NEUROSCIENCE

# Olfactory exposure to late-pregnant and lactating mice causes stress-induced analgesia in male mice

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**In an attempt to improve reproducibility, more attention is being paid to potential sources of stress in the laboratory environment. Here, we report that the mere proximity of pregnant or lactating female mice causes olfactory-mediated stress-induced analgesia, to a variety of noxious stimuli, in gonadally intact male mice. We show that exposure to volatile compounds released in the urine of pregnant and lactating female mice can themselves produce stress and associated pain inhibition. This phenomenon, a novel form of female-to-male chemosignaling, is mediated by female scent marking of urinary volatiles, such as *n*-pentyl-acetate, and likely signals potential maternal aggression aimed at defending against infanticide by stranger males.**

## INTRODUCTION

Mice can discriminate conspecifics and their characteristics via urinary odor cues (*1*) and use those cues for signaling purposes. There are many well-studied examples of adult, intraspecies male-to-female and female-to-female chemosignaling affecting reproductive and other behaviors (e.g., the Bruce, Whitten, Lee-Boot, and Vandenbergh effects); far fewer examples of female-to-male signaling have been documented in rodents [see (*2*, *3*)]. There is also a relative paucity of examples of intersex chemosignaling unrelated to sexual behavior (i.e., reproductive physiology and social attraction). In one intriguing example, a recent study (*4*) described the ability of female mouse lacrimal (tear) fluid to inhibit aggressive behavior in males.

It is commonly assumed that such social signaling is not relevant to standard biomedical experimentation, which has been historically performed overwhelmingly on male subjects (*5*). However, olfactory cues in the laboratory testing environment can robustly influence experimental results. For example, we previously demonstrated that mice of both sexes can respond to male (including human male)–associated olfactory cues with a stress response, leading to stress-induced analgesia (pain inhibition) (*6*). In the process of testing successive cohorts of mice for pain sensitivity, we observed that male mice behaved unusually if pregnant/lactating female mice (being used in a separate experiment) were nearby in the laboratory. Here, we demonstrate stress and stress-induced analgesia in male mice exposed to late-pregnant and lactating female mice, representing both a novel example of female-to-male chemosignaling (with an unexpected urinary volatile mediator) and an unanticipated potential confound of preclinical experiments.

## RESULTS

### Exposure of male mice to pregnant or lactating female mice produces pain inhibition

Mice of both sexes were tested for sensitivity to radiant heat applied to the hind paws immediately before and 30 min after the introduction of a stimulus conspecific to the testing room. The stimulus conspecific—housed in isolation in a lidless cage placed 5 to 30 cm from subject mice individually confined to perforated Plexiglas testing cubicles—was either a single: (i) adult, reproductively naïve male mouse, (ii) adult, reproductively naïve female mouse, (iii) pregnant female mouse in the first 10 days of pregnancy (i.e., early pregnant), (iv) pregnant female mouse in the last 4 days of pregnancy (i.e., late pregnant), (v) lactating female mouse (with pups present), (vi) lactating female mouse (without pups present), (vii) lactating female mouse 24 hours after removal of pups, or (viii) previously pregnant mouse within 7 days after weaning. Figure 1 (A and B) shows hind paw withdrawal latencies before and after exposure to the stimulus conspecific in male and female subjects. Since baseline (preexposure) latencies varied over a considerable range, we performed statistical analyses on the post-pre difference scores. Two-way analysis of variance (ANOVA) revealed a significant sex × stimulus interaction ($F_{7,172} = 3.5$, $P = 0.002$). As shown in Fig. 1 (C and D), withdrawal latencies were statistically unchanged from pre- to post-stimulus exposure in all subject/stimulus groups except for five. In three cases, stimulus exposure rendered mice analgesic (one-sample *t* tests compared to zero difference): (i) male subjects exposed to late-pregnant mice ($t_{11} = 3.8$, $P = 0.003$), (ii) male subjects exposed to lactating mice with pups ($t_9 = 4.2$, $P = 0.002$), and (iii) male subjects exposed to lactating mice without pups ($t_{11} = 2.5$, $P = 0.03$). Male subjects exposed to lactating mice whose pups had been removed 24 hours prior became hyperalgesic ($t_{11} = 2.8$ $P = 0.02$), as did female subjects exposed to female mice after weaning ($t_{11} = 3.1$, $P = 0.01$). Only the male/late-pregnant and male/lactating (with pups) groups displayed statistically significant analgesia at $P < 0.05$ after correction for multiple comparisons. It should also be noted that regardless of the identity of the stimulus conspecific, a strong sex difference emerged in the response of male and female mice to repeated testing (or, perhaps, in their response to the presence of any conspecific), with males overall showing analgesia (repeated measures ANOVA: $F_{1,93} = 11.8$, $P = 0.001$) and females overall showing hyperalgesia (repeated measures ANOVA: $F_{1,93} = 8.1$, $P = 0.005$).

To replicate and generalize the finding to another pain modality, the experiment was repeated with naïve mice of both sexes tested for mechanical sensitivity of the hind paws before and after exposure to naïve female, late-pregnant, or lactating (with pups) stimulus mice.

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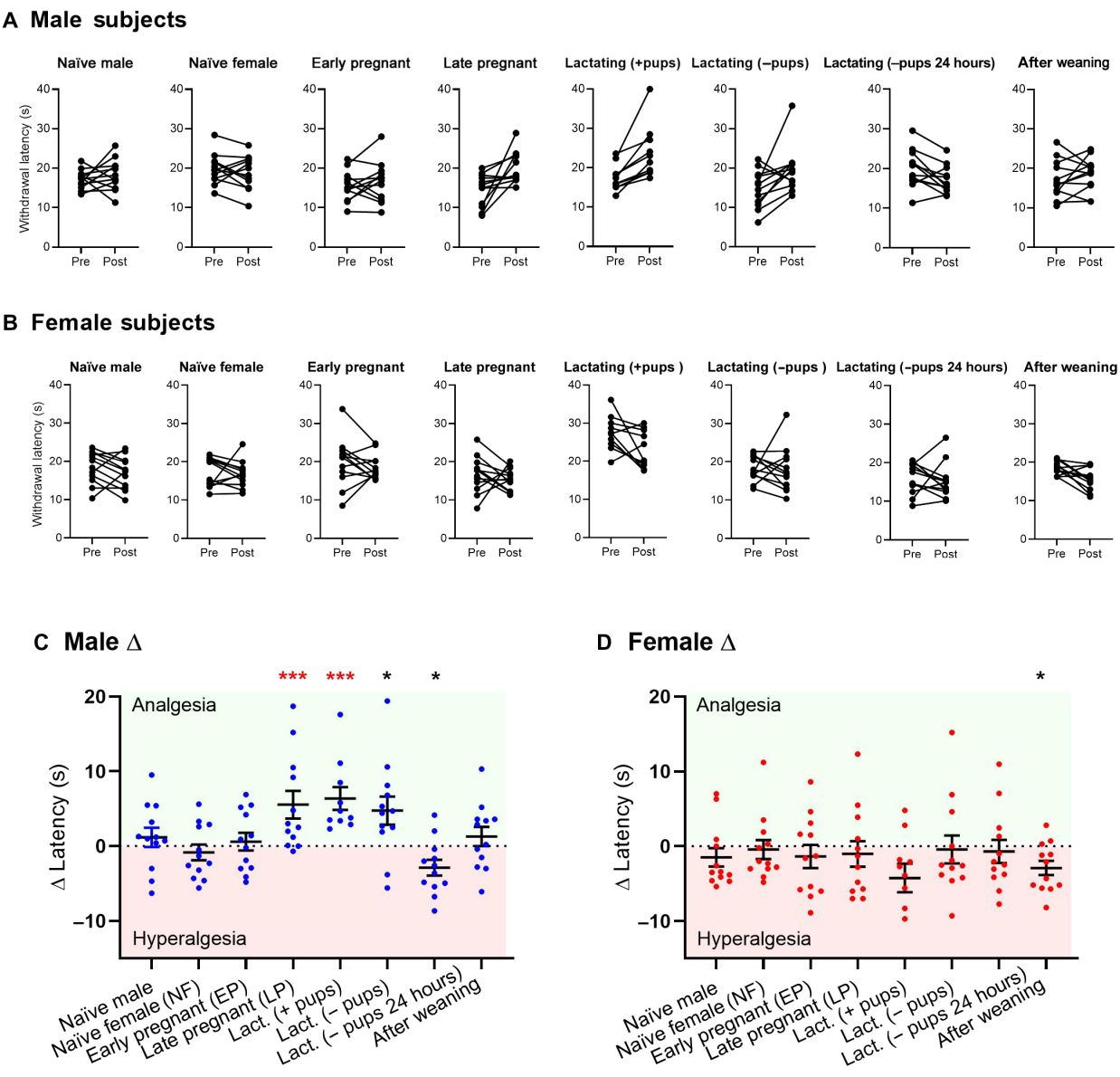


**Fig. 1. Olfactory exposure to late-pregnant and lactating mice produces analgesia in male but not female mice in the radiant heat paw-withdrawal test.** (**A** and **B**) Latency to hind paw-withdrawal (s) from radiant heat of male (A) and female (B) mice before (Pre) and during (Post) exposure to stimulus mice of various reproductive conditions: naïve male, naïve female (NF), early pregnant (EP), late pregnant (LP), lactating (Lact.) [with pups present (+ pups), without pups present (– pups), and with pups removed 24 hours before (– pups 24 hours)], and after weaning. (**C** and **D**) Change (Δ) in hind paw-withdrawal latency (Post-Pre) in all stimulus conditions shown in graphs (A) and (B) in male (C) and female (D) mice. Positive values (green) represent analgesia; negative values (pink) represent hyperalgesia. In all graphs, individual data are shown ($n = 10$ to 12 mice per sex per condition); black bars represent means ± SEM. $^*P < 0.05$, $^{***}P < 0.001$ compared to zero by two-tailed one-sample Student's $t$ test; asterisks are shown in red if the $t$ test result is still statistically significant at $P < 0.05$ after correction for multiple comparisons.

Again, the only groups displaying significant analgesia were male mice exposed to late-pregnant females ($t_{11} = 3.4$, $P = 0.005$) or lactating mice ($t_{10} = 3.0$, $P = 0.01$) (Fig. 2A). Both effects remained significant after correction for multiple comparisons. To provide further generalization, we tested naïve mice of both sexes on the 0.9% acetic acid abdominal constriction (writhing) test. As this test can only be administered once, all mice were tested after exposure to naïve female or late-pregnant stimulus mice. As shown in Fig. 2B, male but not female mice displayed a reduction in writhing behavior (i.e., analgesia) when exposed to late-pregnant mice (subject sex × stimulus: $F_{1,38} = 4.2$, $P = 0.04$). As shown in Fig. 2C, a separate set of male mice were administered 0.9% acetic acid and scored for facial expressions of pain using the Mouse Grimace Scale. Again, only male mice displayed analgesia from exposure to late-pregnant mice (subject sex × stimulus: $F_{1,38} = 4.6$, $P = 0.03$). Overall, then, the phenomenon can be demonstrated using three different noxious stimulus modalities and four different dependent measures of pain behavior.

## The analgesia is olfactorily mediated, testosterone dependent, and stress induced

Next, we attempted to characterize the sensorial, hormonal, behavioral, and stress-related nature of the phenomenon. The analgesic effect

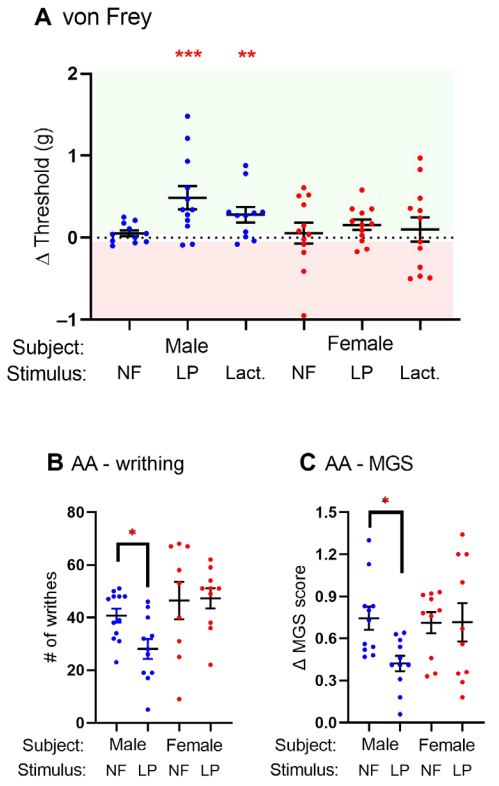


**Fig. 2. Olfactory exposure to late-pregnant and lactating mice produces analgesia in male but not female mice on other assays of pain.** (**A**) Change (Δ) in hind paw-withdrawal threshold (g) from von Frey filaments of male (blue) and female (red) mice caused by exposure to stimulus mice of the following conditions: naïve female (NF), late pregnant (LP), and lactating (Lact., with pups present). Positive values (green) represent analgesia; negative values (pink) represent hyperalgesia. (**B**) Writhing (abdominal constriction) behavior exhibited by male (blue) and female (red) mice receiving an intraperitoneal injection of 0.9% acetic acid (AA) while being exposed to NF or LP stimulus mice. (**C**) Change (Δ) in Mouse Grimace Scale (MGS) scores (0 to 2 scale) immediately before and during exposure of male (blue) and female (red) mice to NF and LP stimulus mice. In all graphs, individual data are shown ($n$ = 11 to 12 mice per sex per condition); black bars represent means ± SEM. *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001 compared to zero by two-tailed one-sample Student's $t$ test or two-sample $t$ test or as indicated. Asterisks are in red if still significant after correction for multiple comparisons (in A).

did not require the actual presence of stimulus animals but was instead mediated olfactorily, as it could be produced in male mice exposed to soiled bedding from a late-pregnant female mouse but not male mice exposed to soiled bedding from naïve female mice (unpaired $t_{19}$ = 3.6, $P$ = 0.002) (Fig. 3A). The sex difference was dependent on testosterone since analgesia was demonstrated by sham-operated but not castrated male mice ($t_{22}$ = 2.7, $P$ = 0.01) (Fig. 3B). Ovariectomized female mice treated acutely or chronically with testosterone also exhibited (nonsignificant) trends toward analgesia. Although we did not observe any direct relationship between male testosterone levels and analgesia (see fig. S1), we investigated the strength of the analgesia in mice displaying relative behavioral dominance or submission as assessed on the "tube test." We observed that subordinate mice displayed more analgesia than dominant mice ($t_{28}$ = 2.1, $P$ = 0.04) (Fig. 3D). Sexual experience (sexually naïve males compared to stud breeder males) did not affect the strength of the analgesia ($t_{30}$ = 1.1, $P$ = 0.28). Although the effect also occurred in male mice who were the sires of the gestating pups, significantly higher levels of analgesia were observed if the pups were sired by another, stranger male ($t_{20}$ = 3.3, $P$ = 0.004) (Fig. 3E). A time course analysis revealed that the analgesia developed as quickly as 5 min after stimulus exposure and abated after 60 min (all $P$s from 5 to 60 min < 0.01 compared to baseline) (Fig. 3F).

The observed analgesia is likely secondary to stress (*7*), as significantly increased levels of plasma corticosterone were observed in male mice exposed to late-pregnant females or lactating females ($F_{3,27}$ = 7.2, $P$ < 0.001) (Fig. 3G). No group differences in plasma corticosterone levels were observed in female subjects ($F_{4,27}$ = 0.5, $P$ = 0.67) (Fig. 3H). To further examine the hypothesis that the observed analgesia was stress-induced analgesia—mediated by descending pathways ultimately inhibiting projection neurons in the spinal cord dorsal horn (*7*)—we examined the expression of the immediate-early gene c-Fos protein in the superficial laminae of the spinal cord in male mice with and without pain (acetic acid) while being exposed to naïve or late-pregnant females. In male mice exposed to naïve females, acetic acid–induced pain caused the expected increase in dorsal horn c-Fos expression ($t_{14}$ = 3.2, $P$ = 0.006); this increase was absent in male mice exposed to late-pregnant females ($t_{14}$ = 0.007, $P$ = 0.93), indicative of stress-induced analgesia in the latter (Fig. 3I).

## Urinary volatiles produce stress-induced analgesia

As chemosignal-related stress has previously been shown to produce analgesia in mice (*6*), we wished to determine which chemosignals might be responsible for the current phenomenon. Gas chromatography–mass spectrometry of urinary volatiles has shown that four aliphatic odorants are increased in concentration in the urine of pregnant (primiparous or multiparous) and lactating female mice compared to nulliparous female mice (*8*): the acetic ester, *n*-pentyl acetate; the ketone, 4-heptanone; and the alcohols, 1-octen-3-ol and 4-penten-1-ol. We tested mice as described above before and after exposure to 100 μl of each pure volatilized compound (placed approximately 3 cm away from each subject) along with distilled water and methyl-hexanoate [which shows no change in concentration in pregnant or lactating mice (*8*)] as negative controls (Fig. 4A). As shown in Fig. 4 (A and B), two of the odorants produced significant analgesia even after correction for multiple comparisons: *n*-pentyl acetate (one-sample $t_{11}$ = 6.2, $P$ < 0.001) and 4-heptanone ($t_{10}$ = 3.6, $P$ = 0.005). 4-Penten-1-ol produced analgesia that was statistically significant before but not after correction ($t_{10}$ = 3.0, $P$ = 0.01), and 1-octen-3-ol produced a trend toward significance before correction ($t_{11}$ = 1.8, $P$ = 0.10). *n*-Pentyl acetate ($CH_3COO[CH_2]_4CH_3$; CAS no. 628-63-7), also known as amyl acetate, is very similar in its chemical structure to isoamyl (or isopentyl) acetate, and both are found in a variety of fruits and used to produce banana oil/extract. As shown in Fig. 4C, supermarket-purchased banana oil itself also produced significant analgesia in male mice ($t_{11}$ = 3.1, $P$ = 0.009). None of these compounds produced significant inhibition of pain in female mice (0.10 < $P$ < 0.99) (see fig. S2).

As it had the strongest effect, we continued to test *n*-pentyl acetate. To see whether effects were concentration dependent, we applied various dilutions of pure *n*-pentyl acetate in a physiologically relevant range [0 to 2 parts per million (ppm)] (*9*, *10*) to the external nasal nares of naïve male mice. As shown in Fig. 4D, this compound produced concentration-dependent analgesia ($F_{4,42}$ = 5.7, $P$ = 0.001),

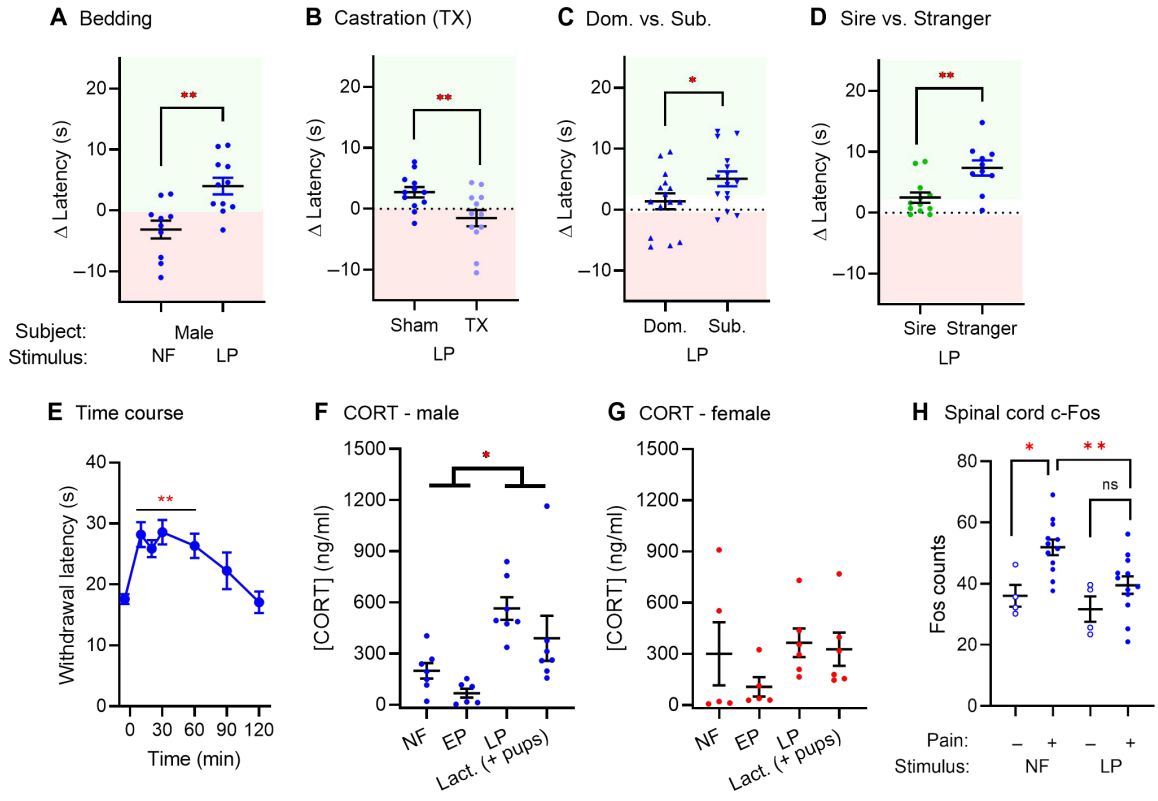


**Fig. 3. Characteristics of late pregnancy/lactation-induced analgesia.** (**A**) Changes from baseline (Δ) in latency to withdraw from radiant heat (s) of male mice exposed to soiled bedding obtained from cages containing naïve female (NF) or late-pregnant (LP) mice. (**B**) Changes from baseline in latency to withdraw from radiant heat of gonadally intact (Sham) or castrated (TX) male mice exposed to LP mice. (**C**) Changes from baseline in latency to withdraw from radiant heat of relative dominant (Dom.) and subordinate (Sub.) mice, as determined by a series of tube test challenges before testing, exposed to LP mice. (**D**) Changes from baseline in latency to withdraw from radiant heat of male mice exposed to LP mice currently carrying their own offspring (Sire) or of another, naive male (Stranger). (**E**) Time course of latency to withdraw from radiant heat of male mice exposed to LP mice over 120 min, where 0 is the start of a 30-min stimulus exposure. (**F** and **G**) Plasma corticosterone concentrations [(CORT); nanograms per milliliter] of male (F) and female (G) mice exposed for 30 min to various stimulus mice (NF, naïve female; EP, early pregnant; LP, late pregnant; lactating + pups). (**H**) Number of c-Fos–positive cells in the dorsal horn of the spinal cord in male mice given an intraperitoneal injection of acetic acid (+) or saline (–) and exposed to NF or LP mice. In all graphs, individual data are shown ($n = 10$ to 12 mice per group in behavioral experiments; $n = 5$ to 7 mice per group in CORT experiments); black bars represent means ± SEM. *$P < 0.05$, **$P < 0.01$ as shown, or compared to baseline in (E). ns, not significant.

displaying an inverted U pattern, with significant analgesia produced by exposing male mice to the 0.5-ppm concentration (Dunnett's multiple comparisons test compared to 0; $P = 0.0013$). A highly similar pattern of *n*-pentyl acetate concentration–dependent increases in plasma corticosterone ($F_{4,79} = 5.4$, $P < 0.001$) was observed (Fig. 4E), with a significant increase produced by the 0.5-ppm concentration (Dunnett's multiple comparisons test compared to 0; $P = 0.001$). Since estrus also features increased urinary concentrations of *n*-pentyl acetate (*11*), we exposed male mice to female mice in estrus but saw no evidence of analgesia ($t_7 = 0.9$, $P = 0.39$). The ability of *n*-pentyl acetate to produce stress was also confirmed behaviorally using the open field test, where male mice exposed to the compound exhibited increased latency to enter ($t_{10} = 2.5$, $P = 0.03$) the center of the open field (Fig. 4F, left) and decreased time spent in the center of the field ($t_8 = 2.8$, $P = 0.02$) over the testing duration (Fig. 4F, right).

## Female aggression and urine marking

To assess the ecological underpinning of this female-to-male chemosignaling, we investigated female aggression toward male mice in a modified resident-intruder paradigm. Gonadally intact stranger male, castrated stranger male, or male partners (i.e., sires) were introduced to a lidless cage containing a late-pregnant or lactating female mouse, and latency to aggression by the female mouse was measured over a 5-min period. Sires and castrated male mice were rarely attacked (Wilcoxon signed rank test compared to the 300-s cutoff latency: $P = 0.50$ and $P = 0.50$, respectively), whereas stranger male mice were often attacked ($P < 0.0001$) (Fig. 5A). Although lactating females have been reported to be aggressive to other intruding female mice (*12*), in our setup, there were zero instances of late-pregnant or lactating female mice attacking an intruder female mouse. A separate group of castrated stranger male mice painted with the attractive male sexual pheromone, darcin (*13*) (male-specific major urinary protein MUP20), were often attacked ($P = 0.004$) (Fig. 5A). As the chemosignaling from female to male mice likely occurs via female urine marking, we tested this in a separate experiment. In an arena where frank aggression was prevented by separating males and females with mesh, we quantified the number of urine marks left by late-pregnant or lactating female mice over a 20-min exposure period. Stranger male and castrated male/darcin groups, but not sires, elicited significantly more urine marks than castrated males ($F_{3,36} = 3.4$, $P = 0.03$) (Fig. 5B).

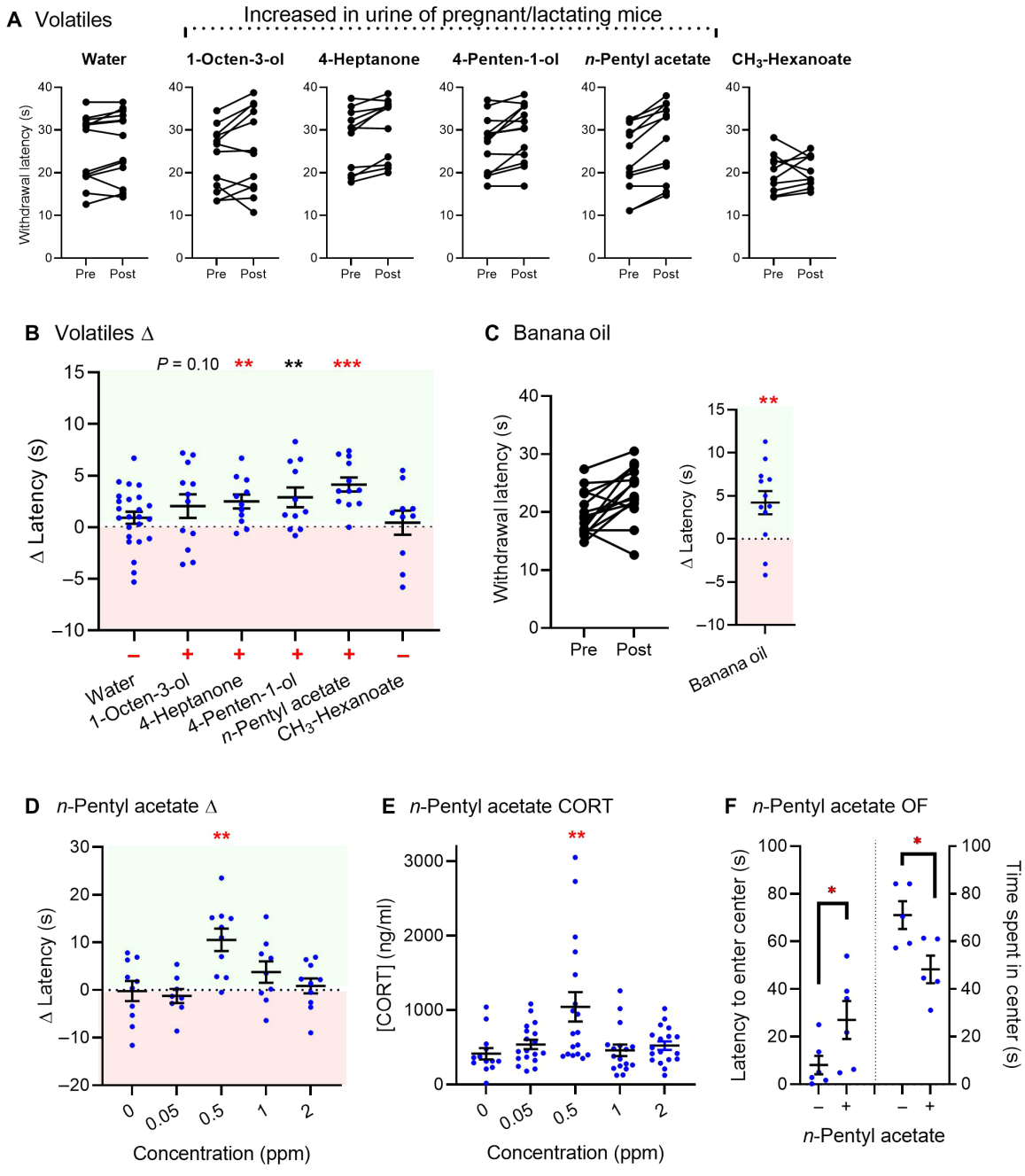


**Fig. 4. Exposure to volatile chemosignals found at higher concentrations in the urine of late-pregnant and lactating mice produce analgesia in male mice.** (**A**) Latency to radiant heat hind paw-withdrawal (s) of male mice following before (Pre) and during (Post) exposure to odorants (all 100 μl volumes) up-regulated in pregnancy and lactation (1-octen-3-ol, 4-heptanone, 4-penten-1-ol, and *n*-pentyl-acetate) and negative controls distilled water and methyl ($CH_3$)–hexanoate. (**B**) Change (Δ) in hind paw-withdrawal latency (Post-Pre) in all stimulus conditions shown in graph (A); up-regulated volatiles indicated by "+"; negative controls indicated by "–"). (**C**) Latency to radiant heat hind paw-withdrawal before and during exposure (left) and change in latency (right) to OliveNation Pure Banana Extract (banana oil). (**D**) Changes from baseline in latency to radiant heat hind paw withdrawal of male mice following exposure to various concentrations (in parts per million diluted in water) of *n*-pentyl-acetate applied to the external nasal nares. (**E**) Plasma corticosterone concentrations [(CORT); nanograms per milliliter] of male mice exposed to various concentrations of *n*-pentyl-acetate applied to the external nasal nares for 1 to 5 min. (**F**) Latency to enter the center region (s; left) or total time spent in the center region (s; right) of an open field (OF) by male mice exposed to water (–) or 100 μl of *n*-pentyl acetate (+). In all graphs, individual data are shown ($n = 8$ to 12 mice per group except for water control; $n = 13$ to 18 per concentration in CORT experiment; $n = 5$ to 6 per group in OF experiment); black bars represent means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, by two-way, one-sample *t* test (compared to 0; uncorrected), or as indicated; asterisks shown are in red if the *t* test result is still statistically significant at $P < 0.05$ after correction for multiple comparisons.

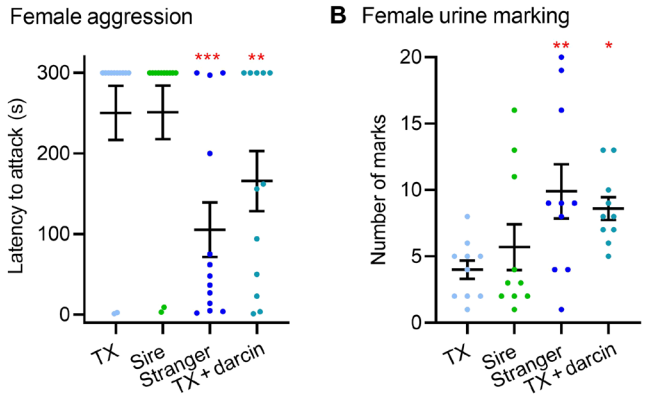


**Fig. 5. Late-pregnant and lactating female mice respond to reproductively active stranger male mice with aggression and urine marking.** (**A**) Latency to attack (s) a stimulus male placed in a modified resident-intruder paradigm by a single late-pregnant or lactating female mouse. Stimulus male mice were either castrated (TX) stranger mice, gonadally intact sire mice (i.e., sires of the female's pups) gonadally intact stranger mice, or TX stranger mice painted with the male sexual pheromone, darcin (1 mg/ml, 20 μl). (**B**) Number of discrete female urine marks after 20-min exposure to male stimulus mice in an arena where female and male mice were separated by mesh. In both graphs, individual data are shown ($n = 10$ to 12 mice per group); black bars represent means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to 300 s by one-sample Wilcoxon signed rank test or by Tukey post hoc comparison test as shown. Data from late-pregnant and lactating mice are shown combined as no significant differences were observed.

## DISCUSSION

We show here that male mice display stress and stress-induced analgesia in the close proximity of late-pregnant or lactating female mice. We also observe in our own hands, as has been seen before, that late-pregnant and lactating female mice respond to gonadally intact stranger male mice with aggression and urine marking. The parsimonious interpretation of our observations is that late-pregnant or lactating female mice are responding to the presence of sexually competent and thus potentially aggressive males by releasing urinary scent marks revealing their current reproductive status to those males, who in turn respond with stress (and stress-induced analgesia) related to potential female aggression. That the lowered sensitivity to pain exhibited by male mice represents stress-induced analgesia is supported by the observations of corticosterone release, inhibition of spinal cord c-Fos expression, and open field behavior.

Male mice, especially virgin males, are well known to engage in infanticidal aggression to advance their genetic fitness (*14*), and this innate aggression has been shown to involve the *Trpc2* gene (*15*) and the actions of submandibular gland protein C and hemoglobin (from the pups themselves and their mothers) acting on a number of vomeronasal receptors in the males (*16*). As a response to protect their parental investment, females will vigorously defend their pups from them. This maternal aggression [see (*17*)], via a mechanism involving the *Irs4* gene (*18*), is observed in rats and mice from approximately day 16 of gestation to day 19 of lactation (*19*). The aggression involves signaling from both the olfactory and vomeronasal epithelia and does not require (but is enhanced in) the presence of pups (*20*, *21*). Although pregnant and lactating females will also attack intruding females (*12*), maternal attack is more often directed at those more likely to harm the pups (i.e., virgin, gonadally intact males) (*22*), and the attacks on such individuals are more vicious and damaging (*23*). The aggression-promoting properties of darcin in lactating females has also been directly demonstrated (*21*). Although maternal attack does not always succeed in preventing male intruders from committing infanticide, any threat of violence is likely to produce stress in both parties, and maternal aggression has been shown to directly produce stress-induced analgesia in males measured after the attacks (*24*). What we demonstrate presently is that stress-induced analgesia in male mice can be observed even in the absence of actual maternal aggression; the mere threat of such aggression is enough and that this threat is communicated via volatile urinary compounds.

In the light of this hypothesis, some of the present findings are curious. For example, we observed a significant (although small) analgesic effect in sires. Under natural conditions, the territorial stud male would participate in the defense of pups with the mother and would obviously be under no threat of attack. An inspection of Fig. 3D suggests, however, that the statistical significance of the sire group was driven entirely by two outlier data points, superimposed on the general tendency of test-retest analgesia of male mice (see Fig. 1C). It is also perhaps counterintuitive that subordinate mice displayed more analgesia than dominant mice, although dominant mice are more likely to engage in infanticide (*25*). The probability of infanticide need not correlate with the observed level of stress-induced analgesia, however, as the latter may be driven more by the male's susceptibility to be effectively intimidated by the pregnant/lactating female. Further experiments will be required to elucidate the factors affecting this novel phenomenon, including the role of testosterone. Although castrated mice did not display analgesia, and ovariectomized female mice given testosterone did, we did not observe any correlation between plasma testosterone levels and analgesia in male mice exposed to late-pregnant mice (fig. S1).

The volatile compound producing the most marked and reliable analgesia in our hands was the ester *n*-pentyl acetate, whose level in the urine of dams right after giving birth is 80% higher than in nulliparous females (*8*). In our hands, *n*-pentyl acetate produced stress and associated pain inhibition at a concentration consistent with its natural urinary concentration (*10*). However, the endogenous olfactory signature in the urinary scent marks indicating to males that a pregnant/lactating female is nearby, and a threat, is probably a mixture of the volatile compounds studied here. Ultimately, such coding likely involves several ligands acting at a much larger number of main olfactory system and/or vomeronasal receptors, as has been shown for sex pheromones triggering mounting behavior (*26*). Presumably, the precise mix of odorants can allow mice to distinguish pregnant/lactating mice from those in estrus. *n*-Pentyl acetate, in particular, is known to stimulate olfactory receptors in rodents leading to evoked potentials (*27*) and has been shown to delay puberty in mice (*10*). In the mouse, *n*-pentyl acetate activates a large number of olfactory receptors (*28*). Furthermore, isopentyl acetate is well known as an alarm pheromone in *Apis mellifera* (honeybees), and in a laboratory experiment, bees exposed to isopentyl acetate displayed stress-induced analgesia to electric shock (*29*). Our data suggest that the widespread use of *n*-pentyl acetate as a nonbiologically active "control" odorant may be misguided.

Because of increasing concerns over reproducibility (*30*), more attention is being paid to laboratory environment factors that may especially affect behavioral experiments; such factors are multitudinous (*31*) and often unexpected (*6*). We believe that part of the reason many such modulatory factors have not been previously identified

by pain researchers is that their actions are too subtle to affect the highly intense noxious stimuli that used to typify preclinical pain research but are perfectly able to modulate the less-intense noxious stimuli commonly used in most modern experiments [see supplementary tables 1 and 2 in (*32*)]. Baseline (preexposure) paw-withdrawal latencies in the current experiments range from 10 to 40 s, with an average across all experiments of ≈20 s. Some of this variability can be accounted for by differential energy output of the radiant heat source over time, some of it by the outbred status of the experimental subjects [but see (*33*)], some of it by competing behaviors being made by subjects during testing (*34*), and some of it by experimenter variation (*35*). There are, no doubt, many other sources of variation in the laboratory environment to uncover, and more attention to this issue would pay off both in terms of more accurate scientific conclusions and improved progress toward the "3Rs" (reduction, refinement, and replacement). The current findings suggest that close proximity of male subjects to reproductively active females is a previously unknown stressor for the males and that stress may even be caused by the proximity of certain foodstuffs.

## MATERIALS AND METHODS

### Subjects

Experiments were performed on naive, young adult (7 to 12 weeks of age) male and female mice. Outbred CD-1 (Crl:ICR) mice were bred in-house from breeders obtained from Charles River Laboratories (St. Constant, QC). Castrated CD-1 male mice were ordered directly from Charles River Laboratories; testing occurred no less than 2 weeks after gonadectomy. Mice were housed with their same-sex littermates (two to four animals per cage) in standard shoebox cages, maintained in a temperature-controlled (20° ± 1°C) environment (12:12-hour light/dark cycle; lights on at 07:00 hours), and received food (Envigo Teklad 2020X) and water ad libitum. Male and female mice were tested in separate within-sex cohorts of up to 12 mice each. Pregnancies were timed on the basis of the presence of a vaginal plug. Although in most experiments, pregnant and lactating mice (see below) were only used as social stimuli, in some experiments, they represented the test subjects themselves.

### Nociceptive assays

Most behavioral experiments used the paw-withdrawal test of noxious thermal sensitivity (*36*). Mice were placed individually in perforated, transparent Plexiglas cubicles (5 cm by 8.5 cm by 6 cm) placed upon a 3/16th-inch-thick glass floor and habituated for 2 hours before any behavioral testing commenced. The stimulus was a high-intensity beam (IITC model 336; setting = 3, ≈45 W) from a projector lamp bulb located 6 cm below the glass floor aimed at the plantar surface of the mid-hind paw of an inactive mouse. Withdrawal latency of each hind paw was measured to the nearest 0.1 s. Reported latencies represent the average of four separate latency determinations at each time point, two per hind paw (before and 30 min after exposure; see below).

Testing of mechanical sensitivity was performed using the von Frey test. Mice were placed individually in transparent Plexiglas cubicles (5 cm by 8.5 cm by 6 cm) placed upon a perforated metal floor and habituated for 2 hours before behavioral testing began. Nylon monofilaments (force range: ≈0.015 to 1.3 g; Touch Test Sensory Evaluator Kit, Stoelting) were firmly applied to the plantar surface of each hind paw of an inactive mouse until they bowed for 0.5 s. The up-down method of Dixon (*37*) was used to estimate 50% withdrawal thresholds. Reported thresholds represent the average of four separate determinations at each time point, two per hind paw. For both mechanical and thermal pain, difference data are presented as the change (Δ) in withdrawal latency or threshold from pre- to postexposure assessments (i.e., post-pre).

In the acetic acid abdominal constriction ("writhing") test, mice were placed individually into Plexiglas observation cylinders (15 cm in diameter; 22.5 cm high) placed on a glass surface suspended over video cameras. Mice were injected into the intraperitoneal cavity with 0.9% acetic acid (10 ml/kg) immediately after being exposed to naïve female or late-pregnant stimulus mice. The number of writhes—lengthwise constrictions of the abdominal musculature—were counted over the next 30 min. A separate group of mice were placed in Plexiglas observation cubicles (9 cm by 5 cm by 5 cm high), videotaped for 30 min, similarly injected and exposed as above, and then videotaped again during exposure for 30 min. Facial expressions of pain were quantified from baseline and post-exposure videos using the Mouse Grimace Scale as described (*38*).

### Social exposures

Sets of 12 same-sex mice were exposed to one of the following social stimuli—single, unfamiliar (i.e., non-cagemate) mice housed in isolation in a standard cage without a lid placed 5 to 30 cm (depending on subject cubicle position) away from the subject mice: (i) adult, reproductively naïve (but gonadally intact) male; (ii) adult, reproductively experienced ("stud" breeder) male; (iii) adult, castrated male; (iv) adult, reproductively naïve female; (v) primiparous pregnant female in the first 10 days of a 21 ± 1–day pregnancy (early-pregnant); (vi) primiparous pregnant female in the last 4 days of pregnancy (late-pregnant); (vii) currently lactating female (with pups present); (viii) currently lactating female (without pups present); (ix) currently lactating female 24 hours after removal of pups; (x) previously pregnant female within 7 to 8 days after weaning (i.e., at 20 to 21 days after parturition); or (xi) ovariectomized female treated with testosterone (400 μg per mouse of testosterone cypionate in 0.1 ml of sesame oil) 1 hour before exposure or daily for 7 days. In some experiments, male mice were exposed (individually) to late-pregnant female mice that they themselves had impregnated (i.e., the sires of the pregnant mouse); otherwise, pregnant female mice were impregnated by (nontested) stud males. In all cases, following habituation, tested mice were exposed to stimulus mice for 30 min before postexposure testing commenced, and the exposure continued during testing, which lasted ≈30 additional minutes. Last, in some experiments, subject mice were exposed instead to soiled cage bedding (from a cage containing four naïve females or one late-pregnant female) or volatile compounds (in sets of six mice) as described below. Note that test mice are isolated in cubicles before and during exposure, such that physical interaction between stimulus and test mice was not possible.

### Dominance testing

Male mice were tested for relative dominance via the confrontation tube test as previously described (*39*). In this test, two mice are introduced into opposite ends of a transparent Plexiglas acrylic tube (3.5 cm in diameter, 30 cm in length) and interact at the center of the tube. The mouse that forces its opponent out of the tube at least 7 of 10 testing trials is considered the "winner" of that dyad. Mice were separated into groups of eight and all possible mouse dyad

combinations within each group were tested, creating a hierarchy of dominance based on the number of "wins" in the tube test. The top three and bottom three in this hierarchy were considered dominant and subordinate mice, respectively. After determining dominance status through this paradigm, mice were tested for baseline and postexposure (naïve or late-pregnant female) thermal paw-withdrawal latencies.

### Corticosterone

Plasma corticosterone levels were measured by enzyme-linked immunosorbent assay (ELISA). After 2 hours of habituation and 30 min of exposure, blood was collected via cardiac puncture and placed into vials with a 1:10 volume of 0.1 M sodium citrate. In concentration-dependent experiments using *n*-pentyl acetate, exposure time after application to the external nares (see above) was 1 to 5 min. Blood samples collected for ELISA were spun down (3000*g*, 4°C) for 10 min, and blood plasma was collected. Plasma samples were diluted 1:100 and ran against a standard curve as part of a validated corticosterone ELISA kit (Abcam, catalog no. ab108821).

### Spinal cord c-Fos immunohistochemistry

Male mice were injected intraperitoneally with 0.9% acetic acid or isotonic saline and exposed to either a naïve or late-pregnant female for 30 min. Immediately thereafter, mice were deeply anesthetized with sodium pentobarbital (120 mg/kg, i.p.) and perfused through the heart with phosphate-buffered saline (PBS) (pH = 7.2), immediately followed by a fixative of 4% paraformaldehyde (Sigma-Aldrich) in PBS. Spinal cords were removed and postfixed with the same fixative solution at 4°C overnight. Lumbar spinal cords were cut into 30-μm-thick coronal sections on a vibratome (Leica VT1000S, Leica Microsystems). For immunohistochemical staining, the sections were blocked for 1 hour at room temperature with 10% normal donkey serum (NDS; Sigma-Aldrich) in PBS supplemented with 0.3% Triton X-100 (PBST) and incubated with rabbit anti–c-Fos antibody (diluted 1:500 with PBST containing 1% NDS) (Cell Signaling Technology) overnight at 4°C. The sections were washed several times and then incubated with Alexa Fluor 568 donkey anti-rabbit immunoglobulin G (diluted 1:500 with PBST) (Thermo Fisher Scientific) overnight at 4°C in the dark. The stained sections were mounted in ProLong Gold antifade reagent (Thermo Fisher Scientific) and then kept at 4°C in the dark until measurements were carried out. The fluorescence was visualized on a Zeiss ApoTome.2 microscope. For each mouse, the total number of Fos-positive cells on both sides of the dorsal spinal cord (laminae I, II and IV, V) was calculated (Fiji/ImageJ, U.S. National Institutes of Health) and averaged from five sections per mouse.

### Odorants

Urinary odorant compounds were chosen on the basis of previously determined concentrations before, during, and after pregnancy (*8*). *n*-Pentyl acetate, 4-heptanone, methyl-hexanoate, 4-penten-1-ol, and 1-octen-3-ol were purchased from Sigma-Aldrich (catalog nos: 109584, 101745, 259942, 111279, and 05284, respectively). Cotton balls containing 100 μl of the selected odorant were placed in empty Plexiglas cubicles between two mice being tested for noxious thermal sensitivity to facilitate equal diffusion among all subjects. All mice were thus approximately 3 cm away from the odor source and were unable to have physical contact with the cotton balls. In concentration-dependent experiments using *n*-pentyl acetate, the compound was diluted in water to concentrations ranging from 0.05 to 2 ppm, and 50 μl was applied with 38-mm (inner) diameter polyethylene tubing married to a 1-cc syringe/26-guage needle directly to the external nasal nares.

### Open field test

Male mice were placed in an open field apparatus, an empty opaque box (38 cm long by 25.5 cm wide by 12 cm high) for 20 min while being exposed to cotton ball impregnated with 100 μl of *n*-pentyl acetate (or saline) attached to the upper edge of the open field box wall. A video recording of the test was made for later behavior analysis. The latency to enter the center (a 10 cm–by–10 cm square) and the time spent in the center versus the outer zone were measured.

### Maternal aggression

The procedure to test for maternal aggression was based on the resident-intruder paradigm (*40*). Late-pregnant or lactating females (resident mice) were tested along with either naive gonadally intact males, castrated males, or their respective sires (intruder mice). An additional group of intruder mice consisted of castrated males treated with the male sexual pheromone, r-darcin (*13*) (recombinant mouse darcin; MUP20; MyBiosource catalog no. MBS638160). Ten microliters of r-darcin (1 mg/ml) was rubbed into the neck, and 10 μl was rubbed into the anogenital region 30 min before testing. When testing lactating females, pups were removed from the home cage before testing and returned after the intruder mouse was removed. The test took place in the resident's home cage with the lid removed. Home cage bedding was not changed for at least 3 days before testing. The intruder mouse was introduced to the resident's home cage, recorded under red lighting, and analyzed with Observer XT software (Noldus) for the latency to the first instance of aggression. If no aggression was observed in the first 5 min, a latency of 300 s was recorded and the test was ended.

### Urine marking

Urine marking was assessed on the basis of a previously published protocol (*41*). Late-pregnant or lactating females were habituated for 1 hour in a wire mesh cage (zone 1: 30 cm by 18 cm by 14 cm) centered inside a larger plastic cage (zone 2: 39 cm by 26 cm by 14 cm) with a sheet of filter paper (Whatman no. 1) as bedding and covered by a perforated Plexiglas lid. After habituation, the bedding sheet was replaced with a new one, and one of the stimulus mice (see Maternal aggression section above) was placed in zone 2, where it was free to roam around (but not enter) zone 1. After 20 min of exposure, both mice were returned to their home cages, and the filter paper sheet under zone 1 was placed under ultraviolet light and urine marks were counted.

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abi9366

View/request a protocol for this paper from *Bio-protocol*.

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**Acknowledgments:** We thank S. Sotocinal for construction of enclosures and M. Laska for useful discussions. **Funding:** This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and the Canadian Institutes of Health Research (J.S.M.). S.F.R. was supported by studentships from the Louise and Alan Edwards Foundation and the Healthy Brains for Healthy Lives program. L.V.L. was supported by a Ron Melzack postdoctoral fellowship from the Louise and Alan Edwards Foundation. **Author contributions:** The phenomenon described here was noticed by S.F.R. S.F.R., L.V.L., and J.S.M. designed experiments. S.F.R., L.V.L., C.C., R.N., M.Z., W.N., E.T., A.S., S.N.T., A.Z., S.G.S., and C.P. performed experiments. S.F.R., L.V.L., and J.S.M. performed data analyses and wrote the paper. **Competing interests:** The authors declare that they have no competing interests. **Data and materials availability:** All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Submitted 13 April 2021
Accepted 24 January 2022
Published 20 May 2022
10.1126/sciadv.abi9366