one phase to another are different for menstrual versus estrous cycles. This could influence Prlr synthesis. Another limitation of our study is the lack of currently available tools to separately study effects mediated by Prlr-L or Prlr-S isoforms. There are no reliable and validated antibodies labeling Prlr-L or Prlr-S, and mouse lines targeting only Prlr-S do not exist. Moreover, there are several Prlr-S isoforms (Freeman et al., 2000). Therefore, our work is unable to address questions related to short- versus long-term effects of Prlr signaling that are mediated by these different isoforms.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.09.039.

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AUTHOR CONTRIBUTIONS

M.P. conducted a majority of experiments and contributed to manuscript writing and revision; S.B., J.M., C.P., P.A.B., and J.T.B performed certain experiments and analysis; A.W. performed certain experiments and analyzed PCR data and prepared corresponding figures and graphical depictions; V.G. generated human PRL and contributed to experimental design, manuscript writing, and revision; D.G. and U.B. generated *Prlr*-cre and Prlr$^{fl/fl}$ mouse lines and contributed to experimental design and manuscript revision; G.D. helped with electrophysiology experiments and experimental design and contributed to the manuscript preparation; T.J.P. and A.N.A designed and directed the project, helped with experiments, wrote the first draft of the manuscript, and prepared the final version of the manuscript.

DECLARATION OF INTERESTS

All authors declare that they have no competing interests.

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ISCI, Volume *20*

Supplemental Information

Prolactin Regulates Pain Responses via a Female-Selective Nociceptor-Specific Mechanism

Mayur Patil, Sergei Belugin, Jennifer Mecklenburg, Andi Wangzhou, Candler Paige, Priscilla A. Barba-Escobedo, Jacob T. Boyd, Vincent Goffin, David Grattan, Ulrich Boehm, Gregory Dussor, Theodore J. Price, and Armen N. Akopian

Transparent Methods:

Ethical Approval

All animal experiments conformed to APS's Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training, and to protocols approved by the University Texas Health Science Center at San Antonio (UTHSCSA) and University of Texas at Dallas (UTD) Institutional Animal Care and Use Committee (IACUC). We followed guidelines issued by the National Institutes of Health (NIH) and the Society for Neuroscience (SfN) to minimize the number of animals used and their suffering.

Key reagents and mouse lines

Experiments were conducted on 8-12-week-old female and male mice, and Sprague-Dawley rats (200-250g) that were purchased from Jackson Laboratory (Bar Harbor, ME) and Charles River, respectively. Estrous phase was determined by vaginal gavage placed on glass slides (Caligioni, 2009). Interpretations of vaginal gavage data is presented in (Caligioni, 2009). *In vivo* estrogen (E2) replacement procedure was performed by systemic (ip) injection of 60 μl E-2 (5 μg/μl) in sesame oil twice a week for 3 weeks (Diogenes et al., 2006).

The $Rosa26^{LSL\text{-}tDTomato/+}$ mouse line on B6.129 background was obtained from the Jackson Laboratory (Bar Harbor, ME). TRPV1-GFP mouse lines were purchased from the GENSAT program (MMRRC services; UNC, NC and UC Davis, CA). The $CGRP^{cre/+\text{-}ER}$ mouse line was kindly provided by Dr. Pao-Tien Chuang (UC San Francisco, San Francisco, CA). The $Prlr^{cre/+}$ mouse line (Prlr-cre) generation by Dr. Ulrich Boehm (University of Saarland School of Medicine, Homberg, Germany) was described (Candlish et al., 2015; Patil et al., 2019). The $Prlr^{fl/fl}$ line generation was described (Brown et al., 2016). $Prlr^{fl/fl}$ has inverse lox sites; hence, cre-recombination ablates the Prlr gene and activates GFP in targeted cells (*Figure S3A*).

Human PRL was generated in an *E.coli* expression system containing plasmid with human PRL. PRL is fully processed, unmodified (i.e. no glycosylation and phosphorylation) and has molecular weight

of ≈23 kDa. The Prlr antagonist is Δ1-9-G129R-hPRL (ΔPRL) (Rouet et al., 2010), which is a modified PRL binding to and blocking the function of Prlr in rat, mouse and human (Bernichtein et al., 2003). We and others thoroughly confirmed the specificity of ΔPRL 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).

Primary DRG neuronal culture

DRG from male and female mice were used. WT or reporter mice, including $Prlr^{cre/+}$/$Rosa26^{LSL\text{-}tDTomato/+}$, were deeply anaesthetized with isoflurane (0.3 ml in 1 liter administered for 60-90 sec) and sacrificed by cervical dislocation. L3-L5 DRG was quickly removed, and neurons were dissociated by treatment with a 1mg/ml collagenase-dispase (Roche, Indianapolis, IN) solution. Cells were maintained in DMEM supplemented with 2% fetal bovine serum (FBS), 2mM L-glutamine, 100U/ml penicillin and 100μg/ml streptomycin and no NGF. The experiments were performed within 6-36 hr after DRG neuron plating. In some experiments, DRG neuronal culture was treated for >4 hr with 1μg/ml estrogen (E2).

CGRP-release assay

Capsaicin (CAP: Fluka, St. Louis, MO) stock prepared in 100% ethanol was diluted in Hank's buffer. 1 spinal cord were used per well. CGRP-release experiments were carried out at 37ºC. Tissues were washed once with Hank's buffer and then soaked in Hank's buffer for 30 min to equilibrate. The supernatant was collected for measurement of baseline CGRP release after 15 min in Hank's. Then, tissues were exposed for 3 min with CAP or CAP+PRL (10μg/ml), and solution was replaced with Hank's buffer. Tissues were maintained for additional 15 min in Hank's buffer. The total evoked CGRP release was measured by pooling the 3 min CAP or CAP+PRL exposure sample with a 15 min vehicle post-exposure sample. Biopsies were only used once and only exposed to one sequence of treatments. The CGRP radio-immuno assay was conducted essentially as described (Ruparel et al., 2008) with primary antibody against CGRP (final dilution, 1:1,000,000; kindly donated by Dr. Michael J. Iadarola

(NIDCR/NIH, Bethesda, MD). CGRP release data was normalized by weight of biopsies. Data was presented as % of release above baseline.

Single-cell RT-PCR

For single-cell RT PCR, single-cell suspensions were prepared from $Prlr^{cre/+}$/$Rosa26^{LSL\text{-}tDTomato/+}$ female or male mouse DRG tissues as described in the section “Primary DRG neuronal culture”. Prlr-cre^{+} cells were manual isolated under an inverted Evos-fl digital fluorescence microscope (cat #1253460, AMG, Bothell, WA) equipped with a manipulator holding pipette (I.D. 0.2 mm). Target genes were amplified using Fluidigm BioMark HD system and Flexsix BioMark 12x12 chip according to the manufacturer protocol. Gene expression with the FlexSix IFC was performed with Fast/Standard Taqman assays (Fluidigm PN 100-7251 C1). In each chip assay, universal mouse RNA (200 pg, cat# R4334566-1, BioChain, Newark, CA), no template (NTC) and pre-amplification controls served as positive and dual negative controls. Taqman primers (all for mice) were the following: short form of Prlr (Prlr-S; Assay# Mm02017047_s1; amplicon size is 109bp within a single exon; Applied Biosystems); long form of Prlr (Prlr-L; Assay# Mm00619170_s1; amplicon size is 135bp within a single exon; Applied Biosystems); TRPV1 (Assay# Mm01246301_m1 amplicon size is 81bp within several exons; Applied Biosystems); CGRP-alpha (Assay# Mm00801462_m1 amplicon size is 84bp within several exons; Applied Biosystems); 5HT3a (Assay# Mm00442874_m1 amplicon size is 90 bp within several exons; Applied Biosystems); NPY2R (Assay# Mm01218209_m1 amplicon size is 86bp within several exons; Applied Biosystems); and GAPDH (Assay# Mm03302249_g1; amplicon size is 70bp within several exons; Applied Biosystems). GE FlexSix Standard v1 program was used to collect the CT values. For quantitative analysis of tissue and single-cell RT-PCR data, comparative delta–delta Ct (ddCt) was utilized to normalize the data based on the endogenous control (i.e. GAPDH), and to express it as the relative fold change, after the exclusion criteria were verified by comparing primer efficiencies (Ruparel et al., 2012).

Electrophysiology

Recordings were made in whole-cell current clamp configurations at 22-24^{o}C. Data were acquired and analyzed using an Axopatch 200B amplifier and pCLAMP10.2 software (Molecular Devices, Sunnyvale, CA). Recorded data were filtered at 5 kHz and sampled at 30 kHz. Borosilicate pipettes (Sutter, Novato, CA) were polished to resistances of 2-3 MΩ. Access resistance (R_s) was compensated (40-80%) when appropriate up to the value of 6-8 MΩ. Data were rejected when R_s changed >20% during recording, leak currents were >50pA, or input resistance was <300 MΩ. Standard external solution (SES) contained (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose and 10 HEPES, pH 7.4. The standard pipette (internal) solution (SIS) contained (in mM): 140 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 D-glucose, 10 HEPES, pH 7.3, 2.5 ATP and 0.2 GTP. Drugs were applied by a fast, pressure-driven and computer controlled 4-channel system (ValveLink8; AutoMate Scientific, San Francisco, CA) with quartz application pipettes.

Small (<30 pF) Prlr-cre^{+} or CGRP^{+}/TRPV1^{+} DRG neurons from Prlr$^{cre/+}$/Rosa26$^{LSL\text{-}tDTomato/+}$ and CGRP$^{cre/+\text{-}ER}$/Rosa26$^{LSL\text{-}tDTomato/+}$/TRPV1-GFP reporter mice were randomly selected for recording as specified in the text. To characterize modulation of Prlr-cre^{+} or CGRP^{+}/TRPV1^{+} DRG neuron excitation by vehicle (control) or drug, the following sequence of recording protocols were applied: (1) a single AP in current clamp configuration was generated with a 0.5ms and 1nA current step to define the type of sensory neurons (Patil et al., 2018); (2) a linear ramp from 0 to 0.3 nA for 1 sec was applied to generate a control AP train; (3) the patched neuron was treated for 2-5 min with vehicle or drug; and then (4) the ramp as in step 2 was re-applied. Data was accumulated from 3-5 independent mouse DRG neuronal cultures. Each culture was generated from one male or estrous female mouse. Changes in neuronal excitability were calculated by dividing AP frequency generated by a current ramp after vehicle or drug-treatment to AP frequency produced by the ramp before treatment. Excitability was determined to be regulated by drug when the drug treatment produced statistically significant increase in AP frequency than vehicle-treatment (i.e. control).

Immunohistochemistry (IHC)

Spinal cord corresponding to L3-L5 levels from perfused female and male $Prlr^{cre/+}$/$Rosa26^{LSL\text{-}tDTomato/+}$ mice were fixed again with 4% paraformaldehyde, cryoprotected with 30% sucrose in phosphate buffer, embedded in Neg 50 (Richard Allan Scientific, Kalamazoo, MI); and 30μm cryo-sections were generated. IHC was carried out as previously described (Belugin et al., 2013). Anti-Prlr rabbit polyclonal (NSJ Bioreagents; San Diego, CA; catalogue R31199; 1:200) (Buteau et al., 1998) or anti-Prlr mouse monoclonal U5 antibodies (Thermo-Fisher Scientific; catalogue MA1-610; 1:100) (Diogenes et al., 2006) were used for IHC detection of Prlr protein. CGRP was detected with anti-CGRP polyclonal antibodies (Sigma; C8198; 1:300) (Patil et al., 2018). Sections were incubated with species appropriate Alexa Fluor secondary antibodies (1:200; Molecular Probes, Eugene, OR). Images were acquired using a Nikon Eclipse 90i microscope (Melville, NY, USA) equipped with a C1si laser scanning confocal imaging system. Images were processed with NIS-elements software (Nikon Instruments, Melville, NY). Control IHC was performed on tissue sections processed as described but either lacking primary antibodies or lacking primary and secondary antibodies. IHC images were obtained from 3-5 independent tissue sections from 3-4 animals. Z-stack IHC images were analyzed using Image J software. Subtractions of background intensity from signal levels were applied.

Behavior experiments

Vehicle or drugs were injected into hind paw (ipl) or intrathecal space of spinal cord (SC). Vehicle for PRL and ΔPRL was 0.9% saline. Vehicle for 4EGI-1 was 0.1% DMSO in 0.9% saline.

Heat hypersensitivity was assessed with Hargreaves' apparatus (Ugo Basile) (Patil et al., 2013). Animals were habituated to the testing environment for at least 1 hour prior to testing. Animals were placed on a glass surface with temperature held constant at ≈20°C. Following habituation, thermal withdrawal latencies to a radiant heat beam were recorded at each time point (3X measurements at each time point, averaged to obtain the data value used in analyses). To prevent tissue damage, the stimulus was terminated after ≈20 sec if the animal did not withdraw the hind paw.

Mechanical hypersensitivity was measured using two approaches. First, animals were habituated for 45-60 minutes and then the baseline readings (three readings per animal) were taken on the right hind paw using the Dynamic Plantar Aesthesiometer (Ugo Basile) to record withdrawal thresholds for mechanical stimulation (Patil et al., 2013). The instrument applies constant ramp of increasing mechanical pressure to the paw (from 0 to 50 grams over 10 second intervals) and the withdrawal threshold was recorded in grams when the paw was withdrawn. Second, in certain experiments, mechanical hypersensitivity was evaluated with von Frey filaments using the standard up-down approach (Chaplan et al., 1994).

Statistics

GraphPad Prism 8.0 (GraphPad, La Jolla, CA) was used for all statistical analyses of data. Data in the figures are mean ± standard error of the mean (SEM), with "n" referring to the number of recorded cells, the number of animals for IHC, and the number of independent animals per group in behavioral experiments. Behavior experiments were blinded such that the experimenter did not know the treatment conditions. We used randomized block designs for behavioral experiments, and tested animals in small groups each time. Differences between groups were assessed by chi-square analysis with Fisher's exact test, unpaired *t*-test, regular 1-way ANOVA with Tukey's post-hoc tests, each column was compared to all other columns, or regular 2-way ANOVA with multiple comparisons "compare cell means regardless of rows and columns" with recommended Tukey's post-hoc test or "compare each cell mean with other cell mean in that raw" with recommended Bonferroni's post-hoc tests. A difference is accepted as statistically significant when $p<0.05$. Interaction F ratios, and the associated p values are reported in the text.

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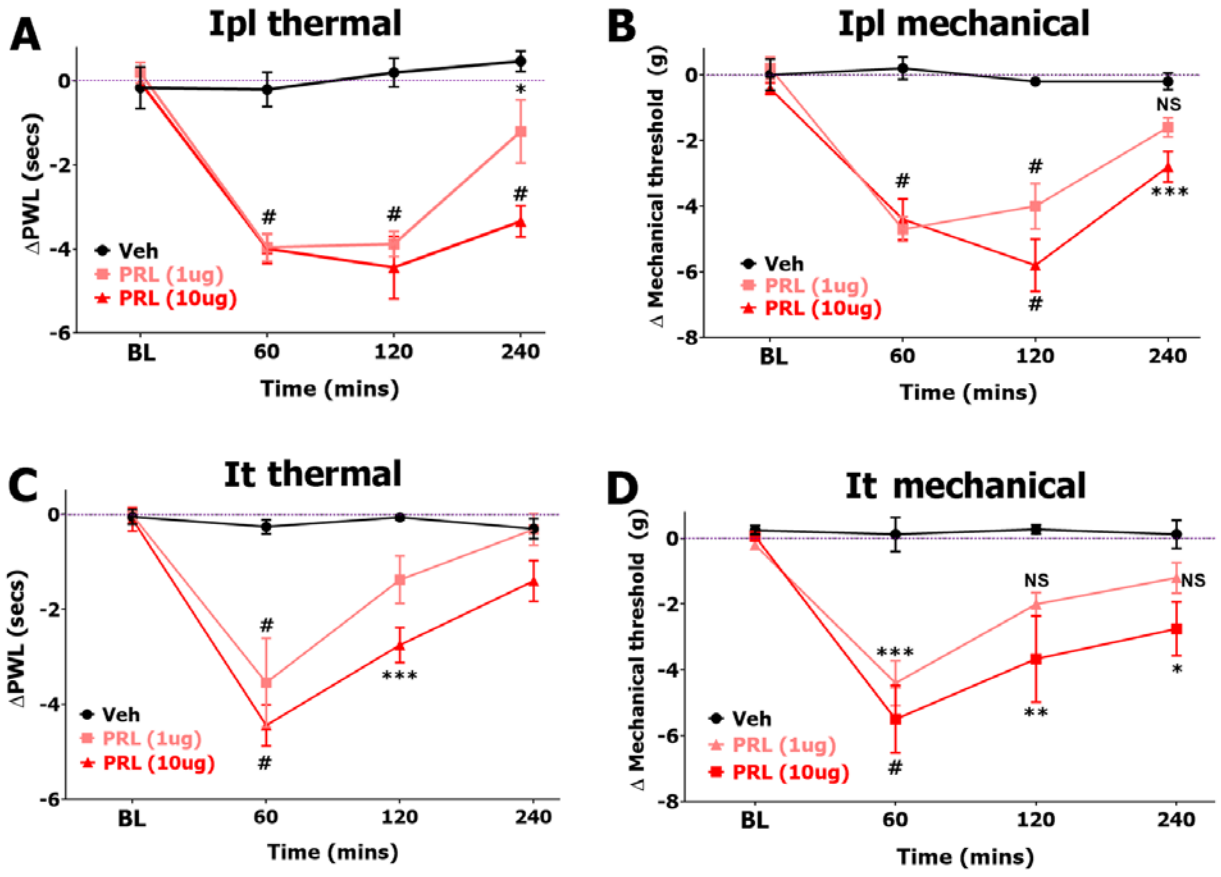
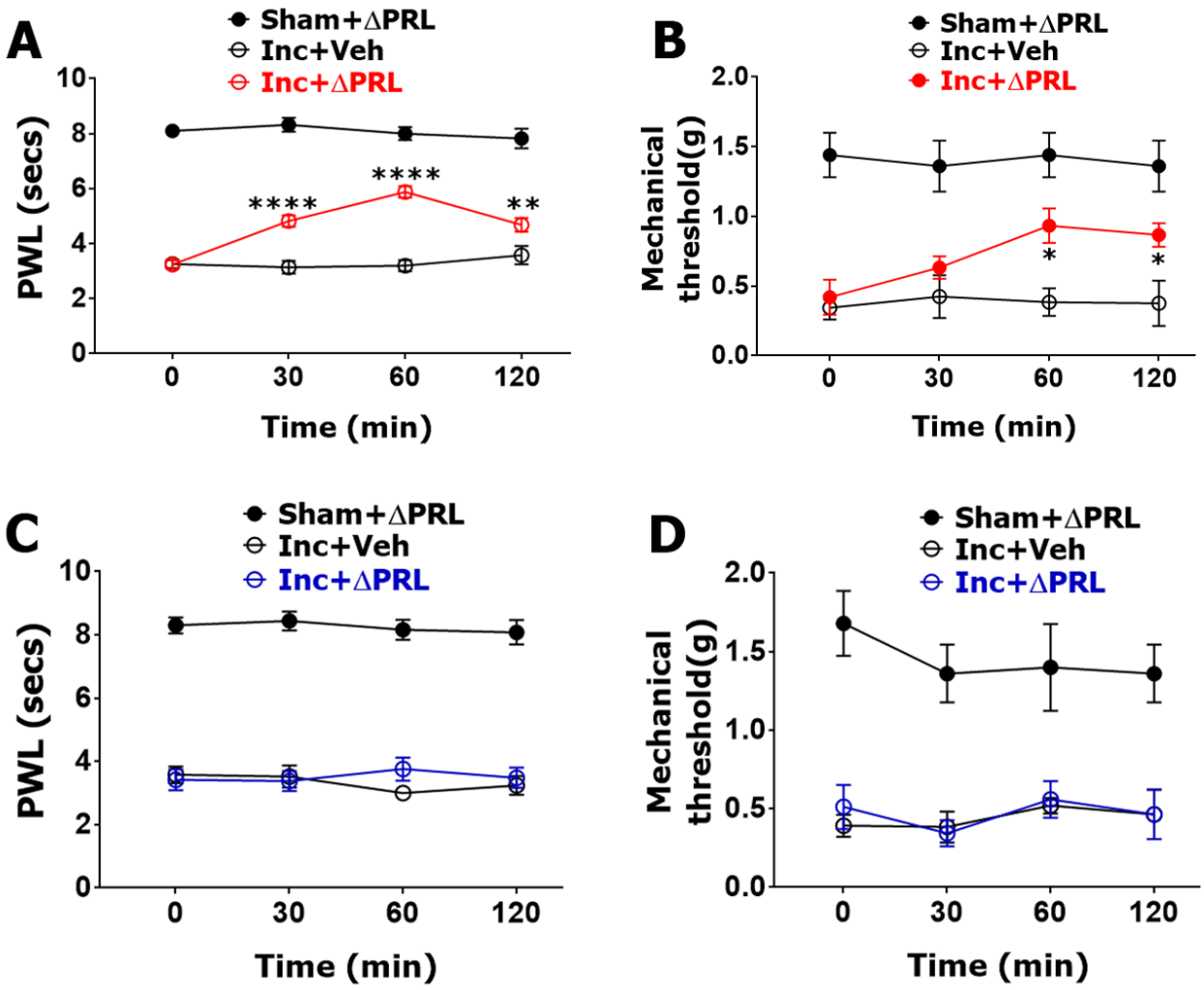


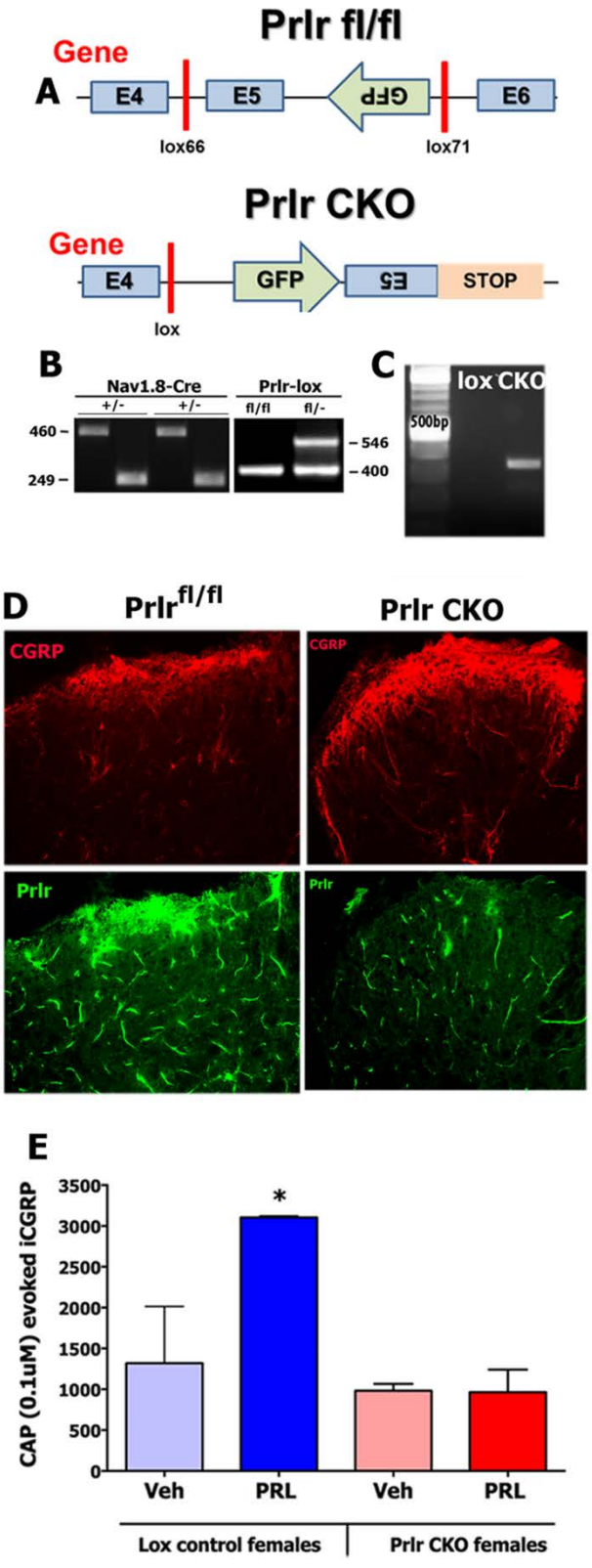
Supplementary Figure 1: Development time-course for exogenous PRL-induced hypersensitivity in female mice, Related to Figure 1

PRL (1 or 10 μg)-induced heat (**A** and **C**) and mechanical (**B** and **D**) hypersensitivity were accessed in female mice in the estrous phase. Data were collected at 1, 2 and 4hr post-PRL. PRL was administrated into hind paw (*ipl*; **A** and **B**), or intrathecal space of spinal cord (*it*; **C** and **D**). Mechanical threshold was measured with the Dynamic Plantar Aesthesiometer. BL – baseline. Data are represented as mean +/- SEM. Statistical test is regular 2-way ANOVA with Tukey's post-hoc test (n=5-6; NS – non-significant; *** $p<0.001$; # $p<0.0001$).

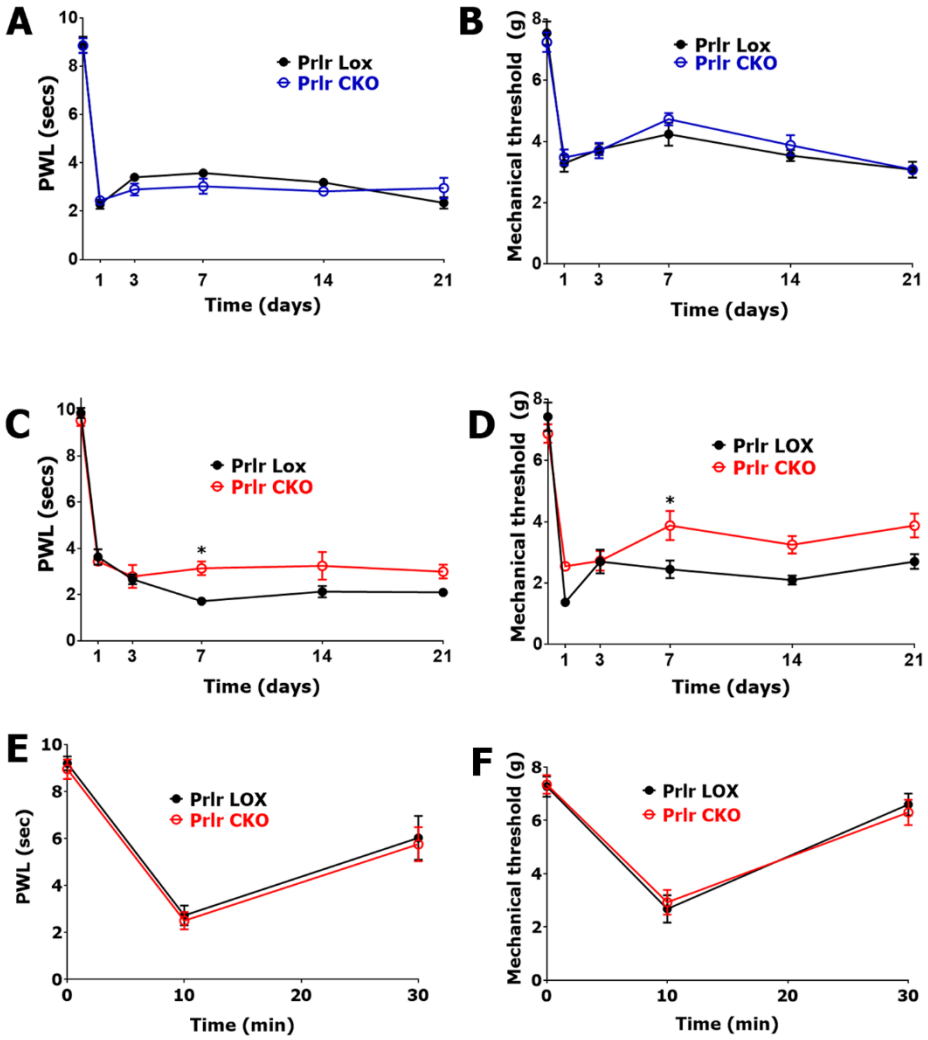


Supplementary Figure 2: Time course of postoperative pain suppression by Prlr antagonist in male and female mice, Related to Figure 2.

Incision (Inc) or sham procedures of hind paws were performed on female and male mice. One day post-incision, heat (**A**) and mechanical (**B**) hypersensitivity were assessed for 2hr post-ΔPRL (5 μg) spinal cord injections in female mice in the estrous phase. (**C, D**) Similar experiments were performed on male mice. Data were collected at indicated post-ΔPRL time points. "0" represent reading at 1d post-surgery, but before ΔPRL injection. Data are represented as mean +/- SEM. Statistical test is regular 2-way ANOVA with Tukey's post-hoc test (n=6; * $p<0.05$; ** $p<0.01$; **** $p<0.0001$).

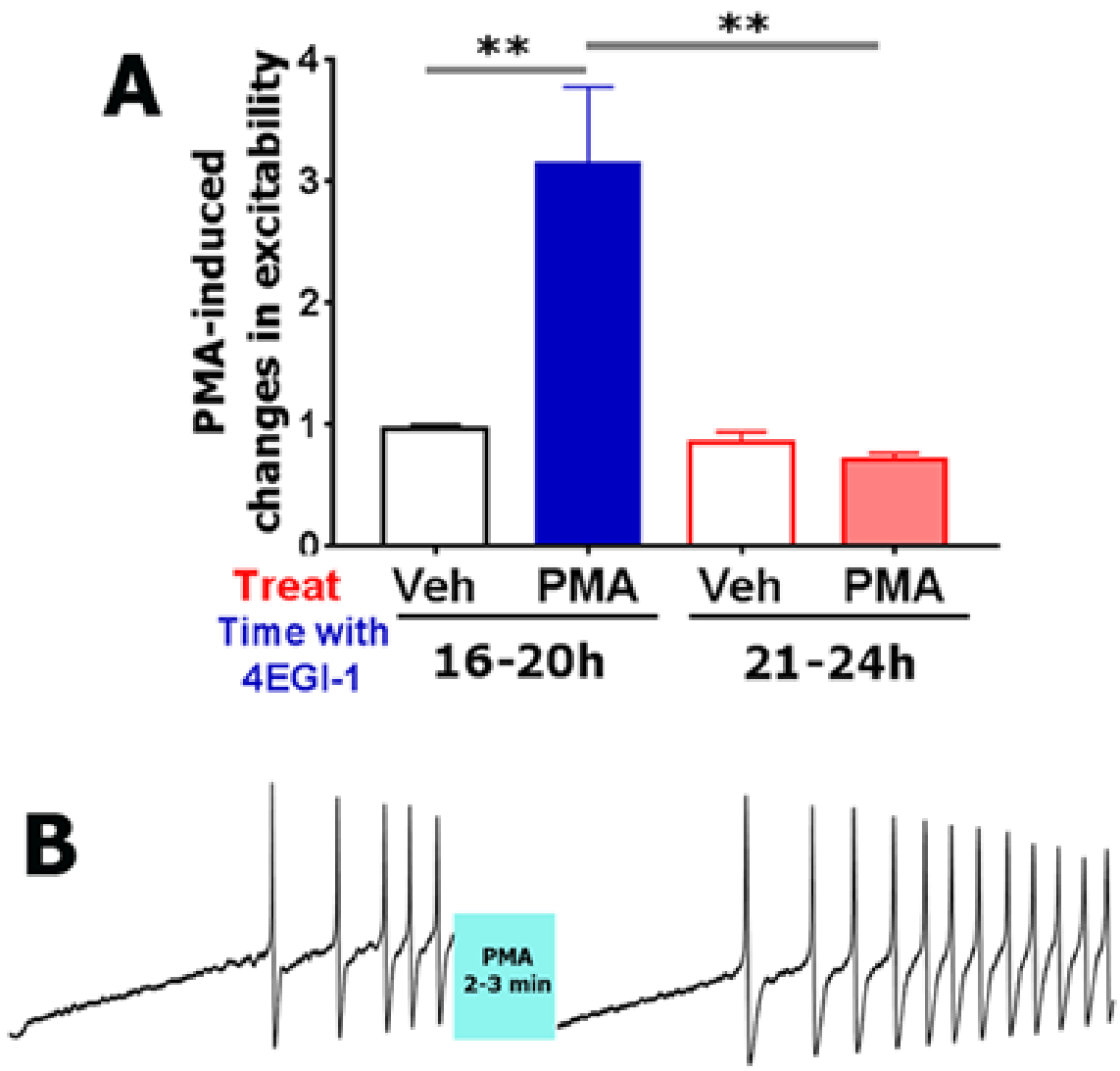


Supplementary Figure 3: Validation of Nav1.8$^{cre/-}$/Prlr$^{fl/fl}$ mice, Related to Figure 4. **(A)** Schematic shows Prlr$^{fl/fl}$ (inverse GFP) construct design, and Prlr CKO post *cre*-recombination. (**B**) PCR genotyping of Nav1.8$^{Cre/-}$/Prlr$^{fl/fl}$ (Prlr CKO) mice. (**C**) PCR of GFP mRNA from Prlr$^{fl/fl}$ (lox) and Nav1.8$^{Cre/-}$/Prlr$^{fl/fl}$ (CKO) female DRG. **(D)** CGRP (red) and Prlr (green) immunohistochemistry (IHC) from spinal cord of Prlr$^{fl/fl}$ and Nav1.8$^{Cre/-}$/Prlr$^{fl/fl}$ (Prlr CKO) female mice. (**E**) Sensitization of CAP-evoked CGRP release in spinal cord slices from Prlr$^{fl/fl}$ (Lox control females) and Nav1.8$^{Cre/-}$/Prlr$^{fl/fl}$ (Prlr CKO females) mice. Data are represented as mean +/- SEM. Statistical test is regular 2-way ANOVA (variables are mouse line and treatments; Tuckey's post-hoc test (* $p<0.05$; n=4).



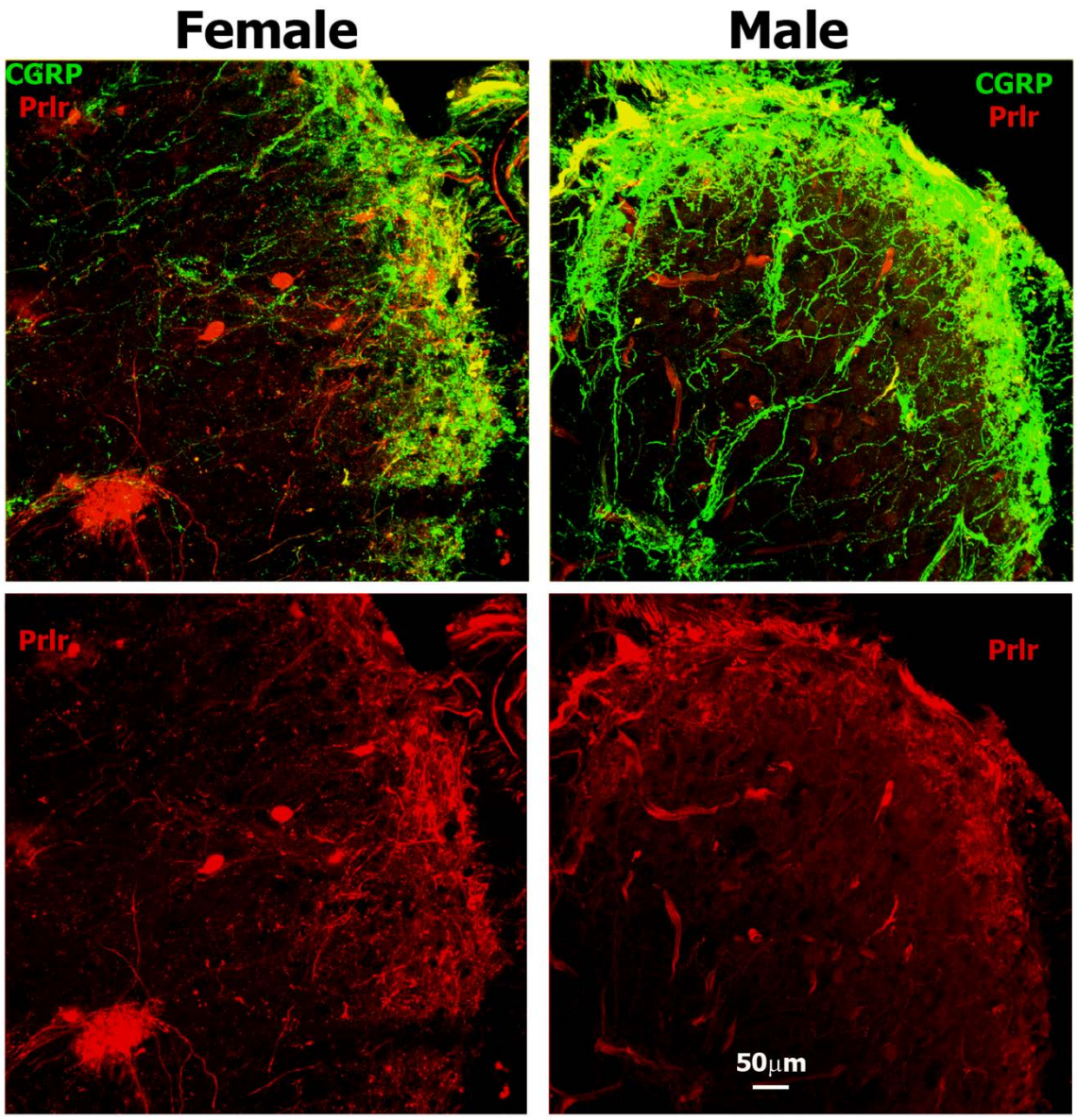
Supplementary Figure 4: Hypersensitivity development in Nav1.8$^{Cre/-}$/Prlr$^{fl/fl}$ males and females in nerve damage- and chemical-induced pain models, Related to Figure 4.

CCI model was performed on Prlr$^{fl/fl}$ (Prlr LOX) and Nav1.8$^{Cre/-}$/Prlr$^{fl/fl}$ (Prlr CKO) male and female mice. Development of heat (**A, C**) and mechanical (**B, D**) hypersensitivity in males (**A, B**) and females Prlr CKO mice (**C, D**). Heat (**E**) and mechanical (**F**) hypersensitivity to mustard oil (10 mM) injected in hind paws of Prlr LOX and Prlr CKO female mice were measured. Mechanical threshold was measured with the Dynamic Plantar Aesthesiometer. Mouse lines are indicated. Data were collected at indicated time points. Data are represented as mean +/- SEM. Statistical test is regular 2-way ANOVA (variables are mouse line and measurement time points) with Tukey's post-hoc test (* $p<0.05$; n=6).



Supplementary Figure 5: effect of a translation inhibitor on PMA-induced excitability in female DRG neurons, Related to Figure 7.

(**A**) PMA (0.5 μM)-induced excitability was evaluated in female CGRP-cre^{+}/TRPV1-GFP^{+} cultured DRG neurons pre-treated for 16-24 hr with 4EGI-1 (1μg/ml). Pre-treatment time is indicated. Data are represented as mean +/- SEM. Statistical test is 2-way ANOVA with Tukey's post-hoc test (variables are treatments with 4EGI-1 and Veh/PMA; **p<0.01; n=4-8). (**B**) Representative traces show PMA-induced increase in CGRP-cre^{+}/TRPV1-GFP^{+} neuron excitability pretreated 18 hr with 4EGI-1.



Supplementary Figure 6: Prlr protein expression in female and male rat spinal cord, Related to Figure 8.

IHC with Prlr antibodies (U5-monoclonal) and CGRP (rabbit polyclonal) on spinal cord sections from WT female in estrous phase and male rats. A representative scale bar of 50μm is shown.