Behavioral/Cognitive

T-Cell Mediation of Pregnancy Analgesia Affecting Chronic Pain in Mice

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It has been reported consistently that many female chronic pain sufferers have an attenuation of symptoms during pregnancy. Rats display increased pain tolerance during pregnancy due to an increase in opioid receptors in the spinal cord. Past studies did not consider the role of non-neuronal cells, which are now known to play an important role in chronic pain processing. Using an inflammatory (complete Freund's adjuvant) or neuropathic (spared nerve injury) model of persistent pain, we observed that young adult female mice in early pregnancy switch from a microglia-independent to a microglia-dependent pain hypersensitivity mechanism. During late pregnancy, female mice show no evidence of chronic pain whatsoever. This pregnancy-related analgesia is reversible by intrathecal administration of naloxone, suggesting an opioid-mediated mechanism; pharmacological and genetic data suggest the importance of δ-opioid receptors. We also observe that T-cell-deficient (*nude* and *Rag1*-null mutant) pregnant mice do not exhibit pregnancy analgesia, which can be rescued with the adoptive transfer of $CD4^+$ or $CD8^+$ T cells from late-pregnant wild-type mice. These results suggest that T cells are a mediator of the opioid analgesia exhibited during pregnancy.

Key words: adaptive immunity; delta-opioid; pain; pregnancy; T cells

Significance Statement

Chronic pain symptoms often subside during pregnancy. This pregnancy-related analgesia has been demonstrated for acute pain in rats. Here, we show that pregnancy analgesia can produce a complete cessation of chronic pain behaviors in mice. We show that the phenomenon is dependent on pregnancy hormones (estrogen and progesterone), δ-opioid receptors, and T cells of the adaptive immune system. These findings add to the recent but growing evidence of sex-specific T-cell involvement in chronic pain processing.

Introduction

Although pregnancy is a risk factor for the development of a number of pain states (Block and Biller, 2014; Casagrande et al., 2015), it is also well known that many women with preexisting chronic pain states experience an attenuation of symptoms during pregnancy (LeResche et al., 2005; Melhado et al., 2007; Ostensen and Villiger, 2007; Block and Biller, 2014). Studies in the rat have shown opioid-receptor-mediated increases in acute pain tolerance during pregnancy that peaks at parturition (Gintzler, 1980) and similar changes in acute pain sensitivity have been demonstrated in pregnant women (Rust et al., 1985; Cogan and Spinnato, 1986; but see Goolkasian and Rimer, 1984; Dunbar et al., 1988). Gintzler and colleagues, in a series of studies in the rat, have established the importance of pregnancy-relevant gonadal hormones and spinal cord opioid receptors in producing pregnancy analgesia (Gintzler and Liu, 2001). Iwasaki et al. (1991) also observed naloxone-reversible analgesia in the late stages of pregnancy in the colorectal distension, tail-flick, and hot-plate tests. Jayaram et al. (1995, 1997), working in mice, provided evidence for enkephalin mediation of pregnancy analgesia with the observation that the enkephalinase inhibitors SCH 32615 and RB 101 enhance the phenomenon. These studies are limited in their clinical relevance in that they used only acute noxious stimuli. Very recently, however, the reduction of mechanical pain hypersensitivity (allodynia) produced by a chronic constriction injury in late pregnancy in the rat was reported (Onodera et al., 2017).

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The bulk of the pregnancy analgesia literature was compiled before it was widely recognized that neurons are not the only cell type processing pain; cells of the immune system such as microglia, macrophages, and T cells are now known to perform important signaling roles (Beggs and Salter, 2010; Grace et al., 2014; McMahon et al., 2015; Ji et al., 2016). We recently demonstrated that females are likely using adaptive immune cells, T cells, to produce pain hypersensitivity after injury, whereas males use microglia to achieve the same ends (Sorge et al., 2015). A possible underlying explanation of this sex difference is that females have a stronger adaptive immune system and more circulating T cells than males (Scotland et al., 2011). During pregnancy, due to changes in sex hormone levels and for the purposes of protection of the fetus, females mount a dampened adaptive immune response (Saito et al., 2010). We wished to investigate the implications for the apparent sex dependence of spinal cord neuroimmune processing of pain in pregnant mice.

Here, we observed that female mice (of two different strains) with a neuropathic or inflammatory injury in the early stages of pregnancy switched from a microglia-independent system of pain signaling to a microglia-dependent system. In late pregnancy, females no longer exhibited any signs of chronic pain whatsoever. We also show that, in the mouse, as in the rat, pregnancy analgesia appears to be mediated by gonadal hormones and opioid receptors, specifically the δ-opioid receptor. Completely novel is the demonstration that this phenomenon is utterly dependent on T cells.

Materials and Methods

Experimental design and statistical analysis. Experiments were performed by investigators blinded to drug or hormone group and mice were assigned to drug groups randomly within cages. Blinding to sex/pregnancy status and genotype was obviously not possible. Most behavioral experiments involved the evaluation of the effects of injuries, drugs, and/or hormones on pain behaviors and were analyzed with repeated-measures ANOVAs as appropriate (Systat version 13). *Post hoc* comparisons were made using the *post hoc* test for repeated measures with Sidak correction. An α criterion of 0.05 was adopted in all experiments. Because these studies were exploratory in nature (Kimmelman et al., 2014) and the phenomenon being studied was entirely novel in the mouse, formal power analyses were not appropriate. Sample sizes were instead based on standard practices in the preclinical pain field (Mogil et al., 2006) and in some cases were dictated by breeding success. In five cases, individual data points were removed from the analysis after being identified as statistical outliers (Studentized residuals >3).

Subjects. Experiments were performed on naive, adult (7–12 weeks of age) male and female mice. Outbred CD-1 (Crl:ICR) and *nude* (Crl:CD1-*Foxn1*^{nu}) mice were bred in-house from breeders obtained from Charles River Laboratories. C57BL/6J mice were purchased from The Jackson Laboratory and *Rag1*$^{-/-}$ (C57BL/6-*Rag1*^{tm1Mom}) mice were bred in-house from breeders obtained from M. Saleh (McGill University). Null mutant mice lacking expression of the *Oprd1* (δ-opioid receptor) gene (*Oprd1*$^{-/-}$; C57BL/6-*Oprd1*^{tm1Kff}) were bred in-house from breeders obtained from L. Diatchenko (McGill University). Mice were housed with their same-sex littermates (2–4 animals per cage) in standard shoebox cages, maintained in a temperature controlled (20 ± 1°C) environment (12:12 h light/dark cycle; lights on at 07:00 h), and received food (Harlan Teklad 8604) and water *ad libitum*. Early-pregnant females were defined as being 5–7 d into a 20 ± 1 d gestation period (i.e., ≈P−14; with P−20 being defined as the day after discovery of a vaginal plug), and late-pregnant females were defined as 16–21 d into gestation (i.e., ≈P−2). Postweaning females were defined as primiparous mice 7–8 d after pup weaning at 20–21 d postparturition (i.e., ≈28 d after birth; P 28).

Gonadectomy and hormone replacement. Ovariectomies occurred under isoflurane/oxygen anesthesia via the dorsal approach 7 d before retesting. Hormonal replacement was achieved via subcutaneous implantation of osmotic minipumps (Alzet model 2002), releasing one of the following: (1) vehicle (0.5 μl/h/d), (2) 17β-estradiol (0.1 mg/kg/d; 0.042 mg total), (3) progesterone sulfate (0.25 mg/kg/d; 0.105 mg total), or (4) hormone-stimulated pregnancy (0.1 mg/kg/d estradiol + 0.25 mg/kg/d progesterone) (Dawson-Basoa and Gintzler, 1993). Pain sensitivity was assessed before and 14 d after pump implantation. Therefore, in all experiments, 3 weeks elapsed between ovariectomy and final behavioral testing.

Inflammatory assays. After testing for baseline mechanical sensitivity on two separate occasions (see below), mice were injected with complete Freund's adjuvant (CFA; 50%, in a 20 μl injection volume) into the left hindpaw. Mice were retested 3 d later to confirm the presence of mechanical allodynia and then (in many but not all studies) at various time points after drug injection up to 120 min after drug. In one study, after baseline mechanical testing, mice were injected with lipopolysaccharide (LPS; *Escherichia coli* serotype 0111:B4; Sigma-Aldrich) intrathecally (0.1 μg; 5 μl injection volume) (Hylden and Wilcox, 1980) and retested 1, 2, 4, 6, and 24 h later.

In two studies, mice were tested before and after CFA for noxious thermal sensitivity or spontaneous pain behavior (see below).

Neuropathic assay. After testing for baseline mechanical sensitivity on two separate occasions, mice were subjected to a unilateral spared nerve injury (SNI) surgery (Decosterd and Woolf, 2000) as adapted for mice (Shields et al., 2003). We spared the sural territory, so von Frey stimuli were aimed at the lateral aspect of the hindpaw. Mice were retested for mechanical allodynia on postoperative day 7 (the earliest time point featuring maximal allodynia) and then at several time points after drug injection.

Algesiometry. Most behavioral experiments used the von Frey test of mechanical sensitivity (von Frey, 1922). Mice were placed individually in transparent Plexiglas cubicles (5 × 8.5 × 6 cm) placed upon a perforated metal floor and habituated for 2 h before behavioral testing began. Nylon monofilaments (≈0.015–1.3 g; Touch Test Sensory Evaluator Kit, Fibers 2–9; Stoelting) were firmly applied to the plantar surface of each hindpaw of an inactive mouse until they bowed for 0.5 s. The up–down method of Dixon (Chaplan et al., 1994) was used to estimate 50% withdrawal thresholds.

Testing of noxious thermal sensitivity occurred using the radiant heat paw-withdrawal test (Hargreaves et al., 1988). Mice were placed individually in transparent Plexiglas cubicles (5 × 8.5 × 6 cm) placed upon a 3/16th-inch-thick glass floor and habituated for 2 h before behavioral testing. 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-hindpaw of an inactive mouse. Withdrawal latency of each hindpaw was measured to the nearest 0.1 s. Reported values are means of two successive latency determinations at that time point (before and 3 d after CFA).

Spontaneous pain was tested using the Mouse Grimace Scale (Langford et al., 2010). Each mouse was placed in an individual chamber (9 × 5 × 5 cm) having transparent Plexiglas outer walls to allow experimental observation, opaque plastic inner walls that isolated each mouse visually from all others, and a wire mesh floor. Mice were habituated for 30 min before behavioral testing. Cameras directed at the front and back of the cubicle recorded 30 min of facial expressions before and 24 h after CFA injection. This time point was used instead of 3 d after injection because facial grimacing subsides from 24–48 h after the stimulus (Matsumiya et al., 2012). One clear facial image was taken for every 3 min interval using Rodent Face Finder Software. The images were scrambled and scored blindly for facial grimacing as described previously (Langford et al., 2010).

Drugs. Minocycline (50 μg, i.t.), naloxone (5 μg, i.t.), naltrindole (5 μg, i.t.), nor-binaltorphimine (5 μg, i.t.), and LPS (1 μg, i.t.) were purchased from Sigma-Aldrich. β-Funaltrexamine (5 μg, i.t.) was purchased from Tocris Bioscience. Minocycline and LPS doses were chosen based on previous findings of efficacy (in males) (Sorge et al., 2011, 2015). Opioid antagonist doses were chosen based on previous demonstrations in the literature of successful blockade of opioid-mediated an-

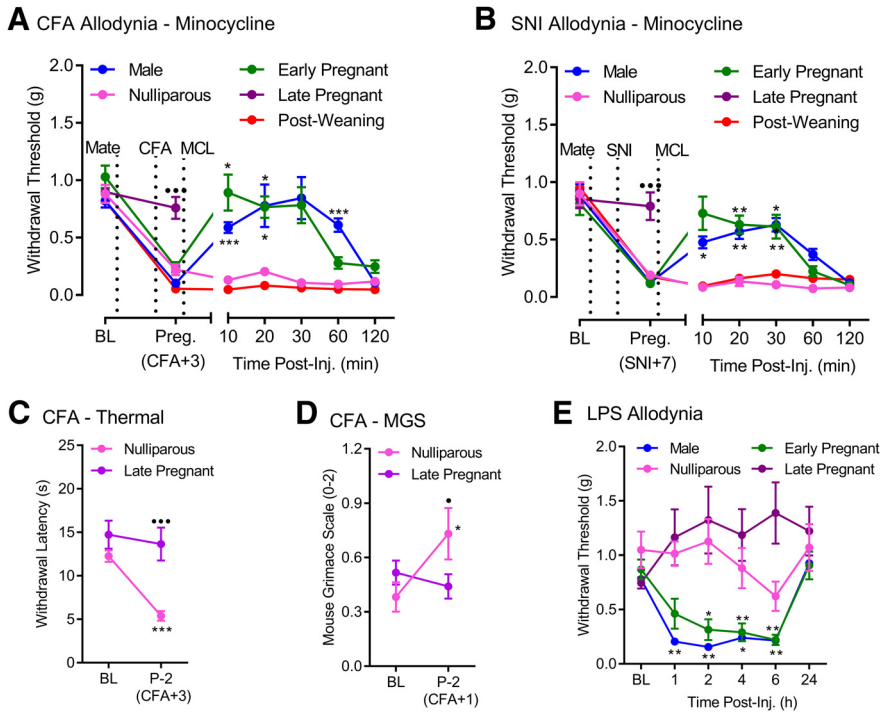

Figure 1. Effect of pregnancy on persistent pain and its sensitivity to reversal by minocycline. ***A***, ***B***, Mechanical allodynia produced by CFA (***A***) and SNI (***B***) is reversed by intrathecal minocycline (MCL) in male and early-pregnant mice (14 ± 2 d before parturition; P−14), but not nulliparous or postweaning (7 ± 1 d postweaning and 28 d postparturition; P28) female mice. Late-pregnant mice (2 ± 2 d before parturition; P−2) display no mechanical allodynia. Symbols represent mean ± SEM ipsilateral hindpaw withdrawal threshold (g) prepregnancy (baseline; BL), 3 d after CFA (pregnancy/CFA+3), or 7 d after SNI (pregnancy/SNI+7), and 10–120 min after injection of MCL; $n = 7–9$ mice/condition. ***C***, CFA produces thermal hyperalgesia in nulliparous female but not late-pregnant (P−2) mice. Symbols represent mean ± SEM ipsilateral hindpaw withdrawal latency (s) prepregnancy (BL) and 3 d after CFA (CFA+3); $n = 8–10$ mice/condition. ***D***, CFA produces spontaneous pain in nulliparous female but not late-pregnant (P−2) mice. Symbols represent mean ± SEM Mouse Grimace Scale score prepregnancy (BL) and 1 d after CFA (CFA+1); $n = 7–8$ mice/condition. In all cases, male and nulliparous female mice were tested with delays between baseline and subsequent testing equivalent to late-pregnant mice. ***E***, LPS produces allodynia in male and early-pregnant (P−14) mice, but not nulliparous and late-pregnant (P−2) mice. Symbols represent mean ± SEM bilateral hindpaw withdrawal threshold (g) at baseline (BL) and 1–24 h after injection of LPS; $n = 6–8$ mice/condition. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with CFA+3 (in graph ***A***), SNI+7 (in graph ***B***), or BL (in graphs ***C***–***E***). $^{\bullet}p < 0.05$ compared to all other groups at the same time point. $^{\bullet\bullet\bullet}p < 0.001$ compared with all other groups at the same time point.

algesia (Qi et al., 1990; Malmberg and Yaksh, 1992; Menendez et al., 1993; Dawson-Basoa and Gintzler, 1996).

Adoptive transfer. Spleens harvested from CD-1 female mice using aseptic technique were placed in sterile, ice-cold RPM1–1640 medium (Sigma-Aldrich) and 10% FBS (Sigma-Aldrich). Cells were then strained into a single-cell suspension. After centrifugation (500 × *g* for 5 min at 4°C), red blood cells were lysed in ACK lysis buffer (Invitrogen) on ice for 5 min. Cells were diluted with RPMI/FBS buffer and centrifuged as described previously. Cells were counted on a hemocytometer using Trypan blue (Sigma-Aldrich). Cells were centrifuged as above and either suspended in sterile 0.9% saline, or further separated into $CD3^+$, $CD4^+$, or $CD8^+$ cell populations using a Qiagen cell sorting kit. Unfractionated splenocytes (10^7 cells in 0.5 ml), $CD3^+$ (5×10^6 cells in 0. 5 ml), $CD4^+$ (4×10^6 cells in 0.5 ml), or $CD8^+$ (3×10^6 cells in 0.5 ml), were injected intraperitoneally into *nude* female mice, which received an intraplantar injection of CFA on the same day. Behavioral testing was performed 72 h after cell transfer.

Quantitative RT-PCR. Dorsal lumbar (L4–L6) spinal cords and dorsal root ganglia (DRG) were removed in freshly killed mice and total RNA isolated (RNeasy Universal Kit; Qiagen) and reverse transcribed (RT^2 First Strand Kit; Qiagen). Amplification of *Oprm1*, *Oprd1*, and *Oprk1* (assay IDs: Mm00445273_m1, Mm01180757_m1, and Mm01230885_m1, respectively; Thermo Fisher Scientific) was performed using Applied Biosystems Quantstudio 3 with TaqMan probes and primers as per the manufacturer's protocol using $n = 3$ technical replicates. Relative quantification was made following the ΔΔCT method. Results were averaged and normalized by dividing mean values of the opioid receptor gene by mean values of the housekeeping gene *18S* (assay ID: Mm03928990_g1; Thermo Fisher Scientific).

Results

In all experiments using minocycline or LPS, vehicle control groups were run concurrently; because no significant repeated-measures effects of vehicle were obtained in any experiment, these data are omitted for clarity. Also omitted for clarity in most graphs are data obtained on the hindpaw contralateral to CFA or SNI; notable effects on the contralateral paw are referred to in the text below.

Inflammatory and neuropathic allodynia in early-pregnant mice shows male-like microglial dependence

Young adult CD-1 male and female mice (nulliparous, in the first week of pregnancy, in the last week of pregnancy, or 1 week postweaning) were tested for mechanical sensitivity, given an inflammatory (CFA) or neuropathic (SNI) injury, and retested 3 or 7 d later, respectively (Fig. 1). With the important exception of late-pregnant mice (see below), no differences in the magnitude of mechanical allodynia were observed among groups (CFA condition × repeated measures, baseline [BL] vs pregnancy: $F_{(3,25)} = 0.5$, $p = 0.68$; SNI condition × repeated measures, BL vs pregnancy: $F_{(3,27)} = 1.6$, $p = 0.21$). Immediately after testing on day 3 after CFA or day 7 after SNI, all mice received an intrathecal injection of minocycline (or saline) and were retested at 10–120 min after injection. Minocycline was effective in reversing allodynia produced by CFA (Fig. 1*A*) and SNI (Fig. 1*B*) in male but not nulliparous female mice, as we have demonstrated previously (Sorge et al., 2015). However, female mice in the early stages of pregnancy were also minocycline sensitive, whereas previously pregnant but not currently lactating mice (i.e., postweaning) were not (CFA, condition × repeated measures, pregnancy-120 min: $F_{(15,125)} = 5.7$, $p < 0.001$; SNI condition × repeated measures, pregnancy-120 min: $F_{(15,135)} = 8.6$, $p < 0.001$).

Late-pregnant mice have no evidence of pain after injury

Mice within 4 d of parturition were observed to have no mechanical allodynia after either CFA (repeated-measures BL vs pregnancy: $F_{(1,6)} = 2.1$, $p = 0.20$; Fig. 1*A*) or SNI (repeated-measures BL vs pregnancy: $F_{(1,7)} = 2.7$, $p = 0.15$; Fig. 1*B*). Late-pregnant mice (and no other group) treated with CFA or undergoing SNI displayed strong trends toward frank analgesia on the contralateral hindpaw as well (CFA: $F_{(1,6)} = 3.7$, $p = 0.10$; SNI: $F_{(1,7)} = 3.1$, $p = 0.12$).

To establish the generalizability of this apparent pregnancy-related analgesia, we compared nulliparous female mice with late-pregnant mice on two other symptom modalities 3 d after

CFA injection. We observed that late-pregnant mice did not display significant thermal hypersensitivity (condition × repeated measures: $F_{(1,16)} = 13.6$, $p = 0.002$; Fig. 1*C*) or spontaneous pain measured via facial grimacing (condition × repeated measures: $F_{(1,13)} = 9.8$, $p = 0.008$; Fig. 1*D*).

Because we demonstrated previously that male but not female mice use TLR4s to process pain in the spinal cord (Sorge et al., 2011), we tested mice for mechanical allodynia produced by intrathecal injection of the TLR4 agonist LPS. LPS produced allodynia for 6–24 h in male and early-pregnant mice, but not nulliparous or late-pregnant mice (condition × repeated measures: $F_{(15,130)} = 5.5$, $p < 0.001$; Fig. 1*E*).

Hormonal and opioid dependence of pregnancy analgesia

These observations are obviously reminiscent of the work of Gintzler (1980), who showed that thresholds to behavioral responding to electric shock in rats increased during pregnancy. His work demonstrated, among other things, that pregnancy analgesia could be mimicked by hormone-stimulated pregnancy (Dawson-Basoa and Gintlzer, 1996) and that the phenomenon was mediated by endogenous opioids (Gintzler, 1980). To establish whether our observations represented a similar process, we performed studies on naive female mice.

First, four groups of mice were ovariectomized (OVX) and then implanted subcutaneously with an osmotic minipump releasing vehicle, estradiol, progesterone, or both hormones together at concentrations observed previously to produce hormone-stimulated pregnancy (HSP) and analgesia in rats (Dawson-Basoa and Gintzler, 1993). Eleven days later, all mice received CFA, such that post-CFA testing occurred on day 14 after hormone treatment. Neither OVX or pump implantation *per se* significantly affected mechanical sensitivity in any group (hormone × repeated measures BL to day +11: $F_{(6,40)} = 2.1$, $p = 0.08$). As shown in Figure 2*A*, CFA allodynia occurred in all groups except the HSP group (hormone × repeated measures day +11 to day +14: $F_{(3,20)} = 4.0$, $p = 0.02$). Similar findings were obtained for SNI. In this experiment, mice were given the nerve injury first, then OVX, and then implanted with a pump. Again, the OVX had no effect on allodynia in any group (hormone × repeated measures +SNI to +OVX: $F_{(3,17)} = 0.2$, $p = 0.86$). As shown in Figure 2*B*, only HSP reversed allodynia (hormone × repeated measures +OVX to day +14: $F_{(3,17)} = 8.5$, $p = 0.001$).

To establish whether spinal cord (or DRG) opioid receptors were mediating the analgesia, we attempted to reinstate mechanical allodynia in pregnant mice with intrathecal naloxone. In the CFA experiment, mice were mated and in late pregnancy injected with CFA. Three days later, immediately after confirming that no allodynia was present, mice were injected with naloxone or saline and retested at 15–120 min after injection. As shown in Figure 2*C*, naloxone reinstated the "hiding" allodynia for at least 60 min (drug × repeated measures pregnancy-120 min: $F_{(4,64)} = 2.8$, $p = 0.03$). In the SNI experiment, mice were given the nerve injury first, and 7 d later (after allodynia was confirmed) were mated. In late pregnancy, immediately after confirming that allodynia was no longer present, mice were injected with naloxone or saline. As shown in Figure 2*D*, naloxone also reinstated SNI-induced allodynia for at least 60 min (drug × repeated measures pregnancy-120 min: $F_{(4,40)} = 3.2$, $p = 0.02$).

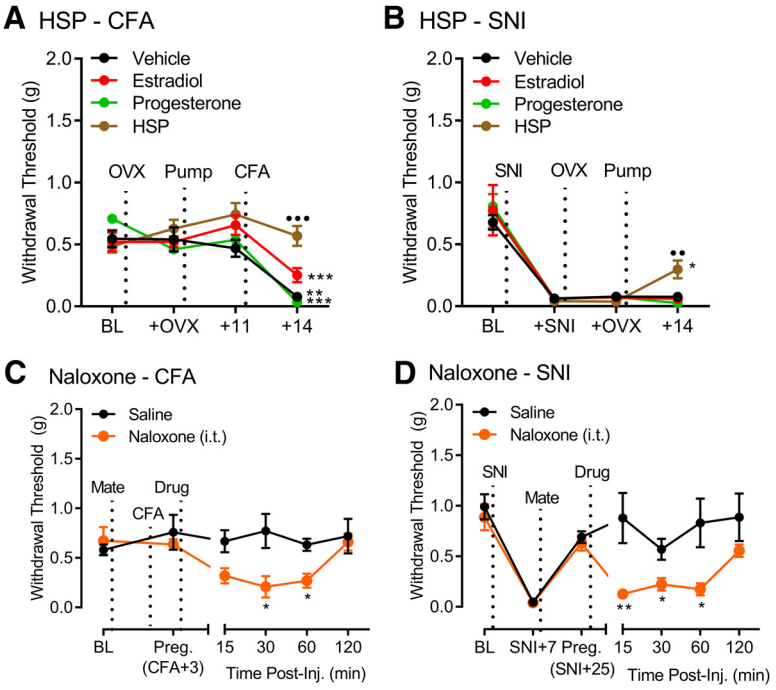

Figure 2. Hormone and opioid dependence of pregnancy analgesia. ***A***, ***B***, Simultaneous adminstration of estradiol and progesterone (HSP) produces abolition of mechanical allodynia from CFA (***A***) and SNI (***B***). Symbols represent mean ± SEM ipsilateral hindpaw withdrawal threshold (g) at baseline (BL), after ovariectomy (OVX) and osmotic minipump (Pump) implantation, and 14 d after SNI or 3 d after CFA (+14 in both cases); $n = 6$ mice/hormone group. ***C***, ***D***, Intrathecal injection of naloxone reveals "hidden" mechanical allodynia after CFA (***C***) or SNI (***D***). Symbols represent mean ± SEM ipsilateral hindpaw withdrawal threshold (g) prepregnancy (BL), on day 3 after CFA at P−2 (pregnancy/CFA+3), or day 7 after SNI (SNI+7) and day 25 after SNI at P−2 (pregnancy/SNI+25), and 15–120 min after injection of drug; $n = 6$–9 mice/drug group. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with +11 (graph ***A***), +OVX (graph ***B***), CFA+3 (graph ***C***), or SNI+25 (graph ***D***). $^{●●}p < 0.01$ compared to all other groups at the same time point. $^{●●●}p < 0.001$ compared with all other groups at the same time point.

T-cell dependence of pregnancy analgesia

To establish more precisely the time course of pregnancy analgesia, we subjected female CD-1 and T-cell-deficient *nude* mice to SNI or sham surgery, confirmed the presence of ipsilateral allodynia on day 7 after surgery, and immediately thereafter mated all mice. Mice were retested for withdrawal thresholds at various time points during pregnancy and after parturition. As shown in Figure 3*A*, mice displayed a progressive loss of ipsilateral SNI allodynia throughout pregnancy (repeated-measures SNI day +7 to P−2: $F_{(6,42)} = 8.3$, $p < 0.001$). Sham-operated mice also displayed a statistically significant frank analgesia developing through pregnancy ($F_{(6,36)} = 2.5$, $p = 0.04$), as did both SNI- and sham-operated mice on the hindpaws contralateral to the injury ($F_{(6,42)} = 4.2$, $p = 0.002$ and $F_{(6,36)} = 3.0$, $p = 0.02$, respectively). At 4 d before parturition, withdrawal thresholds had returned statistically to their presurgery baseline values and, by 3 d after parturition (regardless of whether the pups were removed from the dams), mechanical allodynia was fully reinstated. This progressive loss of mechanical allodynia through pregnancy was entirely absent in T-cell-deficient *nude* mice given SNI (repeated

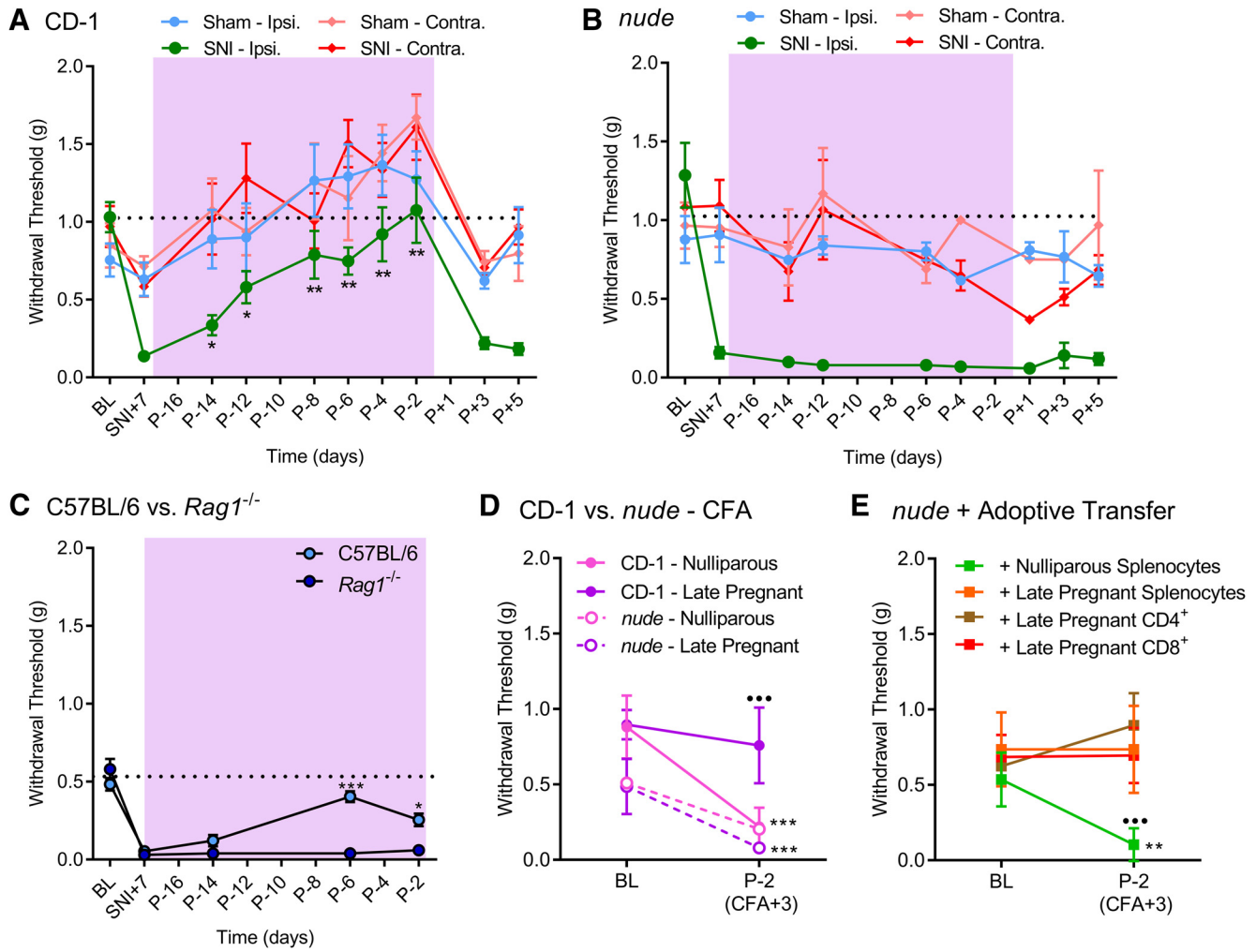

Figure 3. T-cell dependence of pregnancy analgesia. ***A***, ***B***, Progressive attenuation of SNI-induced mechanical allodynia during pregnancy, and reinstatement after parturition, in CD-1 (***A***) but not *nude* (***B***) mice. Symbols represent mean ± SEM ipsilateral (Ipsi.) and contralateral (Contra.) hindpaw withdrawal threshold (g) at baseline (BL), at day 7 after SNI or sham surgery (SNI+7), and at various time points before (P−*x*) and after (P+*x*) parturition; $n = 6–8$ mice/genotype/surgery. Purple shading indicates duration of pregnancy. ***C***, Attenuation of SNI-induced mechanical allodynia during pregnancy in C57BL/6 but not *Rag1*$^{-/-}$ mice. Symbols as in ***A***; $n = 6$ mice/genotype. ***D***, Late-pregnant *nude* mice display CFA allodynia, indicating a loss of pregnancy analgesia. Symbols represent mean ± SEM ipsilateral hindpaw withdrawal threshold (g) at prepregnancy (BL) and on day 3 after CFA at P−2 (CFA+3); $n = 7–10$ mice/genotype/condition. ***E***, Adoptive transfer of splenocytes or isolated CD4$^+$ or CD8$^+$ T cells from pregnant CD-1 mice into late-pregnant *nude* mice blocks CFA allodynia, indicating a restoration of pregnancy analgesia. Symbols as in ***D***; $n = 5–8$ mice/condition. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with SNI+7 (graphs ***A***–***C***) or BL (graphs ***D*** and ***E***). In graph ***A***, asterisks in conditions other than SNI-Ipsi. are omitted for clarity. ●●●$p < 0.001$ compared with all other groups at the same time point.

measures SNI day +7 to P−4: $F_{(4,26)} = 0.1$, $p = 0.98$; Fig. 3*B*). Analgesia in sham-operated mice and on the contralateral hindpaws was also entirely absent in *nude* mice (all $p > 0.40$; Fig. 3*B*).

To confirm that the lack of pregnancy analgesia in *nude* female mice was indeed due to T-cell deficiency, we first replicated the study described above using *Rag1*-null mutant (*Rag1*$^{-/-}$) mice and their wild-type controls, C57BL/6. C57BL/6 mice displayed a significant reduction of allodynia in late pregnancy (although not a complete reversal like CD-1). *Rag1*$^{-/-}$ mice, like *nude* mice, showed no alteration whatsoever of mechanical allodynia through pregnancy (genotype × repeated measures SNI day +7 to P−2: $F_{(3,24)} = 24.2$, $p < 0.001$; Fig. 3*C*).

A lack of pregnancy analgesia in *nude* mice after CFA was also observed (genotype × pregnancy state × repeated measures: $F_{(1,21)} = 6.2$, $p = 0.02$; Fig. 3*D*). To further confirm the involvement of T cells in the production of this analgesia, we used a new cohort of late-pregnant *nude* mice given CFA and adoptively transferred splenocytes from nulliparous CD-1 mice or splenocytes, isolated CD4$^+$ T cells, or isolated CD8$^+$ T cells from late-pregnant CD-1 mice. As shown in Figure 3*E*, adoptive transfer of all cell populations from late-pregnant CD-1 mice were able to restore pregnancy analgesia (i.e., abolish the mechanical allodynia) in *nude* mice (adoptive transfer condition × repeated measures: $F_{(3,24)} = 4.1$, $p = 0.02$).

δ-Opioid receptor mediation of pregnancy analgesia

Because pregnancy analgesia has been shown previously to be opioid receptor dependent (Dawson-Basoa and Gintzler, 1996; Dawson-Basoa and Gintzler, 1997, 1998) and because we also observed reinstatement of CFA and SNI allodynia after intrathecal naloxone administration, we endeavored to define the opioid receptor subtype specificity of the phenomenon. We first performed quantitative RT-PCR of the classical opioid receptor genes, *Oprm1* (μ), *Oprd1* (δ), and *Oprk1* (κ), in the two potential sites of action of intrathecal naloxone: the DRG and the spinal cord. CD-1 nulliparous and late-pregnant mice were compared with *nude* mice of the same status; naive animals were used because the pregnancy analgesia can be seen in uninjured mice. As

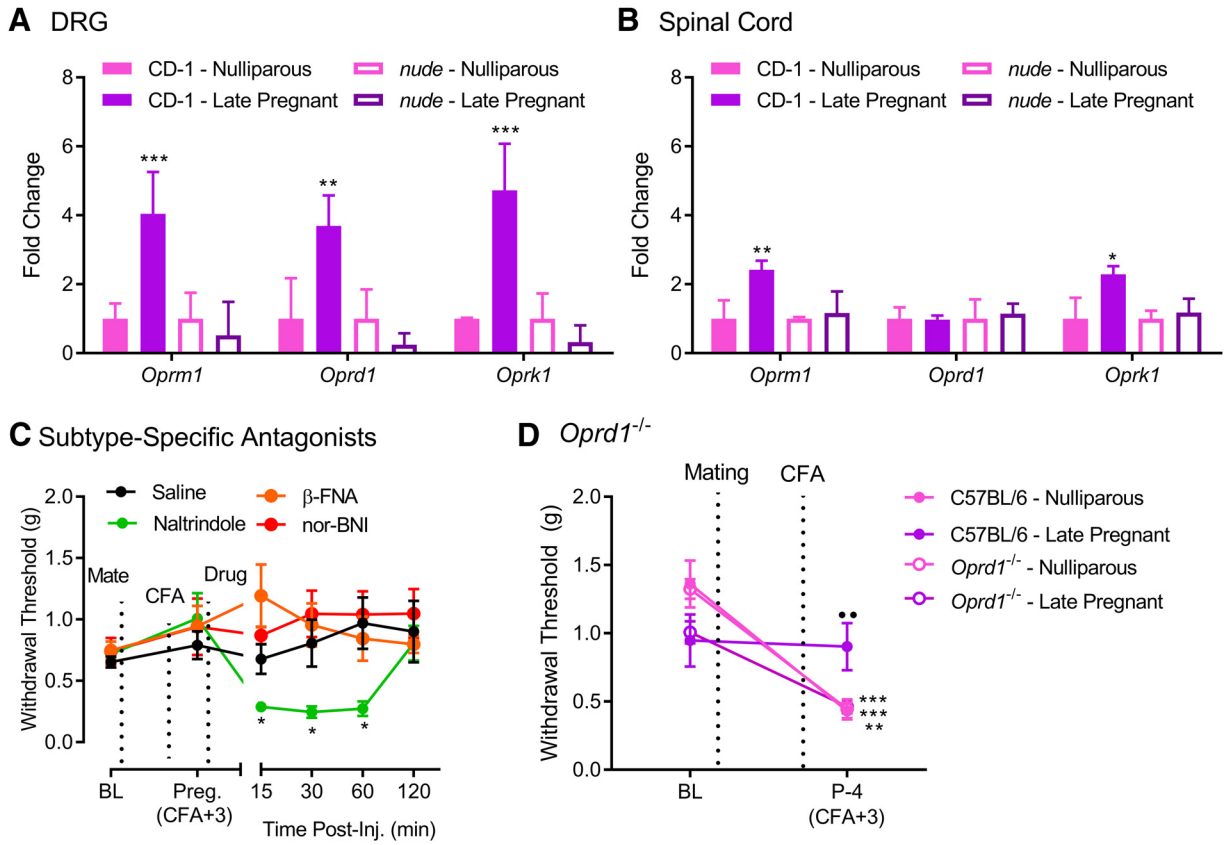

Figure 4. δ-Opioid receptor dependence of pregnancy analgesia. ***A***, ***B***, Relative expression of the *Oprm1*, *Oprd1*, and *Oprk1* genes in the DRG (***A***) and spinal cord (***B***) of nulliparous and late pregnant CD-1 and *nude* mice. Error bars indicate mean ± SEM relative expression normalized to nulliparous CD-1 mice. ***C***, Intrathecal injection of naltrindole, but not β-funaltrexamine (β-FNA) or nor-binaltorphimine (nor-BNI), reinstates "hidden" CFA allodynia in late-pregnant mice. Symbols represent mean ± SEM ipsilateral hindpaw withdrawal threshold (g) prepregnancy (BL), on day 3 after CFA at P−2 (pregnancy/CFA+3), and 15–120 min after injection of drug; $n = 6–9$ mice/drug. ***D***, Pregnancy analgesia is absent in late-pregnant *Oprd1*$^{-/-}$ mice. Symbols represent mean ± SEM ipsilateral hindpaw withdrawal threshold (g) prepregnancy (BL), at P−7, and 3 d after CFA (P−4/CFA+3); $n = 5–8$ mice/genotype/condition. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with nulliparous CD-1 (graphs ***A*** and ***B***), CFA+3 (graph ***C***), or BL (graph ***D***). $^{●●}p < 0.01$ compared with all other groups at the same time point.

shown in Figure 4*A*, pregnancy produced an ≈4-fold upregulation of all three opioid receptor genes in the DRG compared with nulliparous mice (*Oprm1*: $t_{(8)} = 5.3$, $p < 0.001$; *Oprd1*: $t_{(8)} = 4.1$, $p = 0.003$; *Oprk1*: $t_{(8)} = 6.2$, $p < 0.001$); no hint of similar increases were observed in *nude* mice (all $p > 0.7$). A smaller but still statistically significant increase was seen in the spinal cord for *Oprm1* ($t_{(5)} = 4.7$, $p = 0.005$) and *Oprk1* ($t_{(5)} = 3.9$, $p = 0.01$), but not *Oprd1* ($t_{(5)} = 0.2$, $p = 0.88$) (Fig. 4*B*). Because both opioid receptors in the DRG and the spinal cord are capable of producing analgesia, these gene expression findings permit the potential role of all three receptor subtypes. To determine which of them are actually relevant to pregnancy analgesia, we intrathecally injected the subtype-specific antagonists β-funaltrexamine (μ), naltrindole (δ), and nor-binaltorphimine (κ). As seen in Figure 4*C*, only the δ-opioid receptor antagonist naltrindole was capable of reinstating the "hiding" allodynia in a manner similar to naloxone (drug × repeated measures pregnancy-120 min: $F_{(12,108)} = 3.1$, $p = 0.001$). To confirm the relevance of the δ-opioid receptor, we tested nulliparous and late-pregnant *Oprd1*-null mutant (*Oprd1*$^{-/-}$) mice compared with wild-type (C57BL/6) controls for CFA allodynia. Late-pregnant *Oprd1*$^{-/-}$ mice displayed unaltered allodynia compared with nulliparous mutants (genotype × pregnancy status × repeated measures: $F_{(1,18)} = 6.9$, $p = 0.02$), indicating the lack of pregnancy analgesia (Fig. 4*D*).

Discussion

Microglial-dependent and -independent pain processing

The present study documents two separate phenomena related to the neuroimmune processing of chronic pain in pregnant female mice. First, we observed that female mice in the early stages of pregnancy display—like male mice, but unlike nulliparous or postweaning female mice—minocycline-reversible mechanical allodynia. The insensitivity to minocycline reversal of allodynia we interpret, as we have previously (Sorge et al., 2015), as a "switching" over from a microglial-dependent to a parallel, non-microglial-dependent pain-processing mechanism in the spinal cord. That females do not use spinal cord microglia to process allodynia is supported by the insensitivity of female mice (and rats; J. Mapplebeck, R. Dalgamo, O. Moriarty, S. Beggs, Y. Tu, C. Kwok, J. Mogil, T. Trang, and M. Salter, unpublished data) to blockade of allodynia via not only intrathecal minocycline, but also spinal administration of the following: (1) fluorocitrate, (2) propentofylline, (3) the P2X inhibitor TNP-ATP, (4) the p38 MAPK inhibitor SB203580, (5) the NGF/BDNF inhibitor Y1036, (6) the BDNF-sequestering fusion protein TrkB-Fc, (7) the caspase-6 inhibitor ZVEID7; or (8) the microglial toxin Mac-1-saporin (Sorge et al., 2015; Berta et al., 2016; Taves et al., 2016). Also in support of this contention are the male-specific pain phenotypes of *Cx3cr1*^{CreER}-*Bdnf* (Sorge et al., 2015; but see Peng

et al., 2016) and caspase-6-null mutant (Berta et al., 2016) mice. We believe that this parallel "female-specific" mechanism involves T cells, based on the following: (1) the sensitivity of even female T-cell-deficient mouse strains (*nude* and *Rag1*$^{-/-}$) to allodynia reversal by microglial inhibitors; (2) effective reinstatement of microglial inhibitor insensitivity by adoptive transfer of splenocytes; and (3) female-specific reversal of allodynia by pioglitazone, which blocks T-cell-expressed peroxisome proliferator activated receptor-gamma (Sorge et al., 2015). Experiments are ongoing in our laboratory to provide additional evidence of female-specific T-cell involvement in pain processing and these experiments are now including early-pregnant mice. We note that the adaptive immune environement is greatly affected by sex hormones; whereas males have a more dominant Th2 immune population in their circulating CD4$^+$ cells, females have a higher Th1-dominant immune environment (Whitacre et al., 1999). Because our ongoing studies have suggested that it is indeed CD4$^+$ T cells that are responsible for producing hypersensitivity in female mice, known decreases in the Th1/Th2 ratio during pregnancy (Whitacre et al., 1999) might explain the "switching" of early-pregnant mice to an alternate pain processing pathway. Studies in human arthritics have found that an increase in Th2 cytokines during pregnancy positively correlates with improvement of disease activity (Ostensen, 1992).

T-cell dependence of δ-opioid-mediated pregnancy analgesia

Second, and the main focus of this study, we observed that female mice in the late stages of pregnancy do not display any evidence of mechanical allodynia after inflammation, nerve damage, or LPS; thermal hyperalgesia after inflammation; or spontaneous inflammatory pain. Although pregnancy analgesia has been demonstrated in both rats (Gintzler, 1980; Iwasaki et al., 1991) and mice (Jayaram et al., 1995; Jayaram et al., 1997), we are aware of only one prior preclinical demonstration that the phenomenon can be seen in the context of persistent pain. Using the chronic constriction injury (CCI) assay of neuropathic pain, Onodera et al. (2017) very recently reported the loss of mechanical allodynia at 21 d of pregnancy. They also observed that CCI-induced increases in tumor necrosis factor-α, the immediate-early gene *Fos*, activated astrocytes (GFAP), and activated microglia (Iba-1) were absent in pregnant CCI mice (Onodera et al., 2017). However, our finding's of (if not always statistically significant) changes in nociceptive sensitivity on the hindpaw contralateral to the injury and in sham-operated animals suggest that this phenomenon does not represent a specific anti-hypersensitivity process so much as a frank pregnancy-related analgesia. That is, it appears that the reduced mechanical allodynia observed in the late stages of pregnancy simply represents the sum of the allodynia caused by the injury and the analgesia associated with pregnancy itself.

Therefore, we believe the phenomenon under study herein appears to be the same one discovered in 1980 by Alan Gintzler. Indeed, like Gintzler and colleagues (Dawson-Basoa and Gintzler, 1993), we observe that pregnancy analgesia can be produced in nonpregnant, ovariectomized mice by simulating pregnancy-like gonadal hormone conditions; only simultaneous pump administration of estrogen and progesterone were found to block allodynia. Furthermore, we observe that intrathecal injection of the broad-spectrum opioid receptor antagonist naloxone to late-pregnant mice not displaying mechanical allodynia resulted in the temporary unmasking of that allodynia; that is, produced a temporary blockade of the pregnancy analgesia that was counteracting it. Unlike Gintzler, we show here that pregnancy analgesia in the mouse is dependent on T cells and (uniquely) δ-opioid receptors. Pregnancy produced a robust upregulation of all three genes (*Oprm1*, *Oprd1*, and *Oprk1*) coding for the classical opioid receptor subtypes—μ, δ, and κ, respectively—in the DRG, and a smaller upregulation of the *Oprm1* and *Oprk1* genes in the spinal cord. Activation of any or all of these receptors might be expected to produce analgesia. In the rat, Gintzler and colleagues demonstrated the importance of a synergistic interaction between δ-opioid and κ-opioid receptors (Dawson-Basoa and Gintzler, 1998); selective antagonism of the μ-opioid receptor did not affect pregnancy analgesia (Dawson-Basoa and Gintzler, 1996). The implications of this apparent species difference need to be explored in future work, as does the status in the mouse of a parallel mechanism of pregnancy analgesia in the rat involving α_2-noradrenergic activity (Liu and Gintzler, 1999). It is of interest that the only extant work on pregnancy analgesia in the mouse revealed the involvement of enkephalins (Jayaram et al., 1995; Jayaram et al., 1997), which are of course the endogenous ligands of the δ-opioid receptor.

This effect of pregnancy on opioid gene expression appeared to be T-cell dependent because none of these increases appeared in pregnant *nude* mice. Opioid (and especially δ-opioid) receptor regulation of T-cell proliferation and functioning is well known; activated T cells release enkephalins that can modulate T cells via autocrine and paracrine binding to δ-opioid receptors (Sharp, 2006). Antigen-primed CD4$^+$ T cells are well known to release endogenous opioids in the periphery and thereby cause analgesia (Cabot et al., 1997; Verma-Gandhu et al., 2006; Boué et al., 2011). After injury, antigen-presenting cells migrate to the lesion site, followed by T cells, although the exact mechanism of this recruitment is not well understood (Moalem et al., 2004). It is known that injury can activate T cells (Hanschen et al., 2011; Liu et al., 2012), suggesting that the T cells in our experiments may have been primed by the inflammatory or nerve injuries. Experiments aimed at determining the exact mechanism by which T cells (primed or otherwise) affect opioid gene expression are ongoing. CD4+ Th2 cells are known to release anti-inflammatory cytokines such as IL-10 and TGF-β, which themselves can inhibit pain (Echeverry et al., 2009; Jankovic et al., 2010). Interestingly, null mutants of the BMP and activin membrane bound inhibitor (BAMBI) gene, which is known to be a negative regular of TGF-β, show increased μ- and δ-opioid receptor expression in the dorsal horn of the spinal cord after SNI (Lantero et al., 2014).

During pregnancy, the maternal immune system undergoes dramatic changes; this includes a robust phenotypic shift of the T-cell population to produce fewer Th1 cytokines and more Th2 cytokines (Reinhard et al., 1998). This shift has been used to explain maternal protection from autoimmunity during pregnancy (Adams Waldorf and Nelson, 2008; Munoz-Suano et al., 2012). More generally, we note the rapidly increasing evidence of a role of T cells in chronic pain processing and modulation (Costigan et al., 2009; Li et al., 2013; Draleau et al., 2014; Liu et al., 2014; Zhang et al., 2014; Austin et al., 2015; Liu et al., 2015; Luchting et al., 2015; Sorge et al., 2015; Vicuña et al., 2015; Duffy et al., 2016; Krukowski et al., 2016; Massart et al., 2016; Shubayev et al., 2016; Baddack-Werncke et al., 2017). The current observations reinforce the notion that the adaptive immune system is an underappreciated source of pain modulation and one that might be exploited clinically.

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