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Sex Differences in Kappa Opioid Receptor inhibition of Latent Postoperative Pain Sensitization in Dorsal Horn

Lilian Custodio-Patsey¹, Renee R. Donahue^{1,#}, Weisi Fu¹, Joshua Lambert², Bret N. Smith^{1,3}, Bradley K. Taylor^{4,*}

¹Department of Physiology, College of Medicine, University of Kentucky Medical Center, 800 Rose Street, Lexington, KY, USA, 40536-0298

²Department of Statistics, College of Arts and Sciences, University of Kentucky, 302 Multidisciplinary Science Building, Lexington, KY, USA, 40536-0082

³Department of Neuroscience, College of Medicine, University of Kentucky Medical Center, 800 Rose Street, Lexington, KY, USA, 40536-0298

⁴Department of Anesthesiology, Pittsburgh Center for Pain Research, and the Pittsburgh Project to End Opioid Misuse, University of Pittsburgh, Pittsburgh, PA USA

Abstract

Tissue injury produces a delicate balance between latent pain sensitization (LS) and compensatory endogenous opioid receptor analgesia that continues for months, even after re-establishment of normal pain thresholds. To evaluate the contribution of mu (MOR), delta (DOR), and/or kappa (KOR) opioid receptors to the silencing of chronic postoperative pain, we performed plantar incision at the hindpaw, waited 21 days for the resolution of hyperalgesia, and then intrathecally injected subtype-selective ligands. We found that the MOR-selective inhibitor CTOP (1–1000ng) dose-dependently reinstated mechanical hyperalgesia. Two DOR-selective inhibitors naltrindole (1–10 µg) and TIPP[Ψ] (1–20µg) reinstated mechanical hyperalgesia, but only at the highest dose that also produced itching, licking, and tail biting. Both the prototypical KOR-selective inhibitors nor-BNI (0.1–10µg) and the newer KOR inhibitor with more canonical pharmacodynamic effects, LY2456302 (0.1–10µg), reinstated mechanical hyperalgesia. Furthermore, LY2456302 (10µg) increased the expression of phosphorylated signal-regulated kinase (pERK), a marker of central sensitization, in dorsal horn neurons but not glia. Sex studies revealed that LY2456302 (0.3 µg) reinstated hyperalgesia and pERK expression to a greater degree in female as compared to male mice. Our results suggest that spinal MOR and KOR, but not DOR, maintain LS within a state of remission to reduce the intensity and duration of postoperative pain, and that endogenous KOR but not MOR analgesia is greater in female mice.

*Corresponding author: Bradley Taylor, Department of Anesthesiology, University of Pittsburgh School of Medicine, 200 Lothrop Street, Pittsburgh, PA 15213, Office: +1 412 624 2092, bkt@pitt.edu.

#Current address: Saha Cardiovascular research Center, College of Medicine, University of Kentucky, 741 S. Limestone St, Lexington, KY, USA, 40536-0509

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INTRODUCTION

Tissue injury induces a sustained form of neuronal plasticity, termed latent sensitization (LS), that is kept within a state of remission by compensatory pain inhibitory systems that include opioid, neuropeptide Y, and alpha2-adrenergic receptor signaling (Campillo et al., 2011; Corder et al., 2013; Rivat et al., 2002; Solway et al., 2011; Taylor and Corder, 2014; Walwyn et al., 2016)). Evidence for endogenous opioid inhibition of LS exists not only in rodents but also in human experimental pain models (Pereira et al., 2015a; Pereira et al., 2015b). Long-lasting mechanisms of endogenous opioid receptor analgesia include mu-opioid receptor constitutive activity (MOR_{CA}) (Corder et al., 2013; Walwyn et al., 2016), and some studies indicate a contribution of delta-opioid receptor (DOR) and/or kappa-opioid receptors (KOR) as well (Campillo et al., 2011; Walwyn et al., 2016; Xie et al., 2017). However, studies of latent postoperative sensitization were restricted to systemic delivery of a single dose of one drug in a model that included remifentanyl administration in addition to plantar incision (Campillo et al., 2011). To further evaluate the contribution of spinal opioid receptor subtypes to the inhibition of postoperative hyperalgesia, we performed plantar incision at the hindpaw, waited 21 days for the resolution of hyperalgesia, and then intrathecally injected multiple doses of multiple subtype-selective opioid receptor ligands. In addition to behavior, we also assessed touch-evoked changes in the expression of phosphorylated extracellular signal-regulated kinase (pERK), a marker of central sensitization of nociceptive neurons in the dorsal horn (Gao and Ji, 2009).

Numerous studies report sex differences in the ability of *exogenous* KOR ligands to modulate pain in both rodents (Auh and Ro, 2012; Lomas et al., 2007; Mogil et al., 2003; Robinson et al., 2016; Sternberg et al., 2004; Terner et al., 2003a) and humans (Gear et al., 1996a; Gear et al., 1996b, 1999; Pande et al., 1996b). However, questions of sex differences in *endogenous* opioid receptor analgesia have not been rigorously studied. To address this gap, we investigated whether sex is a main factor in endogenous opioid receptor-mediated inhibition of LS by testing both male and female mice.

METHODS

Animals

Experiments were carried out in 8–12 week old male and female C57Bl/6 mice (Charles Rivers Laboratories, Inc, Wilmington, MA). Mice were housed maximum 5 same-sex littermates per cage in a temperature and humidity-controlled room (14:10hr light-dark cycle, lights on at 6:00 am) with *ad libitum* access to food and water. Animals were tested during the lights-ON period, between 8am and 7pm. The Institutional Animal Care and Use Committee at the University of Kentucky approved all procedures following American Veterinary Medical Association guidelines. Mice were acclimated to the colony housing room for at least 4 days and then handled for 5 minutes for 2 days by female experimenters (LCD and RRD) before the initiation of a study.

Plantar Incision Model of Postoperative Pain

Longitudinal incision of the plantar skin plus injury to the underlying plantaris muscle was performed as previously described (Jang et al., 2011; Pogatzki and Raja, 2003). Under isoflurane anesthesia (5% induction followed by 1.5–2% maintenance) and antisepsis of the left hind paw with Chlorascrub® then alcohol, a #11 scalpel blade was used to cut a 5 mm incision through the skin and fascia, beginning 2mm from the proximal edge of the heel and extending towards the digits. Curved forceps were slide underneath the underlying plantaris muscle and extended 4 mm, after which the muscle was incised longitudinally. The overlying skin was closed with synthetic 5–0 sutures (PDS®II, Ethicon) followed by application of antibiotic ointment. Surgery was typically completed within 5–10 minutes. Sutures were removed on post-operative day 10. Sham controls received anesthesia but no surgical incision.

Intrathecal Injection

As previously described (Fairbanks, 2003), the mouse was lightly restrained in a towel, and a 30G × ½ needle (Becton Dickinson) attached to a 25-µl Hamilton microsyringe was inserted into the subarachnoid space between the L5/L6 vertebrae at an angle of 30–45 degrees to the horizontal plane. The needle was advanced until a reflexive tail flick was observed, at which time 5 µl of drug or vehicle was slowly administered. The needle was held in place for 30 sec, withdrawn, and then the mouse was returned to its testing chamber.

Dosing and Timing of Drug Delivery

All drugs or vehicle were injected as a 5 µl bolus. The µ-selective ligand, DPhe-c[Cys-Tyr-Trp-Orn-Thr-Pen]-Thr-NH₂ (CTOP, Cat.#1578 Tocris), was dissolved in sterile saline (0.9% NaCl) and injected at doses of 1–1000 ng based on previous studies (Corder et al., 2013; Gendron et al., 2007). Based on the effects of these initial doses, we established dose-response curves. The delta-selective ligand, naltrindole HCl (Sigma-Aldrich N115), was dissolved in sterile saline (pH = 5.6) and injected at doses of 1.0–10 µg (pH of 10 µg = 5.8). This range is consistent with an intrathecal 5 µg dose used in mouse to block DORs (Rosen et al., 2017). H-Tyr-TicΨ[CH₂-NH]Phe-Phe-OH (TIPP[Ψ], gift from NIDA Drug Supply Program) was sonicated in ddH₂O at 30°C for 30 minutes and injected at doses of 1–20 µg (Riba et al., 2002a; Riba et al., 2002b). The kappa-selective ligand, norbinaltorphimine dihydrochloride (Nor-BNI, 0347 Tocris), was dissolved in saline and injected at doses of 0.1–10 µg. LY2456302 (Rorick-Kehn et al., 2014a; Rorick-Kehn et al., 2014b) was obtained either from the NIDA Drug Supply Program, sonicated in ddH₂O at 30°C for 30 minutes, and injected as doses of 0.1–10 µg, or from Med Chem Express, dissolved 1:1:8 in EtOH: Alkamuls EL-620, Rhodia, Cranbury, NJ: saline, and injected at a dose of 10 µg.

Testing began at the 21-day timepoint in the plantar incision model (PIM 21d mice) to allow for mechanical thresholds to return to pre-incision baseline levels. At this time, animals were assigned to experimental groups by a lab mate who kept the code until experiments were completed. On day 21, animals received appropriate vehicle or MOR, DOR, or KOR drug and then tested by investigators who were blind to experimental groups. After a seven-day washout, animals given MOR or DOR drug on day 21 were given vehicle or higher dose of that same drug on day 28. Conversely, animals given vehicle on day 21 were given drug on

day 28. This cross-over design was not used with the KOR ligands due to their extended half-lives.

Pain-Like Behavioral Assessment: Stimulus-Evoked

All animals were acclimated in a temperature and light controlled room within individual Plexiglas boxes placed on the top of a stainless-steel mesh platform for 30 to 60 min prior to behavioral testing. Mechanical thresholds were assessed using an incremental set of 8 von Frey monofilaments (Stoelting, Illinois), ranging in gram force from 0.008g to 6g. The filaments were applied perpendicularly to the ventral surface of the hindpaw lateral to the suture site with enough force to engender slight bending of the filament. A positive response was characterized as a rapid withdrawal of the paw away from the stimulus fiber within 5 s. Left and right hind paws were tested alternately. Using the up-down method (Chaplan et al., 1994), response patterns were logarithmically converted into 50% withdrawal thresholds (in gram force). Female investigators (LCP and RRD) were blinded to experimental groups and conducted all behavioral experiments.

Touched-evoked phosphorylated Extracellular Signal-Regulated Kinase (pERK)

pERK was evoked by touch stimulation as previously described (Corder et al., 2013). 21 days after surgery, mice received either vehicle or LY2456302 (10 μ g). Two hours later, mice were lightly anesthetized with isoflurane (1.5%), and the ventral surface of the ipsilateral hindpaw was mechanically stimulated with a gentle 2-second stroke with a cotton swab from heel to toe. This was repeated every 5s for 5min. After an additional 5 min pause, mice were more deeply anesthetized with isoflurane and transcardially perfused with cold 0.01M phosphate buffered saline (PBS, Fischer Scientific)/heparin buffer (10,000 USP units/L), followed by 10% phosphate formalin buffer. Lumbar spinal cords were harvested and post-fixed in the same fixative overnight at 4°C, and then cryoprotected with 30% sucrose until total submersion (1–3 days).

Immunohistochemistry

Transverse sections (30 μ m) from L3-L5 were cut on a sliding microtome (Leica, SM 2000R). A series of sections, each 240 μ m apart, was washed in 0.01M PBS, blocked in 3% normal serum (goat or donkey; Gemini Bioproducts) containing 0.3% Triton X-100 (Sigma Aldrich) in 0.01M PBS for 1h, and then incubated with either primary rabbit antibody anti-phosphorylated-ERK1/2 antiserum (1:800, Cell Signaling) at 4°C for 36 hours on a shaker, goat anti-AIF1/Iba1 (Isoforms 1 and 3) (1:500, Sigma Aldrich), and/or chicken anti-GFAP (1:1000, Abcam) at 4°C overnight. The following day, sections were again washed in 0.01M PBS and incubated for 1h at room temperature with secondary conjugated antibodies (Invitrogen: donkey anti-rabbit Alexa Fluor 568, donkey anti-goat Alexa Fluor 488, goat anti-rabbit Alexa Fluor 568, and/or goat anti-chicken Alexa Fluor 488) diluted 1:1000. For neuronal co-staining, FITC-conjugated mouse NeuN (MAB377X 1:500, EMD Millipore) was incubated for 1h at room temperature. The sections were washed in 0.01M phosphate buffer, mounted and cover-slipped with mounting medium with DAPI (Vectashield, Vector laboratory). At least five good quality sections from segment L4 were selected from each subject for microscopy.

Microscopy

Fluorescence Microscopy.—For quantification of pERK immunoreactivity, low magnification images taken with Nikon TE-2000 microscope equipped with a 10X objective were captured and analyzed with NIS-Elements Software AR 4.13.05. An examiner blinded to treatment and sex counted the number of positive pERK cells in laminae I-II and III-V.

Confocal Microscopy.—For double-label immunohistochemistry, confocal images were acquired with a Nikon A1 Confocal Microscope System equipped with a 60X oil-immersion objective (numerical aperture 1.49). Laser excitation and emission windows for the different fluorophores were as follows: Alexa Fluor 568 (Cy3) – excitation 543.5 nm, emission 543–633 nm, Alexa Fluor 488 (EGFP) – excitation 488nm, emission 483–543nm, and DAPI - excitation 405nm, emission 423–483nm. Z-stack images were obtained with 0.5µm steps. Images were processed using Nikon NIS-elements Confocal software AR 4.30.02.

Statistical Analysis

Mechanical thresholds are summarized as mean values quoted with the corresponding standard error (mean $\pm$ SEM). The effects of Drug and Time were analyzed by two-way analysis of variance (ANOVA), followed by Bonferroni multiple correction tests. Data were then re-plotted as area under the curve using the trapezoidal method and analyzed using one-way or two-way ANOVA as appropriate, followed by Bonferroni tests as appropriate using Prism 8 (GraphPad, La Jolla, CA). Non-linear regression analyses of Maximum Possible Effect (%MPE) were used to determine the ED50 for each drug. %MPE was calculated as follows: % MPE = 100* (post-injection threshold – pre-injection threshold) / (post-injury threshold – pre-injection threshold).

When appropriate, sex differences in mechanical threshold were analyzed using repeated measures three-way ANOVA, with Sex and Drug analyzed as between-subject factors, and Time analyzed as a within-subject factor. To assess the Drug by Sex interactions, linear mixed models were used with random intercepts and time slopes for each animal. Pairwise time points were individually analyzed using the mixed model approach to increase the power to detect Sex by Drug interactions. Linear Mixed models were fit using the *lme4* V1.1–15 package in R V3.4.3 (R software). The criterion for the level of significance was set at $P = 0.05$ (*). All graphs were created using Prism 8 (GraphPad, La Jolla, CA).

RESULTS

MOR and KOR provide Postoperative Endogenous Analgesia

To determine which opioid receptors provide endogenous analgesia in the setting of LS, we performed plantar incision and then waited 21 days for mechanical hypersensitivity to completely resolve. At this 21-day timepoint in the plantar incision model (PIM 21d mice), we intrathecally injected opioid receptor subtype-selective inhibitors.

Mu opioid receptor inhibitor.—As illustrated in Fig 1A, intrathecal administration of the selective MOR inhibitor CTOP (1–1000 ng) in male PIM 21d mice dose-dependently reinstated mechanical hypersensitivity as compared to saline ($F_{(28,217)} = 9.43$, $p < 0.0001$,

Drug $\times$ Time). The pronociceptive effect of CTOP was robust, peaking at 30 min (0.14 ± 0.03 g, 0.08 ± 0.02 g at 1ng and 1000ng respectively), and lasted for at least 180 min. As illustrated in Fig 1B, area under the curve analysis (AUC 30–180min) illustrates concentration-dependent effects of CTOP ($F_{(4,29)} = 41.3$, $p < 0.0001$, main effect of Drug). As illustrated in Fig 1C, conversion of the 30 min time point data to %MPE yielded an ED₅₀ value of 1.84 ng.

Delta opioid receptor inhibitors.—Exogenous application of selective DOR agonists inhibit mechanical hypersensitivity associated with injury (Bardoni et al., 2014; Gaveriaux-Ruff et al., 2011; Scherrer et al., 2009). Proposed mechanisms include heterodimerization or other synergistic interactions with MOR (Al-Hasani and Bruchas, 2011; Gendron et al., 2007; Gomes et al., 2000; Hurley and Hammond, 2000), or via autonomous mechanisms in distinct subpopulations of spinal neurons that do not express MOR (Wang et al., 2018). Here we tested the hypothesis that *endogenous* activation of DOR inhibits the longer-lasting vulnerability to postoperative pain associated with LS in male mice. As illustrated in Fig 2A, pre-surgical baseline scores were different between the vehicle group and each of the naltrindole groups ($p = 0.0006$), but this should not alter interpretation of the results because mechanical thresholds 2 and 21 days after surgery were similar between groups ($p > 0.05$). When administered 21 days after incision, naltrindole decreased mechanical threshold ($F_{(2,35)} = 6.28$, $p = 0.0047$, main effect of Drug). Similarly, as illustrated in Fig 2B, TIPP_[Ψ] decreased mechanical thresholds ($F_{(3,22)} = 3.59$, $p = 0.02$, main effect of Drug). The highest dose of naltrindole (10 μg) consistently elicited adverse effects such as itching, licking and tail biting, as did the highest soluble dose of TIPP_[Ψ] (20 μg) in one animal, and it was these doses that significantly decreased mechanical thresholds (Fig 2).

Kappa opioid receptor inhibitors.—We used either a prototypical KOR inhibitor (nor-BNI) or a newer KOR inhibitor that exhibits more canonical pharmacodynamic effects, LY24506302. Compared to nor-BNI, LY24506302 exhibits relatively rapid absorption and a more typical rate of clearance with a 2–4 h plasma half-life (Munro et al., 2012; Rorick-Kehn et al., 2014b). As illustrated in Figs 3A–B, both nor-BNI (0.1–10 μg) or LY2456302 (0.1–10 μg) produced a dose-dependent reinstatement of mechanical hypersensitivity compared to saline (nor-BNI, $F_{(27,216)} = 3.73$, $p < 0.0001$ and LY2456302, $F_{(33,286)} = 5.63$, $p < 0.0001$, Drug $\times$ Time). The effect on hyperalgesia of both drugs peaked at 3h (Nor-BNI, 0.19 ± 0.06 g and LY2456302, 0.30 ± 0.04 g), and this time course of onset is consistent with previous studies using intracerebroventricular injections of nor-BNI (Horan et al., 1992) or systemic administration of LY245606302. The effects of Nor-BNI and LY2456302 lasted at least 72h which is consistent with duration of action of 3–4 weeks for Nor-BNI (Melief et al., 2011) and of several days / less than one week for LY2456302 (Rorick-Kehn et al., 2014b). As illustrated in Figs 3C–D, AUC analysis (1–72h) yielded F values of $F_{(3,24)} = 12.90$ ($p < 0.0001$) for nor-BNI and $F_{(3,26)} = 20.4$, $p < 0.0001$, main effect of Drug) for LY2456302. As illustrated in Figs 3E–F, a focus on the 3h timepoint yielded ED₅₀ values of 0.87 μg for nor-BNI and 1.04 μg for LY2456302.

Postoperative Endogenous KOR Analgesia is Enhanced in Female Mice

As illustrated in Figure 4, plantar incision produced a robust decrease in mechanical threshold on the paw ipsilateral but not contralateral to injury. The timecourse of mechanical hypersensitivity did not significantly differ between male and female mice ($p>0.05$) when tested at either the paw ipsilateral to injury or contralateral to injury. Thresholds returned to baseline by post-operative day 21 in each sex. These results are consistent with previous findings in mouse incision models (Banik et al., 2006).

Postoperative patients exhibit sex differences in endogenous opioid inhibition of pain (Fillingim and Gear, 2004). We previously reported that inverse agonist-induced reinstatement of mechanical hyperalgesia and facial grimace in the CFA model of LS did not differ between male and female mice (Corder et al., 2013); however, these studies were limited to a single dose of naltrexone which can interact with mu, delta, and kappa opioid receptors. Therefore, we asked whether low or high doses of CTOP or LY2456302, as determined by the dose-response curves in Figures 1 and 3, would yield sex differences in mechanical thresholds after injection in PIM 21d male and female mice.

Mu opioid receptor.—As illustrated in Fig 4A, a high dose of CTOP (100 ng) but not vehicle produced robust hyperalgesia in PIM 21 male and female mice at the ipsilateral side ($F_{(1,90)} = 57.25$, $p<0.0001$ main effect of Drug; $F_{(5,90)} = 0.19$, $p=0.96$, Time $\times$ Sex $\times$ Drug). This dose of CTOP produced a slightly less robust and shortened hypersensitivity as compared to Figure 4A, a modest inter-experiment variability that is not unexpected as different experimenters conducted each study. As illustrated in Fig 4B, CTOP (100 ng) also produced robust hyperalgesia at the contralateral side that did not differ between the sexes ($F_{(1,54)} = 48.80$, $p<0.0001$, main effect of Drug; $F_{(5,54)} = 0.87$, $p=0.50$, Time $\times$ Sex $\times$ Drug). As illustrated in Fig 4C, AUC analysis confirmed that CTOP (100 ng)-induced reinstatement of hyperalgesia did not differ between the sexes ($F_{(3,43)} = 0.90$, $p=0.78$, main effect of Drug).

Since this relatively high dose of CTOP may have produced a floor effect that could have masked sex differences, we repeated this study using a minimally effective dose of 1 ng in males (Figure 1). As illustrated in Fig 4D–F, 1 ng CTOP reinstated hyperalgesia ($F_{(1,44)} = 4.40$, $p=0.04$ ipsilateral, main effect of Drug; $F_{(1,14)} = 5.99$, $p = 0.02$ contralateral) to a similar degree in male and female mice (Fig. 4D, $F_{(5,220)} = 0.24$, $p=0.94$, Time $\times$ Sex $\times$ Drug; Fig 4E, $F_{(5,70)} = 0.34$, $p= 0.88$, Time $\times$ Sex $\times$ Drug; Fig 4F, AUC, $F_{(3, 58)} = 0.08$, $p= 0.1$).

Kappa opioid receptor.—Next, we investigated whether sex differences exist in endogenous KOR-mediated inhibition of postoperative pain in our LS model. As illustrated in Fig 5A–B, a relatively high dose of LY24506302 (10 μ g) reinstated mechanical hypersensitivity compared to saline in male and female mice at the sides both ipsilateral ($F_{(1,21)} = 21.80$, $p<0.0001$, main effect of Drug) and contralateral ($F_{(1,21)} = 14.44$, $p=0.001$) to plantar incision. However, the degree of reinstatement did not differ between the sexes ($F_{(8,168)} = 0.13$, $p= 0.99$, 5A; $F_{(8,168)} = 0.26$, $p= 0.97$, Time $\times$ Sex $\times$ Drug), and this is further illustrated in Fig 5C by area under the curve analysis across the 1 to 120 hr timepoints ($F_{(3,43)} = 0.35$, $p= 0.78$).

Similar to CTOP, this relatively high dose of LY24506302 may have produced a floor effect that could have masked a sex difference. Thus, we repeated this study using a lower dose of 0.3 μ g, informed by the dose-response curve in Fig 3F. As illustrated in Fig 5D, LY2456302 (0.3 μ g) reinstated hyperalgesia at the side ipsilateral to incision in both male and female mice, and importantly to a greater extent in female mice (Bonferroni posthoc test $p < 0.05$). Fig 5E illustrates a smaller effect on the contralateral side that did not differ between sex. Further analysis in Fig 5F illustrates that female PIM 21d mice exhibited greater mechanical hyperalgesia at the peak of LY-induced reinstatement at the 2–4h timepoints, albeit modestly (Sex $\times$ Drug Interaction, Satterthwaite: p -value=0.041).

Tonic KOR inhibition of dorsal horn neuron activity is enhanced in female mice

We previously reported that MOR_{CA} inhibits long-lasting sensitization of dorsal horn neurons induced by cutaneous inflammation (Corder et al., 2013). To begin to address whether KOR inhibits neuronal activity as well, we evaluated light touch-evoked expression of pERK in the superficial (I-II) and deep laminae (III-V) of the dorsal horn, two hours after intrathecal administration of LY2456302 or saline in male and female mice, 21 days after either sham or plantar incision surgery. As illustrated in Fig 6, light touch was associated with only a small number of pERK-positive profiles in sham controls that received LY2456302 or vehicle treated mice. By contrast, LY2456302 evoked a larger number of pERK+ profiles in PIM 21d mice at both superficial lamina I-II ($F_{(5,23)} = 1.612$, $p < 0.001$) and deeper laminae III-V ($F_{(5,44)} = 6.60$, $p = 0.001$, Group $\times$ Region). Importantly, LY2456302 was associated with significantly, albeit modestly, more pERK+ profiles in laminae I-II in females as compared to males ($p < 0.05$).

p-ERK can be expressed on neurons, astrocytes, and microglia (Ji et al., 2009; Zhuang et al., 2005). To determine the cell type(s) that express pERK after LY2456302, we co-labelled with NeuN, GFAP, and Iba1. As illustrated in Fig 7, confocal microscopy revealed that pERK was expressed exclusively in NeuN-positive cells in laminae I–V of the dorsal horn obtained from female (Fig 7A–U) or male (Fig 7A'–U') mice.

DISCUSSION

MORs provide Long-lasting Inhibition of Postoperative Pain

Tissue injury induces latent pain sensitization (LS) that is mediated in part by NMDA receptors, AMPA receptors, adenylyl cyclase 1, protein kinase A, and Epac signaling mechanisms in the dorsal horn (Corder et al., 2013; Taylor et al., 2019). These pronociceptive mechanisms are kept within remission by opposing pain inhibitory mechanisms (Campillo et al., 2011; Corder et al., 2013; Rivat et al., 2002; Solway et al., 2011; Taylor and Corder, 2014), that include neuropeptide Y receptors and MOR_{CA} (Corder et al., 2013; Walwyn et al., 2016). Evidence for the contribution of MOR comes in part from behavioral pharmacology studies using MOR-selective inhibitors. For example, when administered 21 days after the intraplantar injection of complete Freund's adjuvant (after the resolution of initial hyperalgesia), intrathecal administration of a single dose of CTOP reinstated hyperalgesia (Corder et al., 2013). The current results not only extend these

findings to the plantar incision model of postoperative pain, but also demonstrate a dose-response curve with an ED50 value of approximately 2 ng.

DORs might provide Long-lasting Inhibition of Postoperative Pain

Both of the DOR inhibitors that we used, naltrindole and TIPP[Ψ], significantly reduced mechanical threshold when administered during the remission phase of LS. However, because the high doses required also produced aversive effects, we cannot conclude with certainty that DOR inhibitors reinstate hyperalgesia. On the other hand, the aversive effects that we observed (itching, licking, and tail biting) might represent additional measures of nociception. Therefore, our results neither support nor refute those of Walwyn and colleagues who reported that subcutaneous administration of naltrindole reinstated hyperalgesia in a mouse CFA model of LS, and that either intrathecal or subcutaneous naltrindole reinstated hyperalgesia in a rat CFA model of LS (Walwyn et al., 2016). Since our studies were limited to the intrathecal route of administration, future studies are needed to investigate the role of endogenous DORs expressed at peripheral terminals of primary afferent neurons (Brederson and Honda, 2015) or at supraspinal sites including those involved in descending pain modulation (Hurley and Hammond, 2000).

KORs provides Long-lasting Inhibition of Postoperative Pain

The current results demonstrate that the KOR-selective inhibitors nor-BNI and LY2456302 dose-dependently reinstated behavioral signs of postoperative pain, and LY2456302 increased stimulus-evoked pERK expression. pERK expression likely represents central sensitization in neurons rather than glia, because we found that it was co-expressed with NeuN but neither GFAP (astrocytes) nor Iba1 (microglia). We conclude that LS is maintained in remission by tonic KOR activity in dorsal horn neurons.

Our results are consistent with and extend previous studies linking endogenous KOR to the inhibition of LS. For example, in a similar plantar incision model that included remifentanyl at time of surgery, nor-BNI reinstated hyperalgesia when systemically administered during the remission phase (Campillo et al., 2011). Furthermore, Marvizon and colleagues reported that nor-BNI reinstated mechanical hyperalgesia in the CFA model of LS (Walwyn et al., 2016). This was observed in prodynorphin-deficient mice, which is inconsistent with a ligand-dependent mechanism, but instead is aligned with the hypothesis that, like MOR_{CA}, inflammation leads to constitutive activity at KOR (Walwyn et al., 2016). Therefore, we have been careful to describe nor-BNI and LY2456302 as KOR inhibitors rather than KOR antagonists, since we do not yet know whether their behavior is indicative of inverse agonism and ligand-independent constitutive activity, or antagonism and ligand-dependent activity.

In addition to the spinal sites of action suggested by our intrathecal injection studies, peripheral and supraspinal sites may also contribute to endogenous KOR inhibition of LS. Regarding peripheral sites, KOR is expressed in the nerve terminals of peptidergic populations of primary afferent neurons arising from the skin, viscera, and synovial tissues, and KOR signaling inhibits nociceptor sensitization and pain (Cunha et al., 2012; Labuz et al., 2007; Moon et al., 2016; Snyder et al., 2018). A recent study indicated that peripherally-

restricted KOR agonists markedly reduced mechanical hyperalgesia at the early inflammatory phase of plantar incision (Snyder et al., 2018), implicating peripheral KOR as a major modulator of acute postoperative pain. Regarding supraspinal sites, microinjection of KOR agonists into the rostral ventral medulla activated descending modulatory neurons to inhibit nociception (Hurley and Hammond, 2000; Schepers et al., 2008a; Schepers et al., 2008b). Furthermore, our current study (Fig 5) demonstrated that KOR inhibitors reinstate hyperalgesia not only at the side ipsilateral to injury but also at the contralateral side, thus further implicating supraspinal modulation of LS as previously proposed for MOR (Corder et al., 2013; Taylor and Corder, 2014) and as suggested by the current CTOP results (Fig 4). Contralateral latent sensitization is poorly understood, and possible mechanisms for future study include not only descending facilitation (Chai et al., 2012; Porreca et al., 2002; Taylor and Corder, 2014) and suppression of descending inhibition from brainstem nuclei (Chen et al., 2018), but also neuroimmune interactions (Cairns et al., 2015) and communication of reactive astrocytes through gap junction connexins (Gao and Ji, 2010), although neither naltrexone (Corder et al., 2013) nor LY2456302 (Figure 7) induced pERK activation in astrocytes.

In contrast to pro-hyperalgesia, KOR inhibitors can exert antihyperalgesic actions. For example, in rats primed with sumatriptan or morphine infusion, delivery of KOR inhibitors to the central amygdala prevented acute stress-induced hyperalgesia, perhaps via MAPK signaling and a descending modulatory circuit (Xie et al., 2017). Indeed, KOR in the central nucleus of the amygdala and the limbic system promotes behaviors suggestive of disinhibition, aversiveness, anxiety (Liu et al., 2019; Narita et al., 2006) and pain facilitation (Navratilova et al., 2018). These studies do not necessarily contradict ours; instead, we propose that the direction of effect depends on the site of modulation: KOR activation at the central terminals of primary afferent or intrinsic dorsal horn neurons leads to pain inhibition, while activation at the amygdala leads to pain facilitation.

Sex Differences in Postoperative Pain

Numerous studies have investigated sex differences in nociception (Greenspan et al., 2007; Hendrich et al., 2012). As described previously (Banik et al., 2006), the current results indicate that male and female mice exhibit similar baseline mechanical thresholds, and responded to plantar incision with a mechanical hyperalgesia of similar intensity and time course. By contrast, other preclinical studies indicate that females present lower mechanical nociceptive thresholds (Barrett et al., 2002b; Chartoff and Mavrikaki, 2015; Hendrich et al., 2012; Rasakham and Liu-Chen, 2011) and take longer to recover from injury than males (Vacca et al., 2014). Furthermore, women commonly report more severe pain than men (Fillingim et al., 2009; Greenspan et al., 2007; Tsang et al., 2008), including higher postoperative pain levels (Storesund et al., 2016), and present greater risk to develop persistent postoperative pain (Kehlet et al., 2006). Likewise, women exhibit lower mechanical nociceptive thresholds and take longer to recover from injury than males (Fillingim and Gear, 2004; Riley et al., 1998).

Sex Differences in KOR (but not MOR) Inhibition of Latent Postoperative Pain

Numerous studies have investigated sex differences in endogenous opioid modulation of pain (Fillingim and Gear, 2004; Fillingim et al., 2009; Tambeli et al., 2001). We report that either low or high doses of CTOP, when administered 21 days after plantar incision, similarly reinstated mechanical hypersensitivity in male and female mice. By contrast, intrathecal administration of a low dose of the KOR inhibitor LY2456302 produced a significantly more pronounced hyperalgesia and pERK activation in the dorsal horn of female mice as compared to male mice. (The higher dose pushed mechanical thresholds down to the limits of detection afforded by von Frey filaments, and thus a floor effect explains failure to demonstrate a sex difference). The sex difference in low-dose LY2456302-induced pain reinstatement was of a small magnitude, but statistically significant and supported by a differences in spinal pERK expression. Therefore, we conclude that tonic endogenous KOR-mediated inhibition of LS is more pronounced in females.

Consistent with our findings, clinical studies suggest a sexual dimorphism in the ability of KOR agonists to manage postoperative pain (Pande et al., 1996a; Pande et al., 1996b). For example, mixed-action MOR/KOR ligands revealed that postoperative KOR analgesia was greater in women following third molar extraction (Gear et al., 1996a; Gear et al., 1996b, 1999). However, the rodent literature of sex differences in the pain-related actions of KOR agonists can be inconsistent and even contradictory. Some studies report that KOR agonists exert greater antinociception in male rodents (Barrett et al., 2002a; Sternberg et al., 2004; Terner et al., 2003a; Terner et al., 2003b), while others suggest greater antinociception in females (Bartok and Craft, 1997; Lawson et al., 2010). Similar mixed effects have been reported in rodent models of inflammatory pain: KOR agonists can exert greater antihyperalgesia in males as compared to females (Auh and Ro, 2012; Lomas et al., 2007; Rasakham and Liu-Chen, 2011), or greater antihyperalgesia and inhibition of spinal Fos expression in females as compared to males (Bereiter, 2001; Clemente et al., 2004; Lawson et al., 2010).

SUMMARY

Our results indicate that surgical incision leads to a long-lasting LS that is tonically opposed by prolonged activation of MOR and KOR (and possibly DOR) in dorsal horn neurons. Both behavioral and spinal neuronal correlates of endogenous KOR inhibition were greater in females. Considering that LS represents a silent state of pain vulnerability, we speculate that failure of KOR signaling could determine the greater predisposition of females to develop persistent postsurgical pain. Further studies are needed to determine the contribution of hormonal (Lawson et al., 2010; Robinson et al., 2016; Sternberg et al., 2004), neurochemical, and genetic (Fillingim and Gear, 2004; Mogil et al., 2003; Rasakham and Liu-Chen, 2011) factors to sex differences in endogenous KOR inhibition of latent postoperative pain. A better understanding of sex-dependent analgesia by spinal KOR signaling is essential for the development of new KOR-based pharmacotherapies that target pain or addiction.

Acknowledgements and Conflict of Interest Statement

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Highlights

Surgery produces a long-lasting kappa opioid receptor analgesia that prevents the transition from acute to chronic postoperative pain, particularly in females.

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Figure 1. The mu opioid receptor (MOR) inhibitor CTOP reinstates mechanical hypersensitivity in a dose-dependent manner.

Mechanical thresholds at baseline (BL), 2 days, and 21 days after plantar incision in male C57Bl/6 male, and then after intrathecal (i.t.) injection of the MOR selective inhibitor CTOP. (A) Time course of mechanical hypersensitivity after CTOP (1–1000ng). CTOP reinstated mechanical hypersensitivity in a dose-dependent manner (n=8 per dose). (B) Area under the curve (AUC, 30–180 min) further illustrates the dose-dependent effects of CTOP. (C) Dose-response analysis of the data at 0.5h after injection revealed an ED_{50} of 1.84ng. MPE = maximum possible effect. ★p<0.05 CTOP vs. vehicle. BL = baseline behavior prior to drug administration. Data are represented as mean ± SEM.

Figure 2. Delta opioid receptor (DOR) selective inhibitors do not reinstate mechanical hypersensitivity.

Mechanical thresholds at baseline (BL), 2 days, and 21 days after plantar incision in male C57Bl/6 male, and then after intrathecal injection of DOR selective drugs (**A**) naltrindole (n=10–18 per dose) and (**B**) TIPP(Ψ) (n=6–8 per dose). ★p<0.05 drug vs. vehicle. Data are represented as mean ± SEM.

Figure 3. Kappa opioid receptor (KOR) selective inhibitors reinstate mechanical hypersensitivity in a dose-dependent manner.

Mechanical thresholds at baseline (BL), 2 days, and 21 days after plantar incision in male C57Bl/6 male, and then after intrathecal (i.t.) injection of KOR selective inhibitors (**A**) Nor-BNI (0.1–10 μ g; n=5–10 per dose) or (**B**) LY2456302 (0.1–10 μ g; n=6–8 per dose). (**C–D**) Area under the curve (AUC, Nor-BNI = 1–72h, LY2456302 = 1–120h) further illustrates drug-induced reinstatement in a dose-dependent manner (**E–F**) Dose-response analysis of the data at 3h revealed an ED_{50} of 0.87 μ g for Nor-BNI, and an ED_{50} of 1.04 μ g for LY2456302. MPE = maximum possible effect. ★p<0.05 nor-BNI or LY2456302 vs vehicle. Data are represented as mean $\pm$ SEM.

Figure 4. CTOP reinstates mechanical hypersensitivity equally in males and females.

(A-B) Time course of mechanical thresholds at baseline (BL), 1, 3, 5, 7, 14 and 21–28 days in male or female C57Bl/6 male, and then after intrathecal injection of CTOP at a dose of 100ng at the paw ipsilateral (A) or contralateral (B) to plantar incision. (C) Analysis of area under the curve (AUC, 0.5–2h) illustrates that CTOP (100ng) reinstated mechanical hypersensitivity at both the ipsilateral and contralateral hindpaws equally in males and females. (D-E) Time course of mechanical thresholds at baseline (BL), 1, 3, 5, 7, 14 and 21–28 days in male or female C57Bl/6 male, and then after intrathecal injection of CTOP at a dose of 1ng at the paw ipsilateral (D) or contralateral (E) to plantar incision. (F) Analysis of area under the curve (AUC, 0.5–2h) illustrates that CTOP (1.0ng) reinstated mechanical hypersensitivity at both the ipsilateral and contralateral hindpaws equally in males and females. n=4–10 per group. ★p<0.05 CTOP vs. vehicle. Data are represented as mean +/- SEM.

Figure 5. LY2456302-induced reinstatement of mechanical hypersensitivity is greater in females. (A-B) Time course of mechanical thresholds at baseline (BL), 1, 3, 5, 7, 14 and 21–28 days in male or female C57Bl/6 male, and then after intrathecal injection of LY2456302 at a dose of 10 μ g at the paw ipsilateral (A) or contralateral (B) to plantar incision. (C) Analysis of area under the curve (AUC, 0.5–2h) illustrates that LY2456302 (10 μ g) reinstated mechanical hypersensitivity at both the ipsilateral and contralateral hindpaws equally in males and females. (D-E) Time course of mechanical thresholds at baseline (BL), 1, 3, 5, 7, 14 and 21–28 days in male or female C57Bl/6 male, and then after intrathecal injection of LY2456302 at a dose of 0.3 μ g at the paw ipsilateral (D) or contralateral (E) to plantar incision. (F) Analysis of area under the curve (AUC, 0.5–2h) illustrates that CTOP (0.3 μ g) reinstated mechanical hypersensitivity at both the ipsilateral and contralateral hindpaws equally in males and females. n=6–8 per group. *p<0.05 LY2456302 vs. vehicle, #p<0.05 male vs. female. Data are represented as mean $\pm$ SEM.

Figure 6. LY2456302-evoked pERK⁺ is greater in females.

(A-F) Representative photomicrographs of light touch-evoked pERK-immunoreactivity in L4 lumbar sections from female (A-C) or male (D-F) mice, 21 days after sham surgery plus 10 μ g LY2456302 (A, D), plantar incision plus vehicle (B, E), or plantar incision plus 10 μ g LY2456302 (C, F). (G-H) Scatterplot and quantification of mean number of touch-evoked pERK⁺ profiles in lamina I-II (G) or lamina III-V (H). * $p < 0.05$ LY2456302 vs. vehicle and sham incision. * $p < 0.05$ LY2456302 vs. sham incision. # $p < 0.05$ male vs female. N = 4–6 mice/group. Data are represented as mean $\pm$ SEM. Scale bar: 100 μ m.

Figure 7. Touch-evoked pERK is expressed in dorsal horn neurons.

Representative transverse lumbar L4 spinal cord sections from mice 21 days after incision. Spinal cords were collected 2h after intrathecal delivery of LY2456302 (10 μ g) and 10 min after initiation of ipsilateral light touch paw stimulation. Sections from females were co-labelled with pERK (A, C, D, G, H, J, K, N, O, Q, R and U), and either NeuN (B-C, E, G), GFAP (I-J, L, N), or Iba-1 (P-Q, S-U). DAPI (F-G, M-N, T-U). Sections from males were co-labelled with pERK (A', C', D', G', H', J', K', N', O', Q', R' and U'), and NeuN (B'-C', E', G'), GFAP (I'-J', L', N'), or Iba-1 (P'-Q', S'-U'). DAPI (F'-G', M'-N', T'-U'). Arrows indicate pERK co-labeling. Light microscopic images in the left three columns were taken with a 10X objective, while confocal images in the right four columns were taken with a 60 $\times$ objective. Scale bars: 50 μ m.