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ORIGINAL ARTICLE

Pregnancy ameliorates neuropathic pain through suppression of microglia and upregulation of the δ-opioid receptor in the anterior cingulate cortex in late-pregnant mice

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Abstract

Purpose Pregnancy-induced analgesia develops in late pregnancy, but its mechanisms are unclear. The anterior cingulate cortex (ACC) plays a key role in the pathogenesis of neuropathic pain. The authors hypothesized that pregnancy-induced analgesia ameliorates neuropathic pain by suppressing activation of microglia and the expression of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and by upregulating opioid receptors in the ACC in late-pregnant mice.

Methods Neuropathic pain was induced in non-pregnant (NP) or pregnant (P) C57BL/6JJmsSlc female mice by partial sciatic nerve ligation (PSNL). The nociceptive response was evaluated by mechanical allodynia and activation of microglia in the ACC was evaluated by immunohistochemistry. The expressions of phosphorylated AMPA receptors and opioid receptors in the ACC were evaluated by immunoblotting.

Results In von Frey reflex tests, NP-PSNL-treated mice showed a lower 50% paw-withdrawal threshold than NP-Naïve mice on experimental day 9. No difference in 50% paw-withdrawal threshold was found among the NP-Naïve, NP-Sham, P-Sham, and P-PSNL-treated mice. The number of microglia in the ACC was significantly increased in NP-PSNL-treated mice compared to NP-Sham mice. Immunoblotting showed significantly increased expression of phosphorylated AMPA receptor subunit GluR1 at Ser831 in NP-PSNL-treated mice compared to NP-Sham mice. Immunoblotting also showed significantly increased δ-opioid receptor in the ACC in P-Sham and P-PSNL-treated mice compared to NP-Sham mice.

Conclusion Pregnancy-induced analgesia ameliorated neuropathic pain by suppressing activation of microglia and the expression of phosphorylated AMPA receptor subunit GluR1 at Ser831, and by upregulation of the δ-opioid receptor in the ACC in late-pregnant mice.

Keywords Neuropathic pain · Pregnancy-induced analgesia · Anterior cingulate cortex · Opioid receptors

Introduction

It is well known that women experience attenuation of pre-existing chronic pain or an increase in the threshold to noxious heat pain during pregnancy, in a phenomenon termed pregnancy-induced analgesia [1–5]. Although several animal studies have reported that pregnancy-induced analgesia is related to inhibition of proinflammatory cytokines, T-cell-mediated immune systems, and immunosuppressive molecular mechanisms, the mechanisms underlying pregnancy-induced analgesia are still not clear [5–7]. Neuropathic pain is a severe chronic pain condition caused by peripheral or central nerve injury [8, 9]. A previous study has reported that the immune cells mediating mechanical pain hypersensitivity after nerve injury differ between male and female mice [10]. In male mice, microglia–neuron interactions are essential for the initiation of neuropathic pain [11, 12]. Although spinal microgliosis after nerve injury is reported to be similar between male and female mice, adaptive immune cells (T cells) are used instead of microglia to produce mechanical allodynia after nerve injury in female mice [10]. Another study has reported that female mice switch from microglia-independent to microglia-dependent

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pain hypersensitivity mechanisms in early pregnancy [7]. The anterior cingulate cortex (ACC) is the cortical area responsible for perception and modulation of nociceptive input and is associated with the pathogenesis of neuropathic pain [12]. The ACC has wide bilateral receptive fields and long-lasting responses to noxious stimuli from the spinal cord via the thalamus [13]. Glutamatergic neurotransmission has been shown to play a critical role in pain processing in the ACC [14]. Previous studies have reported that presynaptic release of glutamate and the function of postsynaptic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in ACC neurons contributed to the development of neuropathic pain [15, 16].

It was previously reported that pregnancy-induced analgesia ameliorated neuropathic pain by suppressing the activation of microglia in the spinal dorsal horn in a rat model of chronic constriction injury (CCI) [6]. In addition, Rosen et al. demonstrated that pregnancy upregulated the genes coding for the opioid subtypes, including κ, μ, and δ, in the dorsal root ganglia, and that intrathecal injection of the δ-opioid receptor antagonist naltrindole counteracted pregnancy-induced analgesia [7]. Although it has been reported that opioid receptors are highly expressed in the ACC and that opioid signaling within the ACC is implicated in modulating neuropathic pain, the role of the ACC in pregnancy-induced analgesia has not yet been investigated [17]. The aim of the present study was to investigate whether pregnancy could ameliorate neuropathic pain, and suppress the activation of microglia and the expression of phosphorylation of glutamate AMPA receptors in the ACC, in neuropathic pain model mice. Furthermore, the expressions of κ-, μ-, and δ-opioid receptors in the ACC were examined to identify those relevant to pregnancy-induced analgesia.

Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Sapporo Medical University (No. 18-040_21-021) and conducted according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD). C57BL/6JJmsSlc female mice [non-pregnant (NP) mice, 8 weeks old] and C57BL/6JJmsSlc female mice on gestational day 10 [pregnant (P) mice, 8 weeks old] were purchased from Japan SLC (Shizuoka, Japan) at the start of the experiments (Fig. 1). Although using only one strain reduces generalizability, mice of a single strain were chosen for homogeneity with our previous study [18], which would facilitate comparisons as needed. Mice were housed (NP-mice, 2–4 animals per cage; P-mice, one animal per cage) in our institutional animal care facility in a temperature-controlled room (22–24 °C) under a 12-h light/dark cycle (lights on from 7 AM to 7 PM) with unlimited access to food and water. Mice were randomly allocated to eight groups, consisting of three control groups (NP-Naïve, NP-Sham, and P-Sham) and five experimental groups [NP-partial sciatic nerve ligation (PSNL), P-PSNL, P-PSNL-Vehicle, P-PSNL-naltrindole (NTI), and NP-PSNL-KNT-127, Figure S1]. The NP-Naïve group underwent no surgery (Fig. 1). The NP-Sham and P-Sham groups underwent sham-treated surgeries, but no PSNL (Fig. 1), whereas the NP-PSNL and P-PSNL groups underwent PSNL-treated surgeries (Fig. 1). The P-PSNL-Vehicle, P-PSNL-NTI, and NP-PSNL-KNT-127 groups underwent PSNL-treated surgeries with guide cannula implantation (Fig. 1). All efforts were made to minimize the number of animals used and the suffering of the animals. After completion of the experiments, the mice were euthanized by decapitation under deep isoflurane anesthesia.

Surgery

Three days after the shipment was received at our institutional animal care facility, the NP-PSNL, P-PSNL, P-PSNL-Vehicle, and P-PSNL-NTI groups were subjected to PSNL, as described in our previous study [18]. In brief, a small skin incision was made at high thigh level in the right hindlimb under 1.5–2% isoflurane anesthesia, and the sciatic nerve was carefully isolated from neighboring connective tissue. The dorsal one-third to one-half of the sciatic nerve was tightly ligated with an 8–0 polypropylene suture on the right. The wound was then closed with two skin sutures (8–0). For the sham operation, the right sciatic nerve was exposed, but not ligated. Naïve mice did not undergo surgery. Postoperative analgesia was not provided so as not to confound the endpoints under study.

Behavioral tests for sensitivity to mechanical stimulation

Behavioral testing was conducted blind with respect to group assignment. Blinding to pregnancy status was obviously not possible. The day before surgery and 5 days after surgery, mechanical allodynia was assessed using a set of calibrated von Frey hair monofilaments (0.16–2.0 g, North Coast Medical, Morgan Hill, CA) to measure the bilateral paw-withdrawal threshold. The mice were placed individually in a clear plastic box on an elevated wire mesh floor and habituated for 1 h to allow acclimatization to the new environment prior to mechanical assessment using von Frey filaments. Beginning with 0.16 g, von Frey filaments were firmly applied to the plantar surface of the right (injured) hind paw of an inactive mouse. The response to each hair force was measured at 5-min intervals. The 50% withdrawal

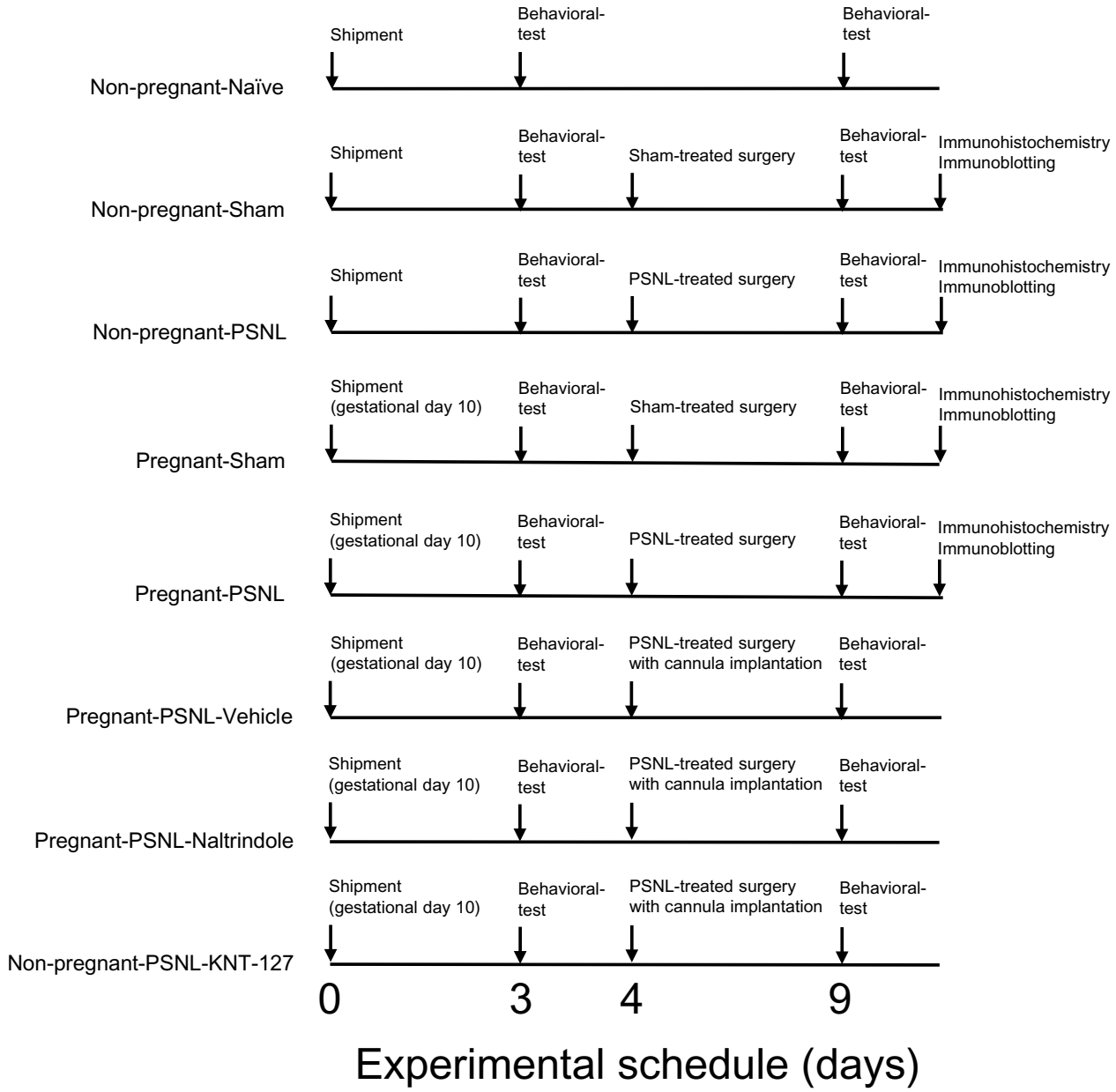

Fig. 1 Schematic illustration of the experimental design of the study. The study design includes behavioral tests, induction of neuropathic pain (PSNL-treated surgery), implantation of guide cannulas, immunohistochemistry, and immunoblotting. *PSNL* partial sciatic nerve ligation

threshold was calculated using the up-down method as described previously [19].

Immunohistochemistry

After completion of behavioral testing, animals were anesthetized with intraperitoneal injections of sodium pentobarbital (50 mg/kg) and transcardially perfused with phosphate-buffered saline followed by 4% paraformaldehyde, and brain tissue samples were harvested. Brains were postfixed in paraformaldehyde overnight and stored in 30% sucrose. Brains were cut into serial 20-µm-thick coronal sections in a cryostat and collected as free-floating sections for immunohistochemistry. Every sixth section randomly selected from intervals of 160 µm cut through the ACC, which was confirmed as from 1.18 mm anterior to the bregma to 0.22 mm posterior to the bregma according to the Mouse Brain in Stereotaxic Coordinates [20], was washed three times in PBS containing 0.1% Tween 20 (PBS-T). Sections were permeabilized and blocked for 30 min with PBS-T containing 5% bovine serum albumin and incubated with primary antibodies for ionized calcium-binding adapter molecule 1

(Iba-1; 1:500, 019-19745; Wako Pure Chemical Industries, Osaka, Japan) and glial fibrillary acidic protein (GFAP; 1:400, AB5541; EMD Millipore, Billerica, MA) overnight at 4 °C. Sections were washed three times in PBS-T and then incubated for 2 h with Alexa Fluor 594-conjugated anti-Iba-1 (Invitrogen, Carlsbad, CA) or Alexa Fluor 488-conjugated anti-GFAP (Millipore, Burlington, MA) at a dilution of 1:500. Nuclei were counterstained with 40,6-diamidino-2-phenylindole dihydrochloride solution (DAPI; 1:1000, D523; Dojindo Laboratories, Kumamoto, Japan). Sections were washed three times in PBS-T and then covered with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA) and the resultant images were observed using confocal laser microscopy (A1; Nikon, Tokyo, Japan). Immunofluorescence of Iba-1 and GFAP was measured using ImageJ software (National Institutes of Health). The ACC area was distinguished according to the Mouse Brain in Stereotaxic Coordinates [20]. The bilateral ACC areas and the areas positive for Iba-1 and GFAP within the ACC areas were measured using ImageJ software. The numbers of Iba-1-positive cells in the ACC were also automatically counted using ImageJ software. Areas showing Iba-1-positive staining are presented as mean percentages of ACC volume (Iba-1-positive signal divided by total volume of the ACC) on each of sections 1–6. The threshold for Iba-1-positive signal in the image was more than 235 per pixel, using ImageJ software. Immunohistochemistry was performed in the NP-Sham, NP-PSNL, P-Sham, and P-PSNL groups.

Immunoblotting

After completion of behavioral testing, mice were euthanized by decapitation under deep anesthesia induced with intraperitoneal injection of sodium pentobarbital (50 mg/kg), and tissue of the whole ACC was dissected on ice and homogenized at 4 °C with carbonate lysis buffer (500 mM sodium carbonate, pH 11.0) containing a protease and phosphatase inhibitor (1:100, #5872, Cell Signaling Technology, Danvers, MA). After homogenization, the samples were sonicated on ice three times for 15 s each. The nuclear components were removed by centrifugation at 1000 g for 10 min, and the protein concentration was quantified by Bradford assay and normalized to 1.2 μg/μL to load the exact equal amount of protein. Equal amounts of protein (9.6 μg/8 μL) were loaded to determine the expression of phosphorylated AMPA receptor subunit GluR1 at Ser831 and Ser845, beta-endorphin, and κ-, μ-, and δ-opioid receptors in the ACC. Electrophoresis was performed on the samples using 4–12% acrylamide gels (Bolt™ Bis–Tris Plus; Thermo Fisher Scientific, Waltham, MA) and transferred to polyvinylidene fluoride membranes (Millipore Sigma, Burlington, MA) by electroelution. Membranes were blocked in blocking solution [20 mM Tris-buffered saline with Tween 20 (TBS-T) (0.1%) containing 3% bovine serum albumin], and then incubated with primary antibody to phosphorylated GluR1 at Ser831 (1:50, N453; Merck Millipore, Darmstadt, Germany), phosphorylated GluR1 at Ser845 (1:50, AB5849; Merck Millipore), beta-endorphin (1:50, #710319; Invitrogen, Waltham, MA), κ-opioid receptor (1:50, ab183825; Abcam), μ-opioid receptor (1:50, ab134054; Abcam, Boston, MA), and δ-opioid receptor (1:50, ab176324; Abcam) overnight at 4 °C. After three washes with TBS-T, the membrane was incubated with a species-specific infrared-dye-labeled secondary antibody (1:3000, #7074; Cell Signaling Technology) for 1 h at room temperature in the dark. After another three washes with TBS-T to remove the remaining secondary antibody, the membrane was washed with Tris-buffered saline to remove the Tween residue to avoid background. All protein expression was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:10,000, NB100-56875; Novus Biologicals, Centennial, CO). Densitometry of the different bands was further quantified using the ImageJ software with normalization by GAPDH. Immunoblotting was performed in the NP-Sham, NP-PSNL, P-Sham, and P-PSNL groups.

Microinjection of a δ-opioid receptor antagonist and a δ-opioid receptor agonist into the anterior cingulate cortex

A guide cannula for micro-injection, targeting the ACC, was implanted in the P-PSNL-Vehicle, P-PSNL-NTI, and NP-PSNL-KNT-127 groups. Mice were subsequently fixed in a stereotactic frame (David Kopf Instruments, Tujunga, CA) under isoflurane anesthesia after PSNL-treated surgeries. The skull was exposed, and a small hole was made using a dental drill. A chronic guide cannula [62004 (outer × inner diameter, 0.41 × 0.25 mm), Neuroscience, Inc., Tokyo, Japan] was implanted into the ACC. The stereotaxic coordinates were 1.0 mm anterior to the bregma, 0.3 mm left lateral to the midline, and 1.4 mm below the outer surface of the skull, according to the method used in a previous study [21]. The guide cannula was secured with dental cement on the surface of the skull. A dummy cannula was inserted after surgery and left in place until the micro-injection day. Postoperative analgesia was not provided so as not to confound the endpoints under study. Five days after the surgeries, the P-PSNL-NTI group was micro-injected [injection needle, 62,204 (outer × inner diameter, 0.21 × 0.11 mm), Neuroscience, Inc.] with the δ-opioid receptor antagonist naltrindole (5 ng/nL in solvent of 0.9% sodium chloride solution, N115, Sigma-Aldrich, St. Louis, MO) in a volume of 100 nL/mouse, into the ACC via the infusion cannula (62,204, Neuroscience, Inc.) using a 0.5-μL Hamilton syringe (2-402-01, AS ONE CORPORATION, Osaka, Japan) and digital infusion pump (1-1591-02, AS ONE CORPORATION) at

an infusion rate of 20 nL/min under isoflurane anesthesia. The P-PSNL-Vehicle group was micro-injected with 0.9% sodium chloride solution. The NP-PSNL-KNT-127 group was micro-injected with the δ-opioid receptor agonist KNT-127 (50 ng/mouse, MedChemExpress, Monmouth Junction, NJ) in a volume of 400 nL/mouse, into the ACC via the infusion cannula using a 0.5-μL Hamilton syringe and digital infusion pump at an infusion rate of 20 nL/min under isoflurane anesthesia. The mice were returned to their home cages after micro-injection. One hour after micro-injection with NTI, vehicle, or KNT-127, mice were tested to assess their sensitivity to mechanical stimulation.

Statistical analysis

Blindness to the treatment group of the specimen was removed only after the analysis phase. The primary outcome of the present study was the 50% withdrawal threshold of the right hind paw. It was estimated that this required eight animals for each of the seven groups [with $\alpha = 0.05$, $1 - \beta = 0.8$, effect size 0.4 g, expected standard deviation (SD) 0.25 g by ANOVA] to demonstrate a 50% decrease in the 50% withdrawal threshold based on our previous experiments with this design and pilot study results. The secondary outcome was the number of Iba-1-positive cells in the ACC on immunohistochemical analysis. Based on our pilot study results, we estimated that a sample of four animals per group was necessary to demonstrate a 40% increase in the number of Iba-1-positive cells in the ACC (with $\alpha = 0.05$ and $1 - \beta = 0.8$ by ANOVA). The sample size '*n*' represents the number of animals for each group. No exclusions for outliers were made in the present study. None of the variables had missing data, and no outliers appeared. All results were examined with animal as the unit of analysis. Statistical analysis was performed with GraphPad Prism 8 software (GraphPad, La Jolla, CA). Normality of the distribution of data was assessed using the Shapiro–Wilk test. When appropriate, statistical analysis of means ± SD of the mean differences between groups was performed by repeated-measures one-way or two-way ANOVA, followed by Tukey's multiple comparison post hoc analysis. All tests were two-tailed, and $P < 0.05$ was considered to indicate significance.

Results

Pregnancy ameliorated neuropathic pain induced by partial sciatic nerve ligation in mice

In the behavioral tests, no mechanical allodynia was observed in any control group (NP-Naïve, NP-Sham, or P-Sham) on experimental day 9 (Fig. 2). In the NP-PSNL group, the 50% ipsilateral paw-withdrawal threshold was significantly decreased compared to that in the NP-Naïve group on experimental day 9 (mean ± SD, 0.05 ± 0.02 vs. 0.95 ± 0.18 g; $F_{1,70} = 15.49$, $P = 0.0002$, Fig. 2a). No difference was found in the 50% ipsilateral paw-withdrawal threshold among the NP-Naïve, NP-Sham, P-Sham, and P-PSNL groups on experimental day 9 ($P = 0.9998$, Fig. 2a). No difference was found in the 50% contralateral paw-withdrawal

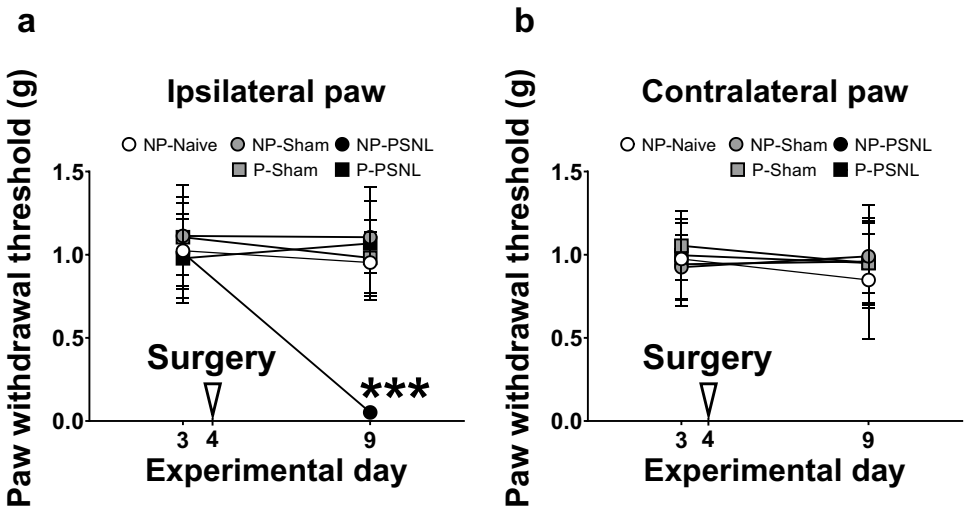

Fig. 2 Pregnancy ameliorates neuropathic pain induced by partial sciatic nerve ligation in mice. **a** In the NP-PSNL group, the 50% ipsilateral paw-withdrawal threshold is significantly decreased compared to that in the NP-Naïve group on experimental day 9. No difference is found in the ipsilateral 50% paw-withdrawal threshold among the NP-Naïve, NP-Sham, P-Sham, and P-PSNL groups on experimental day 9. Data are expressed as means ± SD ($n = 8$ mice/group). ***$P = 0.0002$ vs. NP-Naïve group on day 9. **b** No difference was found in the 50% contralateral paw-withdrawal threshold among the five groups on experimental days 3 and 9. Data are expressed as means ± SD ($n = 8$ mice/group). *NP* non-pregnant, *P* pregnant, *PSNL* partial sciatic nerve ligation

threshold among the five groups on experimental days 3 and 9 ($F_{4,70}=0.3971$, $P=0.8101$, Fig. 2b). These results indicate that pregnancy ameliorated neuropathic pain induced by PSNL in mice.

Pregnancy suppressed the accumulation of microglia in the anterior cingulate cortex in neuropathic model mice

To examine whether neuropathic pain caused by PSNL induced the accumulation of glial cells, microglia, and astrocytes in the ACC, the expression of Iba-1 or GFAP-positivity was measured bilaterally in the ACC 5 days after surgery. Astrocytes positively stained with GFAP were observed in the cingulum and the genu of the corpus callosum, but not in the ACC, in the NP-Sham, NP-PSNL, P-Sham, and P-PSNL groups (Fig. 3a). The number of Iba-1-positive cells in the ACC was significantly increased in the NP-PSNL group compared to that in the NP-Sham group (mean ± SD, 122 ± 5 vs. 68 ± 11; $F_{3,12}=49.77$, $P=0.0001$, Fig. 3b). No difference was found in the number of Iba-1-positive cells in the ACC among the NP-Sham, P-Sham, and P-PSNL groups ($P=0.6856$). Furthermore, the expression of Iba-1-positive areas in the ACC was significantly increased in the NP-PSNL group compared to that in the NP-Sham group (mean ± SD, 2.0 ± 0.3 vs. 1.4 ± 0.2%; $F_{3,12}=8.465$, $P=0.0198$, Fig. 3c). No difference was found in the expression of Iba-1-positive areas in the ACC among the NP-Sham, P-Sham, and P-PSNL groups ($P=0.6338$).

Pregnancy suppressed the expression of phosphorylated AMPA receptor subunit GluR1 at Ser831 in the anterior cingulate cortex in neuropathic model mice

To investigate the protein expression of phosphorylated GluR1 at Ser831 and Ser845 in the ACC, ACC homogenates were obtained. ACC homogenates from the NP-PSNL group expressed higher levels of phosphorylated GluR1 at Ser831, but not phosphorylated GluR1 at Ser845, compared to those from the NP-Sham group (mean ± SD, NP-PSNL: 0.99 ± 0.10 vs. 0.75 ± 0.12; $F_{3,20}=5.646$,

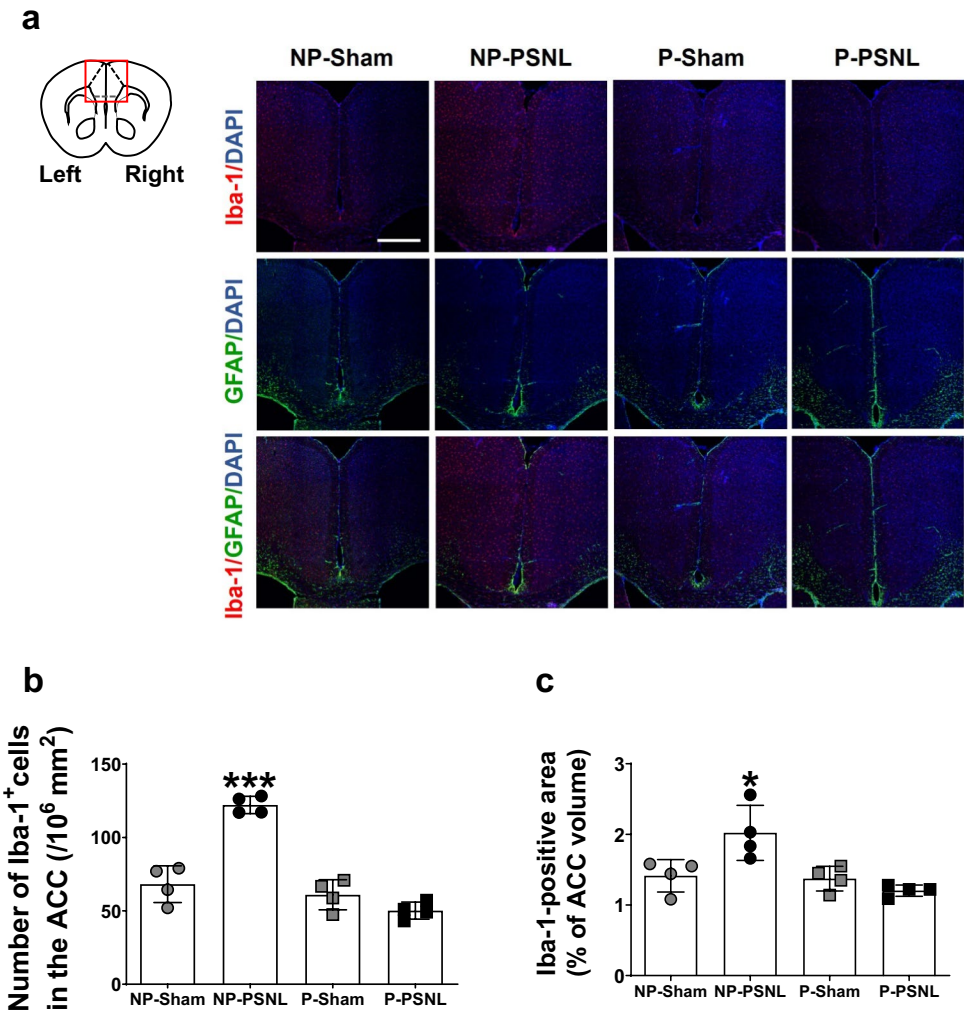

Fig. 3 Pregnancy suppresses the accumulation of microglia in the anterior cingulate cortex in neuropathic pain model mice. **a** Immunofluorescence confocal microscopy for Iba-1 (*red*) or GFAP (*green*)-positive cells in the ACC. The ACC is shown by the dotted line. *Scale bar*, 500 µm. **b** Numbers of Iba-1-positive cells in the ACC in the four groups. Data are expressed as means ± SD ($n=4$ mice/group). ***$P=0.0001$ vs. NP-Sham group. **c** Expression of Iba-1-positive areas in the ACC in the four groups. Data are expressed as means ± SD ($n=4$ mice/group). *$P=0.0198$ vs. NP-Sham group. *NP* non-pregnant, *P* pregnant, *PSNL* partial sciatic nerve ligation, *Iba-1* ionized calcium-binding adapter molecule 1, *DAPI* 40,6-diamidino-2-phenylindole dihydrochloride solution, *GFAP* glial fibrillary acidic protein, *ACC* anterior cingulate cortex

$P = 0.0057$, vs. NP-Sham, Fig. 4b). The P-PSNL group did not show high expression of phosphorylated GluR1 at Ser831 (0.73 ± 0.14 vs. 0.75 ± 0.12; $P = 0.9927$, vs. NP-Sham, Fig. 4a, b).

Pregnancy upregulated the expression of the δ-opioid receptor in the anterior cingulate cortex in mice

It has been previously reported that pregnancy-induced analgesia is dependent on opioid receptors in the dorsal root ganglia (DRG) [7]. To investigate the protein expression of beta-endorphin and opioid receptors (including κ-, μ-, and δ-opioid receptors) in the ACC, ACC homogenates were obtained in the NP-Sham, NP-PSNL, P-Sham, and P-PSNL groups. No significant differences in expression of beta-endorphin in the ACC were seen among the four groups (mean ± SD; $F_{3,20} = 1.117$, $P = 0.3610$, Fig. 5b). The ACC homogenates from the P-Sham and P-PSNL groups expressed higher levels of δ-opioid receptor protein, but not κ- or μ-opioid receptor proteins, compared to those from the NP-Sham group (mean ± SD, P-Sham: 0.88 ± 0.10 vs. 0.69 ± 0.09; $F_{3,20} = 8.8$, $P = 0.0128$, vs. NP-Sham; P-PSNL: 0.92 ± 0.10 vs. 0.69 ± 0.09; $F_{3,20} = 8.8$, $P = 0.0022$, vs. NP-Sham, Fig. 5c). The NP-PSNL group did not show high expression of δ-opioid receptor protein (0.72 ± 0.05 vs. 0.69 ± 0.09; $P = 0.9531$, vs. NP-Sham, Fig. 5a, c).

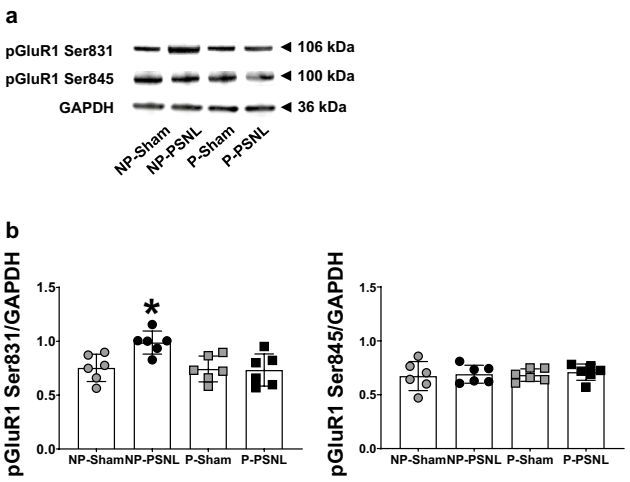

Fig. 4 Pregnancy suppresses the expression of phosphorylated GluR1 at Ser831 in the anterior cingulate cortex in mice. **a** Immunoblot of the phosphorylated GluR1 at Ser831, Ser845, and GAPDH in the anterior cingulate cortex in the four groups. **b** Quantification of data in A. Data are expressed as means ± SD ($n = 6$ mice/group). *$P = 0.0057$ vs. NP-Sham group. *NP* non-pregnant, *P* pregnant, *PSNL* partial sciatic nerve ligation, *pGluR1* phosphorylated AMPA receptor subunit GluR1, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *kDA* kilodalton

The δ-opioid receptor antagonist naltrindole reversed amelioration of neuropathic pain in pregnant mice and the δ-opioid receptor agonist KNT-127 ameliorated neuropathic pain in non-pregnant mice

To examine whether higher expression of δ-opioid receptor protein in the ACC is actually related to pregnancy-induced analgesia, the P-PSNL-NTI group was administered a microinjection of the δ-opioid receptor antagonist naltrindole prior to the behavioral tests. To examine whether δ-opioid signaling within the ACC ameliorates neuropathic pain, the NP-PSNL-KNT-127 group was administered a microinjection of the δ-opioid receptor agonist KNT-127 prior to the behavioral tests. The individual injection sites of cannula placements in the ACC in the three groups (P-PSNL-Vehicle, P-PSNL-NTI, and NP-PSNL-KNT-127) were confirmed based on post-experiment histological examination of cannula placements (Fig. 6a). In the behavioral tests, no mechanical allodynia was observed in the two control groups (NP-Naïve and NP-Sham) on experimental day 9 (Fig. 6b). In the NP-PSNL and P-PSNL-NTI groups, the 50% ipsilateral paw-withdrawal threshold was significantly decreased compared to that in the NP-Naïve group on experimental day 9 (mean ± SD, NP-PSNL: 0.06 ± 0.03 vs. 0.97 ± 0.16 g; $F_{6,98} = 16.85$, $P = 0.0001$, vs. NP-Naïve; P-PSNL-NTI: 0.10 ± 0.05 vs. 0.97 ± 0.16 g; $F_{6,98} = 16.85$, $P = 0.0001$, vs. NP-Naïve, Fig. 6b). No difference was found in the 50% ipsilateral paw-withdrawal threshold among the NP-Naïve, NP-Sham, P-PSNL, P-PSNL-Vehicle, and NP-PSNL-KNT-127 groups on experimental day 9 ($P = 0.9999$). No difference was found in the 50% contralateral paw-withdrawal threshold among the seven groups on experimental days 3 and 9 ($F_{6,98} = 0.1092$, $P = 0.9952$, Fig. 2c). These results indicated that the δ-opioid receptor antagonist naltrindole counteracted pregnancy-induced analgesia in the P-PSNL-NTI group and δ-opioid receptor agonist KNT-127 ameliorated neuropathic pain in the NP-PSNL-KNT-127 group.

Discussion

The present findings demonstrated that pregnancy-induced analgesia ameliorated neuropathic pain by suppressing the activation of microglia and the expression of phosphorylated AMPA receptor subunit GluR1 at the Ser831 site, but not the Ser845 site, in the ACC of PSNL-treated mice. Notably, it was also demonstrated that pregnancy produced the upregulation of the δ-opioid receptor in the ACC, and the δ-opioid receptor antagonist naltrindole counteracted pregnancy-induced analgesia. This finding is consistent with that of a previous report, in which female mice showed reduced mechanical allodynia caused by nerve injury during late

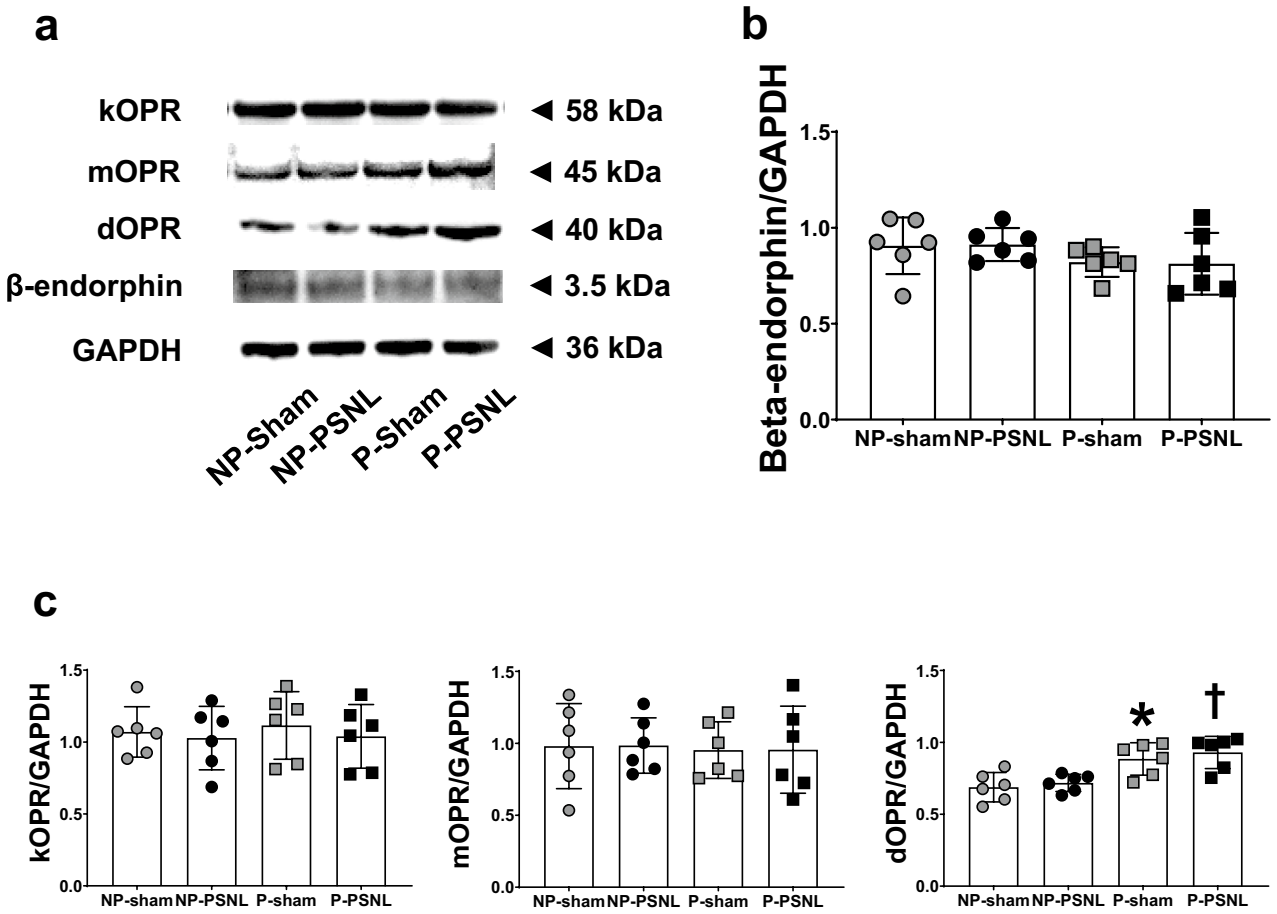

Fig. 5 Pregnancy upregulates the expression of the δ-opioid receptor in the anterior cingulate cortex in mice. **a** Immunoblot of beta-endorphin, the opioid receptors, and GAPDH in the anterior cingulate cortex in the four groups. **b** Quantification of data in beta-endorphin. Data are expressed as means ± SD ($n = 6$ mice/group). **c** Quantification of data in the opioid receptors. Data are expressed as means ± SD ($n = 6$ mice/group). *$P = 0.0128$ vs. NP-Sham group. †$P = 0.0022$ vs. NP-Sham group. *NP* non-pregnant, *P* pregnant, *PSNL* partial sciatic nerve ligation, *kOPR* κ-opioid receptor, *mOPR* μ-opioid receptor, *dOPR* δ-opioid receptor, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *kDA* kilodalton

pregnancy [7]. The results of micro-injection of δ-opioid receptor agonist KNT-127 into the ACC in the NP-PSNL group suggested that activation of δ-opioid receptor within the ACC was responsible for the amelioration of neuropathic pain in non-pregnant animals.

The present study demonstrated that PSNL induced activation of microglia in the ACC and mechanical pain hypersensitivity in the NP-PSNL group. In contrast, activation of microglia in the ACC was suppressed in the P-PSNL group. A previous study has reported that activation of microglia in the ACC was involved in the initiation and development of neuropathic pain in PSNL-treated male mice [15]. In the spinal cord, nociceptive stimulus after nerve injury induces microglial activation, which leads to hyperexcitability of dorsal horn neurons and neuropathic pain [22, 23]. Although the precise correlation of little activation of microglia in the ACC with pregnancy-induced analgesia is still unclear, the present results suggest that in the P-PSNL group, upregulation of the δ-opioid receptor in the ACC might have inhibited nociceptive stimuli from the spinal cord via the thalamus to the ACC and suppressed activation of microglia in the ACC. Previous studies have reported that estrogen and progesterone, which are pregnancy hormones, have the neuroprotective effects on the central nervous system by suppressing the activation of microglia and mediating glutamate excitotoxicity in rodent models of traumatic brain injury [24, 25]. Therefore, estrogen and progesterone would be candidates which have suppressed the activation of microglia and the expression of phosphorylated AMPA receptor subunit GluR1 at the Ser831 in the ACC in late-term pregnant female mice in the present study.

Under neuropathic pain conditions, the subunits of AMPA receptors play important roles in synaptic potentiation, such as long-term potentiation [26]. Previous studies have reported that phosphorylated AMPA receptor subunit GluR1 at the Ser845 site, but not at the Ser831 site, is

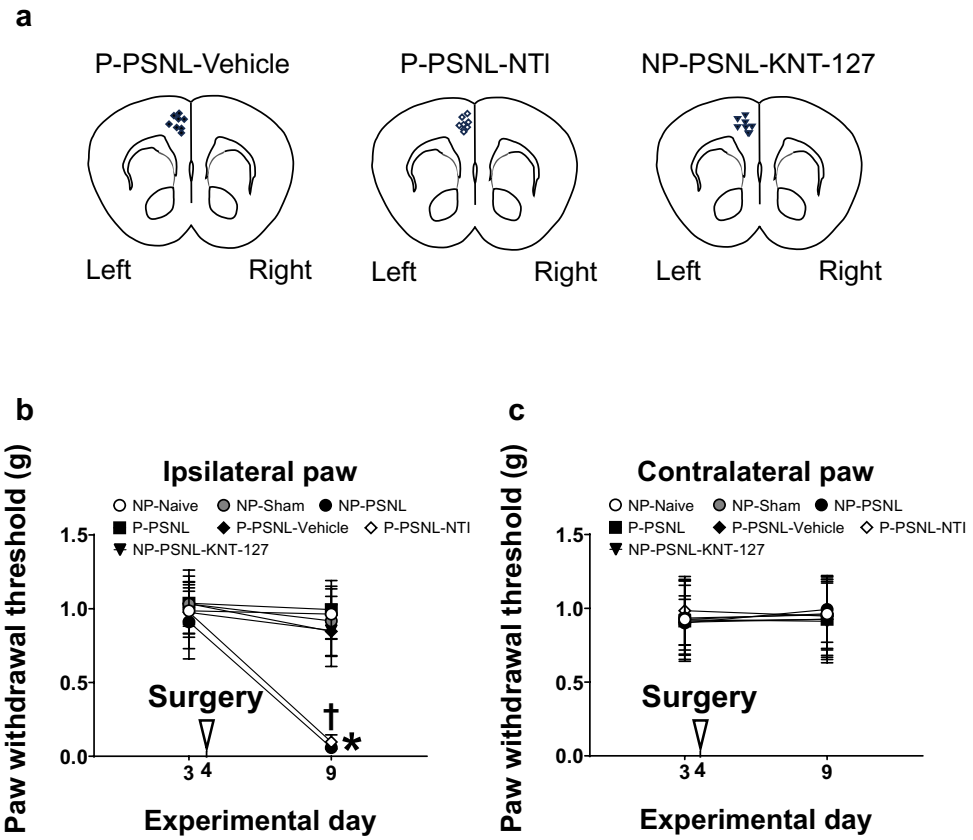

Fig. 6 The δ-opioid receptor antagonist naltrindole reverses amelioration of neuropathic pain in pregnant mice and the δ-opioid receptor agonist KNT-127 ameliorates neuropathic pain in non-pregnant mice. **a** Maps of the individual injection sites of cannula placements in the ACC in the three groups. **b** In the NP-PSNL and P-PSNL-NTI groups, the 50% ipsilateral paw-withdrawal threshold is significantly decreased compared with that in the NP-Naïve group on experimental day 9. No difference is found in the 50% paw-withdrawal threshold among the NP-Naïve, NP-Sham, P-PSNL, and P-PSNL-Vehicle groups on experimental day 9. Data are expressed as means ± SD ($n = 8$ mice/group). *$P = 0.0001$ vs. NP-Naïve group on day 9. †$P = 0.0001$ vs. NP-Naïve group on day 9. **c** No difference was found in the 50% contralateral paw-withdrawal threshold among the five groups on experimental days 3 and 9. Data are expressed as means ± SD ($n = 8$ mice/group). *NP* non-pregnant, *P* pregnant, *PSNL* partial sciatic nerve ligation, *NTI* naltrindole

increased in the ACC of animals with neuropathic pain [16, 27, 28]. In contrast, another study has shown that the expression of phosphorylated AMPA receptor subunit GluR1 at the Ser831 site, but not at the Ser845 site, was increased in the ACC of mice models of neuropathic pain [15]. However, the vast majority of previous studies that implied a correlation of neuropathic pain with phosphorylation of both GluR1 Ser831 and Ser845 in the ACC were conducted only in male animals. The present results suggest that unlike in male animals, phosphorylation of GluR1 Ser831, but not Ser845, in the ACC might be required for mechanical pain hypersensitivity in female mice. Although the mechanisms for suppression of phosphorylation of GluR1 Ser831 in the ACC in late-pregnant mice remain unclear, phosphorylation of GluR1 Ser831 in the ACC would be a new target for ameliorating neuropathic pain in female animals.

The present study demonstrated no positive staining of astrocytes for GFAP in the ACC in the NP-Sham, NP-PSNL, P-Sham, or P-PSNL groups at 5 days after surgery in female mice. Our finding is inconsistent with a previous study, which reported that sciatic nerve injury induced astrogliosis in the ACC at 4 weeks after surgery in male mice [21]. Other studies have reported that glial activation in the brain was dependent on sex and species of animals, brain region, and the experimental time-course after nerve injury [10, 29]. It is possible that differences in the sex of the animals and in the experimental time-course between our study and the previous studies caused the difference in astrogliosis in the ACC after nerve injury.

The present study demonstrated that pregnancy upregulated the δ-opioid receptor, but not beta-endorphin and κ- or μ-opioid receptor, in the ACC in the P-Sham and P-PSNL groups. Furthermore, it was shown that the δ-opioid receptor antagonist naltrindole counteracted pregnancy-induced analgesia in the P-PSNL-NTI group. In addition, to examine whether δ-opioid signaling within the ACC ameliorates neuropathic pain, the δ-opioid receptor agonist KNT-127 was micro-injected into the ACC of non-pregnant mice with

neuropathic pain. Consequently, it was also shown that the δ-opioid receptor agonist KNT-127 ameliorated neuropathic pain in the NP-PSNL-KNT-127 group. The sequential results of the present study suggest that upregulation of the δ-opioid receptor in the ACC might form part of the mechanism of pregnancy-induced analgesia. A previous study has reported that pregnancy produced upregulation of all three genes coding for the μ-, κ-, and δ-opioid receptors in the DRG, and upregulation of the genes coding the κ- and μ-opioid receptors in the spinal cord [7]. The same study also demonstrated that intrathecal injection of the δ-opioid receptor antagonist naltrindole reinstated mechanical allodynia in female mice with neuropathic pain during late pregnancy, and finally demonstrated that upregulation of the δ-opioid receptor in the DRG was related to pregnancy-induced analgesia [7]. We consider that the differences in upregulated subtypes of opioid receptors are due to differences in the evaluated regions of the central nervous system. The present study demonstrated that μ-, κ-, and δ-opioid receptors as well as beta-endorphin existed in the ACC of non-pregnant mice, but mechanical pain hypersensitivity induced by PSNL was not ameliorated in non-pregnant mice. Although a previous study has reported that endogenous opioid signaling in the ACC was implicated in relief of neuropathic pain in male rats [17], the present results suggest that the amounts of μ-, κ-, and δ-opioid receptors, and beta-endorphin, in the ACC of non-pregnant mice would be insufficient to ameliorate mechanical pain hypersensitivity in female mice.

There are important limitations to the current data. First, we evaluated the expression of δ-opioid receptor in the ACC but not in the spinal cord. Evaluation of the expression of δ-opioid receptor in the spinal cord is required to elucidate the precise mechanisms of pregnancy-induced analgesia. Second, the interaction between activation of microglia and phosphorylation of GluR1 Ser831 in the ACC after nerve injury remains unclear in the present study. Further investigation regarding the interaction between activation of microglia and phosphorylation of GluR1 Ser831 in the ACC is required to elucidate the mechanism of neuropathic pain in female mice. Third, the present study was carried out in late-pregnant mice, and not early-pregnant mice. A previous study reported that female mice in early pregnancy displayed microglia-dependent mechanical allodynia, which intrathecal injection of minocycline could reverse, as in male mice [7]. A future investigation regarding the mechanisms of pregnancy-induced analgesia in the ACC of early pregnant mice could enhance our knowledge. Fourth, pain assessment was limited in the present study. A previous study reported that nerve-injured mice developed cold hypersensitivity at 4 weeks rather than at 2 weeks after surgery [30]. Based on this previous report, we did not assess cold hypersensitivity in neuropathic pain model mice, because we conducted behavioral testing at 5 days after surgery. Fifth, we did not assess the role of oxytocin in the ACC in the present study. A previous study has already reported that oxytocin microinjected into the ACC attenuated neuropathic pain by inhibiting presynaptic long-term potentiation [31]. Activation of the paraventricular nucleus to ACC pathway via oxytocin is another candidate for generating pregnancy-induced analgesia.

In summary, pregnancy ameliorated neuropathic pain in female mice in late pregnancy and suppressed the accumulation of microglia and the expression of phosphorylated AMPA receptor subunit GluR1 at Ser831 in the ACC in PSNL-treated mice. Furthermore, upregulation of the δ-opioid receptor in the ACC was related to pregnancy-induced analgesia. The present results significantly extend our knowledge of the mechanisms of pregnancy-induced analgesia, which could lead to a new treatment using δ-opioid signaling within the ACC for neuropathic pain in patients. Thus, we consider that these findings will accelerate translational research to improve clinical outcomes in patients with neuropathic pain.

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Author contributions Sawada designed and conducted the research. Sawada and Yamakage performed data analysis and wrote the manuscript. All authors discussed the results and commented on the manuscript. All authors read and approved the final manuscript.

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Data availability The data that support the findings of this study are available from the corresponding author [A.S.] upon reasonable request.

Declarations

Conflict of interest The authors declare that no conflicts of interest exist.

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