Article

Current Biology

Male-Specific Conditioned Pain Hypersensitivity in Mice and Humans

Highlights

- Re-exposure to a context associated with pain results in pain hypersensitivity

- Conditioned pain sensitivity is only present in males via testosterone

- The phenomenon can be demonstrated in both mice and humans

- The phenomenon is dependent on stress and blocked by zeta inhibitory peptide (ZIP)

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In Brief

Martin et al. develop a new model of pain memory—in both mice and humans—in which re-exposure to a context previously associated with tonic pain results in hypersensitivity to acute pain. Surprisingly, this phenomenon only occurs in males of both species and appears to be linked with testosterone, stress levels, and the atypical protein kinase C.

CellPress

Male-Specific Conditioned Pain Hypersensitivity in Mice and Humans

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SUMMARY

Pain memories are hypothesized to be critically involved in the transition of pain from an acute to a chronic state. To help elucidate the underlying neurobiological mechanisms of pain memory, we developed novel paradigms to study context-dependent pain hypersensitivity in mouse and human subjects, respectively. We find that both mice and people become hypersensitive to acute, thermal nociception when tested in an environment previously associated with an aversive tonic pain experience. This sensitization persisted for at least 24 hr and was only present in males of both species. In mice, context-dependent pain hypersensitivity was abolished by castrating male mice, pharmacological blockade of the hypothalamic-pituitary-adrenal axis, or intracerebral or intrathecal injections of zeta inhibitory peptide (ZIP) known to block atypical protein kinase C (including the protein kinase Mζ isoform). In humans, men, but not women, self-reported higher levels of stress when tested in a room previously associated with tonic pain. These models provide a new, completely translatable means for studying the relationship between memory, pain, and stress.

INTRODUCTION

Chronic pain is by some metrics—for example, disease burden [1] and economic impact [2]—the number one human health problem. Both the persistence of pain after tissue healing and the transition of acute into chronic pain have been attributed to associative learning; that is, chronic pain is increasingly viewed as a form of emotional learning designed to protect against threat [3, 4]. In fact, an increasing number of pain researchers are now referring to chronic pain in both animals [5–7] and humans [8, 9] as "pain memory." Given this trend, rather little is known about the classical (Pavlovian) conditioning of pain (as opposed to fear of pain) [10]. A recent meta-analysis could

only identify nine relevant published studies demonstrating conditioned hypersensitivity (hypersensitivity or allodynia) in humans [11], where the measured response is pain. All extant studies in humans were conducted using repeated pairings of discrete, noxious thermal or electrical stimuli as the unconditioned stimulus (UCS), usually with neutral visual or auditory stimuli as the conditioned stimulus (CS). Although conditioning-based methods are increasingly used in preclinical pain research to quantify pain [12], it has been virtually impossible to demonstrate conditioned hypersensitivity in rodents [13, 14], even though electric shocks are used routinely as the UCS in fear-conditioning paradigms and conditioned stress-induced analgesia is a well-studied phenomenon [15].

The fundamental reliance of neuroscience on appropriate behavioral analysis has recently been highlighted [16]. Understanding the potentially critical phenomenon of conditioned hypersensitivity would require the development of an animal model with credible parallels to the human situation [17], such as using UCSs of more real-world relevance, as opposed to highly artificial trains of acute thermal and electric stimuli. Herein, we develop such a model, requiring but a single pairing of pain and context. Although it was our aim to develop a model of conditioned hypersensitivity, we were surprised to find it to be present only in male mice. Through a comparable study, an analogous and similarly male-specific conditioned hypersensitive response was found in people. Upon further examination of potential hormonal influences, we found that the endocrine stress response and testosterone played a role in context-dependent conditioned pain hypersensitivity in mice. Finally, given the increasing (if controversial) literature documenting the involvement of the atypical protein kinase C (aPKC), including the isoform protein kinase Mζ (PKMζ) in both memory [18] and spinal pain processing [19], and the recently demonstrated sex dependence of aPKCs involvement in referred pain [20], we investigated the effect of aPKC antagonism on the phenomenon.

RESULTS

Sex-Specific Conditioned Hypersensitivity in Mice

Mice were tested repeatedly for sensitivity to hind paw thermal nociception before and 24 hr after an intraperitoneal injection of
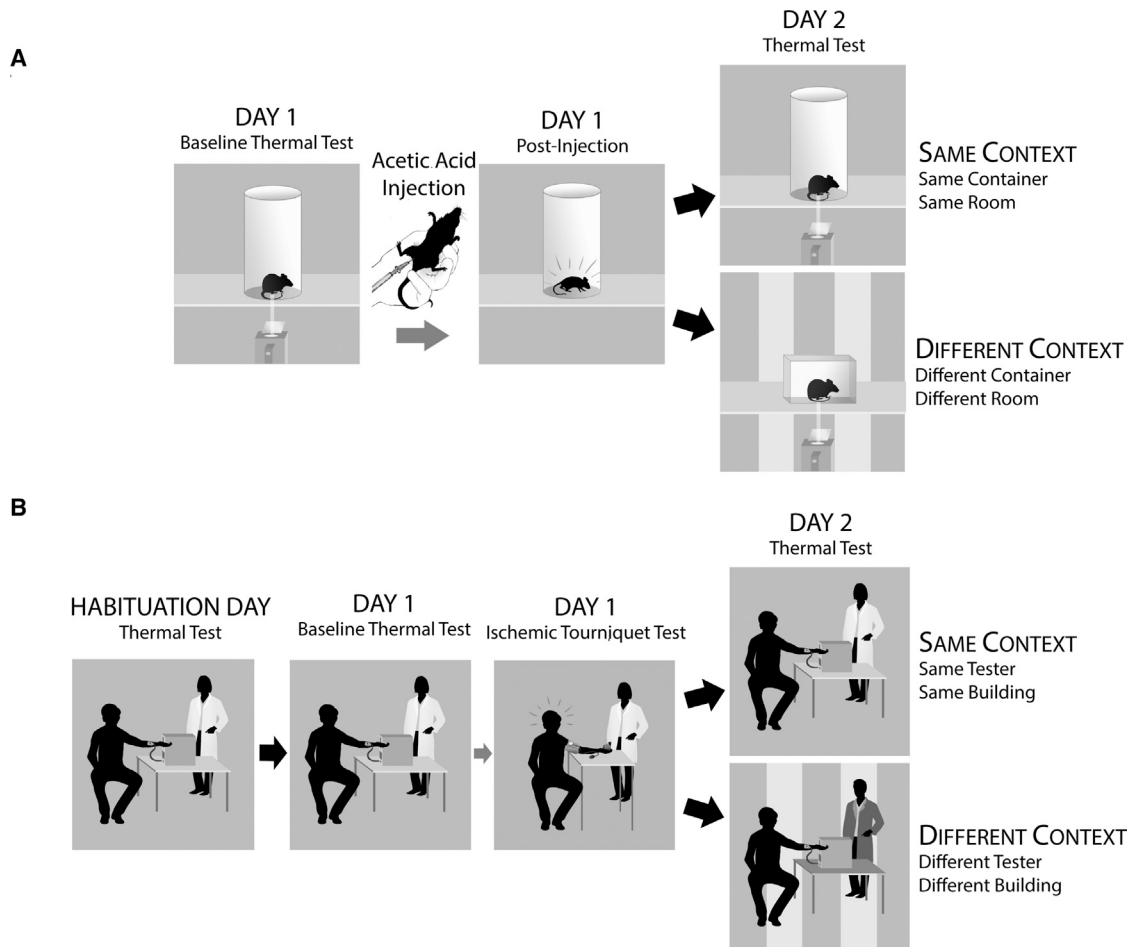
Figure 1. Illustration of the Mouse- and Human-Conditioning Paradigms
(A) As described in STAR Methods, mice of both sexes were tested for thermal pain sensitivity (on day 1) within a transparent cylinder, injected with acetic acid, and then returned to the cylinder for 30 min. The next day (day 2), they are returned to the same cylinder in the same room (same context) or a cubicle in a different testing room (different context) and tested again for thermal pain sensitivity. Half the animals were tested first in a cubicle and then in a cylinder in a different room.
(B) As described in STAR Methods, human participants of both sexes were trained in thermal testing (on the habituation day). The next day (day 1), they were tested for thermal pain sensitivity and underwent the ischemic tourniquet test (on day 1). The day after that (day 2), they returned to the same room and were tested again for thermal pain sensitivity by the same experimenter (same context) or were informed that neither the room nor experimenter were available and tested for thermal pain sensitivity in a different building by a different experimenter (different context).
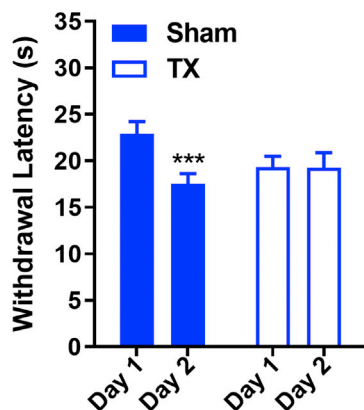
0.9% acetic acid (a noxious stimulus producing abdominal constriction behavior lasting <60 min). On the second day, testing occurred in similar (i.e., same room and testing cubicles or cylinders) or different (i.e., different room and switched cubicles or cylinders) environmental contexts as the previous day (see Figure 1A). Mice tested in the same context (see Figure 2A), but not a different context (Figure 2B), were more sensitive to pain (i.e., hyperalgesic) on day 2 (context × repeated-measure [RM]: $F_{1,156} = 10.2$; p = 0.002). Thus, mice displayed modest but statistically significant context-dependent (i.e., classically conditioned) pain hypersensitivity. Surprisingly, this phenomenon was found to be robust in male mice but entirely absent in female mice (sex × context × RM: $F_{1,154} = 17.2$; p < 0.001; see Figures 2C–2F). An almost identical pattern of results was obtained in a separate experiment, in which a new cohort of mice was tested using mechanical (von Frey fiber) stimuli (sex × context × RM: $F_{1,60} = 9.4$; p = 0.003; see

Figure S1). Furthermore, using both stimulus modalities, male, but not female, mice in the different context condition displayed significant analgesia on day 2 compared to day 1. This phenomenon appears to be novelty-related stress-induced analgesia, as has been previously described [21], of mixed opioid and non-opioid character (see Figure S2). The sex difference in conditioned hypersensitivity was not due to differential response to acetic acid, which produced equivalent abdominal constriction behavior in separately tested mice of both sexes (male: 37.2% ± 4.4% positive samples; female: 38.5% ± 4.6% positive samples; $t_{36} = 0.2$; p = 0.85). A separate concentration-response experiment revealed equivalent levels of hypersensitivity in same-context males at 0.6% acetic acid, but not 0.3% acetic acid (Figure S3A). Female mice displayed no hypersensitivity whatsoever until a concentration of 1.2% acetic was reached, a concentration causing mortality in some subjects. Subsequent testing using male mice only revealed
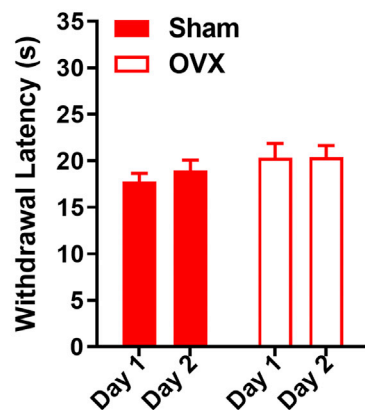
A **Same Context**
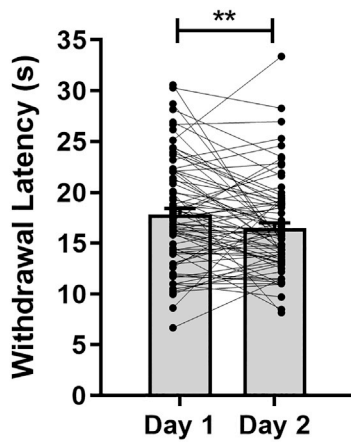
B **Different Context**
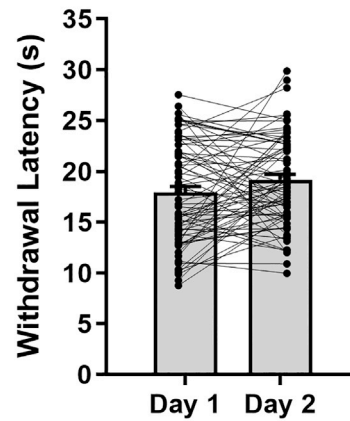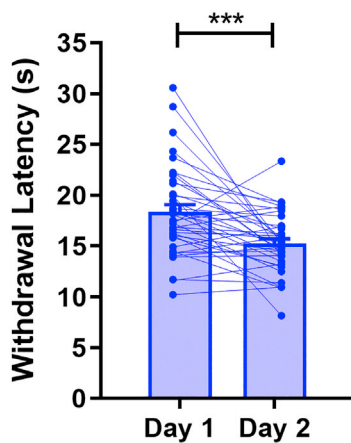
C **Male - Same**
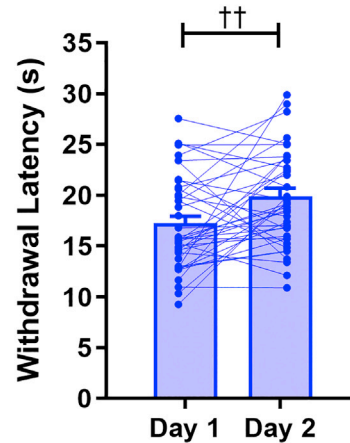
D **Male - Different**
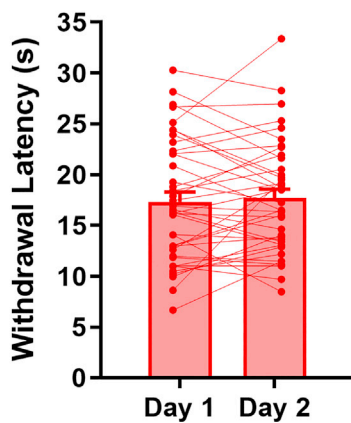
E **Female - Same**

F **Female - Different**
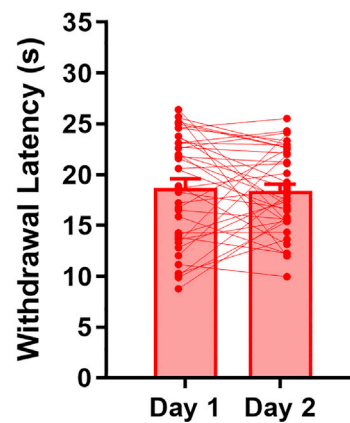
Figure 2. Male-Specific Conditioned Hypersensitivity in Mice

(A–F) Graphs show thermal pain sensitivity of mice of both sexes combined (A and B), male mice only (C and D), or female mice only (E and F) on days 1 and 2 in the same context (same; A, C, and E) or different context (different; B, D, and F) conditions. Bars represent mean ± SEM paw-withdrawal latency (s); symbols represent individual repeated-measures data (n = 38–41 mice/sex/context). **p < 0.01; ***p < 0.001 decreased from day 1. ††p < 0.01 increased from day 1. See also Figures S1, S2, S3, and S4.

A Male - Same Context
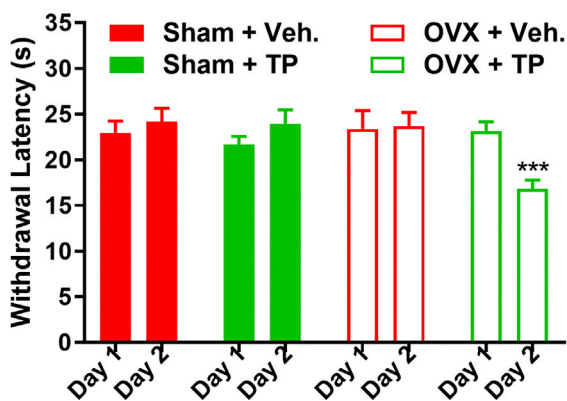

B Female - Same Context

C TP in Females

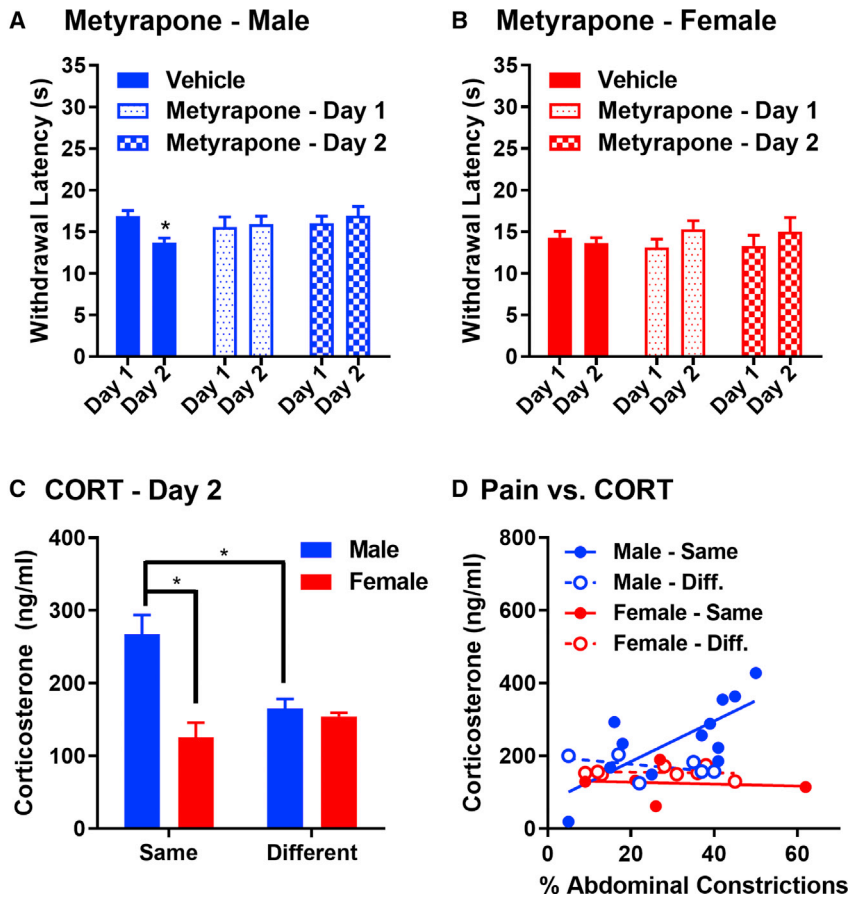**Figure 3. Conditioned Hypersensitivity Is Testosterone Dependent in Mice**

(A) Castration (TX) prevents conditioned hypersensitivity in male mice in the same context condition; the phenomenon persists in sham-operated males. Bars represent mean ± SEM paw-withdrawal latency (n = 19–21 mice/surgical condition).

(B) No effect of ovariectomy (OVX); both sham-operated and ovariectomized female mice do not display conditioned hypersensitivity. Bars represent mean ± SEM paw-withdrawal latency (n = 20 mice/ surgical condition).

(C) Testosterone propionate (TP) reinstates conditioned hypersensitivity in OVX female mice. Bars represent mean ± SEM paw-withdrawal latency (n = 10 mice/surgical condition/hormone). ***p < 0.001 decreased from day 1.

the dependence of the hypersensitivity on associative learning, showing extinction over 24–48 hr, dependence on UCS-CS pairing, and the ability of mice to relearn the conditioning (see Figures S3B–S3D). We also demonstrated the generalizability of the phenomenon, using a different tonic conditioning stimulus (orofacial formalin; see Figure S3E). As an independent measure of associative learning, we assessed conditioned place aversion (CPA). We found that acetic acid exposure produced a sex- and concentration-dependent CPA (Figure S3F), which also extinguished between 5 and 7 days post-injection (Figure S3G). No freezing was produced by acetic acid (Figure S4).

Dependence of Conditioned Hypersensitivity on Testosterone and Stress

To determine the hormonal basis of the sex difference, we repeated the experiment (same context condition only) on sham or gonadectomized mice of both sexes (sex × surgery × RM: $F_{1,75} = 9.6$; p = 0.003). We observed the absence of conditioned hypersensitivity in castrated male mice (surgery × RM: $F_{1,38} = 16.4$; p < 0.001; see Figure 3A). Ovariectomy did not reinstate conditioned hypersensitivity in female mice (surgery × RM: $F_{1,37} = 0.4$; p = 0.53; see Figure 3B), and thus, we conclude that

the relevant steroid hormone is testosterone. To confirm this, we performed a new study on gonadectomized mice given testosterone propionate or vehicle. In ovariectomized females, exogenous testosterone elicited context-dependent hypersensitivity (surgery × hormone × RM: $F_{1,36} = 7.6$; p = 0.009; see Figure 3C).

We reasoned that the observed conditioned hypersensitivity in males was likely a form of stress-induced hypersensitivity. We performed separate experiments (same context condition only) where corticosterone synthesis was blocked using metyrapone—thus preventing activation of the hypothalamic-pituitary-adrenal (HPA) stress axis, either during the conditioning session (day 1) or during the test session (day 2). Compared to vehicle injection, 50 mg/kg metyrapone injected immediately prior to acetic acid on day 1 (the highest dose of metyrapone not itself producing inhibition of abdominal constriction behavior) [22] abolished the context-dependent hypersensitivity in male mice although having no effect on pain sensitivity in female mice (drug × sex × RM: $F_{2,80} = 3.5$; p = 0.03; see Figures 4A and 4B). When metyrapone was instead injected prior to testing on day 2, it also abolished the conditioned hypersensitivity in male mice, with no effect on female mice (drug × sex × RM: $F_{2,82} = 3.3$; p = 0.04; see Figures 4A and 4B).

Mice of both sexes were equally stressed by the acetic acid injection on day 1 as measured by plasma corticosterone levels (male: 531.3 ± 97.3 ng/mL; female: 549.1 ± 71.4 ng/mL; $t_{15} = 0.2$; p = 0.88). However, male mice placed in the same context on day 2 displayed significantly increased corticosterone levels compared to all other groups (sex × context: $F_{1,18} = 8.8$; p = 0.008; see Figure 4C). Furthermore, only in male mice in the same context condition did pain behavior on day 1 predict stress levels on day 2 measured by corticosterone levels (r = 0.74; Bonferroni-corrected p = 0.006; Figure 4D) or fecal boli (r = 0.77; Bonferroni-corrected p = 0.003).

A Metyrapone - Male

B Metyrapone - Female

C CORT - Day 2

D Pain vs. CORT

Figure 4. Conditioned Hypersensitivity Is Stress Dependent in Mice

(A) Inhibition of corticosterone (CORT) synthesis with metyrapone—either injected immediately after testing on day 1 or 30 min prior to testing on day 2—blocks conditioned hypersensitivity in male mice in the same context condition. Bars represent mean ± SEM paw-withdrawal latency (n = 18–21 mice/drug condition).

(B) No effect of metyrapone on pain sensitivity in female mice. Bars represent mean ± SEM paw-withdrawal latency (n = 18–20 mice/drug condition).

(C) Male mice in the same context condition display higher CORT levels than females and males in the different context condition. Bars represent mean ± SEM plasma corticosterone concentration (ng/mL); n = 5–11 mice/sex/context.

(D) Significant correlation between pain behavior on day 1 (% of samples featuring abdominal constrictions over 30 min) and CORT levels on day 2 only in male mice in the same context condition ($r = 0.74$).

*$p < 0.05$ decrease from day 1 (graph A) or as indicated (graph C).

Reversal of Conditioned Hypersensitivity in Male Mice by the aPKC Inhibitor, ZIP

Previously, it was reported that male mice retain spatial information longer than females, a behavioral difference associated with increased expression of synaptic PKMζ in male mice [23]. In addition, genetic and pharmacological inhibition of aPKC reduced referred visceral pain in male, but not in female mice and rats [20]. We therefore tested whether male-specific conditioned pain hypersensitivity is maintained by aPKC via intracerebroventricular (i.c.v.) administration of the aPKC inhibitor ZIP [24] or its scrambled peptide (scr-ZIP), each at 10 nmol/μL 30 min prior to placement in the same or different context on day 2. As shown in Figure 5A, i.c.v. infusion of ZIP completely abolished conditioned hypersensitivity in male mice re-exposed to the same context but did not affect thermal thresholds in the different context condition (drug × context × RM: $F_{1,31} = 59.7$; $p < 0.001$). Further, ZIP did not affect responding in female mice re-exposed to the same context (drug × RM: $F_{1,16} = 0.1$; $p = 0.62$; Figure 5B). Because "pain memory" mechanisms in the spinal cord have been demonstrated [25], we also examined the effects of intrathecal (i.t.) administration of ZIP at the same dose. As shown in Figure 5C, i.t. ZIP completely abolished same-context conditioned hypersensitivity in male mice (drug × RM: $F_{1,18} = 29.7$; $p < 0.001$), with no significant effects in female mice (drug × RM: $F_{1,16} = 3.3$; $p = 0.09$; Figure 5D).

## Sex-Specific Conditioned Pain Hypersensitivity in Humans

Human participants were tested for sensitivity to heat pain before (day 1) and 24 hr after (day 2) experiencing a highly painful tonic stimulus: the 20-min-long submaximal tourniquet test of ischemic pain. On day 2, testing occurred in similar (i.e., same room and experimenter) or different (i.e., different building and experimenter) environmental contexts (see Figure 1B). There were no significant sex differences in thermal pain ratings on day 1 of testing ($F_{1,76} = 0.02$; $p = 0.90$) or systematic differences between experimenters ($F_{1,76} = 0.004$; $p = 0.95$) or testing sites ($F_{1,76} = 1.5$; $p = 0.22$). Before and after pain testing, participants completed state mood measure (SMM) and pain catastrophizing scale (PCS) questionnaires. On day 1, only one psychological variable was found to be correlated with thermal pain ratings: the stress subscale of the profile of the SMM measured immediately before testing ($r = 0.36$; uncorrected $p = 0.007$). Three psychological variables were found to be correlated with muscle ischemic pain ratings: the stress subscale of the SMM measured immediately before testing ($r = 0.29$; uncorrected $p = 0.03$), PCS total score ($r = 0.29$; uncorrected $p = 0.03$), and PCS helplessness subscale ($r = 0.30$; uncorrected $p = 0.03$).

Those tested in the same context (see Figure 6A), but not a different context (see Figure 6B), reported higher, but not significantly so, ratings of pain intensity on day 2 (context × RM: $F_{1,76} = 3.7$; $p = 0.06$). As in mice, this phenomenon was found to be statistically significant in males but entirely absent in females (sex × context × RM: $F_{1,74} = 4.2$; $p = 0.04$; see Figures 6C–6F). A similar pattern was obtained for pain unpleasantness (see Figure S5). The presence of conditioned hypersensitivity in men was not due to sex or gender differences in ratings of ischemic pain on day 1 (intensity: $t_{76} = 0.04$, $p = 0.97$; unpleasantness: $t_{76} = 1.3$, $p = 0.18$; see Figure 7A).

A Male - i.c.v.

B Female - i.c.v.

C Male - i.t.

D Female - i.t.
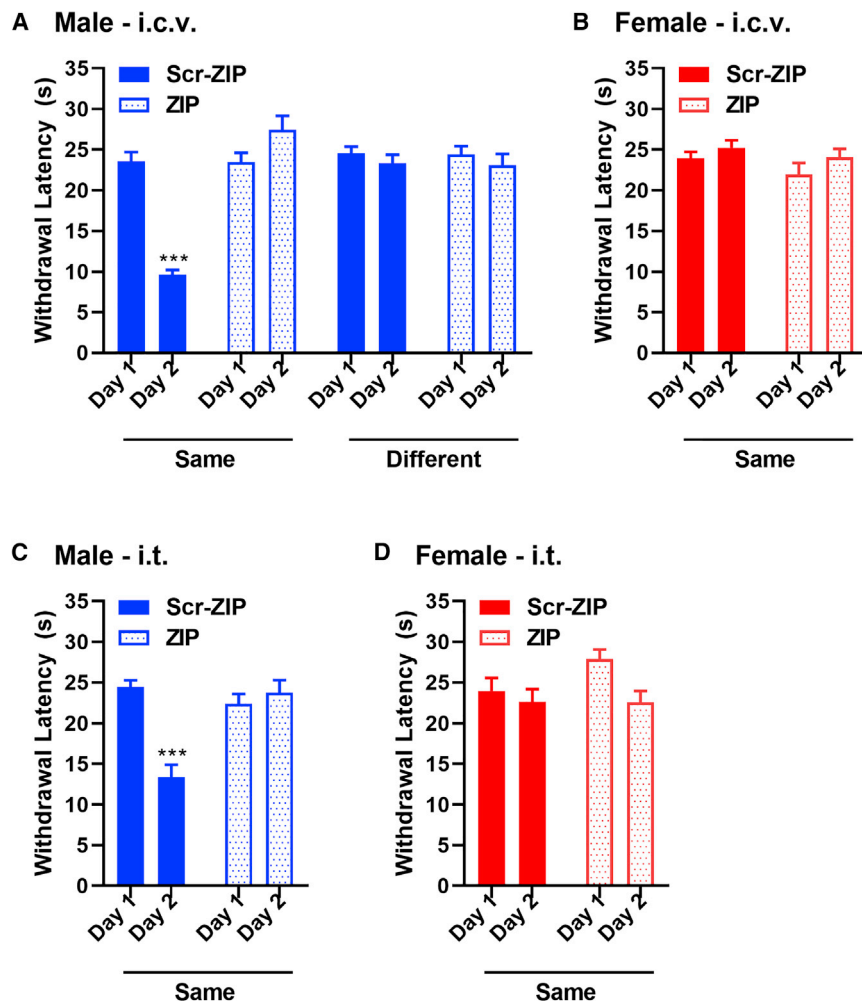
Figure 5. Blockade of Conditioned Hypersensitivity in Males by i.c.v. or i.t. ZIP

(A) Intracerebroventricular (i.c.v.) injection of the aPKC inhibitor, ZIP, but not scrambled ZIP (Scr-ZIP), blocks conditioned hypersensitivity in male mice.

(B) No effect of i.c.v. ZIP on pain sensitivity in female mice.

(C) Intrathecal (i.t.) injection of ZIP, but not Scr-ZIP, blocks conditioned hypersensitivity in male mice.

(D) No effect of i.t. ZIP on pain sensitivity in female mice. Bars represent mean ± SEM paw-withdrawal latency (n = 8–9 mice/route/drug/context/sex). ***p < 0.001 decrease from day 1.

In mice, the male specificity of conditioned hypersensitivity appeared to be stress related, which led us to examine subjective stress ratings during testing sessions on days 1 and 2 in the human cohort. There were no sex differences in subjective stress ratings (using the SMM) at the end of testing on day 1 (t_{74} = 1.5; p = 0.13; see Figure 7B). However, prior to testing on day 2, half of male participants in the same context condition reported being stressed, and the majority of participants in all other groups reported not being stressed or even being relaxed (sex × condition: $F_{1,50}$ = 5.9; p = 0.02; see Figure 7C). Moreover, in males, but not females, subjective stress and pain hypersensitivity (day 2 – day 1 ratings) were strongly correlated (males, r = 0.52, p = 0.003; females, r = −0.17, p = 0.39; Figure 7D).

DISCUSSION

Here, we have demonstrated context-dependent, conditioned pain hypersensitivity following a tonic pain stimulus in both mice and humans. The translation between species was surprisingly direct, with conditioned pain hypersensitivity present in males of both species but absent in females. Based on our findings in mice, the phenom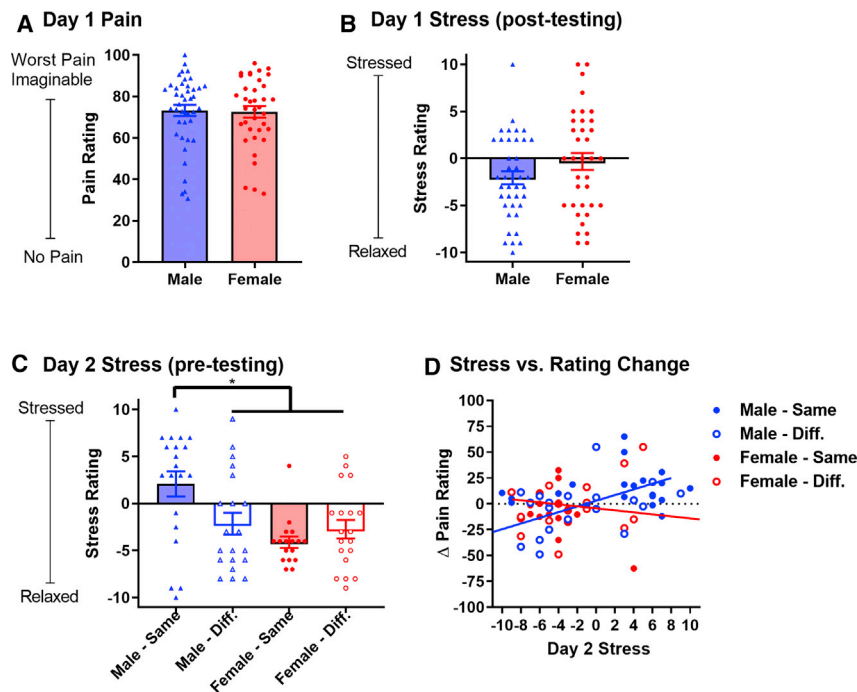enon appears to represent a unique form of stress-induced hypersensitivity that activates the endocrine stress response selectively in males and is dependent on testosterone and aPKC. Psychological factors, such as stress, anxiety, and expectation, play an important role in shaping pain perception both in the clinical setting [26] and in laboratory experiments [27]. These results may have considerable importance for the design of pain experiments, especially in human studies in which pain is assessed over multiple sessions. A single application of a tonic (≈20–30 min) noxious stimulus produced the conditioned pain response rather than multiple exposures to brief noxious stimuli that are usually featured in conditioning studies. Only one pairing of pain with context was required, and no long-lasting pain stimulus was necessary on test day, yielding both interpretative and ethical advantages.

Despite reported sex differences in rodent [28] and human [29] fear conditioning, there was no a priori reason to expect a male-specific hypersensitivity (or, for that matter, a male-specific effect of novelty-related stress-induced analgesia shown in Figure S1). Typically, sex-specific effects of fear-conditioned responses are driven by high levels of estrogen, which may facilitate initial fear acquisition and enhanced extinction and memory recall [29]. In fact, women report more clinical pain and are reliably more sensitive to pain in experimental studies [30]. Thus, any expected sex difference should have involved heightened sensitivity in females. More importantly, sex differences in the underlying spinal and brainstem mechanisms of pain processing have been demonstrated in rodents [31–34] and humans [35]. In this study, both female mice and humans were initially more sensitive to thermal pain than males, just not significantly so. The sex difference in conditioned hypersensitivity might be fundamentally related to stress in both species. Male mice (Figure 4C) and male humans (Figure 7C) exhibited evidence of increased stress on the second day of testing, and for mice, stress measured as plasma corticosterone seemed to be responsible for the observed stress-induced hypersensitivity [36] in males only. Future work might explicitly test cortisol in human

A **Same Context**
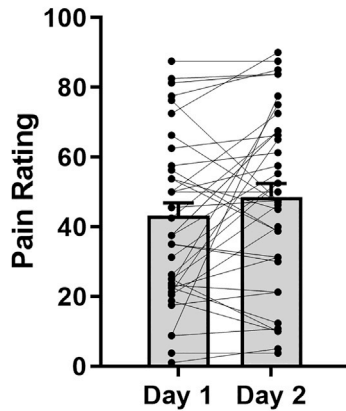
B **Different Context**
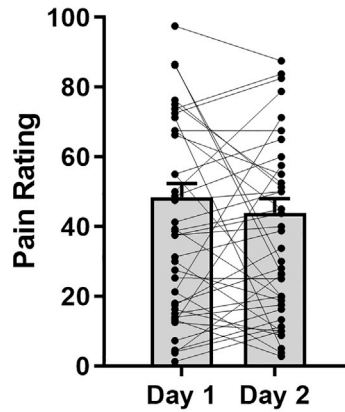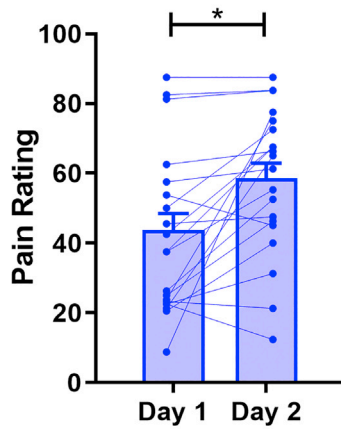
C **Male - Same**
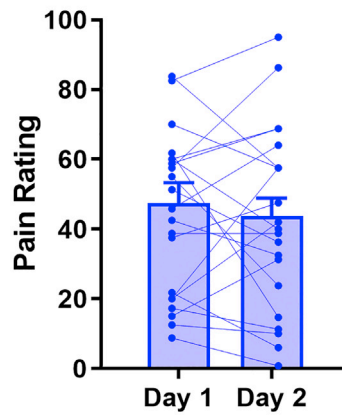
D **Male - Different**

E **Female - Same**
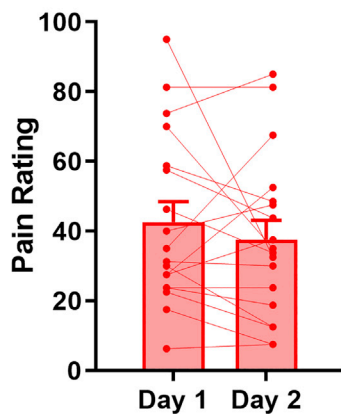
F **Female - Different**
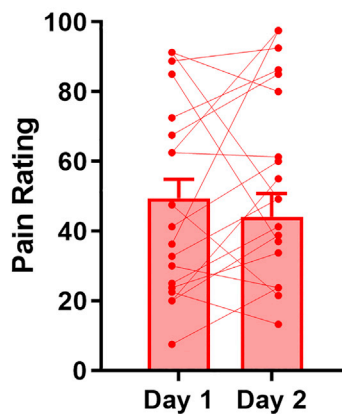
Figure 6. Male-Specific Conditioned Hypersensitivity in Humans

(A–F) Graphs show thermal pain intensity ratings of mice of both sexes combined (A and B), male participants only (C and D), or female participants only (E and F) on days 1 and 2 in the same context (same; A, C, and E) or different context (different; B, D, and F) conditions. Bars represent mean ± SEM pain intensity ratings (0–100 visual analog scale); symbols represent individual repeated-measures data (n = 18–21 participants/sex/condition). **p < 0.05 increased from day 1. Pain unpleasantness rating data are shown in Figure S5.

males to confirm the role of stress beyond self-reported stress.

In both species, the hypersensitivity was context dependent, and considering that pre-treatment with the aPKC antagonist, ZIP, was able to reverse the phenomenon when administered i.c.v., a reasonable interpretation is that males more effectively recalled (or were more emotionally affected by recalling) the stress-inducing properties of the context on day 2 of testing. PKMζ has been implicated in the processing of classical fear conditioning [37], using paradigms that are not dissimilar to those employed here. Further, one study of PKMζ and spatial memory showed that training increased synaptic PKMζ in male, but not female, rats and that synaptic PKMζ levels correlated with memory retention in males, but not females [23]. The direction of sex differences in fear conditioning studies is controversial, with some studies suggesting that male rodents exhibit more conditioned fear [38–42] and others finding no sex difference or suggesting the opposite [43–45]. The human literature is similarly contradictory. Clear conclusions are hampered by complexities related to prior or concurrent stressors, social factors, choice of unconditioned responses, and whether sex differences are attributable to acquisition, retention, and/or extinction of the memory. In one study of conditioned fear using electric shock, for example, women gave higher subjective ratings of fear on day 2 of testing but lower skin conductance responses [46].

The reversal of conditioned hypersensitivity by ZIP administered i.t. suggests a different explanation of the phenomenon, involving aPKC sensitization of spinal pain circuits. At the spinal level, hyperalgesia can undergo a process of modifications that shares characteristics with the phenomenon of memory reconsolidation after reactivation of spinal pathways [25]. Thus, it is possible that repeated thermal pain testing on day 2 reactivated spinal pain pathways in male mice, which

was dependent on aPKCs. Furthermore, a male-specific role of aPKC in pain processing has been reported. Nasir and colleagues [20] observed that pharmacological inhibition (using ZIP in rats) or genetic ablation (in null mutant mice) of PKMζ reduced formalin pain and referred visceral or muscle pain in male, but not female, rodents. The relevance of their nociceptive paradigms to the current assay is not immediately obvious, but it seems unlikely that the male specificity seen by these investigators and in the current study is coincidental.

The neuronal plasticity underlying pain has striking neurophysiological similarities to that underlying memory, with central sensitization in spinothalamic neurons and long-term potentiation in hippocampal neurons increasingly shown to share molecular determinants [47]. Chronic pain may in fact feature an important memory component, as supported by a number of lines of evidence, including phantom limb pain patients [48] and mouse work showing that persistent pain is reduced by targeting spinal memory traces [25, 49]. Further, emotion-processing structures of the brain—including the anterior cingulate, insula, and amygdala—have been found to be important for fear conditioning [50] and affective pain processing [51]. Perhaps the most striking evidence of a relationship between memory systems and chronic pain comes from two case reports of chronic pain patients (one with chronic abdominal pain, the other with sciatica) subsequently suffering amnesia; in both cases, the long-term memory loss was accompanied by dramatic apparent pain relief [52].

With the use of complimentary mouse and human models, such as those described here, we can achieve a better understanding of pain hypersensitivity. This understanding may provide us with an opportunity to examine manipulations that are critical for minimizing these changes. Obviously, aPKC manipulations in humans are not likely to be tried any time soon, but memory-reframing interventions have been attempted to reduce fear of pain from needle injections in children [53] and propranolol administration given directly after retrieval of traumatic memories has been shown to decrease post-traumatic stress disorder symptoms [54].

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Animal Subjects
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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Acetic acid (0.3–1.2% glacial)	Fisher	N/A
Formalin (2.5%)	Fisher	N/A
Metyrapone	Sigma	N/A
Naloxone	Sigma	N/A
AM-251	Sigma	N/A
ZIP (Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu)	R&D Systems	Cat. #2549/1
Scr-ZIP (Myr-Arg-Leu-Tyr-Arg-Lys-Arg-Ile-Trp-Arg-Ser-Ala-Gly-Arg)	R&D Systems	Cat. #3215/1
Experimental Models: Organisms/Strains		
CD-1 mice (both sexes)	Charles River	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jeffrey S. Mogil (jeffrey.mogil@mcgill.ca).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animal Subjects

Most experiments used naive, adult (6-12 week old) CD-1 (ICR:Crl) mice of both sexes. Mice were bred in-house from breeders purchased from Charles River (St. Constant, QC), and housed with same-sex littermates 2–5 per cage after weaning at 18-21 days. The vivarium was temperature controlled and maintained under a 12:12 h light/dark cycle (lights on at 07:00 h), with ad lib access to food (Harlan Teklad 8604) and tap water. Testing occurred both at McGill University and the University of Toronto, by multiple experimenters of both sexes. Experimenters were blinded to drug, but not sex or context condition. Mice were allocated to experimental groups within-cage. All procedures were in accordance with Canadian Council on Animal Care regulations, and approved by local animal care and use committees. No data points were excluded as statistical outliers.

Human Participants

We recruited healthy men and women between the ages of 18 and 40 years on the campus of McGill University. Exclusion criteria were: the presence or history of significant neurological or psychiatric disease, chronic pain, any significant medical condition or sleep disorders; recent use of any pain medication; or regular or frequent night shift work. The study was approved by the local Research and Ethics Board, and written informed consent was obtained from all participants on the first day of testing. Participants were compensated with C$50. The final sample consisted of 41 male (mean age 23.0; SD: 4.2) and 38 female (21.4; SD: 3.3) participants. Participants were allocated to experimental groups using a random-number generator.

METHOD DETAILS

Animal Experiments
Acute nociceptive assays

In most experiments the radiant heat paw-withdrawal test of Hargreaves and colleagues [55] was used to measure sensitivity to acute thermal nociception. Mice were enclosed individually within transparent Plexiglas cylinders (15 cm diameter; 22.5 cm high) or rectangular Plexiglas cubicles (18 × 5 x 5 cm) (see below) atop a glass floor, and habituated for 2 h before any testing commenced. Mice were not able to see other mice that were being tested simultaneously [56]. Testing involved directing a high-intensity heat lamp at the plantar surface of the hind paw; the latency to withdraw from the stimulus was measured to the nearest 0.1 s. Stimulus intensity was 20% of maximal output of the commercial device (IITC Model 336; $\approx$ 45 W) in all experiments. At every time point, both the left and right hind paws were tested once (separated by no less than 20 s), and the latencies averaged.

   In one experiment, a different cohort of mice was tested using an electronic von Frey (Ugo Basile, Dynamic Plantar Aesthesiometer). Mice were placed on a mesh screen floor, under which a movable touch-stimulator unit was placed. The von Frey (0.5-mm diameter) filament was then applied directly to the plantar surface. Force was gradually increased (0–20 g) and the device automatically recorded the force at which paw withdrawal occurred.

Acetic acid abdominal constriction test

The conditioning stimulus (i.e., unconditioned stimulus; UCS) in most studies was tonic pain from dilute acetic acid (0.3%–1.2%; in most experiments, 0.9%) injected intraperitoneally [57] in a volume of 10 mL/kg. Abdominal constrictions were generally not explicitly counted (see, however, main text and Figure 4D), because they were not a relevant dependent measure in these experiments.

Orofacial formalin test

In one study we used a different UCS, orofacial formalin [58]. With mice restrained by hand, 20 μL of 2.5% formalin was injected into the right cheek using a Hamilton microsyringe joined to a 27-gauge needle. Cheek wiping behavior was quantified using a sampling strategy in which the presence or absence of wiping in a 10 s period of every minute was noted over 60 min.

Pain hypersensitivity conditioning paradigm

On the first day of testing (i.e., conditioning day; Day 1), mice were placed in the conditioning context (i.e., Plexiglas cylinders within a particular lab testing room) and their baseline sensitivity to thermal nociception was assessed six times at 5-min intervals on the paw-withdrawal test. After the last baseline measurement, mice were briefly removed from their testing cylinder, injected with the UCS, 0.9% acetic acid (or 2.5% orofacial formalin), and immediately returned to the testing cylinder for 30 min, after which they were placed back in their home cage and returned to the vivarium. The next day (test day; Day 2), some mice were returned to the same cylinder in the same room (*Same Context* condition), and assessed six times at 5-min intervals for paw-withdrawal latencies precisely as on Day 1. Other mice were tested in a *Different Context*, by being placed on Day 2 within rectangular Plexiglas cubicles instead of cylinders, in a different lab testing room. See Figure 1 for an illustration of the mouse conditioning paradigm. As no significant repeated-measures effects were noted on Day 1 ($F_{5,785} = 1.2$, p = 0.29) or Day 2 ($F_{5,780} = 1.4$, p = 0.21), repeated-measures data were collapsed and average Day 1 and Day 2 data were analyzed and reported.

Subsets of *Same Context* mice were retested additionally on Days 3 and 5. A separate Unpaired group of mice were tested for baseline latencies as described above, kept in cylinders for an additional 30 min, returned to their home cages, and 3 h *later* received 0.9% acetic acid in their home cages. On Day 2 they were tested in the same cylinders as on Day 1. Finally, a subset of mice in the *Same Context* group were kept in their home cages for three weeks after the acetic acid injection. On Day 24, they were run through the experimental procedure again (Relearning Experiment), in exactly the same manner as before.

Gonadectomy and testosterone replacement

Gonadectomized and sham-gonadectomized CD-1 mice of both sexes were purchased from Charles River. Castration and ovariectomy (dorsal approach) surgeries were performed by experienced personnel at Charles River no less than 1 week before delivery. In a separate experiment, ovariectomized and sham-ovariectomized mice, were given testosterone propionate (Toronto Research Chemicals, Toronto, ON), dissolved in polyethylene glycol, and administered via subcutaneously implanted osmotic minipumps (Model 2002, ALZET), at a rate of 0.5 μL/h over 14 days in a dose of 250 μg/dL. Mice were anesthetized with isoflurane/oxygen, and osmotic minipumps were implanted via a small (∼1 cm) incision into the upper back. The incision was closed with sterile 9-mm stainless steel wound clips, and animals were placed in a recovery chamber for 30 min before being returned to their home cages. Following the 14-day infusion period, mice were tested as outlined above.

Conditioned place avoidance (CPA)

CPA experiments were conducted using Plexiglas three-chamber boxes with two equal-sized compartments (20 × 20 cm) separated by a neutral gray chamber (20 × 7.5 cm). The chambers were separated by two sliding doors. The two large chambers had different-colored walls (black or white) and different flooring (hard punched metal or stiff wire mesh). On the pre-conditioning day, mice were placed in the center chamber and allowed to freely explore all three chambers for 30-min. These sessions were recorded using a digital video camera, and videos were analyzed using Ethovision software (Noldus, Leesburg, VA) to determine the time spent in each chamber. Conditioning occurred over two days, in which mice were restricted to one chamber, which was either paired with acetic acid (0.9% or 1.2%) or saline. The chamber in which the mouse demonstrated an initial preference (i.e., over 50% of free exploration time during pre-test) was paired with acetic acid injections, whereas the initially non-preferred chamber was always paired with saline. Throughout the conditioning phase, acetic acid and saline injections were counterbalanced between mice, such that half of the mice received acetic acid on the first day of conditioning. Following conditioning, a post-conditioning test was conducted as described for the pre-conditioning day, with free movement between chambers allowed. Data were then calculated as the percentage change in time spent from post-conditioning compared to pre-conditioning for the acetic acid-paired chambers, with negative values representing aversion to that chamber.

Drugs

Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone), naloxone (17-allyl- 4,5α-epoxy- 3,14-dihydroxymorphinan- 6-one) and AM-251 (*N*-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) were purchased from Sigma Aldrich (St. Louis, MO). Metyrapone was dissolved in saline and injected—immediately following testing on Day 1, or 30 min prior to testing on Day 2—subcutaneously at a volume of 10 mL/kg. Naloxone and AM-251 were dissolved in saline and 10% dimethyl sulfoxide (DMSO), respectively. Naloxone and/or AM251 were injected 30 min prior to testing on Day 2, intraperitoneally at a volume of 10 mL/kg. ZIP (Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu) and scrambled (scr)-ZIP (Myr-Arg-Leu-Tyr-Arg-Lys-Arg-Ile-Trp-Arg-Ser-Ala-Gly-Arg) were purchased from R&D Systems (Minneapolis, MN).

Cannula implantation and ZIP infusion

Stereotaxic implantation of a stainless steel injection cannula into the lateral ventricle was performed under isoflurane anesthesia (induction 4%, maintenance, 1.5%–2%). Following exposure of the skull surface, a 23G intracranial guide cannula (length 1.75 mm below the pedestal, HRS Scientific, Anjou, QC) was inserted through a pre-drilled hole (position relative to

bregma: −0.34 mm anterior-posterior; 1.25–1.3 mm medial-lateral) into the right lateral ventricle according to the atlas of Franklin and Paxinos [59]. The cannula was secured using dental cement (K-Dental, Markham, ON). A 23G dummy stylet (HRS Scientific) reaching the tip of the guide cannula was inserted protect the opening of the injection cannula during the remainder of the experiment. Animals were given a 5–7 day post-surgical recovery period before testing commenced. Thirty min prior to contextual exposure on Day 2, the cannulas were connected to a microliter syringe (1700 series Hamilton syringe, 10 μL; Harvard Apparatus) and pump (Pump 11 Elite Nanomite; Harvard Apparatus) via calibrated tubing. ZIP or scr-ZIP (10 nmol) were then infused in a volume of 1 μL over 1 min, after which the injectors were left in position for an additional 2 min. The coordinates for cannula implantations were verified in separate mice by infusing 1 μL of blue dye and confirming the spread of the dye throughout the ventricular space.

In a separate experiment on different mice, 10 nmol ZIP or scr-ZIP was administered through an intrathecal route (5 μL volume, 30 min before testing on Day 2), using the method of Hylden and Wilcox [60], to assess the involvement of the spinal cord.

Plasma corticosterone

Mice were euthanized immediately following 30-min of exposure to the contextual conditions on Day 2, and trunk blood was collected. Blood samples were kept on ice and centrifuged at 4°C and 15,000 rpm for 15 min. Plasma was extracted from the samples and frozen at –70°C until processing. Corticosterone levels were computed using enzyme immunoassay (Cayman Chemical Company, Kit 500655). Samples and standards were assayed in duplicate at a 1:800 dilution according to the manufacturer's protocol. Single absorbance readings for standards and samples were obtained at 405 nm (BioTek Plate Reader), and these values were used for calculation of plasma corticosterone levels (ng/ml) on the basis of linear regression of the standard curve using a log-logit transformation.

Human Experiments

Testing of heat pain sensitivity

Thermal stimulation was performed using a 3 cm x 3 cm contact thermode (Medoc TSA-II NeuroSensory analyzer, Medoc. Advanced Medical System, Israel) applied to the volar aspects of the left or right forearm. Pain threshold of each participant was determined by gradually increasing the temperature of the contact thermode (1.0°C/s). The participant was instructed to press a button as soon as the thermal sensation became painful. The temperature of the thermode returned to baseline (32°C) immediately after the button press and this sequence was repeated four times. A cut-off stimulus intensity of 52°C was used for safety, but no participant approached it. Subsequently, subjective pain intensity and unpleasantness ratings of a 120 s-long heat pain stimulus—delivered at 2°C warmer than their pain threshold obtained on the first testing session (see below), but with minimum intensities of 45.0°C and maximum intensities of 46.9°C—were obtained using visual analog scales.

Visual analog scales (VAS)

After each thermal stimulus, VAS were presented to participants to allow them to evaluate the intensity and hedonic quality (pleasantness/unpleasantness) of the sensation. We explained the differences between stimulus intensity and pleasantness/unpleasantness to the participant using explanations taken from Price et al. [61]. The 200-mm sensation/pain intensity scale was anchored with 0 (no sensation) and 200 (most intense pain tolerable) with a mid-point of 100 defined as the pain threshold [62]. The 200-mm hedonic scale is anchored with –100 (extremely unpleasant) and 100 (extremely pleasant) with a mid-point of 0 labeled "neutral."

Tourniquet-evoked pain

Ischemic muscle pain, the UCS, was produced by a version of the submaximal effort tourniquet test [63, 64]. In this test the arm was elevated above the head and the pressure of a bandage was used to drain blood from the forearm. Then, a blood pressure cuff was inflated above the elbow and the bandage was removed. The participant executed between 20 and 30 squeezes, at 50% of their maximal effort, of a hand-grip dynamometer. Subsequently, the participant rested their arm for a maximum of 20 min with the blood pressure cuff inflated. Subjects rated the intensity and the unpleasantness of the muscle pain sensation throughout the experiment using the same VAS as those used for the thermal stimuli. If the pain sensation started to drop while the blood pressure cuff was inflated, the participant was required to perform more squeezes of the hand-grip dynamometer to ensure that pain levels were kept constant throughout the full 20-min exposure.

Psychophysical Testing Sessions

The human conditioning paradigm is illustrated in Figure 5. All study participants underwent three psychophysical testing sessions at the Alan Edward Center for Research on Pain at McGill University. Testing occurred in Room M/19 of the Strathcona Anatomy & Dentistry Building and/or in Room 3100 of the 740 Docteur Penfield Building. *First testing session (Habituation Day)*: The pain rating scales were explained to the participants, and they were familiarized with the thermal stimuli and testing equipment. Each participant's pain threshold and highest temperature tolerable was determined, as described above. After a short break (approximately 10 min), the subject's heat pain sensitivity to a 120 s-long stimulus was assessed, as described above. In addition, participants completed a state mood measure questionnaire (SMM) at the beginning and end of the test session, and the Pain Catastrophizing Scale (PCS) [65] at the beginning. Using a −10 to +10 numerical rating scale, the SMM assessed the mood dimensions happy-sad, anxious-calm, attentive-distracted, energetic-tired, moody-steady, strong-vulnerable, excited-bored, relaxed-stressed, scared-fearless, and confident-unsure, with instructions modeled after the state subscale of the Spielberger State-Trait Anxiety Questionnaire [66] (see Data S1). The PCS has three subscales: magnification, rumination, and helplessness. *Second testing session (Day 1)*: Participants were assessed for heat pain sensitivity to the 120 s-long stimulus, and then following a 10-min break were subjected to the tourniquet test (the UCS, as described above). All participants completed the SMM at the beginning and at the end of the session. *Third testing session (Day 2)*: Participants were re-assessed for heat pain sensitivity to the 120 s-long stimulus as before. In this

session, half of the participants were tested in the same room and by the same experimenter as on Day 0 and Day 1 (*Same Context* condition). The remainder (chosen via a randomly assigned number sequence) were tested in a different room and by a different experimenter (*Different Context* condition); this change was explained by a ruse involving sickness on the part of the experimenter and flooding in the original testing room. To control for possible systematic effects of room and experimenter, participants in both context conditions were counterbalanced to start in either of the two testing rooms and with either experimenter. All participants completed the SMM at the beginning and at the end of the session.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed by repeated-measures ANOVAs as appropriate following confirmation of the normality and homoscedasticity of all mouse and human datasets. Statistical results can be found in the main text; sample sizes can be found in the figure legends. For analysis of SMM data, raw scores were normalized to *t*-scores. A criterion $\alpha = 0.05$ level was used. Although it was not possible in this experiment to blind experimenters to either condition or sex, the reported data represent the combination of multiple, independent runs by four different experimenters, two of whom were blinded to the hypothesis and all of whom had no *a priori* reason to expect a sex difference. As the phenomenon under study had never before been reported, it was not possible to perform *a priori* power analyses, as effect sizes could not be estimated.

Update

Current Biology

Male-Specific Conditioned Pain Hypersensitivity in Mice and Humans

Loren J. Martin, Erinn L. Acland, Chulmin Cho, Wiebke Gandhi, Di Chen, Elizabeth Corley, Basil Kadoura, Tess Levy, Sara Mirali, Sarasa Tohyama, Sana Khan, Leigh C. MacIntyre, Erika N. Carlson, Petra Schweinhardt, and Jeffrey S. Mogil*
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In the originally published version of this article, a copy-paste error resulted in inaccurate mean and SEM values for some graphs within Figures 2, 6, S1, and S5. The individual values provided in all graphs were accurate, and this error does not affect the conclusions of the paper in any way. New, fully accurate graphs have now been incorporated into the online version of the paper. The authors apologize for any confusion that this error may have caused.
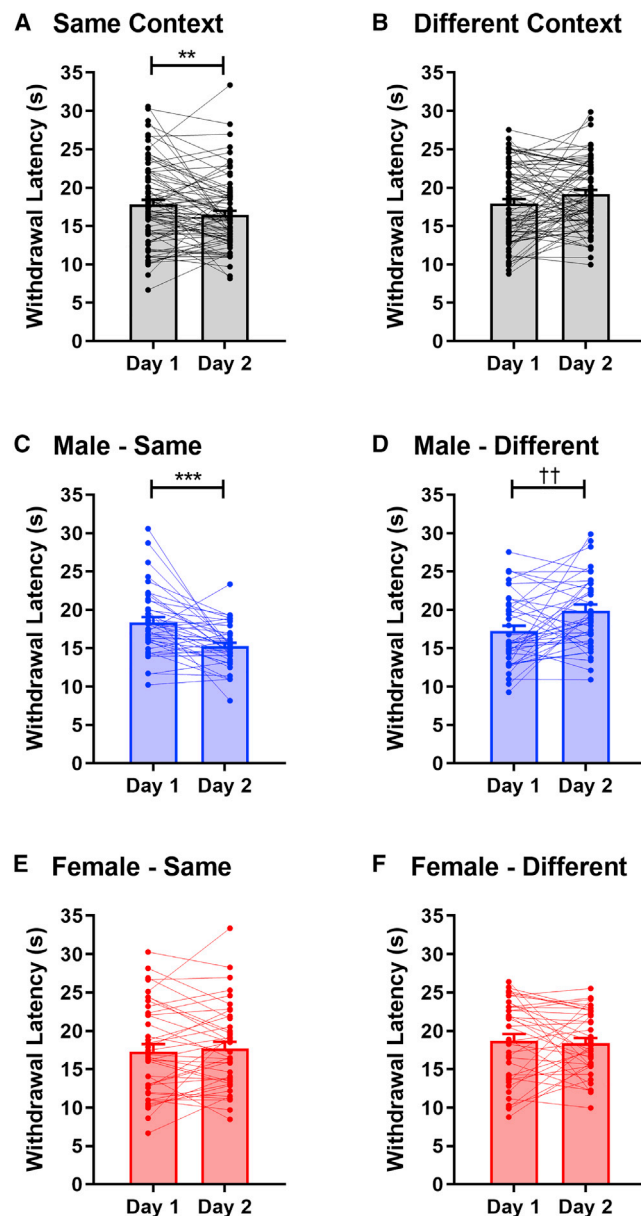

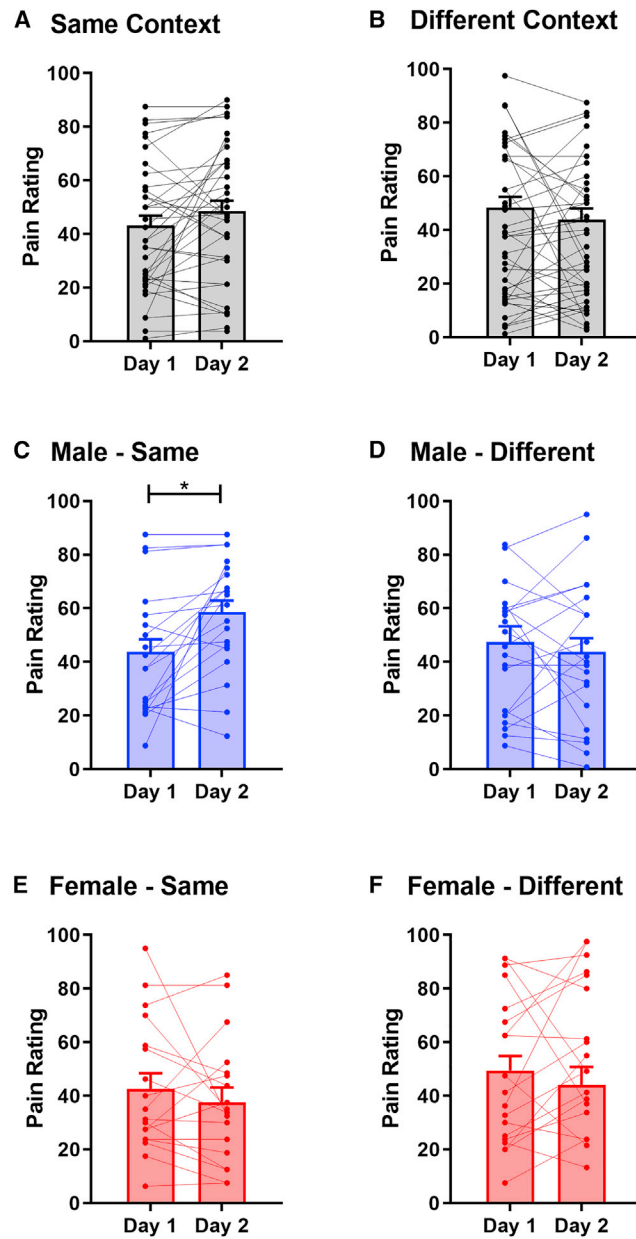Figure 2. Male-Specific Conditioned Hypersensitivity in Mice (corrected)

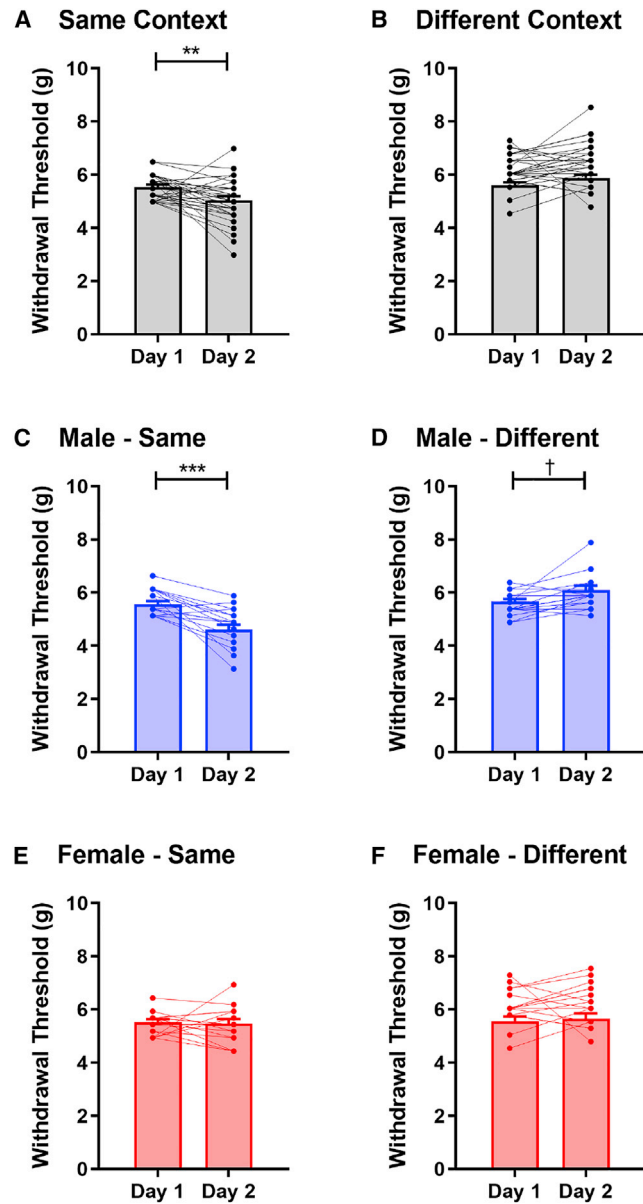**Figure 6. Male-Specific Conditioned Hypersensitivity in Humans (corrected)**

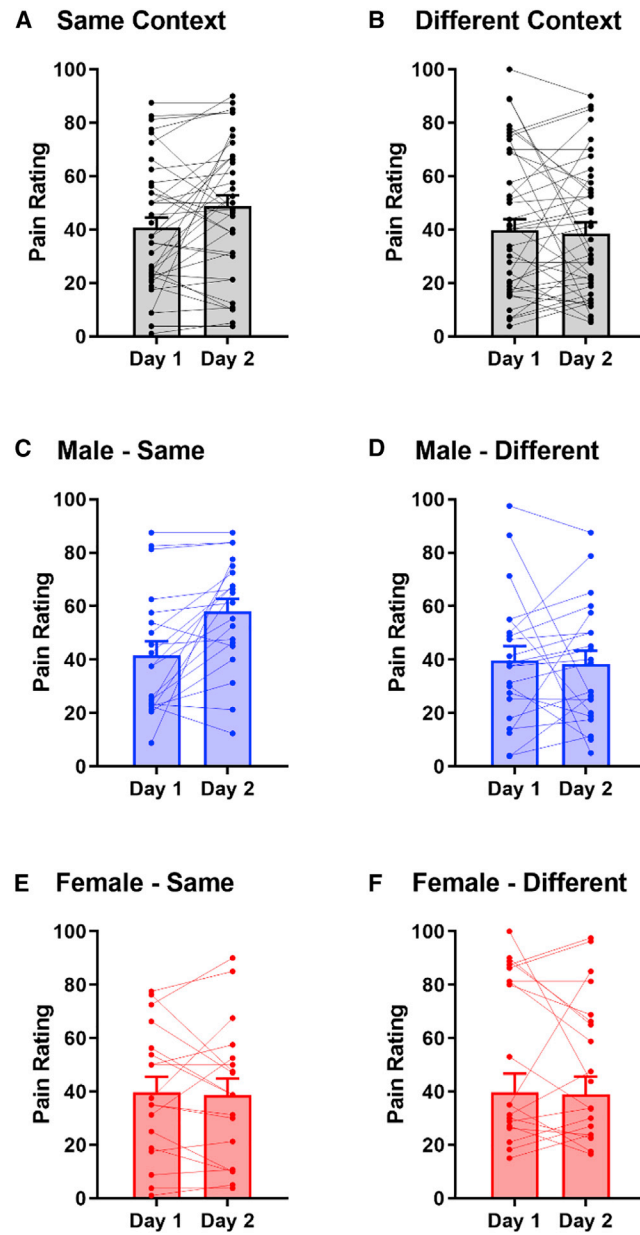**Figure S1. Male-specific conditioned hyperalgesia in mice as measured using the von Frey fiber test of mechanical sensitivity (corrected)**

Figure S5. Human pain unpleasantness data (corrected)