GPR37 activation erases spinal pain memory and resolves increased nociception in murine models

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Abstract

Intense nociceptor inputs from injury induce long-term changes in the spinal nociceptive system - referred to as spinal pain memory - that increase pain in magnitude and duration. We investigated whether activation of G protein-coupled receptor 37 (GPR37) at the spinal level can erase this memory and resolve long-lasting, enhanced pain using two animal models: the capsaicin model and the hyperalgesic priming model. Without altering normal mechanical and heat nociception, a single intrathecal (i.th.) administration of two GPR37 agonists, TX14A and protectin D1 (PD1), dose-dependently inhibited capsaicin-induced increase in nociception not only acutely but also long-term. In the hyperalgesic priming model, a single i.th. injection of either GPR37 agonist, administered after an initial injury-induced priming, dose-dependently prevented increased nociception following a subsequent inflammatory insult, indicating an unpriming effect. Global GPR37 knockout abolished the long-term inhibitory effect and unpriming effect of i.th. TX14A, confirming the receptor at the spinal level as the molecule mediating the effects. Ex vivo Ca^{2+} imaging revealed that i.th. TX14A, given in vivo to the capsaicin model a week before imaging, had rescued dorsal horn excitatory and inhibitory interneurons from long-term potentiation and depression of responsiveness to afferent inputs, respectively, suggesting the erasure of capsaicin-induced spinal pain memory. Conditioned place preference tests indicated no obvious abuse liability for centrally-administered GPR37 agonists. These findings suggest that spinally targeted GPR37 agonism represents a promising therapeutic strategy for resolving persistent pain by erasing spinal pain memory.

Keywords: spinal pain memory, G protein-coupled receptor 37, pain resolution, long-term plasticity

1. Introduction

Chronic pain affects 20% of adults worldwide, with 10% being newly diagnosed with chronic pain each year [14]. This global burden underscores the need for novel therapies that can, for example, resolve pain rather than merely suppressing it temporarily. In this regard, triggering endogenous mechanisms that undo the intense nociceptor input-induced long-term changes in the spinal nociceptive system (referred to as 'spinal pain memory' due to similarities to the memory engram in the brain [18,33,35]) may represent one potential approach. We propose that G protein-coupled receptor 37 (GPR37) at the spinal level holds the key to this approach, based on the literature presented below.

GPR37 is a parkin-associated endothelin-like receptor predominantly expressed in the central nervous system, particularly in the spinal cord [27]. While GPR37 remains classified as an orphan G protein-coupled receptor, prosaposin and protectin D1 (PD1) have been identified as its putative agonists [21,26,28,45]. Importantly, TX14A, a derivative of prosaposin, has been found to alleviate traumatic, diabetic, chemotherapy-induced, and HIV-associated neuropathic pain-like behaviors in rats [19,20,29]; it is noteworthy that both systemic and intrathecal (i.th.) injections of TX14A were effective, but the former took a long time to reach the maximal effect, compared to the latter, suggesting a delayed access of TX14A (or its metabolite) to its central action site from the periphery. PD1 has also been shown to alleviate various forms of pain-like behaviors via GPR37 [4,5,31,42,43]. While the literature mostly focuses on the acute analgesic effects of these GPR37 agonists, it is notable that a single injection of TX14A prevented the development of formalin-induced persistent mechanical hypersensitivity [20], and the spinal application of PD1 abrogated experimentally induced long-term potentiation (LTP) of C-fiber-evoked field potentials in the dorsal horn [31], which collectively suggest that GPR37 agonism at the spinal level may have the potential to resolve pain by erasing spinal pain memory.

In this study, we aimed to test this possibility using two animal models of acute injury-induced long-lasting pain hypersensitivity: the capsaicin model and the hyperalgesic priming

model. In rodents, intraplantar (i.pl.) capsaicin injection induces LTP of excitatory postsynaptic responses to high-threshold afferent inputs in the dorsal horn, which does not require ongoing afferent input for maintenance [17]. This long-term change in the dorsal horn aligns with the properties of hyperalgesia-like mechanical hypersensitivity in the capsaicin model [24,25,36]. The hyperalgesic priming model manifests spinal pain memory in the form of a primed spinal nociceptive system following an initial acute injury, resulting in increased (in the magnitude and/or duration) nocifensive behaviors upon a subsequent, mild inflammatory insult [22,34]. The priming by the initial injury has also been shown to require molecules needed for long-term synaptic plasticity [9,22]. Using these two animal models and two putative GPR37 agonists, TX14A and PD1, we investigated whether spinal-level GPR37 activation resolves long-lasting, enhanced nociception associated with spinal pain memory.

2. Methods

2.1 Animals

Adult male and female C57BL/6N mice (aged 7-12 weeks old) were purchased from Charles River (Houston, TX, USA). GPR37 global knockout (KO) mice (JAX# 005806), SST^{Cre} mice (JAX# 013044), $GAD2^{Cre}$ mice (JAX# 010802), and Ai95D mice (JAX #028865) were purchased from Jackson Laboratory and bred in-house. Each Cre mouse line was crossbred with Ai95D mice to produce offspring expressing GCaMP6f in SST- or GAD2-positive neurons for Ca^{2+} imaging. Mice were group-housed (≤5 per cage) under a 12-h light-dark cycle with ad libitum access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and in accordance with the National Institutes of Health (NIH) guidelines.

2.2. Animal models of pain

Capsaicin model: Under isoflurane anesthesia, a single i.pl. injection of capsaicin (3 µg in 3 µl, in the mixture of 10% ethanol, 10% Tween-20, and 80% saline; Sigma-Aldrich, St. Louis, MO, USA) was given intradermally into the plantar side of one hind paw between the bases of the third and fourth digits using a 30G needle. For ex vivo Ca^{2+} imaging, a piece of gel foam (~5x10 mm) soaked with either vehicle or 0.1% capsaicin was directly applied to the sciatic nerve under isoflurane anesthesia after skin and muscle incision. Mice were kept anesthetized for 45 minutes after the application, with a sterile gauze covering the incision wound. The incision was suture-closed after the capsaicin application, and the spinal cord was harvested for ex vivo Ca^{2+} imaging seven days later.

Hyperalgesic priming model: As an initial priming injury, a single dose of interleukin-6 (IL-6, 25 pg in 5 µl, in saline) was intradermally injected into the center of one hind paw using a 30G needle. After IL-6-induced pain hypersensitivity had completely resolved (7 days post-IL-6), a single dose of prostaglandin E_2 (PGE_2, 50 ng in 5 µl, in saline) was injected at the same site as IL-6. The 'not-primed' group received only a single i.pl. injection of PGE_2.

2.3. Intrathecal drug administration

Mice were anesthetized with 2-2.5% isoflurane, and all intrathecal (i.th.) injections were given between the L5 and L6 vertebrae (lumbar puncture) after removing fur from the injection site using clippers and sterilizing the area using 70% isopropyl alcohol and povidone-iodine wipes. Proper placement of the needle was confirmed by a brief tail-flick response upon insertion. Mice received a single i.th. injection of TX14A (10, 25, or 50 µg in 10 µl, dissolved in saline; cat.# AS-60248-5, Anaspec, Fremont, CA, USA), PD1 (50 or 100 ng in 5 µl, in a mixture of 10% DMSO and 90% saline; item# 10010390, Cayman Chemical, Ann Arbor, MI, USA), or their vehicles 30 min before normal nociception assessment, 30 min after capsaicin injection (in the capsaicin model), or 5 days after IL-6 injection (in the hyperalgesic priming model).

2.4. Behavioral tests

Mice were habituated to the behavioral test conditions and experimenters for 3 days before conducting behavioral procedures.

Von Frey filament (vFF) test: Mice were placed in acrylic chambers (14 cm length x 5 cm width x 4.5 cm height) on a raised metal grid-floor platform and were acclimated for 30 min before testing on the day of the experiment. Mechanical sensitivity of the hind paw was tested using 0.98-mN and 9.8-mN vFFs, which evoke approximately 0–20% and 40–60% nocifensive withdrawal responses at baseline in naïve mice, respectively. The mid-hind paw was probed 10 times, and the percentage of withdrawal responses was recorded.

Radiant heat (Hargreaves) test: Mice were placed on a glass floor, and a radiant heat source was placed under the glass floor directly under the middle of the afflicted hind paw. The latency (sec) to withdrawal from the heat was automatically detected, and the results from three trials (>1 min intertrial intervals) at a given time-point were averaged.

Conditioned place preference (CPP) test: Using a three-chamber apparatus consisting of a bright and dark chamber connected by a smaller grey chamber (place preference LE 892/LE 893, Panlab/Harvard Apparatus, Dover, MA, USA), mice were placed in the small grey chamber and were allowed to freely roam the three-chamber apparatus for 30 min. The amount of time that the mouse spent in each chamber was recorded. Next, conditioning was performed once per day for three days. Mice were given i.th. or intracerebroventricular (i.c.v.) injections of TX14A (50 µg), PD1 (100 ng), morphine (7-10 µg), or saline under anesthesia, then placed in their home cage. Once mice fully awoke from anesthesia (15-20 min post-injection), they were placed into the non-preferred (bright) chamber for 30 min. They were also counter-conditioned for 30 min per day by being placed in the dark chamber after waking from anesthesia without receiving any injection on each conditioning day. After the three days of conditioning, mice were placed in the middle grey chamber and allowed to freely roam through the three-chamber

apparatus. The change in time (Δt) spent in the drug-paired chamber between the post- and pre-conditioning tests was calculated.

2.5. Western Blot

Proteins were extracted using RIPA buffer (1x RIPA with 100x phosphatase inhibitor and 100x protease inhibitor). Protein concentrations were determined using a BCA assay kit (ThermoScientific, Chicago, IL, USA). Equal quantities of protein were subjected to SDS-PAGE (10% gel) and transferred to nitrocellulose membrane. Membranes were blocked in 5% skim milk in T-PBS, then incubated overnight at 4°C with antibodies specific to GPR37 (D4C8H, Cell Signaling Technology, Danvers, MA, USA) and GAPDH (D16H11, Cell Signaling Technology). GPR37 antibodies were diluted to 1:1000 with 5% skim milk, GAPDH antibodies were diluted to 1:2000 with Licor blocking buffer (Cat.# 1610734, Bio-Rad, Hercules, CA, USA). Membranes were then incubated with the corresponding secondary antibodies and developed using a chemiluminescent reagent (ThermoScientific).

2.6. Spinal Cord Slice Preparation

Preparation of spinal cord slices with attached dorsal root was done as described previously [30]. Mice were placed under deep anesthesia using isoflurane, then the lumbar spinal cord was removed and submerged in ice-cold, oxygenated (95% O_2 and 5% CO_2) modified artificial cerebrospinal fluid (ACSF) containing (in mM): KCl 2.5, $NaH_2PO_4.H_2O$ 1.2, $NaHCO_3$ 30, D-glucose 25, HEPES 20, N-Methyl-D-glucamine 93, Na Ascorbate 5, Thiourea 2, Sodium pyruvate 3.0, N-acetyl-L-cystein 5.0, $CaCl_2.2H_2O$ 0.5, and $MgSO_4.7H_2O$ 10 (pH 7.4 and 310–320 mOsm). Sagittal lumbar spinal cord slices (400 μm thick) from both the ipsilateral (to the capsaicin application) and contralateral sides, with at least one attached dorsal root (L3 or L4, 8–12 mm), were prepared using a vibratome (VT1200S, Leica, Germany). These slices were incubated for ~1 h at 35°C in oxygenated ACSF that contained (in mM): NaCl 124, KCl 2.5,

NaH2PO4.H2O 1.2, NaHCO3 15, D-glucose 12.5, HEPES 5, CaCl2.2H2O 2, and MgSO4.7H2O 2 (pH 7.4 and 310–320 mOsm). The slices were transferred into an imaging chamber and perfused with oxygenated ACSF at a rate of 10 ml/min throughout Ca^{2+} imaging at room temperature (22–25°C).

2.7. Ex vivo Ca^{2+} imaging

The offspring of SST^{Cre} x Ai95D and $GAD2^{Cre}$ x Ai95D crossbreeding were used for Ca^{2+} imaging of SST-positive excitatory and GAD2-positive inhibitory interneurons in the superficial dorsal horn, as described previously [30]. The GCaMP6f green fluorescent signal from these neurons was detected using a microscope (BX51W1, Olympus, Japan) equipped with an ORCA Flash4.0LT camera (Hamamatsu, Japan) and PRIOR Lumen 300 LED light source (Cambridge, UK). A series of 512 x 512-pixel fluorescence images was acquired with HCImage software (Hamamatsu) at a 2-Hz sampling rate using a 40X objective immersed in ACSF. A suction electrode was used for electrical stimulation of the dorsal root at increasing intensities (10, 20, 50, 100, 300, and 500 μA; 0.5 ms pulse) to incrementally activate low- to high-threshold afferents. The dorsal root stimulation-evoked Ca^{2+} transients from the ipsi- and contralateral spinal cords were recorded.

ImageJ (version 1.53v, NIH) was used to analyze all image files. Individual neurons showing changes in fluorescence upon dorsal root stimulation were identified, and the degree of change (ΔF) from the baseline fluorescence (F_0) was normalized to F_0 ($\Delta F/F_0$). When comparing dorsal root stimulation-evoked Ca^{2+} transients in the ipsilateral dorsal horn neurons among different groups, their $\Delta F/F_0$ values were first normalized to the averaged maximum $\Delta F/F_0$ in the corresponding contralateral slice.

2.8. Statistical Analyses

The sample size was estimated using the parameters from preliminary studies for a statistical power of 0.8 at an alpha level of 0.05 using G*Power 3.1 (Germany). Data from behavioral experiments were presented as mean±standard error of the mean (SEM), with N, the number of mice. The percent-withdrawal data were analyzed using a generalized linear mixed model (GLMM) - a binary logistic regression using a binomial distribution with a logit link function. The compound symmetry covariance structure was used for repeated measures over time, and degrees of freedom were approximated using the Satterthwaite method. Post hoc pairwise comparisons (vs. the vehicle control) of estimated marginal means on the response scale (i.e., probability) were made using sequential Sidak tests. The withdrawal-latency data were analyzed using 2-way repeated measures (RM) ANOVA followed by Dunnett's tests for pairwise comparisons (vs. the vehicle control). Normalized calcium transients are presented as mean±SEM, with n, the number of neurons, and N, that of mice. Data from multiple neurons in each dorsal horn of individual mice were averaged per side to obtain the subject-level data representing each mouse and analyzed using 2-way RM ANOVA followed by Tukey's tests. CPP data were analyzed using 1-way ANOVA followed by Tukey's tests. GraphPad Prism 8 (Boston, MA, USA) and IBM® SPSS v28 (Chicago, IL, USA) were used for statistical analysis.

3. Results

3.1. Intrathecally injected TX14A and PD1 have no significant effect on normal nociception.

We first determined whether i.th. injection of GPR37 agonists affects normal nocifensive responses in naive mice. We tested three doses of TX14A (10, 25, and 50 μg) and two doses of PD1 (50 and 100 ng). Compared to the vehicle control group, all tested doses of i.th. TX14A (Fig 1) or PD1 (Fig 2) showed no significant effect on nocifensive withdrawal responses to mechanical (9.8-mN) and heat stimulation, suggesting that spinal-level GPR37 activation does not alter normal mechanical and heat nociception. The results also suggested that any changes

in nocifensive behaviors in pain models following i.th. TX14A or PD1 are unlikely to be due to impaired withdrawal motor function.

3.2. Spinal-level GPR37 activation inhibits capsaicin-induced mechanical and heat hypersensitivity.

A single i.pl. capsaicin injection produces mechanical and heat hypersensitivity that persists for >24 hours in C57BL/6N mice [16,24], with which long-term changes in the spinal nociceptive system are associated [17]. When intrathecally given 30 min after the capsaicin injection, TX14A dose-dependently inhibited the hypersensitivity in a biphasic manner; it produced a rapid-onset inhibition that gradually wore off, followed by a long-term effect still present at 24 h post-capsaicin in both sexes (Fig 3). TX14A (50 μg) was more effective against mechanical hypersensitivity in males than in females, with no sex difference for heat hypersensitivity. Specifically, TX14A at this dose virtually resolved capsaicin-induced mechanical hypersensitivity in 24 hours in males but only partially attenuated it in females.

A single i.th. injection of PD1 also dose-dependently inhibited capsaicin-induced mechanical and heat hypersensitivity both acutely and long-term (Fig 4). As for the latter, 100 ng of PD1 virtually abolished mechanical hypersensitivity to 0.98-mN stimulation and partially inhibited that to 9.8-mN stimulation in both sexes. Overall, these findings indicated that spinal-level GPR37 activation resolves or persistently inhibits long-lasting mechanical and heat hypersensitivity in the capsaicin model.

3.3 Spinal-level GPR37 activation prevents exaggerated nociception in the hyperalgesic priming model.

We investigate whether a single i.th. injection of TX14A affects mechanical and heat hypersensitivity development following i.pl. PGE_2 injection in naïve (i.e., not-primed) mice. When given to naïve females two days prior to PGE_2, TX14A (25 and 50 μg) slightly but significantly

reduced PGE_2-induced mechanical, but not heat, hypersensitivity at 4 h post-PGE_2, while it had no effect in naïve males. Next, we examined the effects of i.th. TX14A in mice that had already been primed by i.pl. IL-6 injection. Compared to the vehicle-treated primed mice that developed exaggerated mechanical and heat hypersensitivity following i.pl. PGE_2 injection, TX14A-treated primed mice developed significantly attenuated hypersensitivity following PGE_2 (Fig 5).

When intrathecally given to naïve female mice 2 days prior to PGE_2, 100 ng of PD1 significantly decreased PGE_2-induced heat, but not mechanical, hypersensitivity, whereas it did not alter PGE_2-induced hypersensitivity in naïve male mice. However, further analysis revealed that this female-specific decrease likely resulted from the difference at baseline (BL) in this cohort of mice; specifically, when BL values were subtracted from the dataset, no significant difference was detected between groups in naïve females (Suppl Fig 1).

In primed mice, regardless of sex, both 50 and 100 ng of PD1 dose-dependently prevented the exaggeration of PGE_2-induced mechanical and heat hypersensitivity (Fig 6). These results collectively suggested that spinal-level GPR37 activation erases spinal pain memory underlying hyperalgesic priming.

3.4 Global knockout of GPR37 prevents long-term inhibition of capsaicin-induced mechanical hypersensitivity and unpriming by i.th. TX14A.

While PD1 was shown to be unable to activate GPR37-like 1 (GPR37L1), a close relative to GPR37 [3], TX14A nonselectively activates both GPR37 and GPR37L1 [26,28]. Therefore, to determine whether the above-observed long-term inhibitory and unpriming effects of i.th. TX14A were mediated specifically by GPR37, we repeated our earlier experiments, focusing on mechanical hypersensitivity, in GPR37 global knockout (KO) mice and their wild-type (WT) genotypic control. Western blot of GPR37 in the spinal cord samples confirmed the absence of the receptor in GPR37 global KO mice (Suppl Fig 2).

In the capsaicin model (Fig 7), the effect of i.th. TX14A (50 μg) in the WT control was comparable to that seen in C57BL/6N mice, also showing a sex difference in its long-term efficacy against mechanical hypersensitivity. In GPR37KO mice, by contrast, TX14A failed to inhibit capsaicin-induced mechanical hypersensitivity long-term; it only produced a short-lasting, acute inhibition of mechanical hypersensitivity to 0.98-mN stimulation.

Compared to C57BL/6N mice (Figs 5 & 6), the WT control developed augmented mechanical hypersensitivity following i.pl. PGE_2 injection even in a not-primed condition. However, the impact of hyperalgesic priming was still manifested as prolongation of PGE_2-induced mechanical hypersensitivity in these mice (Fig 8). When intrathecally given after i.pl. IL-6 had primed the spinal nociceptive system (i.e., 5 days after IL-6; 2 days before PGE_2), TX14A effectively prevented such prolongation in the WT control but not in GPR37KO mice. Collectively, these findings indicated that GPR37 mediates the long-term inhibitory and unpriming effects of i.th. TX14A on long-lasting, enhanced nociception associated with spinal pain memory.

3.5. Spinal-level GPR37 activation rescues dorsal horn neurons from capsaicin-induced long-term changes.

To support the behavioral evidence that spinal-level GPR37 activation erases spinal pain memory, we next investigated whether i.th. TX14A abrogates capsaicin-induced long-term changes in neuronal responsiveness to afferent inputs in the superficial dorsal horn (SDH). To this end, we used transgenic mice expressing GCaMP6f either in SST-positive (SST^{Cre} x Ai95D) or GAD2-positive neurons ($GAD2^{Cre}$ x Ai95D). In the SDH, 82±2% of neurons labeled using the SST^{Cre} line express SST gene transcripts [8], and 86±3% of neurons labeled using the $GAD2^{Cre}$ line are immunoreactive for PAX2, a marker of inhibitory neurons in the SDH [30]. In these mice, to maximize the yield of sensitized dorsal horn neurons from each mouse in ex vivo slice preparations, we directly exposed the sciatic nerve to capsaicin (day 0). Afterward, we

intrathecally administered TX14A (50 μg) or saline twice - immediately after the capsaicin application and on the following day. On day 7, we assessed mechanical hypersensitivity (to 9.8-mN stimulation) in both hind paws and then harvested the spinal cord (Fig 9A). The control group received the vehicle of capsaicin on the sciatic nerve and i.th. saline.

In the control group, there was no difference in mechanical sensitivity between the ipsilateral and contralateral hind paws on day 7. However, in mice treated with capsaicin and then i.th. saline (the Cap+Sal group), the ipsilateral hind paw remained hypersensitive to 9.8-mN stimulation compared to the contralateral hind paw. In contrast, in mice treated with capsaicin and then i.th. TX14A (the Cap+TX14A group), there was no mechanical hypersensitivity in the ipsilateral hind paw (Fig 9B). Notably, unlike in our previous experiments where i.th. TX14A was given only once in the i.pl. capsaicin model (Fig 3), here we did not observe a sex difference in the efficacy of TX14A; two i.th. injections of TX14A effectively resolved hypersensitivity to 9.8-mN stimulation in both sexes. Therefore, in the subsequent Ca^{2+} imaging, we pooled data obtained from females and males.

In the SDH harvested 7 days after the capsaicin application, dorsal root stimulation-evoked Ca^{2+} transients in SST-positive excitatory interneurons (SSTn) were different across groups ($F(2,8)=8.977$, $p=0.009$; Fig 9C). Post hoc analyses revealed that their responses to afferent inputs were overall potentiated in the Cap+Sal group, compared to those in the control group ($t(8)=4.085$, Tukey's adjusted $p=0.009$). The Ca^{2+} transients of SSTn in the Cap+TX14A group were overall not different from those in the control group ($t(8)=1.412$, Tukey's adjusted $p=0.38$) but significantly smaller than those in the Cap+Sal group ($t(8)=2.888$, Tukey's adjusted $p=0.048$), suggesting that TX14A had rescued SSTn from capsaicin-induced sensitization. In line with this, when non-normalized Ca^{2+} transients were compared between ipsilateral and contralateral dorsal horns in each group, only the Cap+Sal group showed a trend ($F(1,6)=4.943$, $p=0.068$ by 2-way RM ANOVA) toward a difference in neuronal responsiveness to afferent inputs (Suppl Fig 3A-C).

As for Ca^{2+} transients in GAD2-positive inhibitory interneurons (GAD2n), there was a significant interaction of group x stimulation intensity ($F(58,348)=2.349$, $p<0.0001$; Fig 9D). Pairwise comparisons between groups at each stimulation intensity revealed that their responses to afferent inputs tended to be depressed (Tukey adjusted p=0.058-0.071) at high intensities (≥100 μA) in the Cap+Sal group, compared to those in the control group. Notably, at 500 μA stimulation, the Ca^{2+} transient of the Cap+TX14A group was significantly greater than that of the Cap+Sal group ($t(5.081)=4.748$, Tukey's adjusted p=0.044), suggesting that TX14A had normalized capsaicin-induced long-term disinhibition. In line with this, when non-normalized Ca^{2+} transients were compared between ipsilateral and contralateral dorsal horns in each group, only the Cap+Sal group showed a significant difference ($F(1,8)=7.448$, $p=0.026$ by 2-way RM ANOVA) in neuronal responsiveness to afferent inputs (Suppl Fig 3D-F).

3.6. Activation of GPR37 in the central nervous system has no apparent abuse liability.

As the above findings collectively suggested spinally-directed GPR37 agonists as novel pain therapeutics to erase spinal pain memory without affecting normal nociception, we next evaluated their safety in terms of abuse potential. To this end, we performed conditioned place preference (CPP) tests using saline as the negative control, morphine (7-10 μg) as the positive control, and the GPR37 agonists TX14A (50 μg) and PD1 (100 ng) as GPR37 agonists. As shown in Fig 10, mice conditioned with either GPR37 agonist did not develop place preference for the drug-paired chamber compared to the saline-treated negative control group, suggesting that centrally activating GPR37 agonists may have little to no abuse liability.

Discussion

Intense nociceptive inputs induce long-term changes in the spinal nociceptive system (spinal pain memory), resulting in heightened and long-lasting pain. Aligning with prior findings [6,23,30], the present results of ex vivo Ca^{2+} imaging demonstrate that such changes consist of

neuron type-dependent long-term plasticity in opposing directions: the responsiveness to afferent inputs is potentiated in excitatory neurons but depressed in inhibitory neurons in the SDH. This study provides preclinical evidence that spinal-level GPR37 activation normalizes such long-term changes and thus holds potential as a novel therapeutic approach to resolving pain, instead of temporarily suppressing it. Importantly, GPR37 activation at the spinal level did not alter normal mechanical and heat nociception, suggesting that spinally delivered GPR37 agonists may selectively target maladaptive nociceptive processing in a pathological pain condition. This may be an ideal approach to treating pathological, persistent pain while preserving physiological pain's protective role. In addition, centrally activating GPR37 did not show an apparent abuse potential in the CPP test, further supporting the translational potential of spinal GPR37 activation as a therapy for persistent pain.

Since TX14A is known to be rapidly degraded in the brain and not accumulated after repeated dosing [11,38,39], the long-term effect of TX14A in the capsaicin model and hyperalgesic priming model seems unlikely to be due to the peptide persistently staying (>24 h) in the system. Instead, it suggests that TX14A may trigger a GPR37-mediated event that outlasts the lifespan of the peptide in the nervous system, which subsequently leads to the erasure of spinal memory. The biphasic inhibitory effects of i.th. TX14A in the capsaicin model aligns with this idea, and future pharmacokinetic studies will be necessary to determine whether the initial rapid-onset effect (which wears off in ~3 hours) directly correlates with TX14A concentration within the spinal cord.

As for PD1, it was shown to be readily metabolized in an assay system using human hepatoma cells, with one of its metabolites retaining biological activity to inhibit neutrophil chemotaxis [1]. Therefore, although TX14A and PD1 commonly produced persistent inhibition or resolution of increased nociception in the capsaicin model (except for mechanical hypersensitivity in females, see below) and 'unpriming' in the hyperalgesic priming model,

differences in their metabolic fates and pharmacokinetic properties may account for any observed differences in their effects on nocifensive behaviors. Two potential putative binding sites on GPR37 have been identified using homology modeling: one for small molecules including PD1, and the other for peptides including TX14A [2]. Activating GPR37 through these distinct binding sites may also result in pharmacodynamic differences between PD1 and TX14A. In addition, unlike PD1, TX14A activates both GPR37 and GPR37L1, which may further contribute to differences in the effects of the two GPR37 agonists in pain models. Regarding this, it is noteworthy that TX14A still briefly inhibited capsaicin-induced hypersensitivity to 0.98-mN stimulation in GPR37KO mice. It would be interesting to examine in future studies if GPR37L1 mediates this residual effect. GPR37L1 is primarily expressed in satellite glial cells in DRG [3,10] and astrocytes in the spinal cord [44], playing a critical role in alleviating neuropathic pain [3,44].

A single i.th. injection of TX14A was less effective in inhibiting capsaicin-induced mechanical hypersensitivity in females than in males. As female rodents and humans experience more intense and longer-lasting mechanical pain following capsaicin application [12,13,15,16], a higher dose than 50 μg (or multiple dosing) of TX14A may be necessary in females to produce effects comparable to those in males. This idea is supported by the qualitative observation that the effects of TX14A 50 μg on mechanical hypersensitivity in females (Fig 3A, B) resembled those of TX14A 25 μg in males (Fig 3D, E). Furthermore, when TX14A 50 μg was administered twice at a 24-h interval in females whose sciatic nerve had been directly exposed to capsaicin, mechanical hypersensitivity in the afflicted paw was effectively resolved (Fig 9B).

Speaking of the abovementioned sex difference in the efficacy of TX14A in the capsaicin model, it is noteworthy that no such difference was observed against capsaicin-induced ‘heat’ hypersensitivity (Fig 3C, F). Given that mechanical and heat nociceptions are mediated by two distinct afferent populations in the mouse [7], these results suggest that capsaicin-induced long-

term changes in processing the two distinct afferent inputs are mechanistically different, resulting in sensory modality-dependent efficacy of i.th. TX14A. In this regard, as the exaggeration of PGE_2-induced hypersensitivity of both sensory modalities was effectively prevented by i.th. TX14A in the hyperalgesic priming model, the spinal pain memory underlying heat hypersensitivity may differ across pain models involving different injury types. Conceptually, this idea aligns with the findings that the dorsal horn circuits mediating mechanical allodynia differ between neuropathic and inflammatory pain conditions, and that capsaicin recruits circuits similar to those for neuropathic rather than inflammatory mechanical allodynia [32].

While this study supports the potential of spinal-level GPR37 activation as a novel therapeutic approach to pain treatment, we acknowledge the following limitations. First, it remains to be elucidated what cellular and downstream molecular mechanisms underlie GPR37-mediated spinal pain memory erasure. Previous studies have indicated that GPR37 is expressed in macrophages (but not in microglia), activation of which by TX14A and PD1 triggers phagocytosis and contributes to the resolution of inflammatory pain [4]. Considering the recent finding that a timely resolution of inflammatory pain requires macrophages transferring mitochondria to DRG neurons [41], a future mechanistic study may investigate whether intrathecally injected GPR37 agonists reach DRG and modulate intraganglionic macrophages for pain resolution. Another potential cellular target is the DRG neuron itself. GPR37 is also expressed in DRG neurons [37,40,43], suggesting that their central terminals within the dorsal horn (or cell bodies in the ganglia) may be the action target of i.th. TX14A and PD1. Second, while our CPP results suggest that central GPR37 agonism has little potential for abuse, more rigorous testing, such as the intravenous self-administration test, may be necessary to thoroughly assess the abuse liability of GPR37 agonists, given that most drugs that are commonly abused use routes beyond the intended clinical route. Third, spinal pain memory that maintains chronic pain may differ from that induced by acute injury in its molecular, cellular, and

circuit-level mechanisms. Therefore, to preclinically evaluate the therapeutic potential of spinal GPR37 agonism for chronic pain treatment, we plan to study the effectiveness of i.th. TX14A and PD1 in animal models of chronic pain in future studies.

In conclusion, this study demonstrates that spinal-level GPR37 agonism resolves long-lasting pain hypersensitivity following acute injury by normalizing long-term changes in the spinal nociceptive system (i.e., erasing spinal pain memory), while leaving normal nociception intact. Furthermore, centrally activating the receptor does not show apparent abuse liability in the conditioned place preference paradigm, collectively suggesting that GPR37 holds promise as a potential target for developing novel pain therapeutics.

Acknowledgements

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Figure legends

Fig 1. A single intrathecal (i.th.) injection of TX14A, a peptide agonist of GPR37, does not alter normal nociception. We tested three doses of TX14A (10, 25, and 50 μg; grey broken line) vs vehicle (N=7/group). In both females (A, C) and males (B, D), there was no significant main effect (time or group) or interaction effect (time x group) on nocifensive withdrawal responses to 9.8-mN mechanical stimulation (A, B) or heat stimulation (C, D). BL, baseline.

Fig 2. A single i.th. injection of protectin D1 (PD1), a lipid agonist of GPR37, does not alter normal nociception. We tested two doses of PD1 (50 and 100 ng; grey broken line) vs vehicle (N=7/group). In both females (A, C) and males (B, D), there was no significant main effect (time or group) or interaction effect (time x group) on nocifensive withdrawal responses to 9.8-mN mechanical stimulation (A, B) or heat stimulation (C, D). BL, baseline.

Fig 3. A single i.th. injection of TX14A dose-dependently inhibits capsaicin-induced mechanical and heat hypersensitivity both acutely and long-term. Intraplantar (i.pl.) injection of capsaicin produced mechanical (A, B, D, E) and heat (C, F) hypersensitivity that rapidly develops (in 30 min) and lasts for ≥24 hours in female (A-C) and male (D-F) C57BL/6N mice (purple-shaded area). The nocifensive withdrawal responses of these mice significantly differed among groups at each time point following drug administration (grey broken line), showing a dose-dependent biphasic inhibitory effect of i.th. TX14A on capsaicin-induced hypersensitivity. *$p<0.05$, **$p<0.01$ vs. the vehicle group (Veh) by sequential Sidak tests following generalized linear mixed model (GLMM) analysis (A, B, D, E) or by Dunnett's test following 2-way RM ANOVA (C, F). N=7 per group. BL, baseline.

Fig 4. A single intrathecal (i.th.) injection of PD1 dose-dependently inhibits capsaicin-induced mechanical and heat hypersensitivity both acutely and long-term. I.pl. injection of capsaicin produced mechanical (A, B, D, E) and heat (C, F) hypersensitivity that rapidly develops (in 30 min) and lasts for ≥24 hours in female (A-C) and male (D-F) C57BL/6N mice (purple-shaded area). The nocifensive withdrawal responses of these mice significantly differed across groups at each time point following the drug administration (grey broken line), showing a dose-dependent biphasic inhibitory effect of i.th. PD1 on capsaicin-induced hypersensitivity. *$p<0.05$, **$p<0.01$ vs. the vehicle group (Veh) by sequential Sidak tests following GLMM analysis (A, B, D, E) or by Dunnett's test following 2-way RM ANOVA (C, F). N=7 per group. BL, baseline.

Fig 5. A single i.th. injection of TX14A dose-dependently erases hyperalgesic priming. When intrathecally given to naïve, not-primed females 2 days before i.pl. PGE_2 injection (A, E), TX14A at 25 μg and 50 μg (grey broken line) reduced the degree of mechanical, but not heat, hypersensitivity at 4 h post-PGE_2 (green-shaded area), unlike in males (C, G). Mice that had been primed by i.pl. injection of interleukin-6 (IL-6, orange-shaded area) developed exaggerated mechanical (B, D) and heat (F, H) hypersensitivity in response to a subsequent i.pl. PGE_2 injection. When TX14A was intrathecally given to primed mice 2 days before the i.pl. PGE_2 injection, TX14A dose-dependently prevented the exaggeration of PGE_2-induced hypersensitivity in both sexes (B, D, F, H). *$p<0.05$, **$p<0.01$ vs. the vehicle group (Veh) by sequential Sidak tests following GLMM (A-D) or by Dunnett's test following 2-way RM ANOVA (E-H). N=7 per group. BL, baseline.

Fig 6. A single i.th. injection of PD1 dose-dependently erases hyperalgesic priming. When intrathecally given to not-primed females 2 days before i.pl. PGE_2 injection (A, E), PD1 at 100 ng (grey broken line) reduced the degree of PGE_2-induced heat, but not mechanical,

hypersensitivity (green-shaded area), unlike in males (C, G). Mice that had been primed by i.pl. injection of IL-6 (orange-shaded area) developed exaggerated mechanical (B, D) and heat (F, H) hypersensitivity in response to a subsequent i.pl. PGE_2 injection. When PD1 was intrathecally given to primed mice 2 days before the i.pl. PGE_2 injection, PD1 dose-dependently prevented the exaggeration of PGE_2-induced hypersensitivity in both sexes (B, D, F, H). *p<0.05, **p<0.01 vs. the vehicle group (Veh) by sequential Sidak tests following GLMM analysis (A-D) or by Dunnett's test following 2-way RM ANOVA (E-H). N=7 per group. BL, baseline.

Fig 7. In the absence of GPR37, i.th. injection of TX14A fails to inhibit capsaicin-induced mechanical hypersensitivity long-term. In the wild-type (WT) control (A-D; N=3-5/group), TX14A (50 µg; grey broken line) inhibited capsaicin-induced mechanical hypersensitivity (purple-shaded area) both acutely and long-term. However, in the GPR37 knockout (KO) mice (E-H; N=5/group), TX14A only acutely inhibited hypersensitivity to 0.98-mN stimulation without producing a long-term (24 h post-capsaicin) effect. *p<0.05, **p<0.01 vs the vehicle group (Veh) by sequential Sidak tests following GLMM analysis. BL, baseline.

Fig 8. In the absence of GPR37, i.th. injection of TX14A fails to erase hyperalgesic priming. In the WT genotypic control (A, B; N=7-8/group), IL-6 (orange-shaded area)-produced hyperalgesic priming primarily manifested as prolonged mechanical hypersensitivity following i.pl. PGE_2 (green-shaded area). When intrathecally given to primed WT control mice 2 days before i.pl. PGE2 injection, TX14A (50 µg; grey broken line) prevented such prolongation. However, in the GPR37 knockout (KO) mice (C, D; N=3-7/group), i.th. TX14A had no effect. *p<0.05, **p<0.01 vs the not-primed group by sequential Sidak tests following GLMM analysis. BL. Baseline.

Fig 9. I.th. injection of TX14A resolves long-term changes in dorsal horn neuronal responsiveness to afferent inputs. (A) In mice that express GCaMP6f in somatostatin (SST)-positive excitatory interneurons (SSTn) or GAD2-positive inhibitory interneurons (GAD2n) in the dorsal horn, capsaicin (or its vehicle) was unilaterally applied to the sciatic nerve, and TX14A (vs saline) was intrathecally given twice (days 0 and 1). On day 7, following the measurement of nocifensive withdrawals from 9.8-mN mechanical stimulation, ex vivo Ca^{2+} imaging was performed. (B) Both in females (N=3/group) and males (N=6-8/group), the TX14A treatment (Cap+TX14A) resolved long-lasting mechanical hypersensitivity in the ipsilateral hind paw. **$p<0.01$ vs. the contralateral paw by sequential Sidak tests following GLMM analysis. (C) The dorsal root (DR) stimulation-evoked Ca^{2+} transients were potentiated in SSTn in the ipsilateral dorsal horn harvested from the mice intrathecally treated with saline following the capsaicin application (the Cap+Sal group, n=71, N=4). The Cap+TX14A group (n=53, N=4) showed Ca^{2+} transients comparable to those in the control group (n=41, N=3). (D) In contrast, GAD2n in the ipsilateral dorsal horn of the Cap+Sal group (n=186, N=5) showed a trend toward a decrease in Ca^{2+} transients, compared to the control group (n=129, N=5), whereas the Cap+TX14A group (n=92, N=5) showed no significant difference from the control group. *$p<0.05$, **$p<0.01$ vs. the control group; $^{\#}p<0.05$, $^{\#\#}p<0.01$ between the Cap+Sal and Cap+TX14A groups by Tukey's tests following 2-way RM ANOVA.

Fig 10. **GPR37 agonism in the central nervous system does not show apparent abuse potential.** Unlike morphine (Mor)-treated mice, TX14A- and PD1-treated mice did not develop conditioned place preference for the treatment-paired chamber, compared to the saline (Sal)-treated mice, after intrathecally (A, B; N=6/group) or intracerebroventricularly (C, D; N=6-8/group) receiving treatments for 3 consecutive conditioning days. *$p<0.05$, **$p<0.01$ by Tukey's tests following 1-way ANOVA.

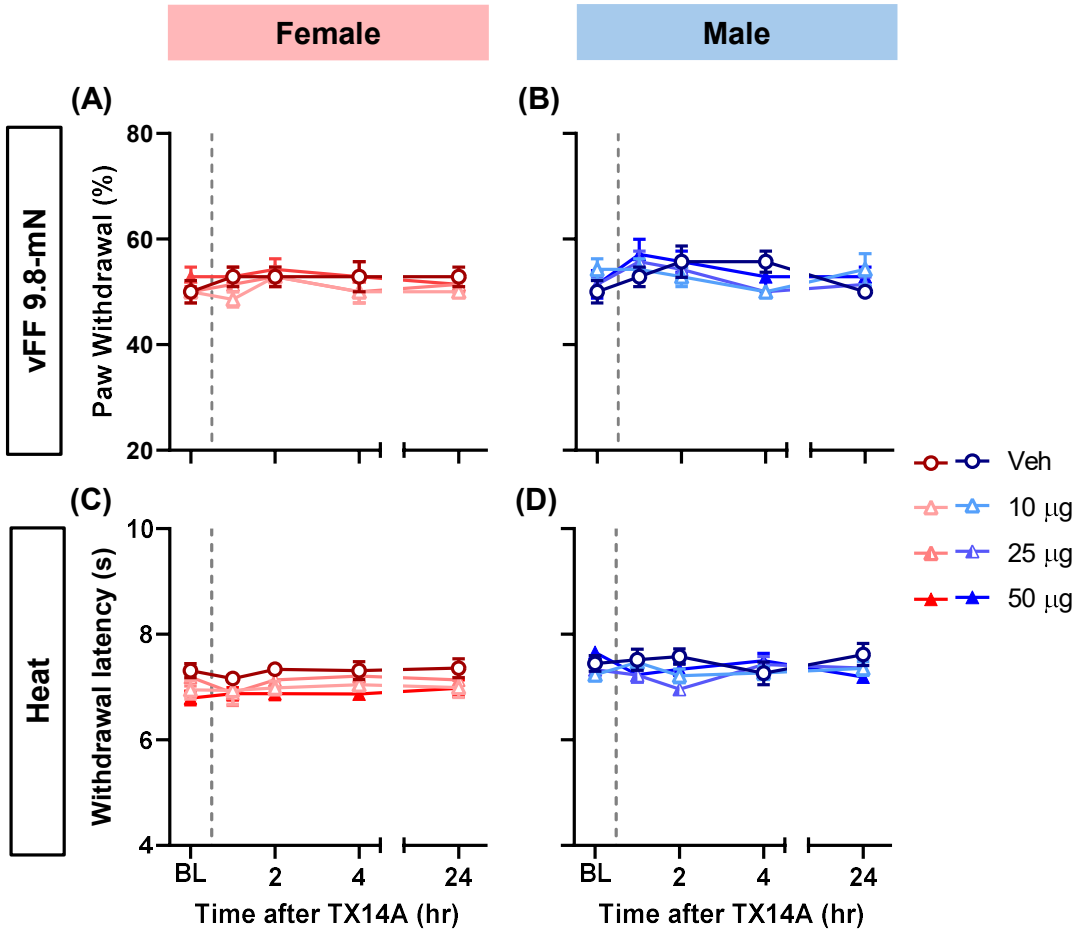
Female
Male
(A)
(B)
(C)
(D)
vFF 9.8-mN
Heat
Paw Withdrawal (%)
Withdrawal latency (s)
80
60
40
20
10
8
6
4
BL
2
4
24
Time after TX14A (hr)
Veh
10 μg
25 μg
50 μg

Figure 1

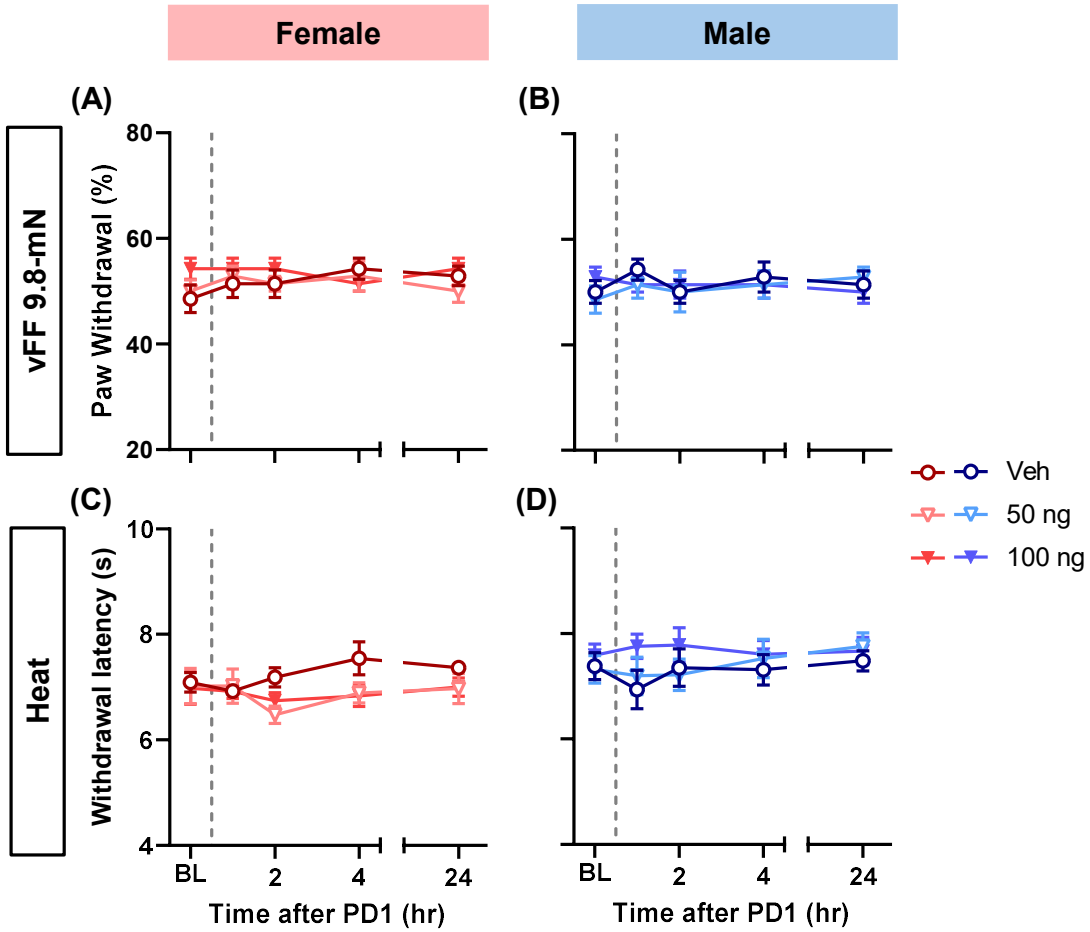
Female
Male
(A)
(B)
(C)
(D)
vFF 9.8-mN
Heat
Paw Withdrawal (%)
Withdrawal latency (s)
80
60
40
20
10
8
6
4
BL
2
4
24
Time after PD1 (hr)
Veh
50 ng
100 ng

Figure 2

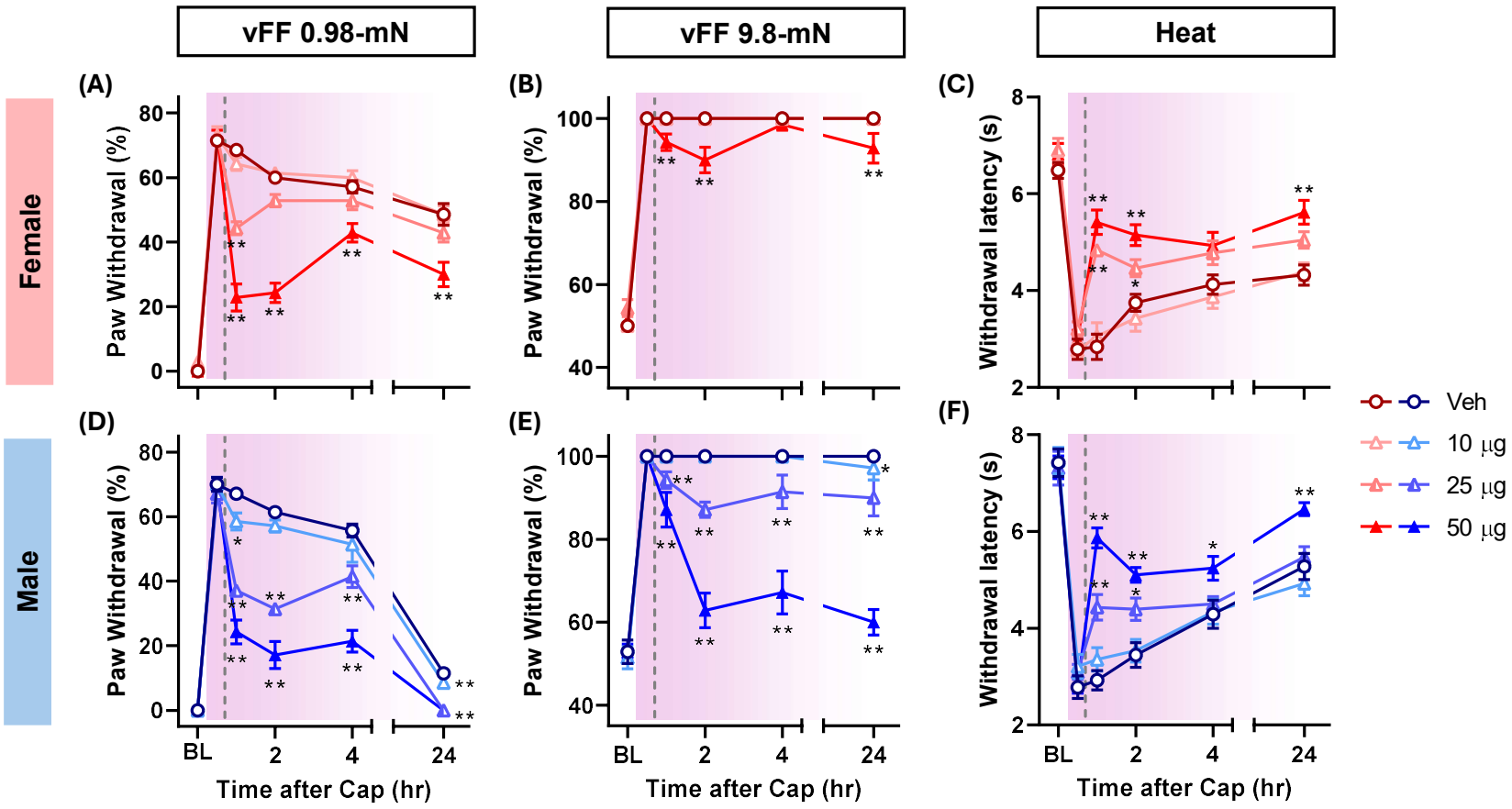
vFF 0.98-mN
vFF 9.8-mN
Heat
Female
Male
(A)
(B)
(C)
(D)
(E)
(F)
Paw Withdrawal (%)
Withdrawal latency (s)
Time after Cap (hr)
BL
2
4
24
Veh
10 µg
25 µg
50 µg

Figure 3

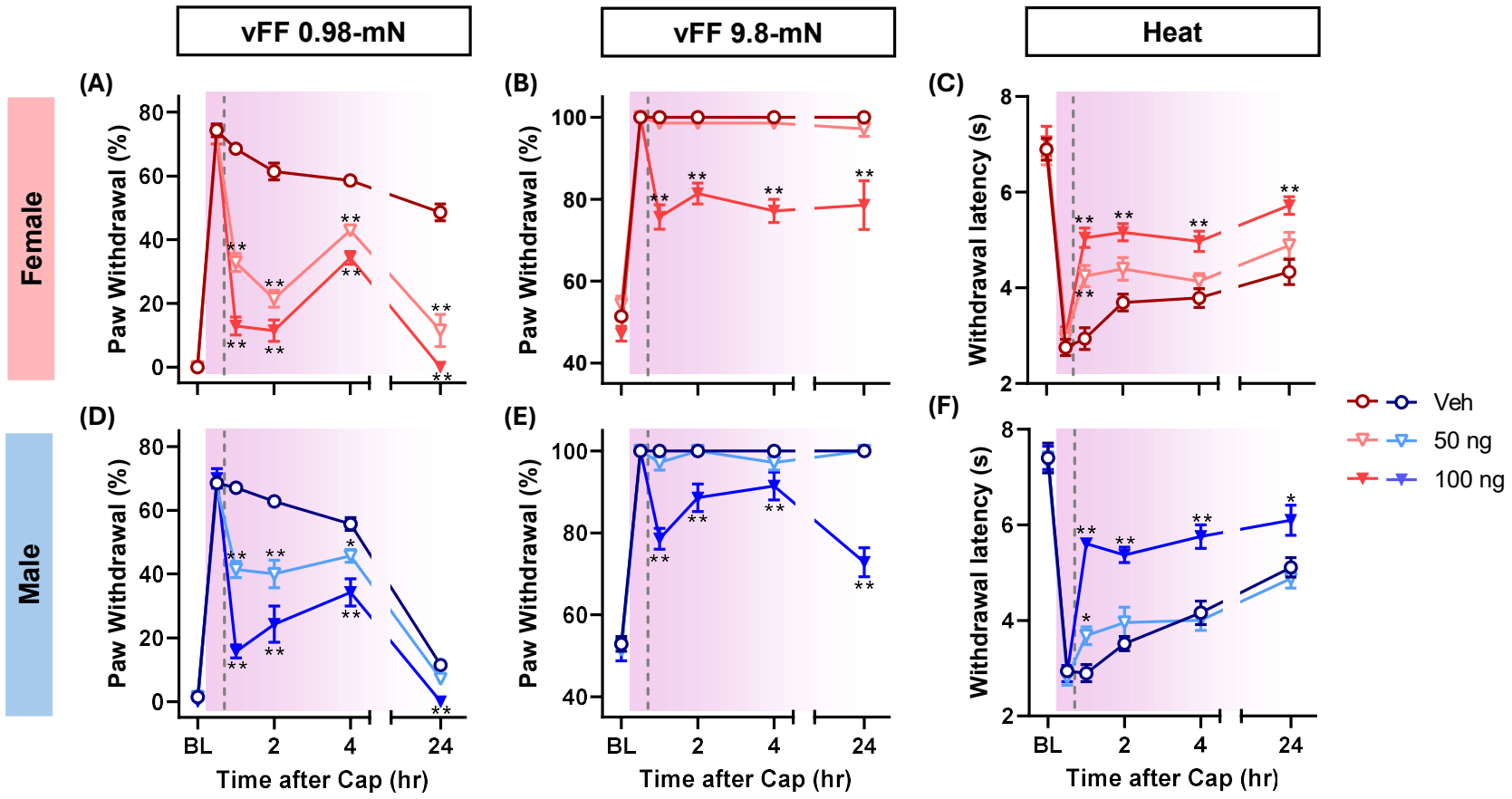
vFF 0.98-mN
vFF 9.8-mN
Heat
Female
Male
(A)
(B)
(C)
(D)
(E)
(F)
Paw Withdrawal (%)
Withdrawal latency (s)
Time after Cap (hr)
BL
2
4
24
Veh
50 ng
100 ng

Figure 4

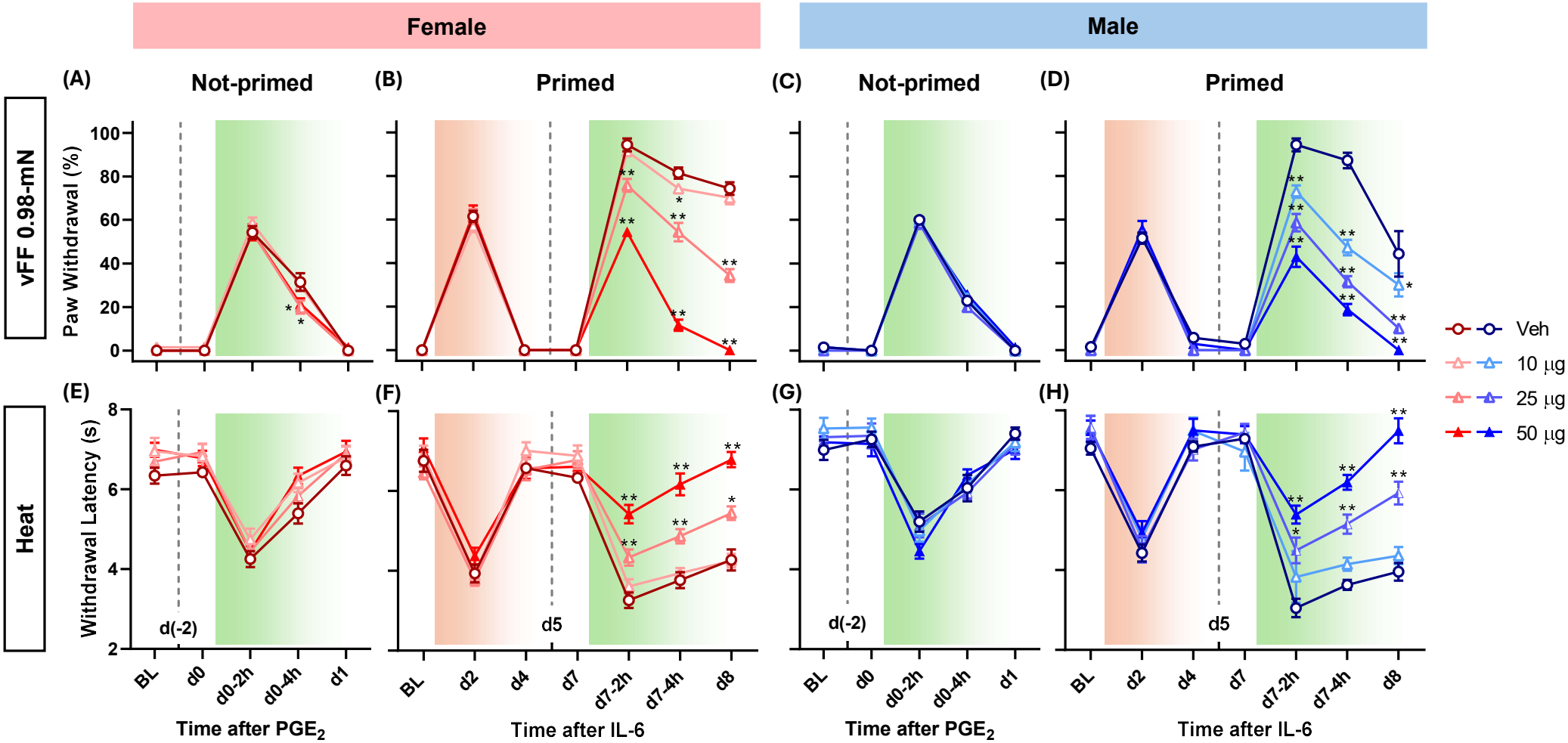
Female
Male
(A)
Not-primed
(B)
Primed
(C)
Not-primed
(D)
Primed
vFF 0.98-mN
Heat
Paw Withdrawal (%)
Withdrawal Latency (s)
(E)
(F)
(G)
(H)
d(-2)
d5
d(-2)
d5
BL
d0
d0-2h
d0-4h
d1
d2
d4
d7
d7-2h
d7-4h
d8
Time after PGE2
Time after IL-6
Time after PGE2
Time after IL-6
Veh
10 µg
25 µg
50 µg

Figure 5

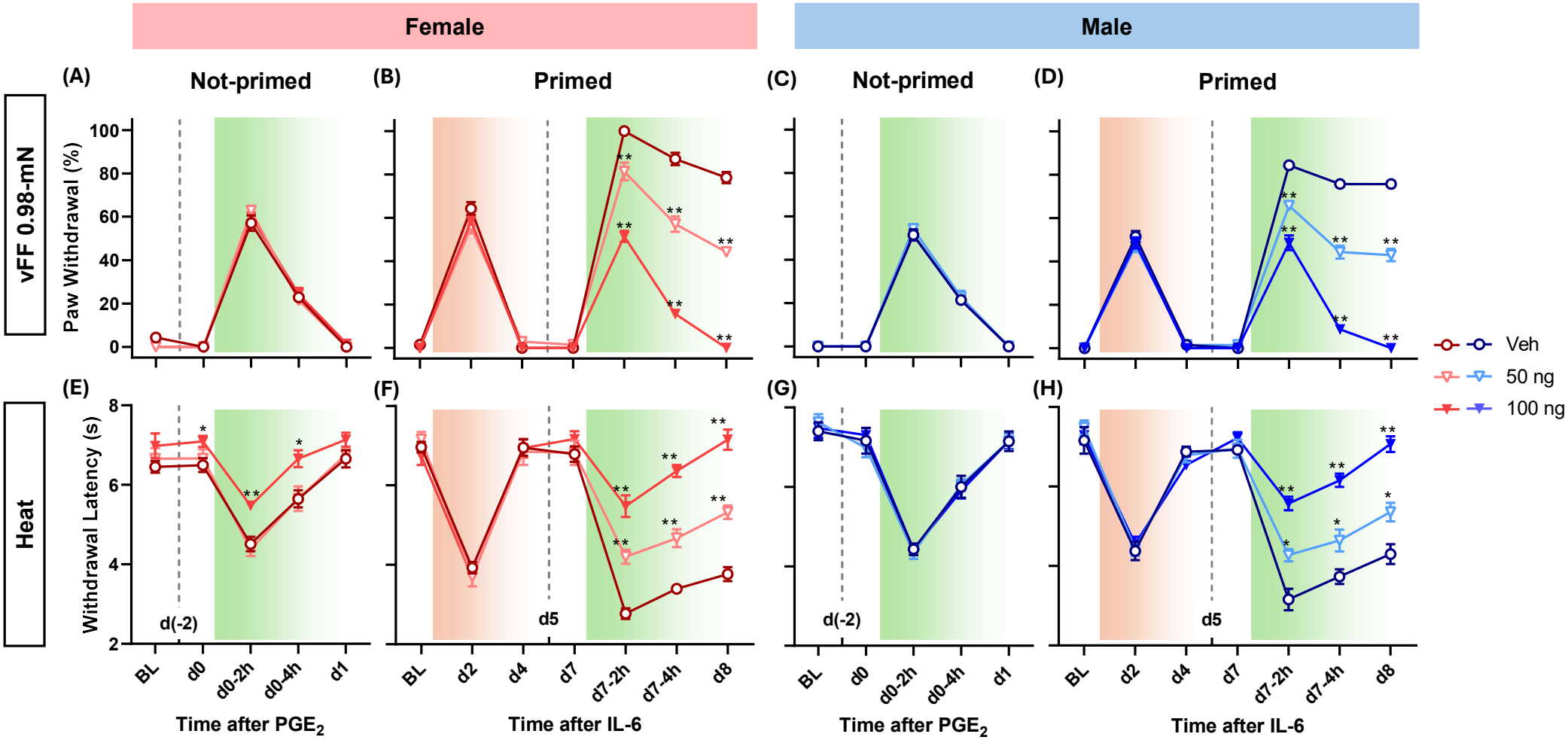
Female
Male
(A)
Not-primed
(B)
Primed
(C)
Not-primed
(D)
Primed
vFF 0.98-mN
Paw Withdrawal (%)
Heat
Withdrawal Latency (s)
(E)
(F)
(G)
(H)
d(-2)
d5
BL
d0
d0-2h
d0-4h
d1
d2
d4
d7
d7-2h
d7-4h
d8
Time after PGE2
Time after IL-6
Veh
50 ng
100 ng

Figure 6

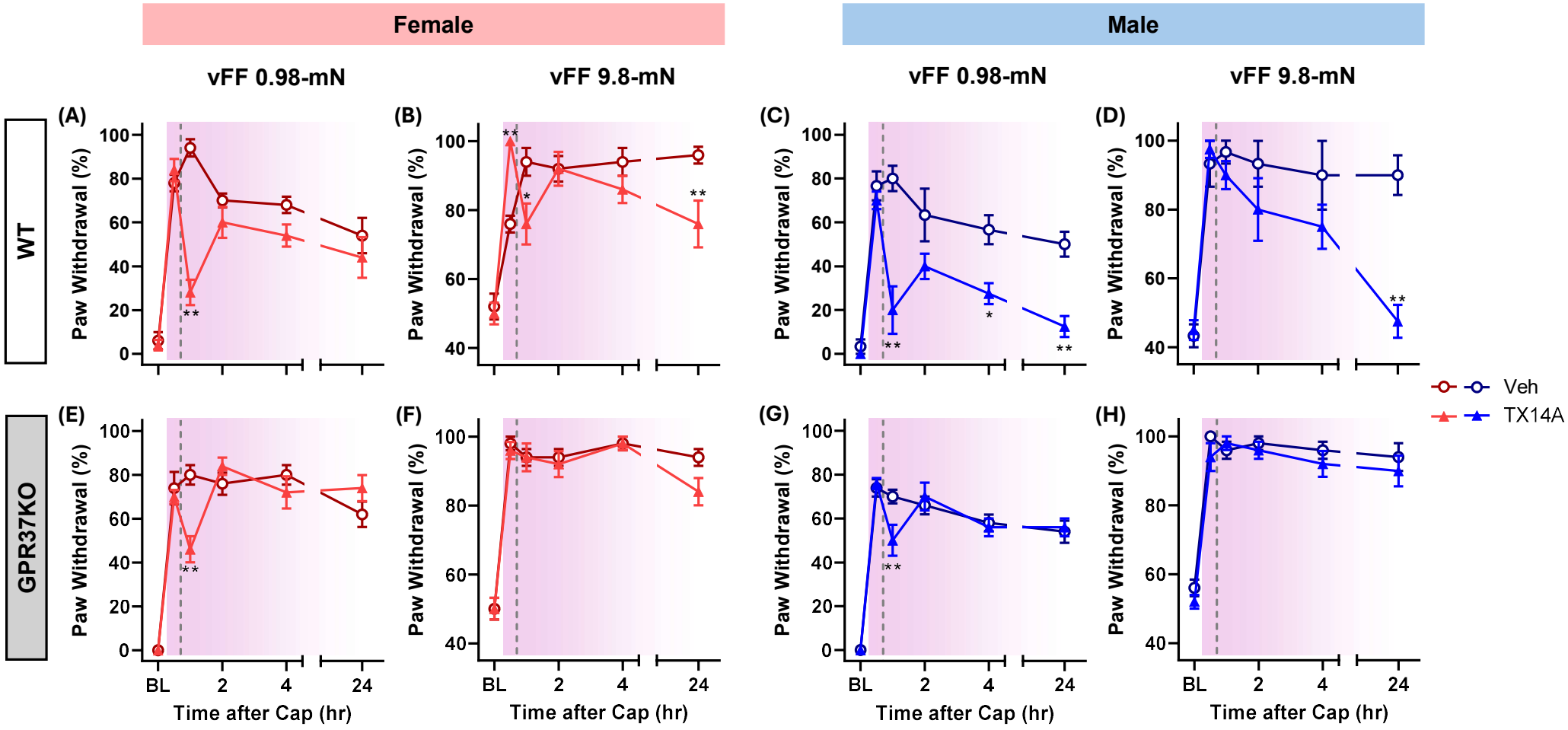
Female
Male
vFF 0.98-mN
vFF 9.8-mN
vFF 0.98-mN
vFF 9.8-mN
WT
GPR37KO
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
Paw Withdrawal (%)
Time after Cap (hr)
BL
2
4
24
Veh
TX14A

Figure 7

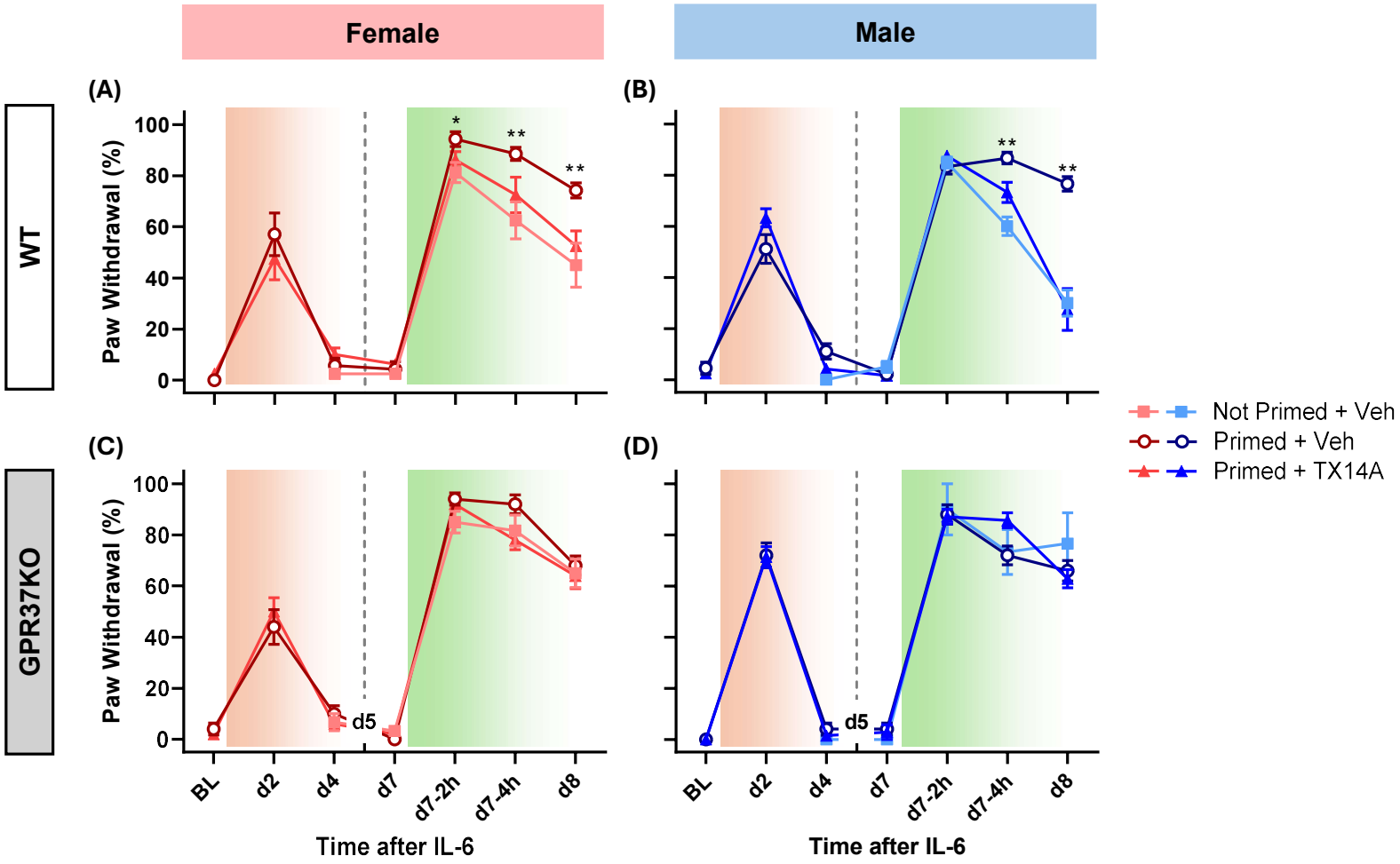
Female
Male
(A)
(B)
(C)
(D)
WT
GPR37KO
Paw Withdrawal (%)
100
80
60
40
20
0
*
**
**
**
**
d5
d5
BL
d2
d4
d7
d7-2h
d7-4h
d8
Time after IL-6
Time after IL-6
Not Primed + Veh
Primed + Veh
Primed + TX14A

Figure 8

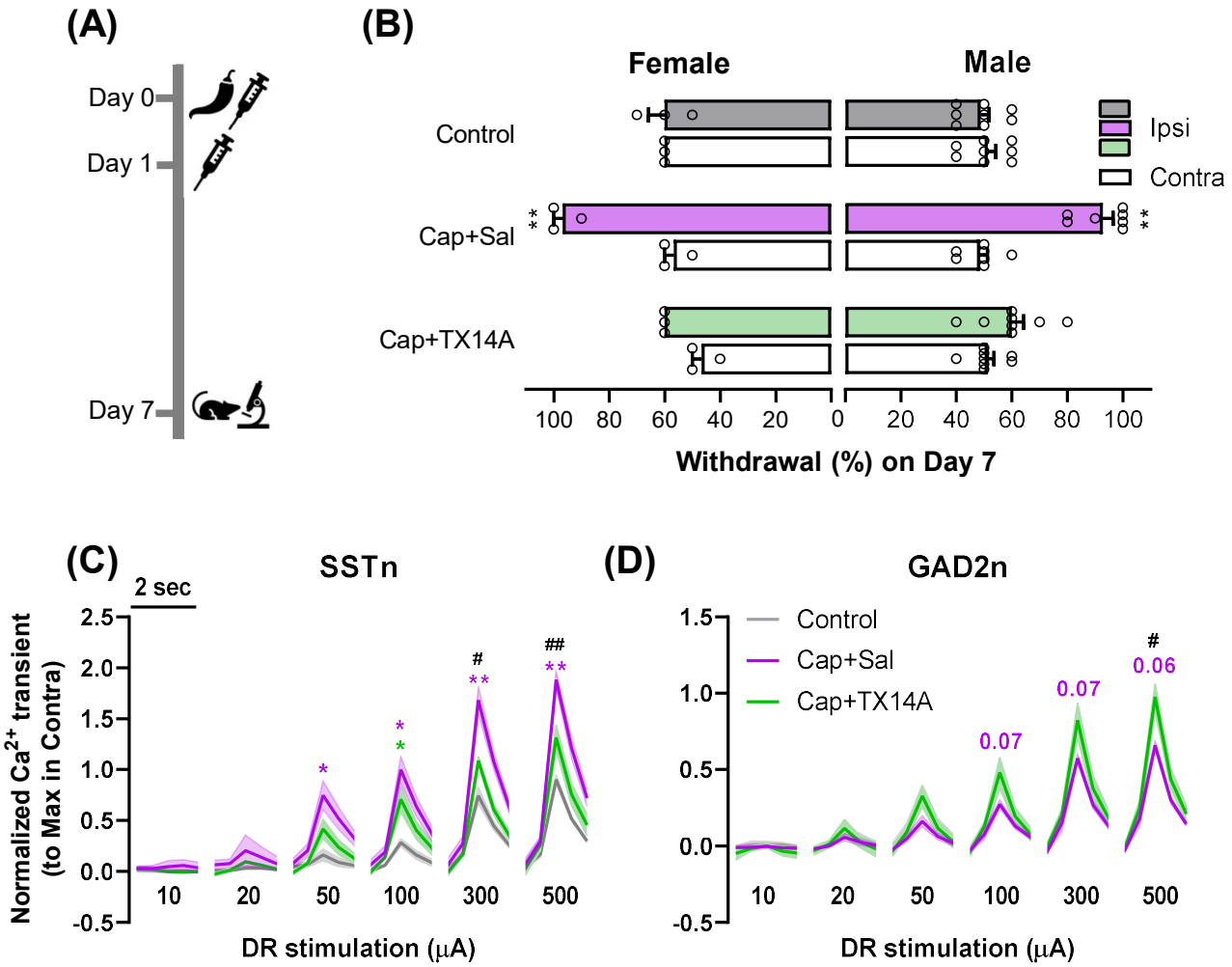
(A)
Day 0
Day 1
Day 7
(B)
Female
Male
Control
Cap+Sal
Cap+TX14A
Ipsi
Contra
100 80 60 40 20 0 20 40 60 80 100
Withdrawal (%) on Day 7
(C)
SSTn
2 sec
Normalized Ca2+ transient (to Max in Contra)
2.5 2.0 1.5 1.0 0.5 0.0 -0.5
10 20 50 100 300 500
DR stimulation (μA)
(D)
GAD2n
Control
Cap+Sal
Cap+TX14A
1.5 1.0 0.5 0.0 -0.5
0.07
0.07
0.06
10 20 50 100 300 500
DR stimulation (μA)

Figure 9

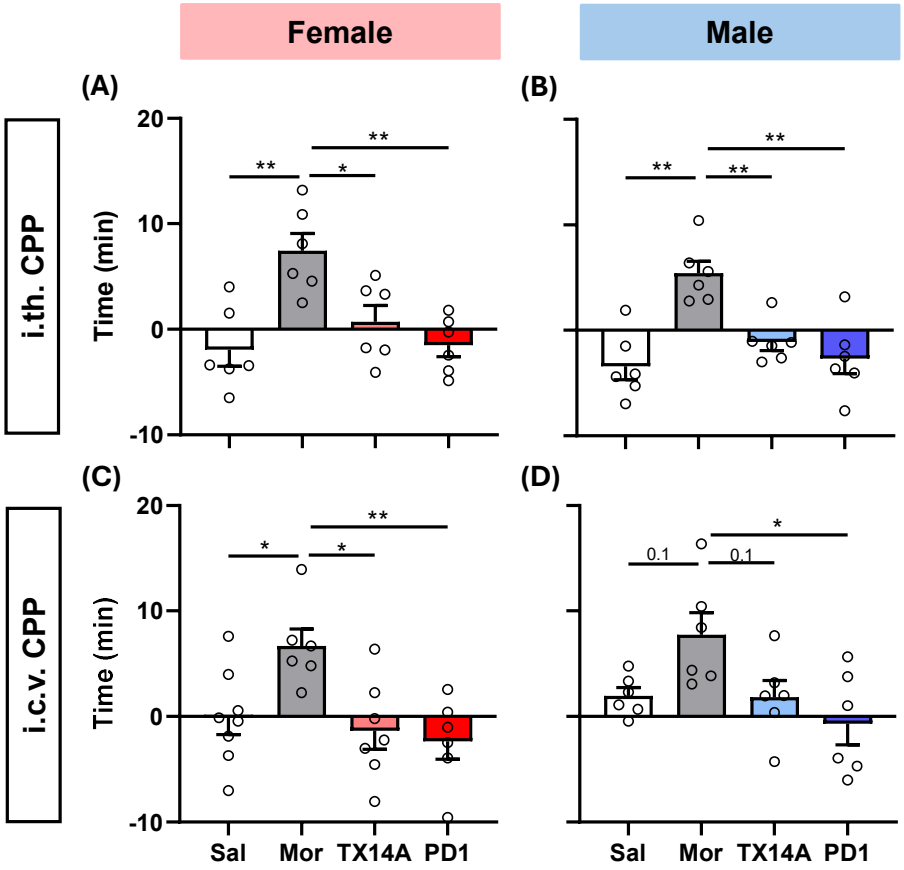
Female
Male
(A)
(B)
(C)
(D)
i.th. CPP
i.c.v. CPP
Time (min)
20
10
0
-10
**
*
0.1
Sal
Mor
TX14A
PD1

Figure 10