NEUROPSYCHOLOGY

Social transfer of pain in mice

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A complex relationship exists between the psychosocial environment and the perception and experience of pain, and the mechanisms of the social communication of pain have yet to be elucidated. The present study examined the social communication of pain and demonstrates that "bystander" mice housed and tested in the same room as mice subjected to inflammatory pain or withdrawal from morphine or alcohol develop corresponding hyperalgesia. Olfactory cues mediate the transfer of hyperalgesia to the bystander mice, which can be measured using mechanical, thermal, and chemical tests. Hyperalgesia in bystanders does not co-occur with anxiety or changes in corticosterone and cannot be explained by visually dependent emotional contagion or stress-induced hyperalgesia. These experiments reveal the multifaceted relationship between the social environment and pain behavior and support the use of mice as a model system for investigating these factors. In addition, these experiments highlight the need for proper consideration of how experimental animals are housed and tested.

INTRODUCTION

Pain is both a sensory and emotional experience and is markedly influenced by psychosocial and environmental factors (*1*–*3*). Clinically significant chronic pain often manifests in the absence of tissue damage, yet most investigations of the neural mechanisms governing these disorders rely upon activation of nociceptive pathways with a noxious stimulus and are only beginning to consider social influences. Like humans, rodents are capable of complex social behaviors, and increasing evidence suggests that social and environmental variables also affect pain responsiveness in these species (*4*–*6*).

Pain is an adaptive process that can serve as a warning of actual or potential injury, enhancing the survival of the individual and its social group. As a social cue, recognition of another's pain can lead to the avoidance of harm or trigger empathy and caregiving behavior. The communication of pain is a complex process, and the spectrum of this behavior ranges from basic alarm cues to empathy, involving multiple sensory modalities. The social communication of pain has been explored in the form of emotional contagion, and previous studies have demonstrated the importance of visual and auditory cues in certain contexts. For example, these foundational studies have demonstrated that the presence of a familiar conspecific that is either responding to an acute noxious stimulus or is in an ongoing state of pain can modulate the behavior of a test animal given the same noxious input with enhanced (*5*, *7*, *8*) or diminished (*9*) pain behaviors, depending on the experimental paradigm. Visual cues are thought to play a primary role in mediating this communication, with paired animals displaying synchronous pain behaviors described as "emotional contagion" (*7*). These findings have been extended with the recent observation that mice housed for several weeks in the same cage as conspecifics subjected to peripheral nerve injury exhibit enhanced responses in the acetic acid–induced writhing test (*10*). This behavior appeared to represent a form of stress-induced hyperalgesia (*11*) because the cagemates of the nerve-injured animals demonstrated changes in behavior on the elevated plus maze (EPM) and in the open-field test, which are thought to measure anxiety-like behavior.

The current studies were designed to further explore the social communication of pain and test whether the presence of "primary" animals in a hyperalgesic state affects "bystander" animals that are housed and tested in the same room but not subjected to any initial noxious stimulus. We observed that bystanders display hyperalgesia, congruent with primary animals subjected to persistent inflammation or withdrawal from opioids or alcohol, as tested by mechanical, thermal, or chemical modalities. The transfer of this hyperalgesia is mediated by olfactory cues, does not involve visually dependent emotional contagion, and cannot be explained as stress-induced hyperalgesia.

RESULTS

Presence of hyperalgesia in bystander mice housed in the same room as mice subjected to persistent inflammation or undergoing opiate withdrawal

To investigate the effect of the social environment on nociceptive behavior, we conducted experiments in which mice were either housed and tested in the same room as those that received a persistent noxious stimulus (Co-Housed) or housed and tested in a separate room (Separate). All mice were individually housed in cages with wire cagetops and assessed at several time points for mechanical responsiveness using calibrated von Frey filaments applied to the plantar surface of the left hind paw. In this first experiment, following testing for basal mechanical thresholds, phosphate-buffered saline (PBS) [vehicle (Veh)] or complete Freund's adjuvant (CFA) was injected into the plantar surface of the tested paw (Fig. 1A). CFA is well known to induce long-lasting, localized inflammation and hyperalgesia (*12*, *13*). Injection of vehicle led to modest hypersensitivity that was resolved by the third test session in mice housed in their own separate room (Veh/Separate; Fig. 1B). As expected, CFA-treated animals demonstrated a robust and persistent mechanical hypersensitivity for the entire 2-week time course (CFA/Co-Housed; Fig. 1B). However, mice injected with PBS but housed in the same room as the CFA-injected mice (Veh/Co-Housed) also displayed pronounced hypersensitivity that was evident for 2 weeks (Fig. 1B). This experiment indicates that bystander mice that are housed in the same room as mice that experience CFA-induced hypersensitivity exhibit congruent hypersensitivity.

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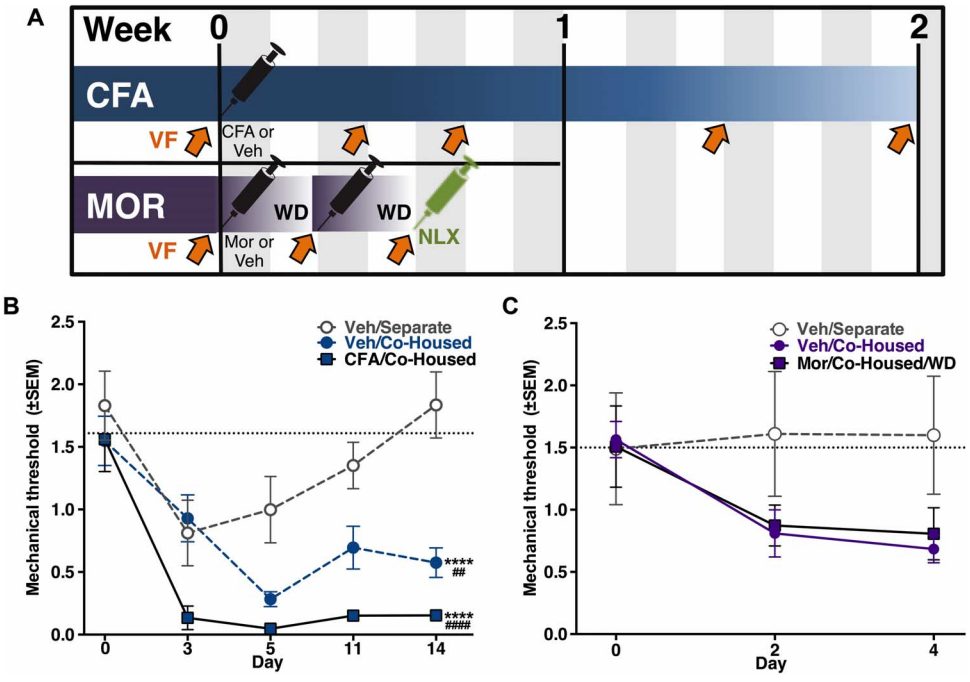

Fig. 1. Social transfer of CFA and morphine withdrawal–induced pain. (**A**) Experimental timeline of experiments presented in (**B**) and (**C**). Von Frey (VF, thick orange arrows) injections of morphine/CFA (Mor/CFA, black syringes) or naloxone (NLX, green syringe). (B) Mice subjected to intraplantar CFA injection showed a robust and persistent decrease in mechanical sensitivity for all test sessions (CFA/Co-Housed; $n = 8$) compared to vehicle-injected mice housed in a separate room (PBS/Separate; $n = 8$). Vehicle-injected mice housed in the same room as CFA-injected mice (Veh/Co-Housed; $n = 8$) demonstrated significantly decreased mechanical thresholds compared to Veh/Separate mice during the last three test sessions. This resulted in significant differences between groups ($F_{2,21} = 30.0$, $P < 0.0001$) across time ($F_{4,84} = 27.6$, $P < 0.0001$) and a significant interaction between these variables ($F_{8,84} = 9.1$, $P = 0.003$) according to repeated-measures analysis of variance (ANOVA). (C) Co-Housed mice injected with either a slow-release morphine emulsion (Mor/Co-Housed/WD; $n = 7$) or vehicle emulsion (Veh/Co-Housed; $n = 8$) every other day demonstrated significant decreases in mechanical thresholds on the two test sessions compared to vehicle-injected mice housed in a separate room (Veh/Separate; $n = 7$). Repeated-measures ANOVA showed a significant effect of treatment ($F_{2,19} = 7.4$, $P = 0.004$) and a significant effect of time ($F_{2,38} = 5.7$, $P = 0.006$). Following a significant interaction, Bonferroni's post hoc analyses were conducted. Differences compared to control are represented by *, and differences compared to baseline are represented by #. Mean basal responses of all groups are represented by dotted lines.

To determine the generalizability of this acquired hypersensitivity in bystander mice, we examined the potential for the transfer of alternate hyperalgesic states. Hyperalgesia is known to occur during opiate withdrawal, and therefore, we investigated the ability of bystanders to acquire hypersensitivity when housed and tested in the same room as primary mice in a state of morphine withdrawal–induced hypersensitivity. Mechanical sensitivity was assessed during two sessions of spontaneous withdrawal from morphine (48 hours after injection; see Fig. 1A). Accordingly, immediately after the baseline test, a subcutaneous injection of morphine base [300 mg/kg; Mor/Co-Housed/withdrawal (WD)] or vehicle in a sustained-release emulsion (Veh/Co-Housed/WD) was given. The first mechanical test occurred 48 hours later, immediately followed by the second injection of morphine or vehicle. This treatment regime has been demonstrated to induce profound physical dependence in rodents (*14*–*16*). Two days after each injection, withdrawal from morphine led to evident hypersensitivity compared to basal mechanical thresholds or to vehicle-treated mice housed in a separate room (Veh/Separate; Fig. 1C). As in the previous experiment, vehicle-treated mice that were housed in the same room as mice that experienced hyperalgesia also demonstrated significant mechanical hypersensitivity (Veh/Co-Housed/WD; Fig. 1C). To confirm that the morphine-treated mice developed dependence, an intraperitoneal injection of naloxone (10.0 mg/kg) was given 24 hours after the final test session. This dose of naloxone precipitated withdrawal in morphine-treated mice, leading to jumping, wet dog shakes, and paw tremors (fig. S1), confirming that this dose of morphine is sufficient to induce physical dependence. This experiment indicates that thetransfer of hyperalgesia from primary experimental mice to vehicle-treated bystanders is not specific to inflammatory stimuli but can also be demonstrated during morphine withdrawal–induced hyperalgesia.

Presence of hyperalgesia in bystander mice housed in the same room as mice undergoing alcohol withdrawal

If transfer is a general phenomenon that occurs with any hyperalgesic state, then it should be seen in conditions in which the treatment does not specifically target pain transmission (CFA) or pain modulation (morphine) systems. Therefore, we tested animals undergoing alcohol withdrawal because hyperalgesia and spontaneous pain are well documented during alcohol withdrawal in humans, although they are understudied in rodents (*17*, *18*). Thus, we used a standard voluntary drinking protocol to test whether alcohol withdrawal would lead to hypersensitivity in alcohol-withdrawn and control (water-drinking) mice housed in the same room. We exposed mice to a 24-hour access, two-bottle choice drinking procedure (*19*, *20*). In the initial experiment, mice were individually housed in cages with wire tops containing water and introduced to

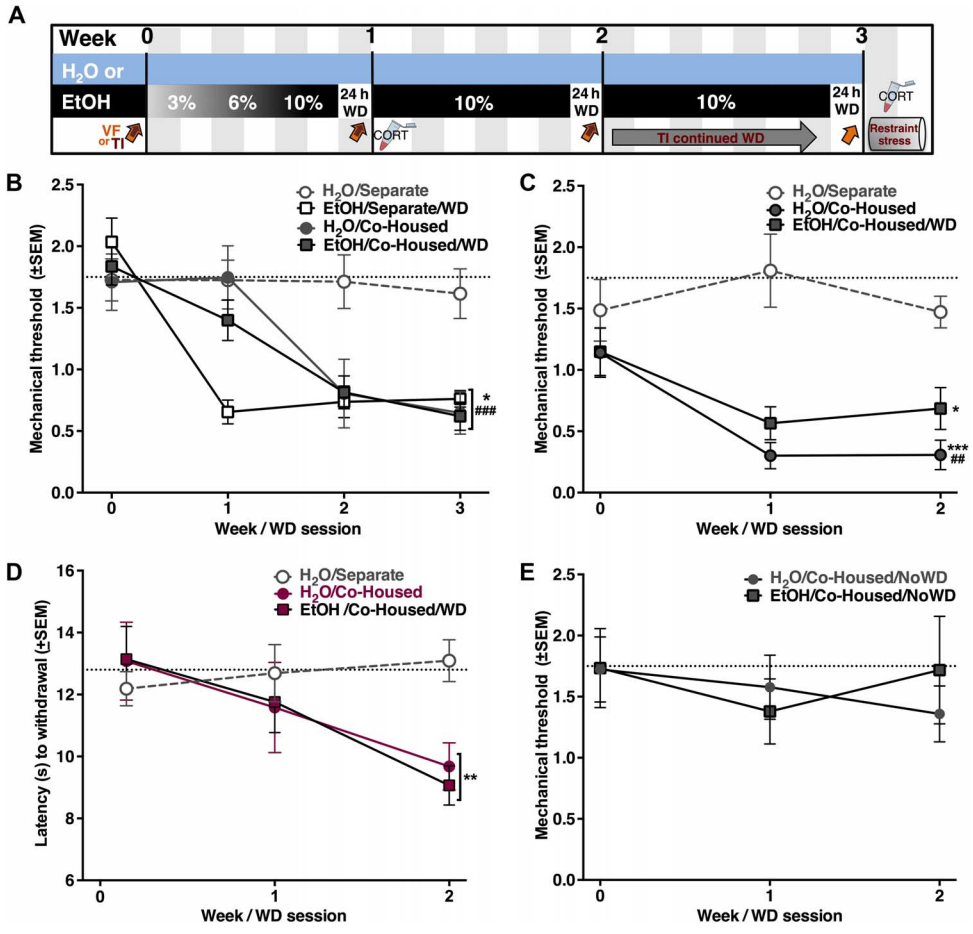

Fig. 2. Social transfer of alcohol withdrawal–induced mechanical sensitivity to nearby water-drinking controls. (**A**) Experimental timeline of experiments presented in (**B**) to (**E**). Von Frey (thick orange arrows); tail immersion (TI, small maroon arrows); ethanol [EtOH, 3 to 10% (v/v)]. h, hours. (B) Ethanol-drinking mice (EtOH/Co-Housed/WD; n = 14 males per group) demonstrate a significant decrease in mechanical thresholds following one withdrawal session that is matched by water-drinking control mice housed in the same room (H_2O/Co-Housed; n = 10 males) by the second withdrawal session. Ethanol-drinking control mice housed in an adjacent room (EtOH/Separate/WD; n = 12 males) also demonstrate enhanced mechanical sensitivity between 1 and 3 withdrawal sessions. Water-drinking mice in an adjacent room (H_2O/Separate; n = 14 males) display stable mechanical thresholds across the time course. Repeated-measures ANOVA that compared mechanical sensitivity of male mice over time revealed significant main effects of week ($F_{3,138}$ = 26.16, P < 0.0001), treatment ($F_{3,46}$ = 6.69, P = 0.0008), and a significant interaction ($F_{9,138}$ = 4.97, P < 0.0001). Bonferroni's post hoc analysis revealed significant differences between H_2O/Separate and H_2O/Co-Housed, EtOH/Co-Housed/WD, and EtOH/Separate/WD. (C) In a separate experiment that used female mice (n = 7 to 8 per group), H_2O/Separate mice (n = 8) never significantly deviated from baseline. Both Co-Housed groups demonstrated decreased mechanical thresholds during the first and second withdrawal sessions, with the bystander group (H_2O/Co-Housed; n = 7) reaching the lowest level. Repeated-measures ANOVA demonstrated significant main effects of treatment ($F_{2,19}$ = 13.0, P = 0.0003), week ($F_{2,38}$ = 7.1, P < 0.002), and a significant interaction ($F_{4,38}$ = 4.4, P < 0.005). Bonferroni's post hoc analysis revealed significant differences between H_2O/Separate and H_2O/Co-Housed and EtOH/Co-Housed/WD. (D) When tested for thermal sensitivity by immersing the tail into a hot water bath, Co-Housed EtOH mice (n = 8) and H_2O mice (n = 8) demonstrate significantly shorter withdrawal latencies on the second withdrawal session compared to H_2O/Separate mice according to one-way ANOVA on the second withdrawal session ($F_{2,21}$ = 9.8, P = 0.001). (E) Ethanol-drinking mice with continuous access/no withdrawal sessions (EtOH/Co-Housed/NoWD; n = 7) and H_2O mice housed in the same room (H_2O/Co-Housed/NoWD; n = 7) did not demonstrate any alterations in mechanical sensitivity following 2 weeks of ethanol exposure. There were no significant differences between groups according to repeated-measures ANOVA (P > 0.05). Significant changes (P < 0.05) from baseline according to Bonferroni's post hoc analyses are represented by #. Significant differences compared to control (P < 0.05) are represented by *. Mean basal responses of all groups are represented by a dotted line.

increasing concentrations of ethanol (EtOH, 3 to 10%) with weekly 24-hour sessions of imposed abstinence from ethanol (withdrawal; Fig. 2A). Mice that were given ethanol (EtOH/Co-Housed/WD) voluntarily drank 9.4 ± 0.9 g/kg per day (mean ± SEM; table S1) and were housed and tested in a room with ethanol-naïve control mice that only drink water (H_2O/Co-Housed). Additional ethanol- and water-drinking control groups were individually housed and tested in separate rooms (EtOH/Separate/WD and H_2O/Separate groups, respectively). Basal nociceptive thresholds were determined at the beginning of the protocol, and each group was tested weekly thereafter (Fig. 2A).

At the end of the first session of abstinence, mice in the EtOH/Co-Housed/WD group exhibited significant mechanical sensitivity relative to baseline (Fig. 2B). This hypersensitivity was maintained in subsequent withdrawal sessions, and mechanical thresholds were decreased by 68 ± 2% relative to baseline (mean ± SEM) at the third withdrawal session. Notably, H_2O/Co-Housed mice demonstrated equivalent hypersensitivity by the second week of testing

and an overall 62 ± 2% decrease at the third and final test session (Fig. 2B). Animals that drank ethanol but were housed in a separate room without a water-drinking group (EtOH/Separate/WD) also displayed significant hypersensitivity during withdrawal, demonstrated by an overall decrease of 65 ± 0.9% in mechanical thresholds by the final session (Fig. 2B). However, control mice that only drank water and were housed without an ethanol group in the same room (H_2O/Separate) did not develop hypersensitivity at any point (Fig. 2B).

We repeated this experiment in female mice and found that, similar to males, females developed significant hypersensitivity during alcohol withdrawal (EtOH/Co-Housed/WD; Fig. 2C). Again, congruent mechanical sensitivity was also observed in water-drinking control mice housed in the same room (H_2O/Co-Housed/WD), and in this case, mechanical thresholds exhibited by the bystanders were significantly lower than those displayed by the primary mice in alcohol withdrawal. As with males, female mice in water but were housed in a separate room maintained stable mechanical thresholds for the 3 weeks of testing (H_2O/Separate; Fig. 2C). These data demonstrate that, following voluntary drinking in both male and female mice, episodes of acute withdrawal lead to reduced mechanical thresholds in both alcohol-withdrawn and water-consuming control mice housed in the same room.

To further investigate nociceptive responsiveness in this paradigm, we assessed thermal sensitivity in an additional set of male mice by immersing the tips of their tails into a 46°C water bath. As with mechanical thresholds, both the EtOH/Co-Housed and H_2O/Co-Housed groups demonstrated significant hypersensitivity (decreased withdrawal latency) compared to H_2O/Separate mice by the second 24-hour withdrawal session (Fig. 2D). Thus, alcohol-withdrawn and bystander mice display abnormal responses to non-noxious mechanical and thermal stimuli.

Additional experiments were conducted to further characterize hyperalgesia in both the primary (alcohol-exposed) and bystander (water-drinking) mice. First, we verified that the mechanical hypersensitivity in the Co-Housed groups was related specifically to withdrawal from ethanol and not merely the consumption of ethanol or the presence of ethanol-related olfactory and/or behavioral cues. In this experiment, we gave constant ethanol access [EtOH/Co-Housed/no withdrawal (NoWD)] to an independent set of mice. Neither this group nor water-drinking mice housed in the same room (H_2O/Co-Housed/NoWD) displayed changes in mechanical sensitivity at any point (Fig. 2E). The lack of changes in nociceptive thresholds indicates that alcohol-drinking mice do not primarily demonstrate alcohol-related neuropathy (*21*) at these time points because the displayed hypersensitivity is contingent upon withdrawal. These data further indicate that the hypersensitivity displayed by water-drinking mice cannot be attributed to the odor of alcohol, presence of alcohol metabolites, or the cues related to behavioral intoxication in the alcohol-drinking mice. Thus, the behavior in both groups is specific to the hypersensitivity experienced during alcohol withdrawal.

Next, we tested recovery of normal mechanical responses in Co-Housed alcohol- and water-drinking groups. Access to ethanol was discontinued after the third withdrawal session, and nociceptive thresholds were tested daily for the next 7 days. Nociceptive thresholds returned to basal levels in both ethanol groups (EtOH/Co-Housed/WD and EtOH/Separate/WD) over the course of 4 days, and recovery from hypersensitivity in the H_2O/Co-Housed group resembled that of the two ethanol-drinking groups (fig. S2). Mice in the H_2O/Separate control group remained at baseline throughout this period (fig. S2). These findings indicate that a continued signal from the ethanol-withdrawn animals is required to maintain hypersensitivity in the bystander animals.

We also tested whether familiarity between mice contributed to the development of congruent hyperalgesia in bystanders. Accordingly, C57BL/6J mice were used as the primary group (EtOH/Co-Housed/WD-Familiar), and we investigated whether two groups of bystanders would develop congruent hyperalgesia. The first group consisted of C57BL/6J mice (H_2O/Co-Housed/Familiar) that arrived in the same shipment as the primary mice, and the second bystander group consisted of wild-type (WT) C57BL/6J mice from our animal colony (H_2O/Co-Housed/Stranger). All groups developed mechanical hypersensitivity following the first withdrawal session (fig. S3), indicating that unfamiliar stranger mice display the same level of socially transferred hypersensitivity as mice that are familiar with each other.

Hyperalgesia is communicated to bystanders via olfactory cues

The lowered nociceptive threshold exhibited by the bystander mice suggests that these mice acquired hypersensitivity due to cues within the social environment. To determine the sensory channel mediating this communication, we used the alcohol withdrawal paradigm and assessed the ability of olfactory cues to provoke hyperalgesia. Accordingly, a group of naïve animals housed in a separate room were exposed to bedding from the primary and bystander (Co-Housed) mice; that is, following a single session of withdrawal, and daily for the next week (during drinking and the second withdrawal session; Fig. 3A), small amounts of bedding from EtOH/Co-Housed/WD and H_2O/Co-Housed mice, which both displayed hypersensitivity, were placed in empty cages without cagetops in a separate room containing control mice (H_2O/Olfactory-WD). Exposure to bedding from the hypersensitive Co-Housed mice induced significant mechanical hypersensitivity in the otherwise treatment-naïve mice within 24 hours (H_2O/Olfactory-WD; Fig. 3B). This hypersensitivity cannot be attributed merely to cues associated with novel mouse bedding because exposure to bedding from unfamiliar but experimentally naïve mice had no effect on the behavior of a separate group of water-drinking mice housed in an adjacent room (H_2O/Olfactory-CTRL; Fig. 3B). This finding demonstrates that olfactory cues released into the social environment by mice experiencing hyperalgesia are sufficient to rapidly provoke congruent hypersensitivity in nearby mice.

Alcohol-withdrawn and bystander mice demonstrate nonsynchronous hyperalgesia

To further confirm that the abnormal nociceptive responsiveness in alcohol-withdrawn and bystander mice represents hyperalgesia, we administered a noxious chemical stimulus to mice that had previously demonstrated mechanical hypersensitivity. Therefore, at the completion of the mechanical testing, subsets of mice from previous experiments (n = 6 to 8; Figs. 2D and 3B) were subjected to the formalin test (*22*, *23*). Briefly, formalin was injected into the plantar surface of the hind paw, and nocifensive paw-licking behavior was quantified during the two phases of the formalin test. A low concentration of formalin (1.5%) was used to avoid ceiling effects. We found that all groups that previously displayed mechanical

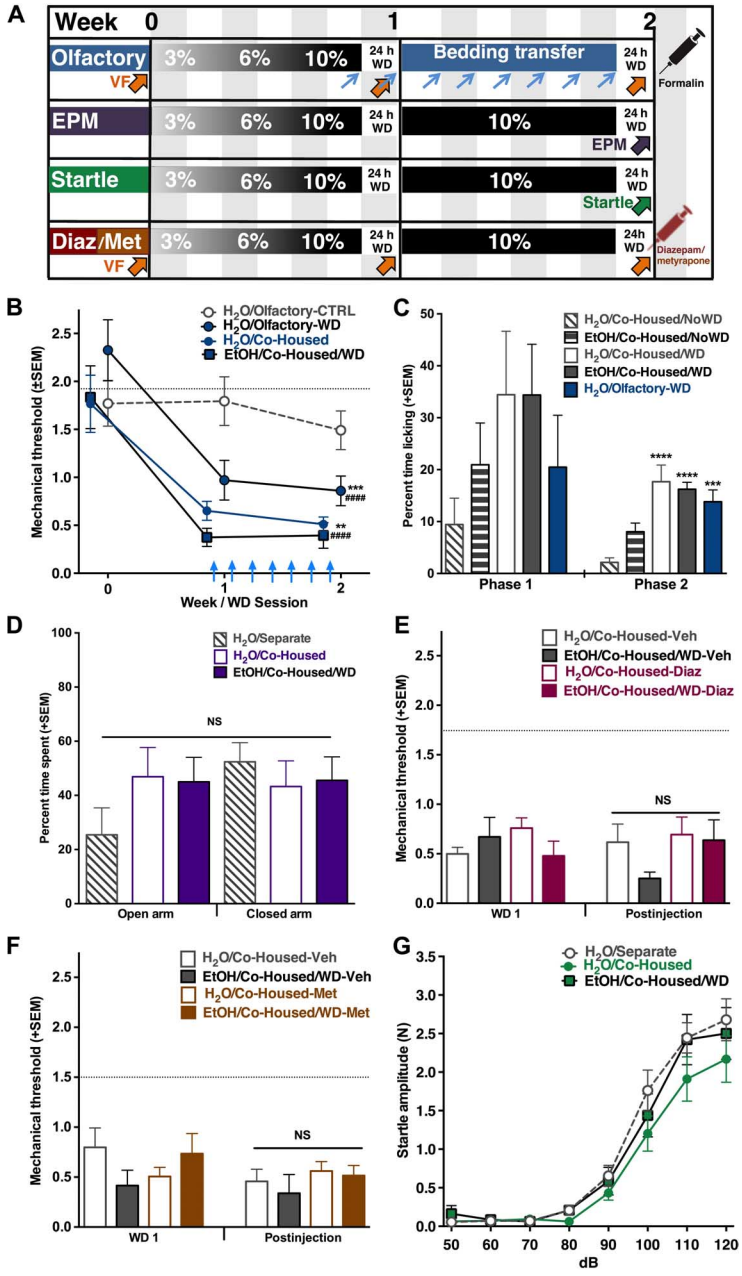

Fig. 3. Social transfer occurs via alcohol withdrawal–specific olfactory cues, and this state leads to chemical and thermal hyperalgesia. (**A**) Experimental timeline for (**B**) to (**G**). Von Frey (thick orange arrows); ethanol [EtOH, 3 to 10% (v/v)]. (B) When a group of mice housed in a separate room (H_2O/Olfactory-WD; $n = 8$) was exposed to bedding from the cages of H_2O/Co-Housed mice ($n = 9$) and EtOH/Co-Housed/WD mice ($n = 8$), they demonstrated significant decreases in mechanical thresholds within 24 hours. Mice exposed to bedding from naïve water-drinking mice maintained baseline levels of sensitivity (H_2O/Olfactory-CTRL; $n = 16$). H_2O/Co-Housed and EtOH/Co-Housed/WD mice began the experiment 1 day before H_2O/Olfactory-WD mice, and transfer of bedding is represented by thin blue arrows. Repeated-measures ANOVA revealed a significant effect of treatment ($F_{3,37} = 7.3$, $P = 0.0006$) and test session ($F_{2,74} = 26.7$, $P < 0.0001$), as well as a significant interaction ($F_{6,74} = 3.3$, $P = 0.0068$). (C) The mechanical hypersensitivity in groups of mice from the olfactory experiment (H_2O/Co-Housed, EtOH/Co-Housed, and H_2O/Olfactory-WD) and the no withdrawal experiment (Fig. 1D) manifests as hyperalgesia following a low concentration (1.5%) of formalin (black syringe) in a pattern that was significant during the second phase of the formalin test according to one-way ANOVA ($F_{4,30} = 10.19$, $P < .0001$). (D) There were no significant differences in the percent of time spent on closed or open arms for any group (H_2O/Separate, $n = 9$; EtOH/Co-Housed, $n = 9$; and H_2O/Co-Housed, $n = 9$) according to ANOVA ($P > 0.05$). (E) H_2O/Co-Housed mice ($n = 14$) and EtOH/Co-Housed/WD mice ($n = 14$) were treated with diazepam (Diaz; 1.0 mg/kg; maroon syringe; $n = 7$) or vehicle (Veh; $n = 7$) 20 min before the second von Frey test. Diazepam had no effect on mechanical thresholds in any group, according to ANOVA ($P > 0.05$). (F) H_2O/Co-Housed and EtOH/Co-Housed/WD were treated with metyrapone (Met; 50.0 mg/kg; maroon syringe) or vehicle (Veh) 20 min before the second von Frey test. Metyrapone had no effect on mechanical thresholds in any group (EtOH/Co-Housed, $n = 5$; H_2O/Co-Housed, $n = 7$) compared to vehicle (EtOH/Co-Housed, $n = 4$; H_2O /Co-Housed, $n = 8$), according to ANOVA ($P > 0.05$). (G) Acoustic startle responses did not differ between Co-Housed ($n = 8$/group) and Separate ($n = 8$) mice according to repeated-measures ANOVA ($P > 0.05$). Significant changes ($P < 0.05$) from baseline according to Bonferroni's post hoc analyses are represented by #. Significant differences compared to control ($P < 0.05$) are represented by *. Nonsignificant differences are represented by NS. Mean basal responses of all groups are represented by a dotted line.

hypersensitivity (EtOH/Co-Housed/WD, H_2O/Co-Housed, and H_2O/Olfactory-WD) also exhibited enhanced nocifensive behavior in the second phase of the formalin test compared to controls, which had exhibited normal mechanical thresholds (EtOH/Co-Housed/NoWD and H_2O/Co-Housed/NoWD). The latter groups had been directly or indirectly exposed to ethanol but never experienced withdrawal or been housed with animals that underwent withdrawal (Fig. 3C). Socially transferred hyperalgesia is thus observed across three distinct modalities of nociception (chemical, thermal, and mechanical).

To test whether the mice exhibited visually dependent emotional contagion during the formalin test, we examined the synchrony of nocifensive behaviors in mice tested within the same sessions (*7*). We estimated whether licking behavior was correlated across time within groups of six to eight mice tested within proximity of each other (fig. S4A). Licking behavior among animals tested together was not synchronized, and the between-subject variance was comparable to that of randomly grouped mice (fig. S4B). This analysis indicated that these mice do not exhibit synchronized behavior during testing.

To further determine whether the hyperalgesia demonstrated by the Co-Housed water-drinking group represented emotional contagion based on sensory cues and/or temporally matched behavior during the test session, we restored ethanol access to a separate group of animals that underwent withdrawal. Following 4 hours of ethanol access, mechanical thresholds returned to baseline in EtOH/Co-Housed/WD mice (fig. S5A), and this reversal of hypersensitivity was correlated with the amount of ethanol consumed over the 4-hour period (fig. S5B). However, hypersensitivity was not reversed in the simultaneously tested H_2O/Co-Housed animals. These results denote a lack of synchronized behavior and suggest a lack of emotional contagion because responses to chemical and mechanical stimulation were incongruent between the two groups tested within the same sessions.

Hyperalgesia in alcohol-withdrawn and bystander mice does not depend upon a concurrent state of anxiety or enhanced corticosterone levels

To determine whether the hypersensitivity exhibited by the H_2O/Co-Housed or EtOH/Co-Housed/WD mice was dependent on a state of generalized anxiety or could be described as stress-induced hyperalgesia (*11*, *24*), we conducted several independent experiments. First, we examined behavior on the elevated plus maze (EPM), one of the most widely used measures of anxiety-like behavior (*25*). The EPM consisted of two white open arms (anxiety-producing) and two black opaque high-walled arms, and the amount of time spent in each of these areas was recorded, as previously reported by our laboratory (*26*). During the second withdrawal session, no differences were observed between the groups in any measure on the EPM (Fig. 3D and fig. S6). The lack of differences suggests that the hypersensitivity displayed by both groups at this time point does not occur in conjunction with a state of ongoing anxiety.

In the next experiment, we treated groups of H_2O/Co-Housed and EtOH/Co-Housed/WD mice with a prototypical anxiolytic (diazepam; 1.0 mg/kg) or vehicle before the second mechanical test session. Diazepam had no effect on mechanical threshold in any group (Fig. 3E), although this dose of diazepam was sufficient to reverse another phenotype [handling-induced convulsions (HICs)] triggered by acute ethanol withdrawal in a separate group of mice (fig. S7) and has been previously shown to reverse anxiety-like behavior on the EPM in C57BL/6J mice (*27*). The inability of diazepam to alter the hypersensitivity exhibited in either the primary mice undergoing alcohol withdrawal or the bystander mice further argues that the presence of anxiety is not necessary for the presentation of hyperalgesia in either group. In addition, the lack of sensitivity to a pharmacologically appropriate dose of diazepam implies that the neural mechanisms underlying hyperalgesia during alcohol withdrawal and HICs are distinct.

To determine whether the hypothalamic-pituitary axis (HPA) was activated during alcohol-induced or socially transferred hyperalgesia, we examined plasma corticosterone (CORT) levels (Table 1) at several time points (Fig. 2A). Blood was taken immediately after the final pain sensitivity test session. There were no differences between groups in plasma CORT levels in separate groups of mice during one to three withdrawal sessions, following reversal of hypersensitivity after 4-hour drinking, or after 7 days of extended withdrawal (Table 1). The lack of altered plasma CORT indicates that activation of the HPA axis is not the primary underlying mechanism for the abnormal pain behavior exhibited by either mice that experience alcohol withdrawal or socially influenced bystanders. To determine whether enhanced CORT levels are required for the expression of hyperalgesia, a CORT inhibitor (metyrapone, 50.0 mg/kg) was administered to groups of H_2O/Co-Housed and EtOH/Co-Housed/WD mice before the second mechanical test session. Inhibition of CORT had no effect on mechanical threshold in any group during the second test session (Fig. 3F), suggesting that this behavior was not representative of stress-induced hyperalgesia.

Table 1. No changes between groups in plasma CORT levels at several time points. When examining plasma CORT (taken immediately postmortem) in separate groups of mice, there were no changes in the mean (±SEM) plasma CORT levels ($P > 0.05$) between groups ($n = 5$ to 12) following 1 week of drinking and one withdrawal session (WD 1), 3 weeks of drinking and three withdrawal sessions (WD 3), following restored access to EtOH during the fourth withdrawal session or after 4 weeks of drinking and four withdrawal sessions followed by 7 days of extended withdrawal (xtend), or following 30 min of restraint stress on the eighth day after recovery from hyperalgesia.

Time of sacrifice	H_2O/Co-Housed	EtOH/Co-Housed/WD	EtOH/Separate	H_2O/Separate
WD 1	—	204.4 ± 15.63	—	192.9 ± 24.91
WD 3	279.3 ± 44.57	310.7 ± 63.68	337.4 ± 37.2	381.3 ± 44.33
WD 4/restored	202.5 ± 14.07	178 ± 25.67	—	—
xtend WD	288.4 ± 22.4	319.8 ± 45.5	271.5 ± 30.39	314.8 ± 41.06
After restraint	629.4 ± 67.39	615.9 ± 55.47	—	569.3 ± 46.65

Because acute measurement of CORT does not assess stress responsivity in these mice, we tested the CORT response to 30-min restraint stress following 8 days of extended withdrawal (Fig. 2A). As expected, all groups displayed an enhancement in CORT in response to restraint stress, but there were no differences between the Co-Housed and H_2O/Separate groups in the CORT response (Table 1). This indicates that these mice demonstrate normal responses to stress, as measured by plasma CORT levels. Together, these experiments indicate that, although Co-Housed mice demonstrate mechanical, thermal, and chemical hyperalgesia, it is not dependent on a state of concurrent anxiety or simultaneous activation of the HPA axis and does not lead to long-term adaptations in the stress response.

Alcohol-withdrawn and bystander mice demonstrate normal responses to acoustic startle

Finally, it could be theorized that EtOH/Co-Housed/WD and H_2O/Co-Housed/WD groups display hyperreactivity to novel stimuli across multiple sensory systems (for example, auditory). To investigate this possibility, we examined acoustic startle responses as a measure of hyperacusis (*28*) and sensory hyperreactivity. The acoustic startle procedure consisted of exposure to 18 trials of 60- to 120-dB tones in 10-dB increments in a random order, with variable intertrial intervals. There were no differences between any of the groups in acoustic startle responses (Fig. 3G), indicating that EtOH-withdrawn and bystander mice do not demonstrate hyperacusis or an exaggerated response to a novel, startling stimulus. This finding shows specificity of this phenotype to pain-related systems and argues against an overall sensory hyperreactivity.

DISCUSSION

Our findings reveal that exposure to olfactory cues from primary mice experiencing hyperalgesia can trigger hyperalgesia in mice housed and tested in the same environment (bystanders). These bystander mice demonstrate hypersensitivity that does not require injury or noxious stimulation but that is acquired following exposure to olfactory cues in the social environment. Under the current experimental conditions, this phenomenon reliably occurs during multiple pain states, including local inflammation (CFA) and hypersensitivity during drug withdrawal (morphine- or alcohol-induced). This socially transferred hyperalgesia can be measured by standard mechanical, thermal, and chemical pain tests. Furthermore, we demonstrate that the phenomenon of social transfer can occur via an olfactory mechanism because 24 hours of exposure to bedding from hyperalgesic mice was sufficient to induce hyperalgesia in otherwise naïve mice. However, we cannot eliminate the possibility that other sensory modalities could also play a role. By examining the social communication of pain, these findings highlight the importance of environmental and social variables in conducting and interpreting preclinical pain research. At the same time, they help elucidate the relationship between alcohol abuse and pain.

The hyperalgesia demonstrated by bystanders is nearly identical to that seen in animals subjected to withdrawal (from either an opioid or an alcohol) but is not as severe as that seen in mice subjected to persistent localized inflammation induced with CFA. This indicates that differences among groups can be maintained in some paradigms and may be related to the magnitude of hyperalgesia in the primary animals. The magnitude of socially transferred hypersensitivity was greater in female compared to male bystanders. This is intriguing because females demonstrate higher levels of empathy than males (*29*), and thus, social transfer may play a role in the overrepresentation of females in many chronic pain conditions such as migraine and fibromyalgia (*30*). However, the current studies exclusively examined reflexive responses and did not investigate whether the pain experience (which includes emotional components) is identical in these groups of mice, and therefore, it will be important to compare the affective states of bystander mice in future studies.

It is well known that social and environmental factors influence pain in humans, and these variables have also been shown to modulate pain behaviors in preclinical models, leading to analgesia (*9*) or hyperalgesia (*5*, *7*, *8*), depending on the paradigm. As such, the social communication of pain has also been explored in the form of emotional contagion, which is considered an endophenotype of empathy (*31*). For example, Langford and colleagues (*7*) showed that, when pairs of mice are given identical noxious stimuli and tested together, they display increased pain behaviors compared to being tested alone or with another mouse that has not received the noxious stimulus. This "social modulation of pain" was dependent on visual cues and the familiarity of the dyads. These findings have been extended with the recent observation that mice housed for several weeks in the same cage as conspecifics subjected to peripheral nerve injury exhibit enhanced responses in the acetic acid–induced writhing test (*10*). This behavior appeared to represent a form of stress-induced hyperalgesia (*11*), but the sensory channel mediating this social communication of pain was not investigated. These studies indicate that the presence of a conspecific in pain can have a physiological and behavioral effect through social cues. The current results differ from previous findings (*7*, *8*) in that the hypersensitivity exhibited by bystander animals is not associated with emotional contagion acquired via visual cues (*7*) nor does it represent modification of an existing pain state (*5*, *7*, *9*). Specifically, previous studies have relied on a nociceptive trigger and contemporaneous visual cues or explicitly stressful stimuli, whereas the current results demonstrate a socially induced pain state that occurs in the absence of tissue damage, visually dependent emotional contagion/synchronous behavior, concurrent anxiety, or simultaneous activation of the HPA axis.

The present studies also support the idea that other sensory modalities (beyond visual cues) are likely to play a role in the long-range social communication of pain. It has been previously demonstrated that olfactory cues can act as the channel of social communication because exposure to chemical cues from tumor-bearing mice leads to behavioral and neuroimmune changes in cagemates (*32*). In humans, fear-related chemosignals can influence associative learning (*33*, *34*). Although the social modulation of behavioral and physiological states through chemical communication has been documented, the olfactory communication of pain has not been studied extensively. However, olfactory cues, like visual cues, can communicate information capable of altering nociceptive response. For example, rats display analgesia following exposure to olfactory chemosignals from a conspecific that had received an electric shock (*4*), indicating the activation of endogenous pain control mechanisms following a social-olfactory cue. In addition, neuropathic pain behavior can also be increased by cohousing with rats that exhibit high levels of neuropathic pain behavior following nerve injury (*5*). Nevertheless, the current studies differ from previous research because we display the long-range olfactory communication (throughout a room, rather than within a cage) of hyperalgesia.

Olfactory cues in the social environment have been shown to induce physiological and behavioral changes that are not accompanied by measurable changes in CORT or a concurrent state of anxiety [as assessed by standard measures such as EPM (*32*)]. Although others have reported a social influence on pain as a form of stress-induced hyperalgesia (*10*), the hyperalgesia observed in bystanders in the present studies was not contingent upon a simultaneous state of anxiety, enhanced CORT levels, or long-term changes in stress-induced activation of the HPA axis. This follows from the absence of altered CORT levels, the inability of a CORT inhibitor or an anxiolytic to attenuate the expression of mechanical hypersensitivity, the lack of changes in the EPM and acoustic startle behavior, and the normal response to restraint stress. That said, we cannot rule out the possibility that the HPA axis is activated or anxiety is present in the bystander animals at some point during hyperalgesia. For example, the hyperalgesia displayed by bystanders could be triggered by stress, leading to neuroadaptations that maintain hyperalgesia in the absence of ongoing HPA axis activation (*35*, *36*). In summary, we were unable to demonstrate evidence for the involvement of the HPA axis or anxiety in the expression of hyperalgesia in primary and bystander mice. However, our studies do not fully negate the involvement of stress and/or anxiety in the social transfer of hyperalgesia at some point (for example, during acquisition of hyperalgesia).

The lack of changes in the response to restraint stress in the present experiments agrees with the lack of evidence for increased anxiety during withdrawal from voluntary alcohol self-administration in mice (*37*). Drinking in the standard two-bottle choice procedure in mice has been argued to be a poor model of alcoholism, in part because of the lack of overt signs of pathological effects after prolonged history of drinking (*38*). The observation of hyperalgesia displayed during abstinence from voluntary drinking in the present study provides a potentially translational sign of withdrawal following that developed within a single week of alcohol drinking in the two-bottle choice procedure. Previously, hyperalgesia during alcohol withdrawal has only been demonstrated in the rodent after prolonged self-administration (*39*), forced alcohol exposure (*17*, *40*–*42*), or dependence-inducing escalated drinking procedures (*43*–*45*). Thus, we speculate that previous studies did not detect hypersensitivity during withdrawal from standard two-bottle choice drinking because it was communicated via olfactory cues to nearby water-drinking mice, the typical control group. This social transfer could obscure any between-group differences. Regardless, the current observations illuminate the relationship between alcohol abuse and pain disorders, which has been amply demonstrated in humans (*46*) but understudied in animal models despite apparent similarities in neuroanatomical substrates (*17*). For some individuals, alcohol abuse precedes the development of chronic pain, whereas in others, alcohol consumption occurs as a mechanism for coping with chronic pain. Moreover, chronic drinking can lead to severe pain during and following the withdrawal process (*47*, *48*). Although pain is often reported as a symptom of withdrawal in humans, it has never been reported following voluntary drinking in the C57BL/6J mouse. Finally, the short time course used in the current studies and the lack of changes in nociceptive response in the absence of withdrawal indicate that the hyperalgesia seen in alcohol-drinking mice does not represent alcohol-induced neuropathy. In summary, the current studies provide additional evidence for the relationship between pain and alcohol use, which has previously been theoretically considered (*17*, *18*).

The current findings also have broader methodological implications for rodent studies. It is common for experimental groups to be housed and tested with or near their respective comparison groups to control for environmental confounds. The present findings demonstrate that a physiologically relevant behavioral state can be transmitted between rodents housed throughout a room via olfactory cues. Although the experimental conditions used here may have maximized the potential for social transfer via an olfactory channel (cages had wire tops with no filter lids to permit access to drinking bottles, and the mice were tested in the room in which they were housed), the manner in which the experimental animals are housed and tested should be considered as a factor in the experimental design. Our findings expand the concern raised by a recent study, which has suggested that mice undergoing neuropathic pain can induce hypernociception in cagemates (*10*). It will be important in future studies to determine the various environmental and test conditions in which social transfer of pain occurs. For example, it is possible that filter tops or cage filtration could reduce the exposure to olfactory cues and, in turn, attenuate the development of hyperalgesia in bystanders.

The current studies elucidate the complex relationship between social-environmental cues and pain behavior while supporting the use of rodents as models for understanding the multidimensional aspects of chronic pain and alcoholism. Finally, further investigation of the social transfer of pain may prove to be relevant to chronic pain disorders in human patients that have no obvious noxious cause and are highly influenced by social and environmental factors.

MATERIALS AND METHODS

Animals

A total of 289 adult C57BL/6J mice were used in all experiments ($n = 7$ to 16 per group), with the exception of two experiments. The first was conducted to examine HICs in male DBA/2J mice ($n = 12$), and in the second, 10 male mice from our animal colony were used as the bystander comparison group (see description of "Familiarity" experiment below). Male mice were used in all experiments, with the exception of the experiment represented in Fig. 1C, in which females were used. The C57BL/6 mouse strain was chosen because this strain voluntarily drinks high levels of alcohol (*49*), is highly sociable, and is sensitive to the social transfer of fear (*50*). Mice were delivered from the Jackson Laboratory at 7 to 8 weeks of age, housed (three to five per cage), and spent at least 1 week acclimating to our colony room (12:12 schedule; lights on: 6:00 a.m.) before being individually housed and transferred to the experimental room (12:12 schedule; lights off: between 9:30 a.m. and 10:30 a.m.) for an additional 7-day acclimation period before the initiation of the experiment. For all experiments, mice were housed in cages containing wire cagetops and no filter lids in a temperature- and humidity-controlled environment with ad libitum access to food (LabDiet 5001; LabDiet) and tap water. All protocols were approved by the Oregon Health and Science University animal care and use committee and performed within the National Institutes for Health Guidelines for the Care and Use of Laboratory Animals as well as the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research.

Experimental rooms

Four separate experimental rooms were used in the current studies. These rooms exist within an isolated 70 sq. m suite and were connected

via a common hallway containing a sink and supplies. Each room had an adjustable light cycle and was enclosed from the common room by a door. For each experiment, treatment groups were rotated among physical rooms in the suite to prevent room-specific environmental factors from confounding the results. For all sets of experiments, one room contained "Co-Housed" experimental and "bystander" control (vehicle-treated or water-drinking) groups, and adjacent rooms contained "Separate" control groups of mice tested concurrently. Overall, there was no effect of any single housing room on behavior because the behaviors were predictable according to the treatment/social condition and were unaffected by the physical room the experiment took place in.

Cage details

Mice were individually housed in standard polycarbonate "shoebox" cages (18.4 cm W × 29.2 cm D × 12.7 cm H) with wire cagetops and no filter lids. Bedding was fresh at the beginning of the experiment and was not exchanged during the course of the experiment. Within each room, individual cages were placed 5 to 15 cm apart on metal housing racks. A range of 8 to 64 mice were housed in a single room during a given experiment, although in most cases, only 8 to 24 mice were in a single room. The number of mice in a room did not lead to any obvious changes in the measured behaviors.

Drugs

CFA contained 1 mg of *Mycobacterium tuberculosis* (H37Ra, American Type Culture Collection 25177) per milliliter of emulsion in 85% paraffin oil and 15% mannide monooleate. The vehicle in this experiment was the same volume of PBS. Morphine base (300 mg/kg) was delivered subcutaneously in an emulsion that consisted of 50 mg of morphine base suspended in 0.1 ml of Arlacel A (mannide monooleate), 0.4 ml of light liquid paraffin, and 0.5 ml of 0.9% (w/v) NaCl, and the vehicle for these experiments was the suspension lacking morphine. Naloxone (10.0 mg/kg; Sigma) was dissolved in saline and injected intraperitoneally. Solutions of ethanol (EtOH) for drinking (w/v) were prepared from 95% ethyl alcohol in tap water for drinking and in saline for injection [20% (v/v)]. For acute EtOH withdrawal, a dose of 4 g/kg was injected intraperitoneally. Diazepam (Sigma) was injected intraperitoneally at a dose of 1.0 mg/kg. Diazepam was dissolved in Tween 20 until it produced a clear solution and was then diluted with saline. The final concentration of Tween 20 in the solution was 1%. The vehicle used in the diazepam experiment contained 0.9% saline with 1% Tween 20. Formalin was made from paraformaldehyde (PFA; Sigma) and diluted into PBS for a final concentration of 1.5% formalin or 0.56% PFA.

Noxious stimuli

CFA-induced inflammatory pain.

To examine the social transfer of chronic inflammatory pain, mice were housed in two adjacent rooms, tested for basal mechanical thresholds to von Frey stimulation, and then lightly restrained and immediately injected with either PBS (PBS/Co-Housed or PBS/Separate) or 10 μl of CFA (CFA/Co-Housed) into the intraplantar surface of the left hind paw, which is known to reliably induce long-lasting pain (*51*). Mice were then tested on days 3, 5, 11, and 14 after injection.

Morphine withdrawal.

To determine whether withdrawal from a drug of abuse would lead to the social transfer of pain, mice were individually housed and tested in two neighboring rooms (Co-Housed or Separate). Mice were tested for basal mechanical sensitivity to von Frey stimulation of the hind paw. Immediately following the baseline test, mice were injected with either a slow-release morphine base (300 mg/kg, Mor/Co-Housed/WD, n = 8) or vehicle suspension lacking morphine (Veh/Co-Housed/WD, n = 8 or Veh/Separate, n = 8). Forty-eight and 96 hours after injection, mice were tested again and then injected with their assigned treatment. Immediately following the final test on day 5, all mice were injected with naloxone (10 mg/kg) and rated for morphine withdrawal–related behavior such as jumping, wet dog shakes, paw tremor, and diarrhea by an experimenter who was blind to treatment assignments during scoring.

Alcohol withdrawal.

To examine whether withdrawal from alcohol resulted in increased pain sensitivity and its social transfer, mice were given continuous access to two bottles: one containing water and the other containing a solution of EtOH. Once weekly (2 hours into the dark cycle), EtOH bottles were removed and replaced with bottles containing water for 24 hours. Thus, for the first week of drinking, all mice received 3 and 6% EtOH each for 2 days and 10% EtOH for 1 day followed by 24 hours of withdrawal. On each following week (in relevant experiments), the mice were allowed access to 10% EtOH for 6 days followed by 24 hours of withdrawal.

Pain tests

Mechanical sensitivity.

Responses to mechanical stimulation by von Frey hairs (0.01 to 2 g of plastic fibers) were determined in the plantar surface of the left hind paw. Normal response was considered as withdrawal, shaking, or licking of the paw. Mechanical thresholds were tested using the up-down technique (*52*). This method uses stimulus oscillation around the response threshold to determine the median 50% threshold of the response. Mice were allowed to acclimate to the plexiglass enclosure on top of a wire testing rack for 40 min on 2 days before the start of the experiment and for 10 to 20 min before each test session. All testing occurred during the dark cycle, with illumination via a dim red lamp. A standard testing rack was placed on top of a cart/table, and consisted of 50-cm posts holding a 91.4-cm × 50-cm platform that contained 6.35-mm metal mesh flooring. Experimental boxes were made of clear plexiglass (length: 20.3 cm × width: 20.3 cm × height: 15.2 cm split into four quadrants). The testing rack was located on the top of a table within each testing room near the housing rack and illuminated with a dim red lamp. Mechanical sensitivity was assessed before treatment exposure (baseline), and mice were then assigned to treatment group based on the basal mechanical thresholds. All experimental timelines are detailed in panel A of each Figure. Preliminary research determined that, following 4 to 5 weeks, social isolation had a significant effect on mechanical threshold (fig. S8). All mechanical testing was conducted by a single experimenter. During testing, the experimenter was blind to the individual treatment assignments within each room.

Thermal sensitivity.

Mice were tested for thermal nociceptive sensitivity at baseline and during two weekly withdrawal sessions using the heat-evoked tail withdrawal reflex. Two days before the first test session, mice were habituated to handling (light restraint in a soft cloth), and the tip of their tail (5 cm from the end) was immersed into room temperature water. On the test days, mice were lightly restrained, and the tail was submerged into 46°C water to detect the response (flicking the tail

out of water), which provided baselines of approximately 15 s. Two tail withdrawal measurements were taken 10 min apart and averaged for a single data point for each animal. A stopwatch was used to determine the latency to flick the tail (*53*). All mice from this experiment were also used in the restraint stress experiment (described below).

Chemical sensitivity.

A subset of mice from (i) EtOH/Co-Housed/WD and H_2O/Co-Housed, (ii) H_2O/Olfactory-WD, and (iii) H_2O/Co-Housed/NoWD and EtOH/Co-Housed/NoWD groups (see Experimental procedures) received a formalin test following the second 24-hour withdrawal session. Immediately following the final mechanical test, mice were injected with 1.5% formalin (Sigma) into the plantar surface of the left hind paw. A low dose of formalin was chosen to avoid a potential ceiling effect. Following injection, the mice were placed into individual plexiglass chambers on the testing rack and digitally videotaped for 60 min for later analysis. Because no nocifensive behaviors were demonstrated between 46 and 60 min, these time points were excluded from the analysis. Using a stopwatch, an experimenter who was blind to the group assignment sampled video files for 5 s at 1-min intervals for pain behavior. Nocifensive behavior was defined as licking/biting of the injected paw. These data were analyzed as percent time spent licking during every 5-s interval. The first phase was defined as 0 to 5 min after injection, and the second phase was defined as 11 to 45 min after injection. To determine synchrony of licking behavior [as described elsewhere (*7*)], we calculated all possible correlations between mice tested during the same session that were in visual range of each other. This led to three to five correlations per mouse, depending on testing conditions, because six to eight mice were tested during each experimental run. We then took the average of those correlations (R; fig. S4) and calculated the grand average of $R \pm$ SD across the three experimental runs ($R = 0.107 \pm 0.22$). The data were then permuted 100 times, creating random pairings of mice and allowing for calculations of the grand mean and SD for these data. We found that the actual SD of the mice tested together was not significantly different from that of randomly grouped mice (permuted data; $R = 0.108 \pm 0.007$, $P = 0.97$), suggesting a lack of synchrony in licking behavior.

Experimental procedures

Ethanol intake procedures.

During the 7-day acclimation period, mice received 24-hour access to two bottles with metal sipper tubes (containing water) on either side of the cage, with food evenly distributed along the wire cage top. Following acclimation and/or baseline testing, mice either received access to two bottles of water only (H_2O mice) or one bottle of water and one bottle of alcohol (EtOH mice). For the 24-hour access, two-bottle choice, EtOH mice received 24-hour access to two bottles: one containing tap water and the other one containing increasing concentrations of EtOH (3 to 10%) that was dissolved in tap water. Both 3 and 6% were available for 2 days, after which the animals had access to 10% EtOH for the remainder of each experiment. Fluid levels from each of the two bottles were recorded on a daily basis during the second hour of the dark cycle. The locations of the bottles on the cages (left versus right) were alternated every other day to avoid the potential confound of an inherent side preference. Further, when multiple treatment groups were housed in a single room, the treatment was randomly assigned across the cage locations to avoid any confound related to the treatment of neighboring cages.

No withdrawal.

To examine whether the mere presence of (i) alcohol cues in the room or (ii) cues related to the behavior of intoxicated neighbors was enough to elicit mechanical hypersensitivity in the water-drinking mice, we co-housed water-drinking mice with an EtOH-drinking group that did not experience any forced abstinence (H_2O/Co-Housed/NoWD and EtOH/Co-Housed/NoWD; Fig. 2B). Mechanical testing occurred on the 7th and 14th days, 2 hours into the dark cycle. This experiment was conducted at the same time as the olfactory experiment (Fig. 3B, described below), and these mice were subjected to the formalin test (described below) immediately following their final mechanical test.

Olfactory stimuli.

To examine the sensory method of social transfer, three neighboring rooms were used. One room contained EtOH/Co-Housed/WD and H_2O/Co-Housed mice (Fig. 2, A and D). In two adjacent rooms, mice were given access to water only (H_2O/Olfactory). The Co-Housed mice (which received either ethanol and water or water only) began their schedule 1 day before the H_2O/Olfactory mice, and thus had been in 24 hours of withdrawal when the first bedding was collected. This experiment followed the same timeline as all other 2-week experiments, with the exception that, on the seventh day, dirty bedding was removed from the cages (~5 g per cage) of all mice in the Co-Housed room—for example, EtOH/Co-Housed/WD and H_2O/Co-Housed/WD mice ($n = 32$ per group; both groups displayed hypersensitivity at this time)—or from the cages of water-drinking mice in the animal colony ($n = 45$). Bedding from each set of mice (Co-Housed or colony; ~100 to 150 g total/day) was mixed and placed into three empty cages with wire cagetops. The three cages that contained bedding from Co-Housed mice were set (evenly spaced) on the housing rack of one of the rooms containing water-drinking mice (H_2O/Olfactory-WD). As a control for novel mouse bedding cues, the three cages that contained dirty bedding from mice in the animal colony were placed on the housing rack of water-drinking mice in the final room (H_2O/Olfactory-CTRL). Bedding from both sets of mice (Co-Housed and colony) was continually removed, combined, and placed into these cages each day for 1 week. This was done to match the experience of continuous exposure to olfactory cues experienced in the Co-Housed room. H_2O/Olfactory-WD and H_2O/Olfactory-CTRL mice were tested for mechanical sensitivity 24 hours after the first bedding exposure and 1 week later. The Co-Housed/Olfactory-CTRL experiment was run twice in two separate rooms to ensure the reliability of this effect. There were no statistical differences between the groups in the first and second experiments; thus, these were combined to create single groups of 16 mice.

Elevated plus maze.

To explore the possibility that anxiety was present in Co-Housed mice, we examined EPM activity in groups of Co-Housed mice (H_2O- and EtOH-drinking) and H_2O/Separate mice following the second 24-hour withdrawal session. Testing occurred in the experimental/housing rooms. The EPM apparatus (Med Associates Inc.) consisted of two black opaque high-walled arms and two white open arms (51 cm long × 8 cm wide) elevated 60 cm off the ground. Small lamps were placed over the open arms, and the closed arms remained unlit, resulting in respective lux values of 95 and 2. Mice were placed in the center platform facing a closed arm, and the following variables were scored live by an experimenter who was blind to the treatment group assignment during a 5-min test: entries and time spent in open arms, closed arms, and rearing

behavior, grooming, urination, and fecal boli. Between each session, the EPM was cleaned with water and a sponge and thoroughly dried with paper towels. Data were presented as percent time spent or number of occurrences (+SEM).

Diazepam treatment.

In a separate group of Co-Housed (H_2O- and EtOH-drinking) mice, following baseline testing, subjects were counterbalanced into four groups: H_2O mice that received vehicle (H_2O/Co-Housed-Veh) or diazepam (H_2O/Co-Housed-Diaz) and EtOH mice that received vehicle (EtOH/Co-Housed/WD-Veh) or diazepam (EtOH/Co-Housed/WD-Diaz). For habituation, saline injections were given immediately before the first test session (in 24-hour withdrawal). Following the second 24-hour session of withdrawal, mice were weighed, injected, and placed on the testing rack. The mechanical test took place 20 min later (Fig. 3, A and E).

Handling induced convulsions.

To verify that a dose of diazepam (1.0 mg/kg) would successfully reverse another commonly used alcohol withdrawal phenotype (*54*), we examined the ability of diazepam to attenuate HICs in DBA/2J mice, which reliably display this behavior (fig. S7) (*55*). We used this strain of mice because C57BL/6J mice (used in all other experiments) do not reliably display this behavior [HICs (*56*)], yet display similar anxiolysis as DBA/2J in both the EPM and light-dark box following a dose of diazepam (1.0 mg/kg) (*57*). In addition, the same dose of diazepam actually leads to lower brain concentrations in DBA/2J mice compared to C57BL/6J mice, suggesting that C57BL/6J mice should be more sensitive to the same dose of diazepam (*58*). Following an intraperitoneal injection of EtOH (4.0 g/kg), DBA/2J mice were scored for HICs, as reported in detail elsewhere (*59*). Individual baselines were subtracted from HIC scores, and data were shown as mean (±SEM) group response across time (hours).

Acoustic startle.

To test for auditory hypersensitivity, we conducted a separate experiment in which acoustic startle responses were investigated on the second withdrawal session (Fig. 3G). The same drinking/withdrawal protocol was used, as described for all other alcohol-drinking experiments, with the following exceptions: on the second withdrawal session (24 hours after removal of EtOH bottles), mice were removed from home cages and placed into the acoustic startle chambers (Kinder Scientific) present in the housing/testing room. For the first 5 min, mice were not subjected to any tone (habituation). All tones were separated by random intertrial intervals (15 to 30 s). Following habituation, the session began (and ended) with three no-tone trials. Following the three no-tone trials, 60- to 120-dB tones were played (10-dB increments) in a randomized order for a total of 24 trials. Data were plotted as group mean ± SEM of acoustic startle response to increasing intensity tones.

Restraint stress.

Mice from the tail immersion experiment were allowed 1 week of recovery in their experimental/housing room. On the eighth day after the last tail immersion test, mice were removed from home cages and placed in standard plexiglass restrainer tubes on a table in their respective housing rooms for 30 min. Immediately following removal from the restraint devices, mice were sacrificed via CO_2 inhalation, and trunk blood was taken for CORT analysis.

Familiarity.

To test for the potential effect of familiarity between mice on social transfer of pain, we used the alcohol withdrawal paradigm and measured mechanical sensitivity at baseline and following a single withdrawal session. Mice were assigned to three groups, with one primary group and two bystander groups. Accordingly, 24 C57BL/6J mice from the Jackson Laboratory were assigned to receive either primary (EtOH/Co-Housed/WD-Familiar; n = 9) or bystander (H_2O/Co-Housed/Familiar; n = 15) treatment. These mice arrived in the same shipment, and three to five mice per cage were housed for a maximum of 1 week upon arrival at our facility. In addition, it is likely that at least some of these mice were also cagemates and/or littermates in the supplier's colony. The third group consisted of "Stranger" mice from our animal colony. These mice received bystander treatment (H_2O/Co-Housed/Stranger; n = 10) and were housed and tested in the same room as the mice ordered from the Jackson Laboratory. The Stranger mice were WT littermates from our urocortin 1 knockout colony (*19*). These mice were originally generated on a 129X1/SvJ × C57BL/6J background and then backcrossed onto C57BL/6J background in our colony for at least 17 generations. There were no statistical differences between the behaviors of WT Stranger and Jackson mice during baseline or withdrawal; thus, all animals were included in analysis.

CORT analysis.

Immediately after the final mechanical test or following 30-min restraint stress, mice were sacrificed via CO_2 inhalation, and trunk blood was collected for CORT analysis (Table 1). Samples were kept on ice and then centrifuged, and plasma was removed and stored at −20°C until analyzed. CORT was assayed using a commercially available radioimmunoassay kit (MP Biomedicals), with plasma samples diluted at a ratio of 1:200 and run in duplicate. The intra-assay coefficient of variation was 4.67%, and the inter-assay coefficient of variation was 5.5%.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/10/e1600855/DC1

fig. S1. Naloxone precipitates withdrawal behaviors in morphine-treated mice.
fig S2. Recovery of mechanical thresholds returns to baseline after 4 days of extended withdrawal.
fig S3. Stranger mice develop socially transferred hypersensitivity.
fig S4. Nonsynchrony of nocifensive behavior in primary and bystander mice.
fig S5. Alcohol access reverses mechanical hypersensitivity in EtOH-withdrawn mice.
fig. S6. No differences in behavior on EPM.
fig. S7. Diazepam attenuates HICs following acute EtOH withdrawal.
fig S8. Four to 5 weeks of isolation/individual housing leads to mechanical hypersensitivity.
table S1. Average mechanical thresholds and alcohol intake.

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