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A prolactin-targeting antibody to prevent stress-induced peripheral nociceptor sensitization and female postoperative pain

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Scheduled surgeries elicit stress in many patients. Levels of preoperative stress, anxiety, and female gender are known risk factors for increased and prolonged postoperative pain. The mechanisms by which psychological stress increases postoperative pain, especially in women, remain unknown. We hypothesized that stress amplifies postoperative pain by sensitizing dorsal root ganglion (DRG) nociceptors. Prolactin (PRL) is a female-predominant neurohormone that is controlled by estrogen and stress. PRL signals at the prolactin receptor long (PRLR-L) and short (PRLR-S) isoforms to induce gene transcription and nociception, respectively. Critically, prolactin sensitizes female, but not male, murine, Macaque and human nociceptors, revealing an evolutionarily conserved mechanism with high translational potential for human therapy. Prior restraint stress (RS) increased the magnitude and duration of incisional injury–induced postoperative pain hypersensitivity in both male and female mice. In females, RS or incisional injury downregulated PRLR-L and increased PRL-dependent nociceptor excitability. Female selective inhibition of postoperative pain hypersensitivity was produced by a) pharmacological inhibition of pituitary PRL b) overexpression of DRG PRLR-L to bias PRL signaling away from PRLR-S and c) CRISPR/Cas9 editing of PRLR isoforms. PL200,019, our recently discovered monoclonal antibody against human PRL (hPRL), prevented hPRL-induced sensitization of human female nociceptors. Using female mice genetically modified to express hPRL, rather than murine PRL, PL200,019 prevented both stress and incisional injury–induced hypersensitivity. Preemptive inhibition of stress-induced nociceptor sensitization with a monoclonal antibody to sequester PRL can improve female postoperative pain, diminish the need for postoperative opioids and decrease the risks of transition to chronic pain.

sex differences | sensitization | pain | prolactin | therapy

Significance

Psychological stress is a risk factor for worsening and chronification of postoperative pain. Stress responses and postoperative pain disproportionally affect women. Stress increased circulating prolactin (PRL), sensitized rodent and human nociceptors, and increased postoperative pain hypersensitivity in female mice. Pharmacological or genetic targeting of PRL, or its receptor, inhibited nociceptor sensitization, and reduced stress-related postoperative hypersensitivity in female mice. We developed a monoclonal antibody that sequesters human PRL, prevents sensitization of female human nociceptors and decreases stress-related postsurgical pain in female mice modified to produce human PRL. Preemptive inhibition of stress-induced nociceptor sensitization with a novel monoclonal antibody to sequester PRL can improve postoperative pain, diminish the need for postoperative opioids, and decrease risks of transition to chronic pain in females.

Risk factors for worsening postoperative pain include high levels of stress, anxiety in anticipation of a scheduled surgery, and female gender (1–4). Compared to men, women report greater responses to acute stress (5–8), have a higher incidence of chronic pain (9–11), and are more likely to experience longer lasting postoperative pain (12–15). Sexual dimorphism in pain can be quantitative reflecting differences in prevalence (9) as well as qualitative where underlying mechanisms are different in men and women (16). Qualitative sex differences offer opportunities for precision medicine allowing therapy optimization based on patient sex.

Recent studies have highlighted female-selective pronociceptive effects of prolactin (PRL), a circulating neurohormone, that is also produced locally by numerous cell types (PRL) (17–23). PRL is released from the anterior pituitary into the bloodstream and is under tonic inhibitory control of tuberoinfundibular dopamine (TIDA) neurons in the hypothalamus (24, 25). Dopamine from TIDA neurons binds to D2 receptors on pituitary lactotrophs to inhibit PRL release (26). PRL has been linked to increased migraine in patients as well as to opioid induced hyperalgesia, both of which occur more frequently in women (18, 19, 21, 22). Furthermore, relative to men, women have higher levels of circulating PRL (27). Critically, serum PRL is enhanced by external factors that inhibit hypothalamic TIDA neurons, including psychological stress (28–30). PRL may also be increased from extrapituitary sources by trauma (31, 32).

PRL exerts its cellular effects by binding to PRL receptors (PRLR), which are expressed on nociceptive sensory neurons in the dorsal root ganglia (DRG) and trigeminal ganglia (TG) (22, 33). There are two isoforms of PRLR in mice, the long (PRLR-L) and short (PRLR-S) receptors that couple to divergent intracellular signaling pathways (34, 35).

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Activation of PRLR-L is associated with nuclear transcription through Jak2/Stat5 signaling pathways (36). In contrast, signaling from the PRLR-S isoform is pronociceptive through modulation of pain relevant surface receptors and ion channels, such as transient receptor potential channels (i.e. TRPA1, TRPV1) (37). Importantly, coexpression of PRLR-L and PRLR-S is mutually inhibitory, meaning that outcomes of PRL signaling may depend on the relative expression of PRLR isoforms (34).

We have previously shown that stress induces an increase in pituitary PRL release and downregulation of PRLR-L expression (19). This downregulation shifts neuronal activity toward a pronociceptive state mediated by PRLR-S, which is characterized by allodynia in uninjured female mice and hypersensitivity of DRG neurons to low concentrations of PRL (38, 39). In the present study, we determined whether prior stress could act as a female selective PRL-dependent mechanism of peripheral nociceptor sensitization. We theorized that this would enhance the magnitude and duration of postoperative pain, while providing an opportunity for preemptive presurgical intervention.

We used a well-established model of postoperative pain induced by hindpaw incision either alone, or with prior restraint stress (RS) priming (40). We employed multiple approaches to prevent signaling at the pronociceptive PRLR-S through a) pharmacological inhibition of PRL secretion; b) transient overexpression of PRLR-L; and c) CRISPR-Cas9 editing of both PRLR isoforms. Last, we evaluated our recently discovered monoclonal antibody targeting human PRL (hPRL) and characterized its protective effects on PRL-induced hyperexcitability of human dorsal root ganglion neurons. We further validated this antibody in stress-related postoperative pain using mice genetically engineered to produce human, rather than murine, PRL. Collectively, these studies support preemptive prevention of stress-induced peripheral nociceptor sensitization as a strategy that can be implemented to improve postoperative pain hypersensitivity associated with scheduled surgeries in females.

Results

Pituitary PRL Promotes Injury-Related Postoperative Pain Hypersensitivity Selectively in Female Mice. We first explored whether pituitary PRL could be a determining factor in worsening of postoperative hypersensitivity. Female mice were subjected to incisional hindpaw injury (41) and expression of DRG PRLR isoforms was assessed using western blot. Incisional injury down-regulated levels of DRG PRLR-L protein without altering expression of PRLR-S (Fig. 1 *A*–*C*). To determine whether the injury-related change in PRLR-L was due to pituitary PRL, mice were treated with cabergoline, a dopaminergic D2 receptor agonist that inhibits pituitary PRL release and upregulates expression of PRLR-L, prior to incisional injury (Fig. 1*D*). Cabergoline increased PRLR-L protein expression in female mice receiving incisional injury without affecting PRLR-S expression (Fig. 1 *E*–*G*).

We then assessed potential functional consequences of inhibiting circulating PRL and downregulation of PRLR-L by evaluating postoperative pain hypersensitivity following incisional injury (Fig. 1*D*). Postoperative pain hypersensitivity induced by incisional injury was evident for approximately 3 to 4 d and was prevented by prior treatment with cabergoline (Fig. 1 *H* and *I*). In the same mice, capsaicin was injected into the hindpaw contralateral to the incisional injury and spontaneous nocifensive pain behaviors reflected by flinching and biting were measured. Mice with prior cabergoline treatment showed significantly reduced spontaneous pain behaviors elicited by capsaicin injection (Fig. 1*J*).

Pituitary PRL Does Not Alter Injury-Related and Capsaicin-evoked Postoperative Pain Hypersensitivity in Male Mice. Though expression of PRLR is barely detectable in DRG from male mice (22), we nevertheless assessed possible protective effects of cabergoline in male animals receiving incisional injury. Incisional injury–induced postoperative pain hypersensitivity in male mice was unaffected by treatment with cabergoline (Fig. 1 *K* and *L*). In these same mice, nocifensive behaviors elicited by capsaicin injection in the paw contralateral to the incision were similarly unaffected by prior treatment with cabergoline (Fig. 1*M*).

Transient Overexpression of PRLR-L Protects From Injury Induced Postoperative Pain Hypersensitivity in Female Mice. The effects observed with cabergoline suggested that decreasing circulating PRL to increase PRLR-L and bias signaling away from the pronociceptive PRLR-S could protect against incisional injury–induced postoperative pain hypersensitivity. This possibility was directly tested by genetically increasing PRLR-L protein expression followed by evaluation of injury-induced postoperative pain hypersensitivity (Fig. 2*A*). The gene for PRLR-L fused to a GFP reporter was cloned into a cDNA plasmid as reported previously (22). The PRLR-L plasmid was mixed with an in vivo transfection reagent and injected intrathecally to male and female mice. Intrathecal delivery of the PRLR-L encoding plasmid transiently increased the expression of PRLR-L protein in the DRG of female mice compared to mice treated with a control plasmid (Fig. 2*B*). Mice injected with either PRLR-L overexpression or the control plasmid were then subjected to incisional injury and incisional injury–related postoperative pain hypersensitivity was evaluated. In female mice, intrathecal treatment with the PRLR-L plasmid prevented the development of postoperative pain hypersensitivity for approximately 24 h, and as overexpression is transient, postoperative hypersensitivity returned on day 2 as expected (Fig. 2 *C* and *D*). In contrast, overexpression of PRLR-L protein in male mice did not affect incisional injury–induced postoperative pain hypersensitivity (Fig. 2 *E* and *F*). To confirm the distribution of expression of the intrathecally delivered constructs we performed immunohistochemistry on DRG tissue sections and observed robust signal for the GFP reporter in neurons (*SI Appendix*, Fig. S1). These data are consistent with the view that signaling at PRLR-L is protective, likely by inhibiting signaling at the pronociceptive PRLR-S, and further that this is sufficient to protect from postsurgical hypersensitivity in female, but not male, mice.

Restraint Stress Induces Mechanical Hypersensitivity and Impairs Recovery From Incisional Injury. Repeated restraint stress increases levels of circulating PRL and has been shown to decrease expression of PRLR-L (19). This paradigm was used to model the potential influence of preoperative stress that is known to promote poor outcomes following surgery. Mice were subjected to restraint stress (RS) for 2 h per day over 3 consecutive days (Fig. 3*A*). In both female and male mice, RS produced hindpaw allodynia that resolved by approximately day 7 relative to mice that underwent a sham stress procedure (Fig. 3 *B* and *E*). Incisional injury was then performed in these mice on day 15 following the first stress episode, a timepoint when mice showed baseline sensory responses. Female mice demonstrated significantly increased postoperative pain hypersensitivity that also took longer to return to baseline when compared to mice that received sham stress treatment followed by incisional injury (Fig. 3*C*). The AUC for female mice that received incisional injury with prior stress was significantly higher than for mice receiving injury without prior stress (Fig. 3*D*).

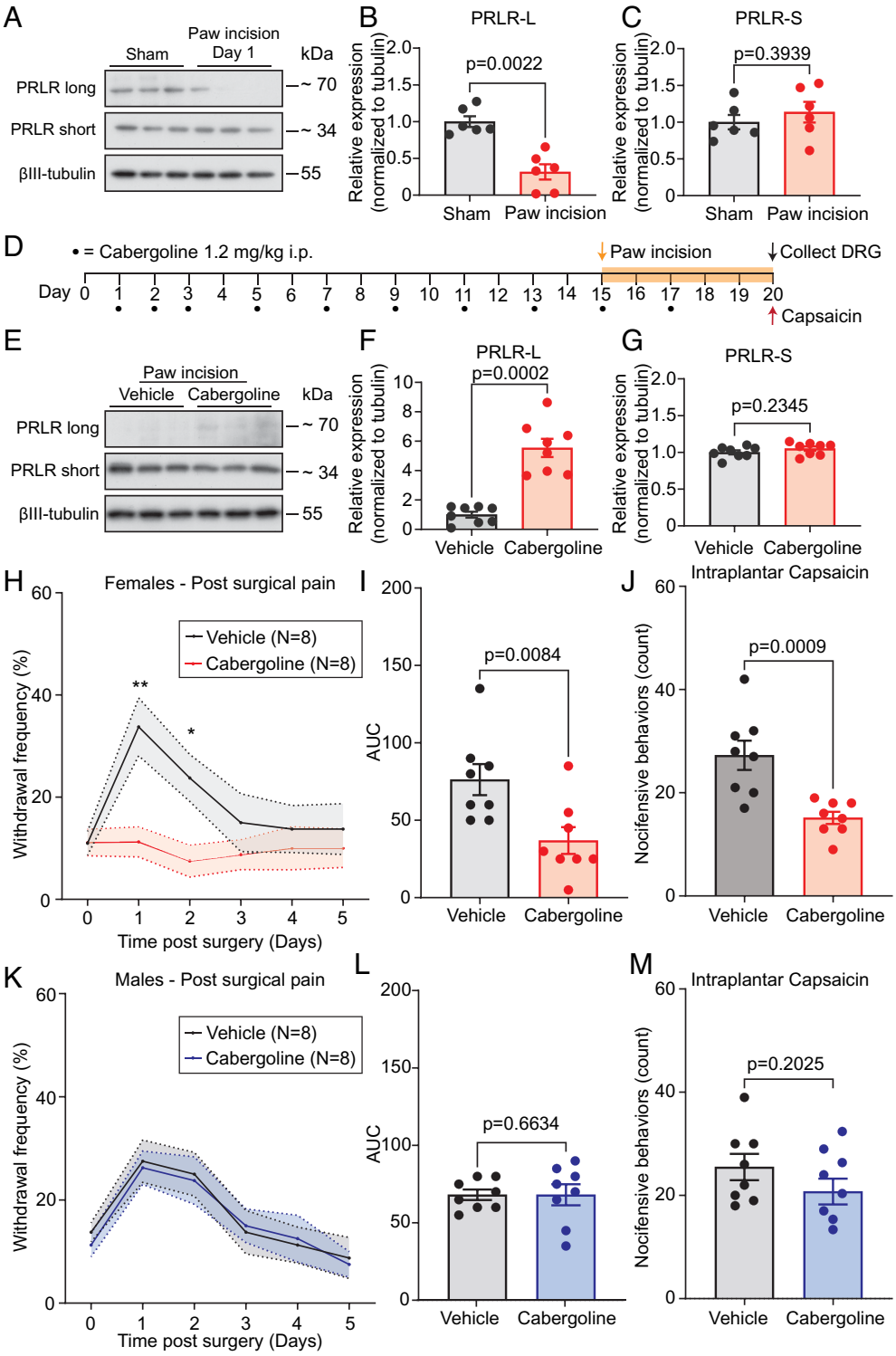


Fig. 1. Pharmacological inhibition of pituitary PRL release prevents dysregulation of PRLR isoforms and blocks mechanical hypersensitivity following incisional injury. (*A*) Western blot showing downregulation of PRLR-L in female mouse DRG 1 d after paw incision surgery. (*B*) Quantification of PRLR-L expression downregulation after paw incision. (*C*) Quantification showing no change in the expression of PRLR-S. (*D*) Timeline of cabergoline treatments and experimental manipulations pertaining to panels *E*–*M*. (*E*) Western blot showing expression of PRLR-L and PRLR-S in cabergoline treated female mouse DRG after paw incision. (*F*) Cabergoline treatment increases the expression of PRLR-L relative to vehicle-treated mice after paw incision. (*G*) There was no change in the expression of PRLR-S following treatment with cabergoline after paw incision. (*H*) Treatment with cabergoline blocks the emergence of postoperative pain hypersensitivity in female mice. (*I*) Quantification of the area under the curve (AUC) for female mice after incisional injury. (*J*) Spontaneous nocifensive behaviors after injection of capsaicin into the contralateral paw relative to the incisional injury. (*K*) Treatment with cabergoline had no effect on postoperative pain hypersensitivity in male mice following incisional injury. (*L*) Quantification of the AUC for male mice after incisional injury. (*M*) Evaluation of spontaneous nocifensive behaviors in male mice after injection of capsaicin into the contralateral uninjured paw. Data are displayed as mean ± SEM. Each data point represents an independent animal. N = 6–8 mice per group for biochemistry. Two-way repeated measures ANOVA with Sidak's test for multiple comparisons (*H* and *K*). Other comparisons used the Mann–Whitney test. $^{*}P < 0.05$ and $^{**}P < 0.01$. Additional statistical details can be found in *SI Appendix*, Table S2.

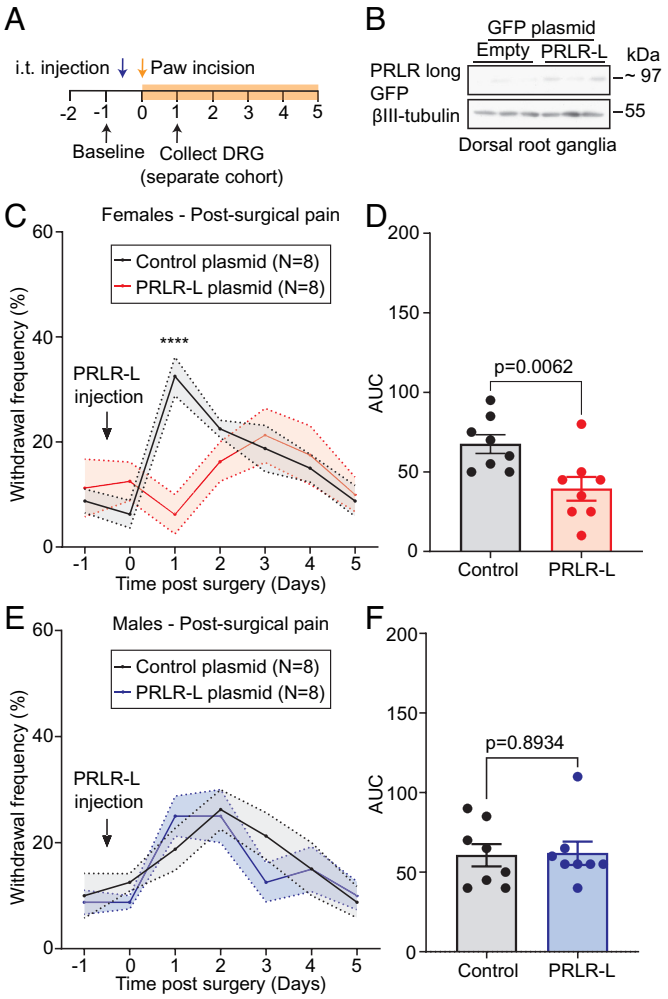


Fig. 2. Overexpression of PRLR-L protects from incisional injury–induced hypersensitivity in female mice. (*A*) Timeline of experimental manipulations including intrathecal injection of PRLR-L-GFP plasmid to overexpress the receptor. (*B*) Western blot showing increased expression of PRLR-L protein in the DRG of female mice after intrathecal injection of a plasmid encoding the receptor. (*C*) Intrathecal injection of PRLR-L plasmid in female mice 1 d before incisional injury followed by mechanical hypersensitivity testing. (*D*) Quantification of the AUC for the female mice in panel *C*. (*E*) Male mice were given an intrathecal injection of a plasmid encoding PRLR-L 1 d before incisional injury followed by mechanical hypersensitivity testing. (*F*) Quantification of the AUC for the animals shown in panel *E*. Data are shown as mean ± SEM. The number of animals used is indicated in the figure. Two-way repeated measures ANOVA with Sidak's test for multiple comparisons (*C* and *E*). All other comparisons used the Mann–Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Additional statistical details can be found in *SI Appendix*, Table S2.

Similarly, male mice receiving incisional injury with prior RS had increased postoperative pain hypersensitivity and a longer duration of recovery compared to mice without prior RS (Fig. 3*F*). The AUC for paw hypersensitivity in male mice receiving incisional injury with prior RS was also significantly increased compared to unstressed mice (Fig. 3*G*). These data indicate that prior exposure to stress significantly increases and prolongs the recovery from postsurgical pain hypersensitivity consistent with clinical outcomes where stress can prolong recovery from surgery.

Primary Sensory Neurons From Female, but Not Male, Mice Are Hyperexcitable and Sensitized to a Low Concentration of PRL Following Stress-Induced Downregulation of PRLR-L. We have previously shown that in vitro exposure to PRL at a concentration of 50 nM leads to sensitization of primary sensory neurons and produces robust hyperexcitability in female, but not male, DRG neurons from the mouse, Macaque, and human (20). However, incubation with a lower concentration of PRL (5 nM) did not induce hyperexcitability in DRG sensory neurons. We hypothesized that RS could prime sensory neurons so that they would now be responsive to a low concentration of PRL. DRG neurons from the same mice subjected to either RS and paw incision or a sham procedure and paw incision, as described above, were cultured overnight with 5 nM mouse PRL. We then performed patch clamp electrophysiological recordings of small diameter (<30 μm) sensory neurons to evaluate excitability. PRL increased action potential firing in DRG neurons from female mice that had been exposed to RS and paw incision, but not in mice receiving incisional injury without prior RS (Fig. 4*A*). These neurons also fired more action potentials per current step than the non-stress-treated group when exposed to low concentration PRL (Fig. 4*B*). There was also a significant reduction in the rheobase of the RS primed female mice compared to those not exposed to RS (Fig. 4*C*) while the resting membrane potential was not significantly different between these groups of female mice (Fig. 4*D*).

In DRG neurons cultured from male mice there was no significant difference in the number of action potentials fired between mice subjected to RS prior to incisional injury when cultured with the low concentration of PRL overnight (Fig. 4*E*). The excitability profile was nearly identical and prior exposure to RS had no effect on responses to low concentration PRL (Fig. 4*F*). Additionally, the rheobase and resting membrane potential were also not altered by treatment with low concentration PRL in the stress primed male mice compared to those without prior RS (Fig. 4 *G* and *H*). These data suggest that preoperative RS can act to prime female sensory neurons to low concentrations of PRL. Since our biochemical studies showed that stress leads to reduced PRLR-L expression in vivo, it is possible that this increased excitability could be through this mechanism.

Inhibition of Pituitary PRL Prevents Stress and Injury Induced Mechanical Hypersensitivity Selectively in Female Mice. Decreased levels of pituitary PRL could inhibit incisional injury–induced postoperative pain hypersensitivity possibly by increasing expression of PRLR-L. However, whether this protective effect would be observed in the presence of RS priming was unknown. Female and male mice were treated once daily with cabergoline prior to each RS episode and every other day for 14 d thereafter to decrease circulating PRL and upregulate PRLR-L prior to receiving incisional injury (Fig. 5*A*). An almost complete blockade of RS-induced hindpaw allodynia was observed during the priming period in female mice treated with cabergoline (Fig. 5*B*). Incisional injury–related postoperative pain hypersensitivity in these female mice was also blocked by cabergoline (Fig. 5 *C* and *D*). While male mice exhibited typical RS-induced allodynia and incisional injury–induced postoperative pain hypersensitivity, cabergoline treatment had no effect (Fig. 5 *E*–*G*). To determine a potential mechanism that could explain this protective effect, protein expression of PRLR isoforms in DRG obtained from mice treated with cabergoline alone, or with cabergoline and RS, was quantified in female mice (Fig. 5*H*). Consistent with the blockade of pain hypersensitivity, cabergoline treatment significantly increased expression of PRLR-L protein regardless of RS without altering expression of PRLR-S (Fig. 5 *I* and *J*). Additionally, as observed previously, female mice receiving RS in the absence of cabergoline demonstrated lower levels of PRLR-L protein though this did not reach significance in this experiment (Fig. 5*I*). These data demonstrate that decreased circulating PRL and upregulation of PRLR-L is protective against both RS-induced allodynia as well

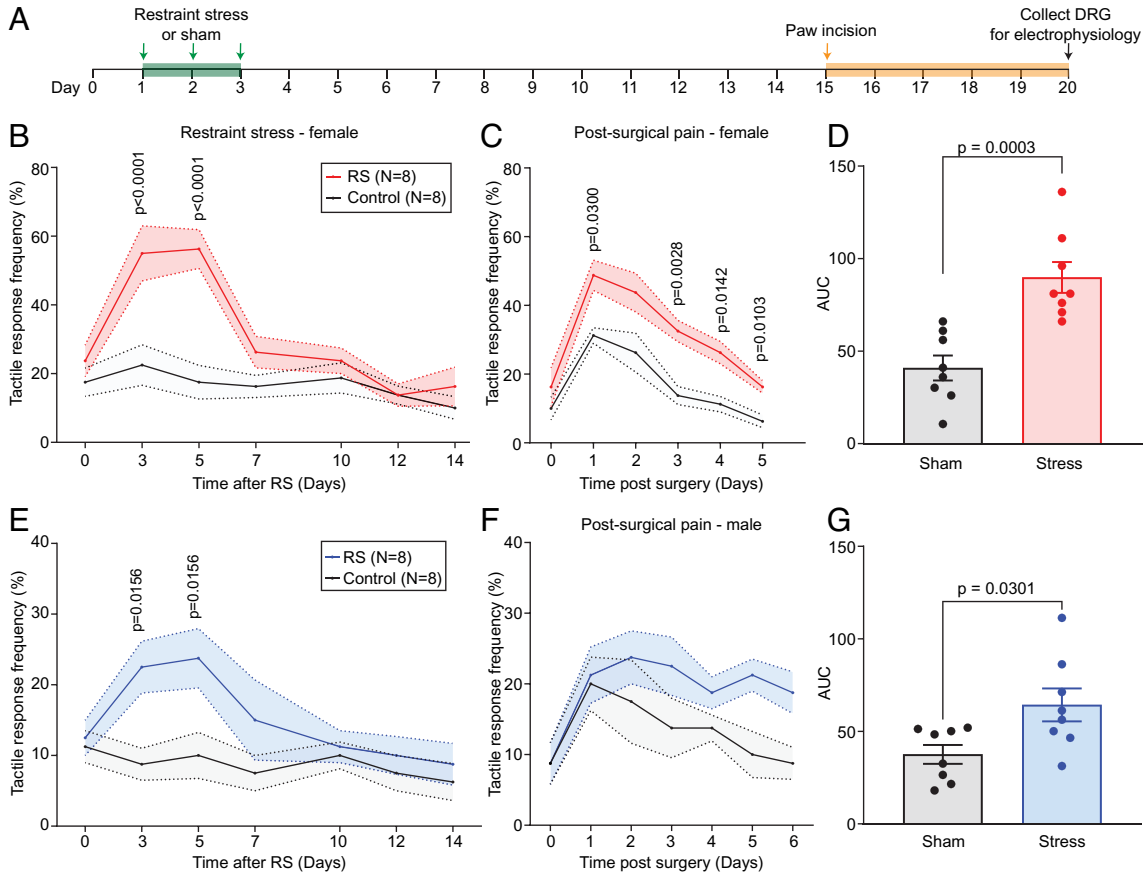


Fig. 3. Restraint stress causes mechanical hypersensitivity in male and female mice and prolongs recovery from incisional injury–induced pain. (*A*) Experimental timeline. (*B*) Restraint stress (RS) produces transient hindpaw allodynia in female mice that returns to baseline by approximately day 7. (*C*) Prior exposure to restraint stress prolongs the recovery and enhances incisional injury–induced pain hypersensitivity in female mice. (*D*) Quantification of the AUC for female mice after incisional injury. (*E*) RS produces transient hindpaw allodynia in male mice that resolves by approximately day 7. (*F*) Prior exposure to stress also prolongs the recovery from incisional injury and increases the magnitude of incisional injury–related pain hypersensitivity. (*G*) Quantification of the AUC for male mice after incisional injury. Data are mean ± SEM. Two-way repeated measures ANOVA with Sidak's test for multiple comparisons (*B*, *C*, *E*, and *F*). All other comparisons used the Mann–Whitney test. Additional statistical details can be found in *SI Appendix*, Table S2.

as incisional injury–related postoperative pain hypersensitivity regardless of previous perioperative stress. Lack of an effect due to cabergoline treatment in males also demonstrates that this outcome is not due to direct agonist action of cabergoline on nociception.

Inhibition of Pituitary PRL Prevents RS-Induced DRG Hyperexcitability Elicited by Low Concentrations of PRL Selectively in Female Mice. We next evaluated whether RS induced dysregulation of PRLR isoforms during priming could increase PRL-induced hyperexcitability in mice with incisional injury. DRG neurons from the same mice treated with RS and incisional injury described above were harvested to determine whether they exhibited a primed phenotype with increased excitability to low concentrations of PRL. DRG neurons isolated from RS/incisional injury female mice treated with vehicle and cultured with low dose PRL (5 nM) overnight demonstrated a robust hyperexcitability profile (Fig. 6*A*). Increased responses were observed across the full excitability curve in DRG neurons obtained from female mice of the control group (Fig. 6*B*). In contrast, increased excitability was absent in female mice receiving RS/incisional injury and treated with cabergoline (Fig. 6*B*). Significant differences in rheobase were not observed in these neurons regardless of cabergoline treatment (Fig. 6*C*). However, the resting membrane potential in DRG neurons from female RS/incisional injury mice treated with cabergoline was more negative than that of control animals (Fig. 6*D*). Further, the number of action potentials fired at the maximum current injection was lower in sensory neurons from female RS/incisional injury mice previously treated with cabergoline (Fig. 6*E*).

In contrast to observations of increased DRG excitability in neurons from female mice, in male mice, there was no difference in action potential firing between groups receiving vehicle or cabergoline (Fig. 6 *F* and *G*). Rheobase and resting membrane potential were similarly unaffected by cabergoline treatment in DRG cells from male mice (Fig. 6 *H* and *I*) and the number of action potentials fired was comparable between the two treatment groups (Fig. 6*J*). These data suggest that the perioperative stress-induced increase in pituitary PRL is sufficient to promote dysregulation of PRLR isoforms to drive increased neuronal excitability selectively in female mice.

Prevention of PRLR Signaling Protects Against Female Neuronal Hyperexcitability and Mechanical Hypersensitivity. As restraint stress down-regulates expression of PRLR-L, we asked whether deletion of all PRLR isoforms (22) could prevent RS-induced allodynia, injury-induced DRG hyperexcitability, and postoperative pain in female mice to directly demonstrate the pronociceptive effect of signaling at PRLR-S. We previously developed and validated a CRISPR construct that targets the first exon common to both PRLR isoform mRNA transcripts, which we call *Prlr*-total CRISPR (22). Female mice received the *Prlr*-total CRISPR construct, or an empty control plasmid, and mechanical response frequency was evaluated before and after injection (Fig. 7*A*). No change in tactile hypersensitivity was observed, indicating

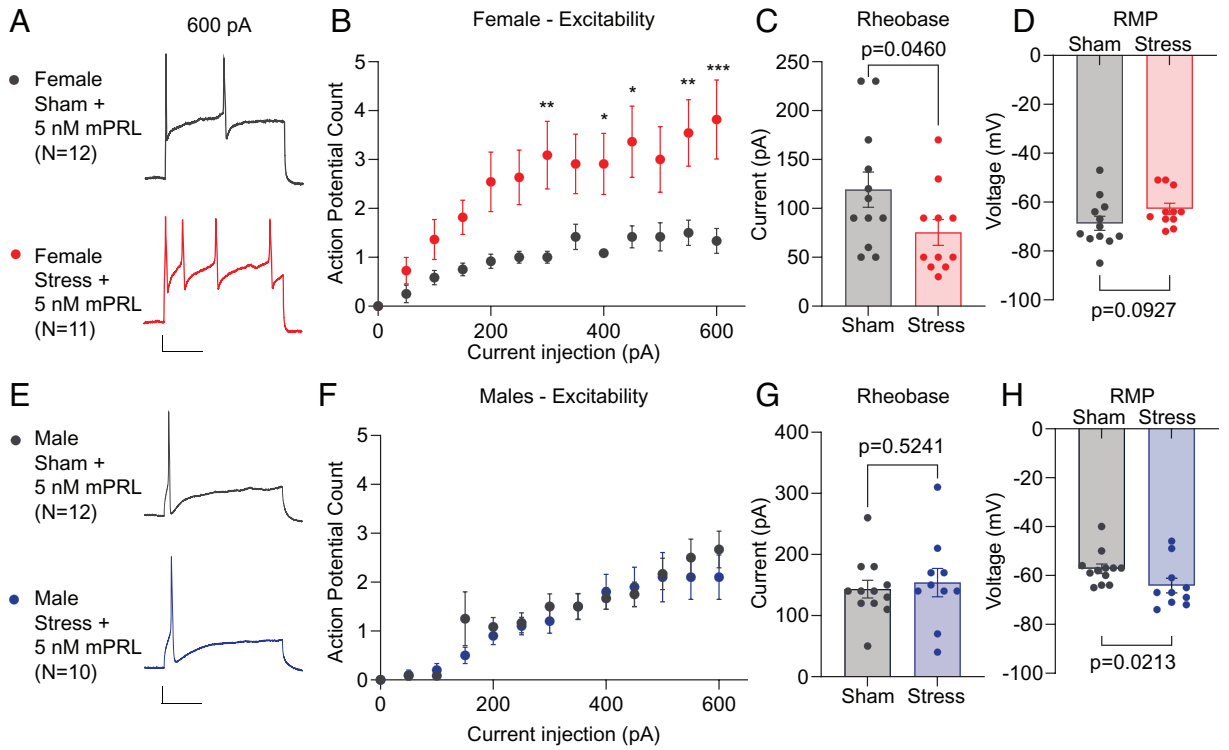


Fig. 4. Downregulation of PRLR-L following stress and injury triggers enhanced responses to a low concentration of PRL selectively in female mouse DRG neurons. (*A*) Representative traces from small diameter female mouse DRG neurons during current clamp recordings while injecting 600 pA of current. These recordings are from the same mice used for behavioral experiments in figure 3. (*B*) Plot showing that DRG neurons of mice previously exposed to stress had increased excitability to a low dose of PRL. (*C*) Rheobase, the minimum current to fire a single action potential, was reduced in female mice from the group exposed to stress and treated with PRL in vitro. (*D*) The resting membrane potential (RMP) was not different between the treatment groups. (*E*) Representative current clamp traces from male mouse DRG neurons treated with low doses of PRL evoked at 600 pA. (*F*) Excitability plot showing that increasing numbers of action potentials are fired per current step but that low concentration PRL treatment does not affect male sensory neurons. (*G*) The rheobase was not affected in male sensory neurons treated with PRL. (*H*) The resting membrane potential was slightly more negative in male mouse DRG neurons that had previously received stress exposure. Data are mean ± SEM. (Scale bars are 20 mV and 200 ms.) The number of cells recorded is indicated in the figure. Two-way repeated measures ANOVA with Sidak's test for multiple comparisons (*B* and *F*). All other comparisons used the Mann–Whitney test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. Additional statistical details can be found in *SI Appendix*, Table S2.

that these treatments had no effect on baseline response profiles (Fig. 7*B*). Mice then received RS, as described above, and those treated with the control plasmid displayed robust RS-mediated hindpaw allodynia (Fig. 7*B*). However, a complete blockade of mechanical hypersensitivity that lasted for the duration of the 2 wk test period was observed in mice treated with *Prlr* -total CRISPR (Fig. 7*B*). Both cohorts of mice then underwent incisional injury, and postoperative pain hypersensitivity was assessed over a 5 d recovery period. Mice treated with the control plasmid demonstrated typical transient increases in injury-induced postoperative pain (Fig. 7*C*). However, mice treated with the *Prlr* -total CRISPR had significantly reduced response frequencies following injury (Fig. 7*C*). The reduced postoperative pain hypersensitivity responses are demonstrated by the significantly reduced AUC values in mice treated with *Prlr* -total CRISPR relative to mice treated with the empty plasmid (Fig. 7*D*).

Following resolution of the transient RS/incisional injury–induced postoperative pain hypersensitivity, the DRG neurons from these mice were harvested and treated overnight with a low concentration of PRL (5 nM). Sensory neurons from RS mice treated with the control plasmid showed increased action potential firing frequency after overnight incubation with low concentration PRL while neurons from RS mice treated with *Prlr* -total CRISPR were unchanged (Fig. 7*E*). The summary excitability curve shows a higher rate of evoked action potential firing in the empty plasmid treated group compared to the group treated with the *Prlr* -total CRISPR (Fig. 7*F*). Rheobase was also significantly higher in the CRISPR treated group, suggesting increased firing thresholds (Fig. 7*G*). There was no difference in the resting membrane potential between sensory neurons obtained from mice treated with the empty CRISPR or the *Prlr*-total CRISPR construct following treatment with low concentration PRL (Fig. 7*H*).

PL 200,019, a Monoclonal Antibody Against hPRL Sequesters hPRL to Prevent Increased Excitability in Female Mouse DRG. While pituitary PRL effectively promotes stress-related postoperative pain hypersensitivity, it remains possible that PRL produced by local sources, including immune cells, could also contribute to this type of pain. We recently reported the discovery of PL 200,019, a murine monoclonal antibody able to neutralize hPRL, but not murine PRL (42). As hPRL is a potent agonist at murine PRLR (43), we first determined whether PL 200,019 could prevent hPRL-induced sensitization of mouse DRG neurons in vitro. DRG neurons from female mice were cultured and treated overnight with a maximal concentration of hPRL (50 nM), PL 200,019 (25 nM), or a combination of hPRL and PL 200,019 and neuronal excitability was evaluated in small diameter sensory neurons (*SI Appendix*, Fig. S2*A*). hPRL increased excitability of female mouse DRG neurons though to a lesser extent than observed with mPRL (*SI Appendix*, Fig. S2*A*). PL 200,019 treatment alone did not affect neuronal excitability but prevented the hPRL-induced increase in excitability of female sensory neurons (*SI Appendix*, Fig. S2*B*). There were no significant differences in the rheobase between the treatment groups (*SI Appendix*, Fig. S2*C*). The resting membrane potential was slightly more negative in the two groups

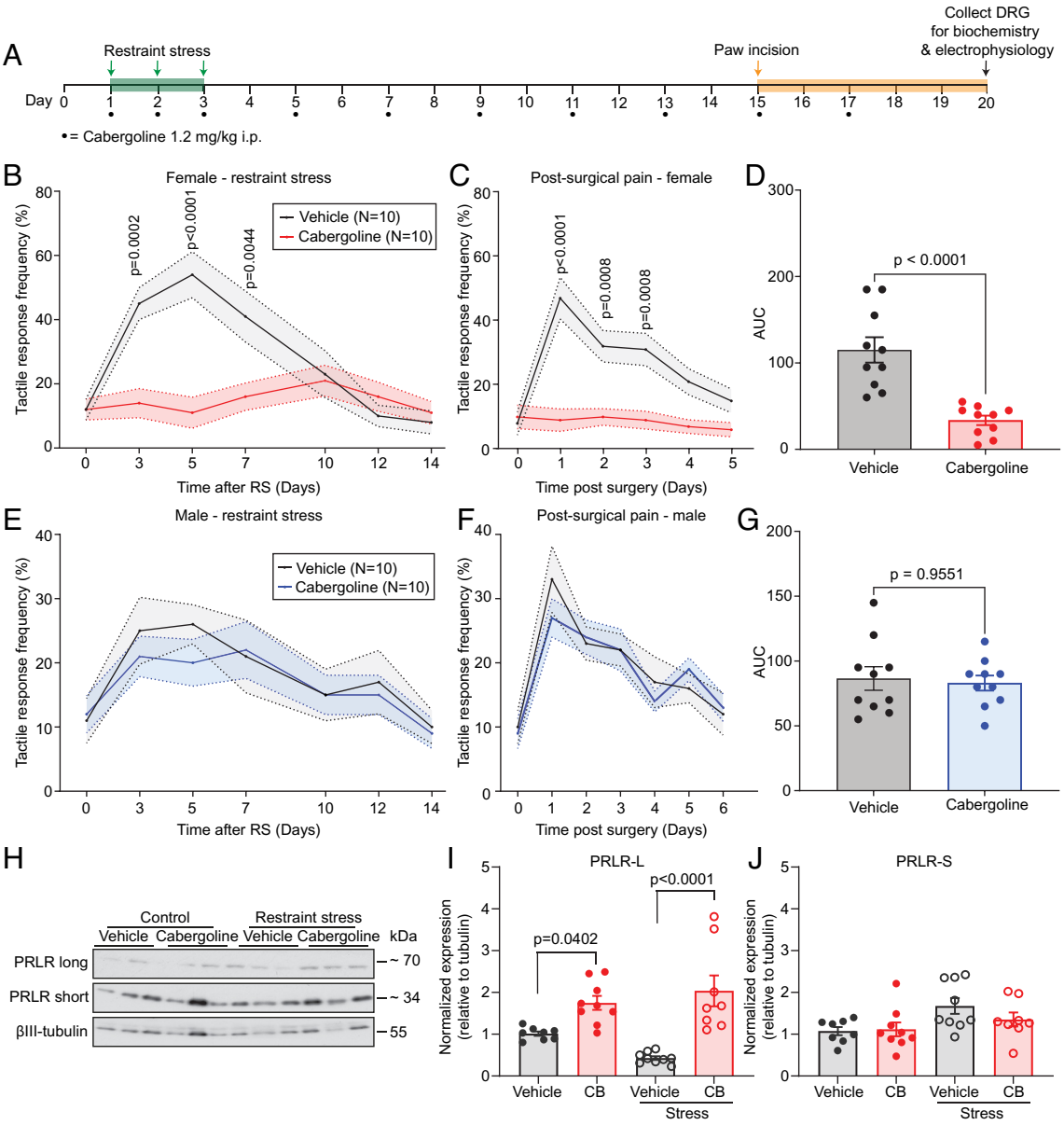


Fig. 5. Treatment with cabergoline to upregulate PRLR-L protects from stress priming and injury-induced pain hypersensitivity in female mice. (*A*) Experimental timeline showing when RS was performed, when cabergoline was administered, and when DRG were harvested for further analysis. (*B*) Evaluation of hindpaw allodynia in female mice treated either with vehicle or with cabergoline. (*C*) Treatment with cabergoline blocks the development of incisional injury–induced pain hypersensitivity compared to vehicle treated controls. (*D*) Quantification of the AUC for female mice after incisional injury. (*E*) Evaluation of hindpaw allodynia in male mice treated with cabergoline or vehicle after restraint stress. (*F*) Treatment with cabergoline does not affect the development of incisional injury–related pain hypersensitivity. (*G*) Quantification of the AUC for male mice after incisional injury. (*H*) Western blot showing the relative expression of PRLR-L and PRLR-S after treatment with vehicle or cabergoline (CB) in sham- or RS-treated female mice in the absence of incisional injury. (*I*) Quantification of the expression of PRLR-L showing that RS decreases expression of PRLR-L, treatment with cabergoline upregulates PRLR-L and that RS-induced reduction can be blocked by cabergoline treatment. (*J*) There were no changes in the level of PRLR-S expression with RS or cabergoline treatment. Data are mean ± SEM. The number of animals used for behavioral experiments is indicated in the figure. N = 8–9 mice for biochemistry. One-way ANOVA with Sidak's test for multiple comparisons (*I* and *J*). Two-way repeated measures ANOVA with Sidak's test for multiple comparisons (*B*, *C*, *E*, and *F*). All other comparisons used the Mann–Whitney test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. Additional statistical details can be found in *SI Appendix*, Table S2.

treated with the monoclonal antibody (*SI Appendix*, Fig. S2*D*). These results confirm that PL 200,019 can sequester hPRL to prevent sensitization of mouse DRG neurons in vitro.

PL 200,019 Prevents hPRL Induced Hyperexcitability in Female Human Sensory Neurons. We have recently reported that hPRL sensitizes sensory neurons recovered from female, but not male, human consented donors (20). Since our ultimate goal is to develop a strategy for treating postoperative pain in patients, we evaluated whether PL 200,019 could sequester hPRL and prevent neuronal sensitization of female human DRG neurons. Overnight treatment with high dose hPRL (50 nM), to mimic the high concentrations of PRL released during stress, produced robust action potential firing in female human sensory neurons (Fig. 8*A*). Coincubation with PL 200,019 blocked this hPRL-induced increase in neuronal excitability (Fig. 8*B*). There was an increase in the rheobase of sensory neurons treated with the neutralizing antibody compared to those treated with hPRL alone (Fig. 8*C*). There was no difference in the resting membrane potential between the treatment groups (Fig. 8*D*). These data demonstrate that PL 200,019 effectively prevents sensitization of female human DRG neurons by hPRL suggesting there is

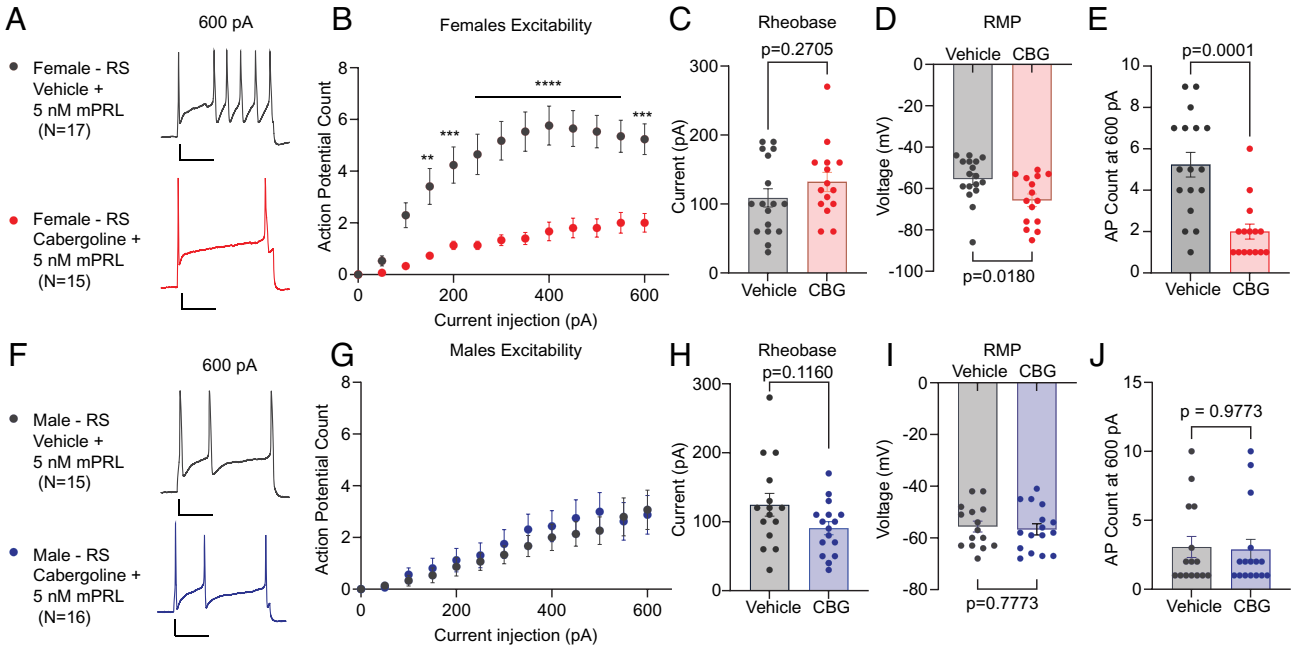


Fig. 6. Treatment with cabergoline prevents hyperexcitability following overnight incubation with a low concentration of PRL. (*A*) Representative traces evoked at 600 pA from female mouse DRG neurons following treatment with low concentration PRL. These neurons were isolated from the same animals in which behavior was performed in Fig. 5. (*B*) Treatment with cabergoline reduced the excitability of female mouse DRG neurons in response to low concentration PRL compared to mice treated with vehicle. (*C*) Rheobase was not affected following treatment with low concentration PRL in cabergoline treated animals. (*D*) The resting membrane potential was slightly more negative in DRG neurons from female mice treated with PRL. (*E*) The action potential count at maximum current injection was significantly lower in female mice following treatment with cabergoline. (*F*) Representative traces evoked at 600 pA from male DRG neurons treated with low concentration PRL. (*G*) The excitability of male DRG neurons was not affected by PRL treatment in mice treated with either vehicle or cabergoline. (*H*) The rheobase and the (*I*) RMP of male mouse DRG neurons was not affected by PRL treatment. (*J*) The maximum number of action potentials fired was not significantly different between groups. Data are mean ± SEM. (Scale bars are 20 mV and 200 ms.) CBG, Cabergoline. The number of cells recorded is indicated in the figure. Two-way repeated measures ANOVA with Sidak's test for multiple comparisons (*B* and *G*). All other comparisons used the Mann–Whitney test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. Additional statistical details can be found in *SI Appendix*, Table S2.

significant translational potential in using this antibody to decrease stress-related postoperative pain in patients.

PL 200,019 Sequesters hPRL to Prevent Increased DRG Excitability and Postoperative Pain Hypersensitivity in Female Mice Genetically Engineered to Produce hPRL. We next tested whether PL 200,019 could be effective in vivo against circulating and local hPRL to prevent sensitization after RS and injury. We used a transgenic mouse engineered to produce hPRL, as previously described (43), and that expresses the mouse PRLR (Fig. 8*E*). A single dose of PL 200,019 was delivered subcutaneously at 20 mg/kg per mouse 24 h before the first RS session and again before the paw incision surgery (Fig. 8*F*). Repeated RS produced mechanical hypersensitivity in mice that had been treated with the vehicle but not in mice receiving the neutralizing antibody (Fig. 8*G*). We then subjected these same female mice to paw incision surgery. Treatment with PL 200,019 significantly reduced stress-related postoperative pain hypersensitivity compared to mice that were treated with vehicle (Fig. 8 *H* and *I*). These data suggest that sequestration of hPRL protects female mice from stress-related postoperative pain hypersensitivity likely by preventing signaling at PRLR-S.

Discussion

Preoperative anxiety, catastrophizing, and stress are well-established predictors of worse postoperative pain outcomes and delayed recovery especially in women (2, 44). Stress is commonly associated with many female prevalent pain disorders and women exhibit greater stress responses (45–47). Sexually dimorphic mechanisms promoting excitability of animal and human nociceptors have recently been identified (20, 48). Nevertheless, consideration of patient sex, a fundamental form of precision medicine, is not generally considered in pain management. Our studies reveal a qualitative sex difference that directly links psychological stress to nociceptor effectors to promote hypersensitivity in females. Improved postoperative pain outcomes in women may be achieved by breaking the neurohormonal link between psychological stress and injury-induced nociceptor sensitization in scheduled surgeries. Preemptive targeting of a female-selective molecular mechanism may prevent peripheral neuronal sensitization from preoperative stress prior to surgical tissue injury and improve postoperative pain in females. Additionally, preemptive intervention of stress-related peripheral nociceptor sensitization is clinically feasible and would be expected to decrease acute postoperative hypersensitivity resulting from a scheduled surgical injury, diminish the need for use of pain medications including opioids, shorten hospital stays, and prevent the transition of acute to chronic pain. Critically, prevention of stress-induced peripheral nociceptor sensitization in settings of scheduled surgeries could be accomplished while maintaining the protective role of acute pain, which typically results from activation of nociceptors by high intensity, tissue-damaging stimuli. To our knowledge, this preemptive approach to diminish postoperative pain hypersensitivity has not previously been explored.

Surgical procedures are almost always accompanied by pain and injury-related allodynia that collectively present hurdles to patient recovery (49, 50). Allodynia in areas at, or near, surgical injuries can cause significant patient discomfort, inhibit return to function and extend inpatient duration with increased risks of infection and other comorbidities. Postsurgical allodynia can be attributed, in part, to sensitization of peripheral nociceptors, where lower

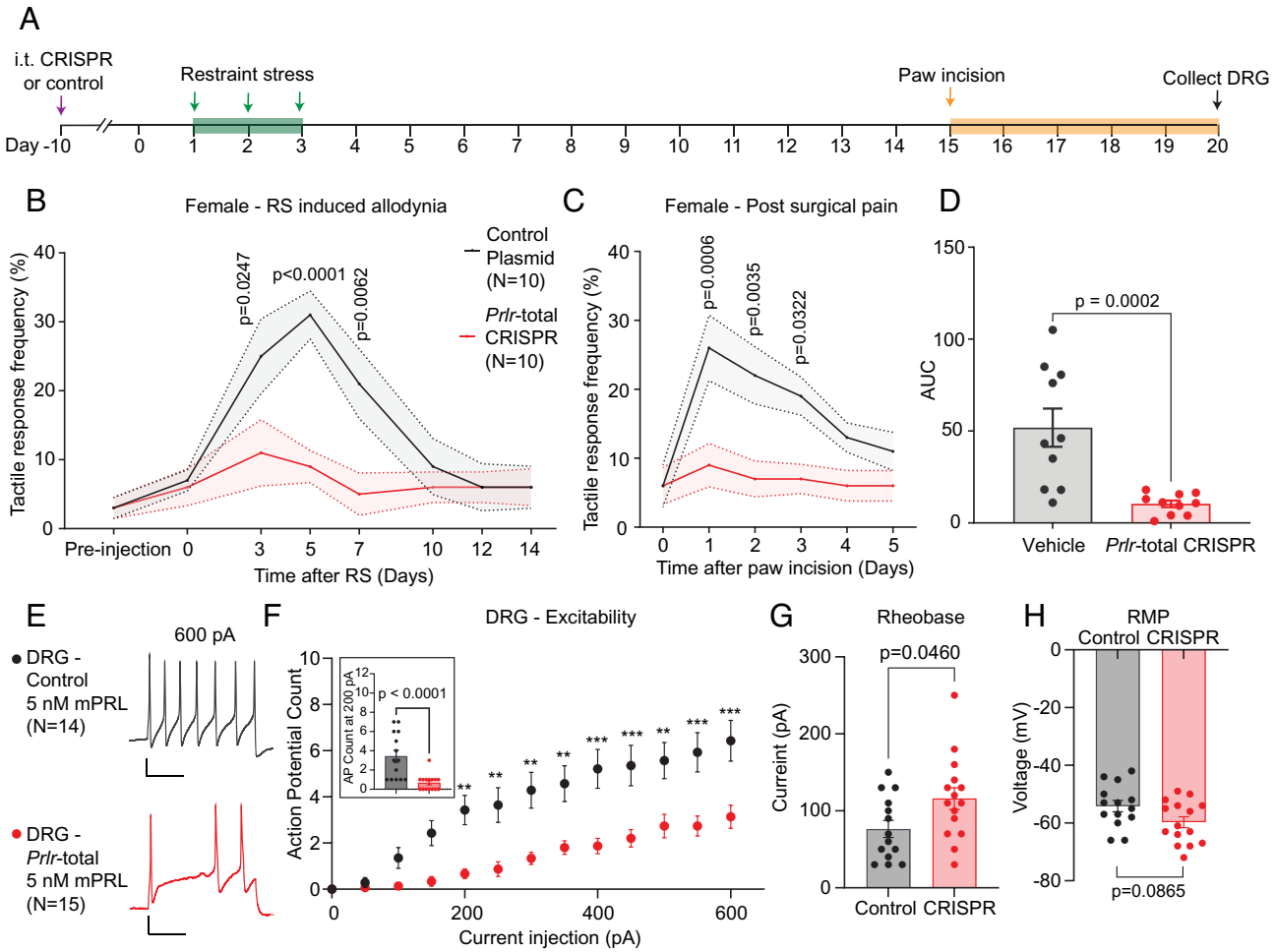


Fig. 7. Treatment with PRLR-total CRISPR elicits female-selective prevention of incisional injury–related pain hypersensitivity and DRG hyperexcitability in response to low concentration PRL treatment. (*A*) Experimental timeline. (*B*) Female mice were given an intrathecal injection of a CRISPR construct that deletes the PRL receptor (*Prlr*-total CRISPR) and this treatment blocked the development of RS-induced hindpaw allodynia. (*C*) Treatment with *Prlr*-total CRISPR also prevented the incisional injury–induced postoperative pain hypersensitivity following stress priming in female mice. (*D*) The AUC quantifying the differences observed in panel B following incisional injury. (*E*) Representative traces from female mouse DRG sensory neurons recorded after overnight treatment with a low dose of PRL and obtained from mice treated with control CRISPR or *Prlr*-total CRISPR and subjected to RS and incisional injury. (*F*) The excitability of female sensory neurons from mice treated with *Prlr*-total CRISPR was significantly lower relative to control CRISPR mice. *Inset* shows the 200 pA current step. (*G*) The rheobase was increased in sensory neurons from mice treated with *Prlr*-total CRISPR. (*H*) There was no change in the resting membrane potential in sensory neurons from mice treated with *Prlr*-total CRISPR compared to those of control treated mice. Data are mean ± SEM. (Scale bars are 20 mV and 200 ms.) The number of cells recorded is indicated in the figure. Two-way repeated measures ANOVA with Sidak's test for multiple comparisons (*B*, *C*, and *F*). All other comparisons used the Mann–Whitney test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001. Additional statistical details can be found in *SI Appendix*, Table S2.

neuronal activation thresholds increase the probability of pain from normally subthreshold stimuli (51, 52). Prolonged hypersensitivity of peripheral nociceptors may also contribute to establishing and sustaining chronic pain (53). Peripheral nociceptor sensitization can result from tissue factors associated with the surgical injury (52, 54). Additionally, neurohormonal mechanisms, including PRL, can promote sensitization of nociceptors that can lead to enhanced hypersensitivity (20, 22).

PRL exerts a tonic inhibitory effect on expression of PRLR-L and pharmacological treatment with cabergoline, a dopaminergic D2 agonist, to inhibit pituitary PRL secretion, can increase expression of PRLR-L in uninjured female mice (22). In the present experiments, cabergoline prevented postoperative pain hypersensitivity in female, but not male, animals experiencing incisional injury in the absence of stress. This suggests that decreased pituitary PRL signaling, in combination with upregulation of PRLR-L, is sufficient to protect against incisional injury–induced postoperative pain hypersensitivity. Interestingly, these experiments revealed a global effect of decreased PRL signaling, as pain from capsaicin injection in the contralateral uninjured paw was also decreased. In this case, capsaicin induced pain was assessed by evaluation of spontaneous nocifensive behaviors suggesting a potential role for PRL signaling in ongoing pain as well as injury-related allodynia. These data are notable as cabergoline, and other dopaminergic D2 agonists, are FDA approved and used clinically for treatment of hyperprolactinemia resulting from pituitary adenoma (55).

An important goal of these studies was to determine whether stress could act as a priming stimulus to increase the excitability of DRG neurons providing a mechanism that could lead to enhanced responses to subsequent incisional injury. We previously reported that repeated RS activates a hypothalamic circuit that inhibits dopaminergic (TIDA) neurons to disinhibit the release of pituitary PRL, which promotes downregulation of PRLR-L and leads to female-selective sensitization of primary afferent neurons (20, 32, 56). This finding suggested that this paradigm would be appropriate to model the consequences of preoperative stress on pain hypersensitivity following incisional injury. In both sexes, repeated RS alone produced a period of transient hindpaw allodynia and subsequent incisional injury–related postoperative pain was greater and longer lasting than in animals without prior stress exposure. The priming effect of RS was demonstrated using

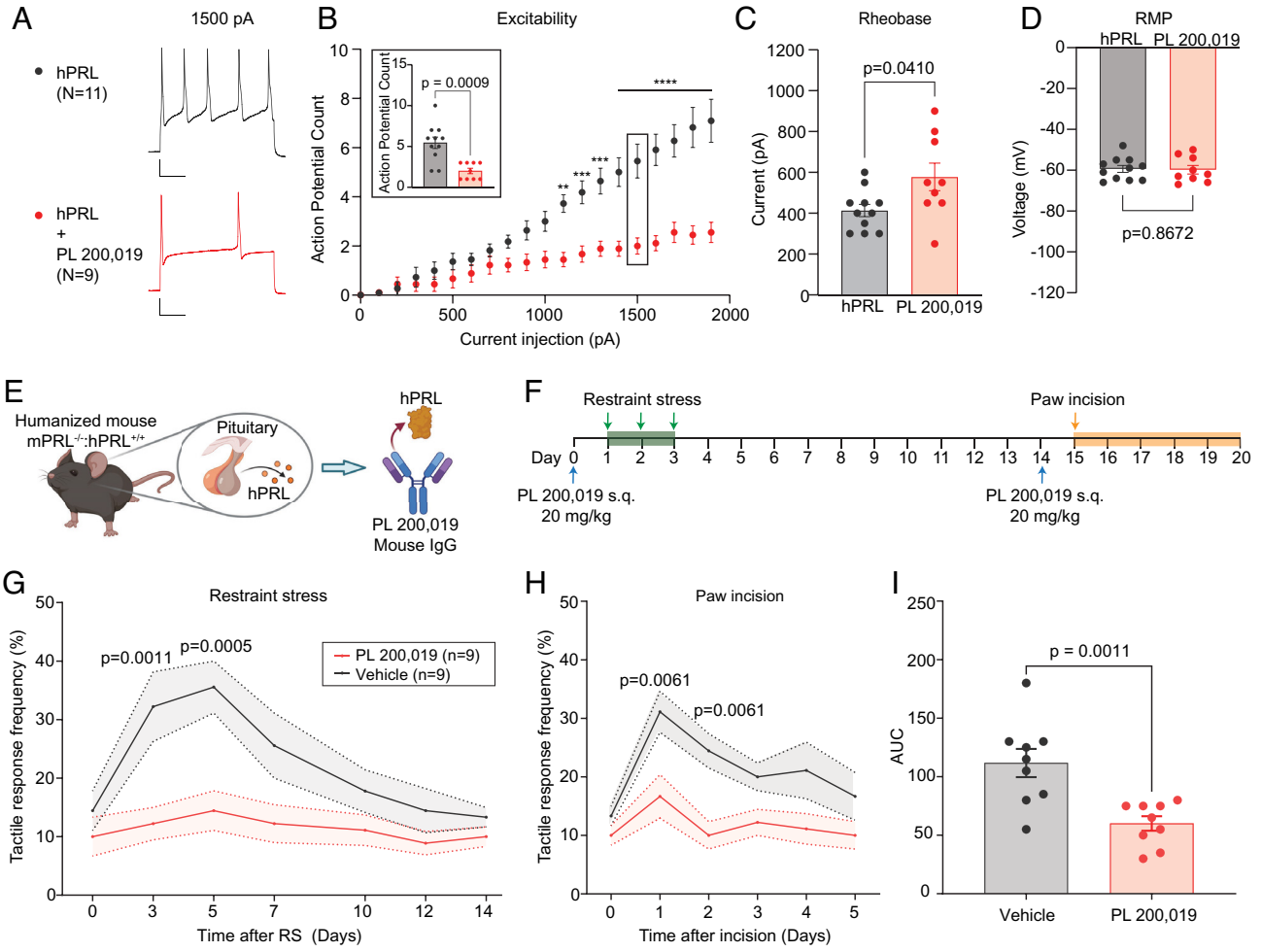


Fig. 8. Sequestration of PRL prevents RS induced hindpaw allodynia, incisional injury–related pain hypersensitivity and in vitro sensitization in human female sensory neurons. (*A*) Representative action potential traces of human DRG neurons treated with hPRL (50 nM) overnight or hPRL with the neutralizing antibody (25 nM). (*B*) Excitability of human sensory neurons showing that treatment with the neutralizing antibody blocks hPRL induced hyperexcitability. *Inset* shows the individual data points for the action potentials evoked at the 1,500 pA current step. (*C*) The rheobase was significantly higher in the group treated with the neutralizing antibody. (*D*) The resting membrane potential was unaffected by treatment with the neutralizing antibody. Data are shown as mean ± SEM. (Scale bars are 20 mV and 200 ms.) (*E*) Diagram showing the humanized mouse that releases hPRL instead of mouse PRL. The *Right* hand side shows the neutralizing antibody built on a mouse IgG backbone with complementarity determining region that recognizes hPRL. (*F*) Experimental timeline for stress, antibody, and incisional injury treatments. (*G*) Treatment with the neutralizing antibody PL 200,019 (20 mg/kg s.q.) in female humanized mice blocked the development of RS-induced hindpaw allodynia. (*H*) These humanized mice also demonstrated reduced postoperative pain hypersensitivity following treatment with PL 200,019. (*I*) The AUC was lower in the humanized mice treated with the neutralizing antibody. Two-way repeated measures ANOVA with Sidak's test for multiple comparisons (*B*, *G*, and *H*). All other comparisons used the Mann–Whitney test. Data are mean ± SEM. The number of animals or cells is indicated in the figure. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$. Additional statistical details can be found in *SI Appendix*, Table S2.

electrophysiological recordings from small diameter DRG neurons obtained from the same animals receiving RS and incisional injury. DRG neurons from female animals showed increased excitability in the presence of a low concentration of PRL, chosen to mimic the presence of endogenous PRL. No effect was observed, however, in DRG neurons from male animals consistent with a female-selective action of PRL-induced sensitization. We note that we also observed a small, but significant, change in rheobase in DRG neurons of female animals consistent with decreased firing threshold resulting from the effects of stress priming and injury.

In order to determine whether the priming effects of stress followed by incisional injury were due to circulating PRL, we treated the animals with cabergoline and demonstrated that RS induced transient hindpaw allodynia and subsequent incisional injury–related postoperative pain hypersensitivity were both blocked selectively in female animals. We also found that cabergoline treatment prevented the increased excitability of DRG neurons taken from female animals subjected to RS and incisional injury. However, we did not observe a change in either rheobase or resting membrane potential between neurons taken from control or cabergoline-treated animals. Importantly, no differences in neuronal excitability were observed in neurons from male animals. These data suggest that RS promotes priming of female DRG neurons to incisional injury that results from increased pituitary PRL and are consistent with clinical reports that preoperative stress can amplify the magnitude and duration of postoperative pain and tenderness (3, 54).

To confirm that the outcomes of pharmacological inhibition of pituitary PRL secretion were due to dysregulation of PRLR isoforms, we overexpressed the PRLR-L receptor and evaluated possible effects on incisional injury–induced postoperative pain hypersensitivity in mice with RS priming. Similar to results with cabergoline-induced inhibition of pituitary PRL, overexpression of PRLR-L blocked postoperative pain hypersensitivity observed in mice with stress priming and incisional injury. This suggests that biasing PRL signaling away from the pronociceptive PRLR-S is protective against injury-induced pain hypersensitivity in female mice regardless of prior stress exposure. We note that the overexpression of PRLR-L was transient, as expected, since the injected plasmid is not incorporated into the genome of the host and is

degraded within the intracellular environment. A similar plasmid packaged into a lentivirus capable of host genome incorporation could be considered in the future as a potential gene-therapy strategy to maintain increased expression of PRLR-L and decreased signaling at PRLR-S to reduce nociception. These findings were also consistent with experiments designed to prevent signaling at both PRLR isoforms by CRISPR-Cas9 editing of the *Prlr* gene with a *Prlr*-total CRISPR plasmid in female animals. In these studies, we found that preventing PRL signaling at PRLR-S inhibited both transient RS allodynia as well as subsequent postincisional allodynia. Additionally, the increased excitability observed to a low concentration of PRL in neurons from animals subjected to RS and incisional injury was fully prevented by editing the expression of both PRLR isoforms.

We then explored the therapeutic potential of PL 200,019, our recently discovered novel murine monoclonal antibody that is optimized to sequester hPRL allowing for inhibition of PRL signaling arising from either pituitary or local sources. Our data show that inhibition of pituitary PRL with cabergoline is effective in inhibiting both stress-induced allodynia as well as postsurgical allodynia. However, surgical procedures are known to increase local levels of PRL produced by immune cells in the periphery (29, 31, 32). This could be important in humans where the gene encoding PRL contains an alternative transcription start site that is thought to be responsible for increased extra pituitary PRL production (57). We employed PL 200,019 in a genetically engineered mouse that produces human, rather than mouse, PRL. Systemic treatment with PL 200,019 effectively blocked stress and incisional injury–induced hypersensitivity in these mice. We note that the pharmacokinetic properties of PL 200,019 have yet to be fully characterized due to compound availability. However, our data suggest that this antibody is likely stable in circulation for an extended period of time accounting for the long-lasting effects we observed across the 20 d study period. A single treatment with PL 200,019 prevented the hypersensitivity observed in female mice subjected to RS, which lasted for two weeks. A second injection of PL 200,019 blocked incisional injury–induced hypersensitivity when evaluated at 6 d after administration. Additionally, we demonstrate that PL 200,019 blocked hPRL-induced hyperexcitability in cultured human sensory neurons obtained from two female donors, indicating the high translational relevance of our approach.

In summary, these data suggest a preemptive strategy to break the link between psychological stress and postoperative pain in women by preventing peripheral nociceptor sensitization. This can be accomplished using treatments that decrease pituitary PRL or short-term administration of a PRL sequestering monoclonal antibody prior to a planned surgery where perioperative stress levels are high. A benefit of employing a PRL monoclonal antibody would be the ability to sequester both circulating PRL, released due to perioperative stress, as well as any contributions of local PRL released due to tissue trauma during the surgery itself. Furthermore, prevention of stress-related peripheral nociceptor sensitization with a monoclonal PRL antibody would provide benefit by reducing the burden for post operative analgesia including the need for opioids as well as for nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen. Reducing opioid consumption can reduce the risk of respiratory depression, physical dependence, addiction, and constipation. Postoperative pain and tenderness are commonly treated with NSAIDs (58, 59). This drug class is among the most commonly used analgesics in the world and their effectiveness validates the clinical significance of inhibiting nociceptor sensitization produced by prostaglandins (60). NSAIDs carry significant risks that limit their use in management of postoperative pain including prevention of blood clotting and inhibition of bone healing (61, 62). Approximately 16,000 people die each year from the use of NSAIDs (63). Identification of novel mechanisms preventing peripheral nociceptor sensitization would therefore address a significant, unmet medical need. Inhibiting female nociceptor sensitization with a PRL antibody could therefore also have significant advantages when compared to NSAIDs. This is especially true given that significant sex differences have been reported in prostaglandin signaling pathways (64, 65). Male subjects have reported greater pain control from NSAIDs when compared to female subjects (66). This suggests that an alternative to NSAIDs, such as targeting PRL signaling, could be advantageous for limiting nociceptor sensitization in women. Finally, as our monoclonal antibody was shown to inhibit sensitization of female human DRG neurons by hPRL, there is a high likelihood of successfully translating this therapy into the clinic. The potential therapeutic strategy described here may also be useful for treatment of other stress-related female prevalent pain syndromes including migraine (67), irritable bowel syndrome (46), temporomandibular disorder (68), fibromyalgia (69) and endometriosis (70). Some of these syndromes, including migraine and endometriosis, have a known link to PRL so that an antibody therapy targeting PRL could be of direct utility in preventing stress-related pain attacks.

Materials and Methods

Detailed descriptions of each experiment and any associated references are available in SI Materials and Methods. All electrophysiology, behavioral, and biochemical experiments were conducted according to standard protocols in the field. Electrophysiological recordings were performed on small-diameter sensory neurons obtained from the dorsal root ganglia and most displayed a prominent inflection on the falling phase of the action potential, which is indicative of $Na_v1.8$ expression (71). Sex was considered as a primary biological variable in this study and animals of both sexes were included in experiments where possible. In some cases, we did not have reason to expect an effect in male animals, and therefore they were not included in order to reduce the overall number of animals we used in our study consistent with the Animal Research Reporting of In Vivo Experiments guidelines. We designed our experiments such that animals were tested for a behavioral phenotype before tissues were harvested and further experiments were conducted to measure electrophysiological or biochemical outcomes. This allowed us to draw powerful associations between the changes in electrophysiological parameters and behavioral phenotypes.

Data, Materials, and Software Availability. All unique materials produced for this study are available in Zenodo (72). All other data are included in the article and/or *SI Appendix*.

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Zosano, Upjohn (Division of Pfizer), Pieris, Revance, Equinox. Honoraria: CME Outfitters, Curry Rockefeller Group, DeepBench, Global Access Meetings, KLJ Associates, Academy for Continued Healthcare Learning, Majallin LLC, Medlogix Communications, MJH Lifesciences, Miller Medical Communications, Southern Headache Society (MAHEC), WebMD Health/Medscape, Wolters Kluwer, Oxford University Press, Cambridge University Press., David Dodick - Stock Options/Shareholder/Patents/Board of Directors: Ctrl M (options), Aural analytics (options), ExSano (options), Palion (options), Healint (Options), Theranica (Options), Second Opinion/Mobile Health (Options), Epien (Options/Board), Nocira (options), Matterhorn (Shares/Board), Ontologics (Shares/Board), King-Devick Technologies (Options/Board), Precon Health (Options/Board), David Dodick - Nuvie Bio. Patent 17189376.1-1466:vTitle: Botulinum Toxin Dosage Regimen for Chronic Migraine Prophylaxis., David Dodick - Research Support: Department of Defense, NIH, Henry Jackson Foundation, Sperling Foundation, American Migraine Foundation, Patient Centered Outcomes Research Institute (PCORI).

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