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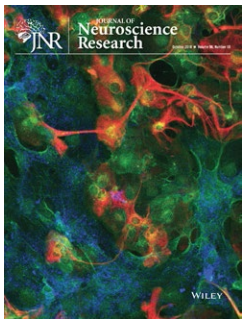
RESEARCH ARTICLE



Sex differences in kappa opioid receptor antinociception is influenced by the number of X chromosomes in mouse

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Abstract

Kappa opioid receptor (KOR) agonists produce robust analgesia with minimal abuse liability and are considered promising pharmacological agents to manage chronic pain and itch. The KOR system is also notable for robust differences between the sexes, with females exhibiting lower analgesic response than males. Sexually dimorphic traits can be due to either the influence of gonadal hormones during development or adulthood, or due to the complement of genes expressed on the X or Y chromosome. Previous studies examining sex differences in KOR antinociception have relied on surgical or pharmacological manipulation of the gonads to determine whether sex hormones influence KOR function. While there are conflicting reports whether gonadal hormones influence KOR function, no study has examined these effects in context with sex chromosomes. Here, we use two genetic mouse models, the four core genotypes and XY*, to isolate the chromosomal and hormonal contributions to sex differences in KOR analgesia. Mice were treated with systemic KOR agonist (U50,488H) and thermal analgesia measured in the tail withdrawal assay. We found that KOR antinociception was influenced predominantly by the number of the X chromosomes. These data suggest that the dose and/or parental imprint on X gene(s) contribute significantly to the sexually dimorphism in KOR analgesia.

KEYWORDS

analgesia, kappa opioid receptor, sex chromosomes, sex difference, sex hormones

1 | INTRODUCTION

Activation of the kappa opioid receptor (KOR) produces analgesia without euphoria and is emerging as a target for chronic pain and itch without abuse potential. The KOR system is also notable for a significant sex difference, with females less responsive to KOR agonists than males (Barrett, Smith, & Picker, 2002; Craft & Bernal, 2001; Negus, Zuzga, & Mello, 2002; Stoffel, Ulibarri, Folk, Rice, & Craft, 2005). This is important because females are more likely to suffer from chronic pain and require pain treatments (Sorge & Totsch, 2017). Understanding the biological basis for the sex difference in KOR agonist efficacy is necessary to promote analgesic effects of agonists for treatment of pain in females.

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The biological origins of the sex differences in KOR analgesia remain elusive. Sexually dimorphic traits can be due to either the influence of gonadal hormones during development or adulthood (Arnold, 2017). In addition, the complement of genes expressed on the X or Y chromosome can drive, or contribute to, sexually dimorphic phenotypes (Arnold, 2017). The influence of gonadal hormones on KOR function is complex. For example, gonadectomy has been shown to decrease KOR antinociception in both males and females, and fluctuating female gonadal hormones influence KOR spinal antinociception (Abraham et al., 2018; Negus & Mello, 2002; Stoffel et al., 2005). However, additional reports have indicated that KOR activation and agonist-induced dysphoria do not vary with estrous cycle nor following gonadectomy of either ovaries or testes (Conway et al., 2019; Harris & Drake, 2001; Russell et al., 2014).

The studies above have relied on surgical or pharmacological manipulation of the gonads, which is unable to isolate underlying sex chromosome influences. In fact, it is a challenge to isolate these chromosomal effects, given that sex chromosomes are always expressed in the presence of their respective gonads. To overcome this limitation, we use two genetic approaches to manipulate the expression of sex chromosomes independent of gonadal hormones. In the "four core genotypes" (FCG), the testes determining gene, *Sry*, is removed from the Y chromosome and reinserted as a transgene on chromosome 3 (Burgoyne & Arnold, 2016). This produces four genotypes comprised of males and females each with either XX or XY sex chromosomes. Because gonadal sex is independent of sex chromosomes, conclusions can be drawn about the role of gonadal hormones and sex chromosomes. If a sex difference is found to vary based on sex chromosomes, the FCG model cannot differentiate whether this difference is due to genes on the X or Y chromosome. The latter question can be answered by using the XY* model in which male mice have a variant Y chromosome (Y*) that recombines abnormally with the X chromosome during meiosis. Mating an XY* mouse with a WT XX female produces four genotypes in which the dose of X and Y genes varies independently (i.e., XX, XO, XY, and XXY). This model provides information on whether the sex difference varies with the number of X or Y chromosomes (Burgoyne & Arnold, 2016).

In this study, we use the FCG and XY* genetic lines to determine the biological basis for the sex differences in KOR antinociception. By manipulating sex chromosomes and gonadal hormones independently, we identify the role of sex chromosomes, gonadal hormones, and the interaction between the two in mediating KOR analgesia.

Significance

Kappa opioid receptor (KOR) agonists are known to have a notable sex difference; however, the biological basis for this sex difference remained elusive. Previous work relying on surgical or chemical manipulation of gonadal hormones have produced conflicting results on the relative contribution of gonadal hormones to the KOR sex difference. Here, we were able to show that sex chromosomes (and particularly the X chromosome) contribute significantly to the KOR sex difference. This discovery carries meaningful clinical implications given KOR agonists are currently being developed as novel analgesics, and women are significantly more likely to require pain treatments in their lifetime.

2 | METHODS

2.1 | Animals

All animal care and experimental procedures were conducted in accordance with the National Institutes of Health *Guide for the Care and use of Laboratory animals* and were approved by the University of California Los Angeles Institutional Animal Care and Use Committee and the University of Alberta Health Sciences Animal Care and Use Committee.

Wild-type male and female C57BL/6J (8 weeks, total 44 mice equal males and females) mice were ordered from Jackson Laboratories. FCG (total 53 mice six to eight per genotype) mice on a C57BL/6J (B6) background (B6.Cg-Tg(Sry)2Ei Sry^{dl1Rlb}/ArnoJ, Jackson Laboratories stock 10905; backcross generation greater than 23) bred at UCLA. In this model, the testis-determining gene *Sry* is deleted from the Y chromosome and inserted as a transgene on chromosome 3 (Itoh et al., 2015). FCG mice comprise four genotypes: XX and XY females (without *Sry*, with ovaries) and XX and XY males (with *Sry* and testes). Because gonadal sex is independent of sex chromosome complement, conclusions can be drawn about the role of gonadal hormones and sex chromosome complement (and interactions between the two) in the observed sex difference. In this study, "male" refers to a mouse with *Sry* (with testes) and "female" refers to a mouse without *Sry* (with ovaries) and is independent of sex chromosome complement (XX or XY). In some animals, gonadectomy was performed at 75 days of age. Following isoflurane anesthesia (1.5%–3%), gonads were exposed and excised. Animals were allowed to recover in their home cages for at least 2 weeks prior to testing.

The XY* model (total 35 mice, 6–11 per genotype), backcrossed to C57BL6/J, was used to isolate which sex chromosome was responsible for the sex differences in KOR antinociception (Burgoyne, Mahadevaiah, Perry, Palmer, & Ashworth, 1998). XX gonadal females were bred with XY* gonadal males. This produces four genotypes XY^{*X}, XX, XY^{*}, and XX^{Y*} that are similar to the genotypes XO, XX, XY, and XXY, respectively (Burgoyne & Arnold, 2016; Burgoyne et al., 1998; Chen et al., 2008; Eicher et al., 1991). While XX and XO mice have ovaries (female), XXY and XY mice have testes (male). The genotypes allow measurement of the effects of one versus two X chromosomes (XO vs. XX; XY vs. XXY) and effects of one versus no Y chromosome (XY vs. XO; XXY vs. XX).

In both models, mice were separated by gonadal sex and housed in groups of four. All experiments were performed with mice between 8 and 12 weeks of age (25–35 g). All animals were housed in ventilated plastic cages in groups of four with standard bedding, maintained on normal 12-hr light/dark cycle (temperature at 22-C and 60% humidity) with lights on a 07:00 hr, and allowed free access to standard rodent chow (Teklad, Harland, Indianapolis, IN, USA) and water.

2.2 | Genotyping

For the FCG, ear punches were collected to isolate DNA. Extracted DNA was amplified using polymerase chain reaction (PCR) using a commercial PCR mix (Promega) and specific primers for the *Sry* gene, the Y chromosome, and internal control (Burgoyne & Arnold, 2016). For the XY* mice, the number of X chromosomes and the presence of the Y chromosome were determined by interphase fluorescent *in situ* hybridization using gene markers specific for the X and Y chromosome (Kreatech KI-30505 kit, Leica Biosystems). Some genotypes were confirmed by anatomical assessment, including anogenital distance and testis size.

2.3 | Drugs

U50,488H hydrochloride (U50) was purchased from R&D Systems (Minneapolis, MN, USA). Ethylketocyclazocine (EKC) was purchased from Sterling Drug Inc (Rensselaer, NY, USA). All drugs were dissolved in saline (0.9% sodium chloride) and given by subcutaneous (s.c.) injection (0.22–0.32 ml per injection, titrated to body weight).

2.4 | Tail withdrawal assay

Antinociception was measured by the tail withdrawal assay. Briefly, animals were gently retrained in a soft plastic conical sleeve and 2.5 cm of the tail was immersed in 49°C water. The time to tail withdrawal was measured. After three baseline measurements, mice received an injection of U50,488H (10 mg/kg, s.c.) or EKC (1 mg/kg, s.c.). The latency to tail withdrawal was measured every 10 min for 30 min. A cutoff of 15 s was imposed to avoid tissue damage. For the dose response curve, mice were injected with escalating doses of U50,488H (1–30 mg/kg, s.c.) or EKC (0.1–10 mg/kg, s.c.), and tail withdrawal latencies were measured after 20 min, immediately prior to the next injection. Withdrawal latencies were converted to percent maximum potential effect (%MPE) calculated as [test latency − baseline latency]/[cutoff latency-baseline latency] × 100.

2.5 | Data analysis

Results are shown as means ± *SEM*. Data were tested for normality (Shapiro–Wilk normality test) and means compared with one-, two-, or three-way ANOVA, as required. Differences were considered significant when $p < 0.05$.

3 | RESULTS

3.1 | Sex-dependent KOR antinociception

Female C57BL/6J mice exhibited significantly lower baseline tail withdrawal thresholds (Figure 1a; $t = 2.38$ (18), $p = 0.03$, $n = 10$) and significantly lower U50,488H (10 mg/kg, s.c.) antinociception (Figure 1b; $F_{Sex}(1, 10) = 8.7$, $p = 0.01$, $n = 10$). This sex difference remained when data were normalized to baselines and expressed as the percent maximum potential effect (%MPE, $F_{Sex}(1, 18) = 8.12$, $p = 0.01$). Following cumulative dose response testing, females exhibited a reduction in maximal effect of U50,488H (Figure 1c; $F_{Sex}(1, 30) = 10.87$, $p = 0.003$, $n = 6$; E_{max} females: 4.5 vs. males: 11.13, $p = 0.044$). To determine whether this sex difference was unique to U50,488H, another KOR agonist (EKC; 1 mg/kg, s.c.) was used under the same conditions. This resulted in a similar sex difference, with females exhibiting a significantly lower antinociceptive response than males (Figure 1d, $F_{Sex}(1, 10) = 1.68$, $p = 0.22$, $n = 6$, Figure 1e: $t(10)2.58$, $p = 0.02$, $n = 6$, Figure 1e; $F_{Sex}(1, 32) = 7.44$, $p = 0.01$, $n = 6$).

3.2 | Hormonal versus chromosomal contribution to sex difference

To determine whether the observed sex difference in U50,488H antinociception is due to gonadal hormones or sex chromosome genes, the sex difference in KOR function was further investigated using FCG mice. Baseline tail withdrawal thresholds were significantly higher in XY animals than XX, regardless whether they were male (testes, +Sry) or females (ovaries, −Sry) (Figure 2a; $F_{Chromosome}(1, 22) = 24.50$, $p \leq 0.0001$; $F_{Hormone}(1, 22) = 0.33$, $p = 0.56$; $F_{Interaction}(1, 22) = 7.56$, $p = 0.01$, $n = 6$–8). Following U50,488H administration (10 mg/kg, s.c.), XY animals exhibited greater tail flick antinociception when compared to XX animals (Figure 2b; $F_{Chromosome}(1, 22) = 11.84$, $p = 0.002$; $F_{Hormone}(1, 22) = 0.20$, $p = 0.20$, $F_{Interaction}(1, 22) = 0.65$, $p = 0.43$, $n = 6$–8). This effect remained when data were transformed to %MPE ($F_{Chromosome}(1, 22) = 7.32$, $p = 0.01$, $F_{Hormone}(1, 22) = 1.23$, $p = 0.28$, $F_{Interaction}(1, 22) = 0.006$, $p = 0.93$, $n = 6$–8) Although there was no main effect of gonad type, the interaction between sex chromosome complement and gonad type on baseline tail withdrawal thresholds (Figure 2a) suggests that ovarian hormones may augment the sex chromosome effect, or testicular hormones may blunt it, or both. Nevertheless, removing the gonads from the FCG mice did not reduce the overarching sex difference seen in the tail flick latencies (Figure 2c; $F_{Gonadectomy}(1, 43) = 0.87$, $p = 0.35$, $F_{Chromosome}(1, 43) = 13.5$, $p < 0.0001$; $F_{Hormone}(1, 43) = 0.92$, $p = 0.34$, no significant interactions detected, $n = 5$–8).

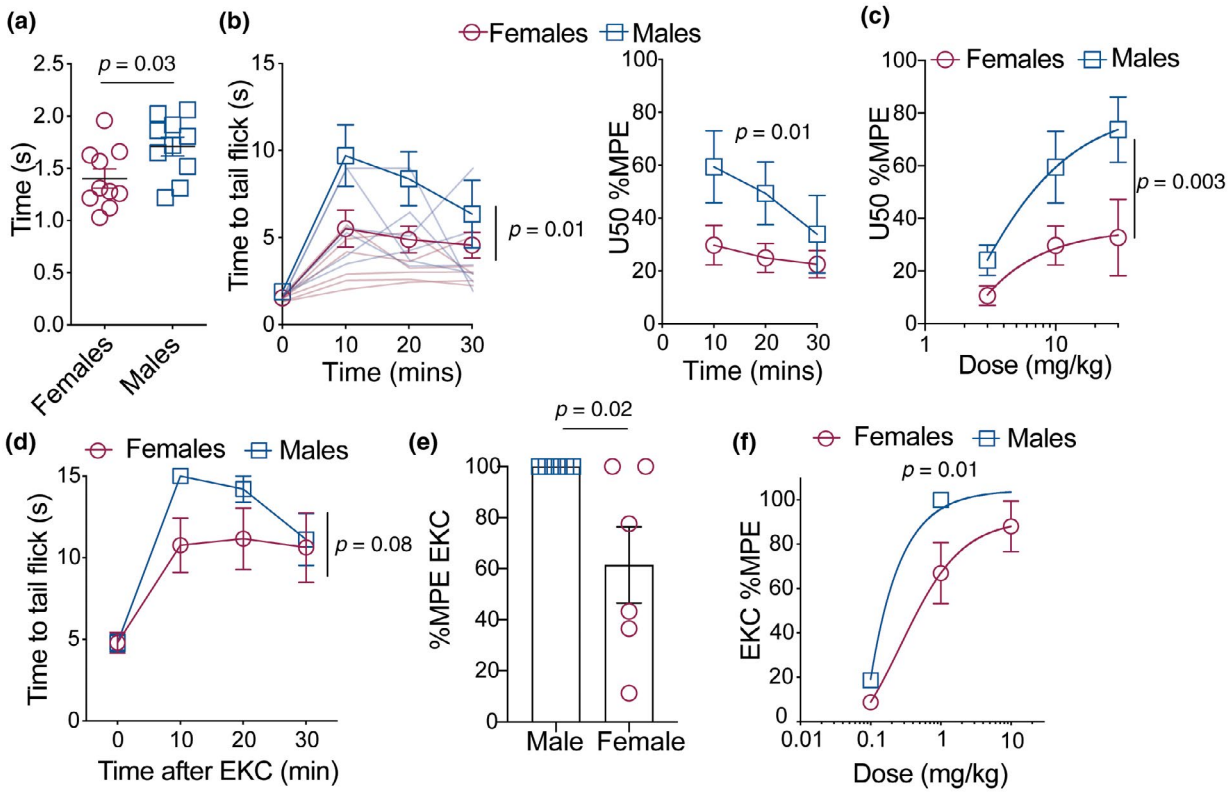

FIGURE 1 KOR antinociception is lower in female mice. (a) Baseline tail withdrawal thresholds are significantly higher in male C57BL/6J mice, $n = 10$. (b) U50 antinociception (10 mg/kg) is significantly lower in female mice. Left graph is raw data indicating tail withdrawal thresholds from 49oC water. Right graph is transformed data (Percent maximum potential effect, %MPE), $n = 10$. (c) U50 dose response curve indicates rightward shift in females, $n = 6$ (d) Sex differences exist with another KOR agonist, ethylketocyclazocine (EKC, 1 mg/kg, s.c.). Tail withdrawal thresholds from 49oC water were measured before and after injection with EKC (e) Transformed data (%MPE) of tail withdrawal thresholds from animals treated with EKC (1 mg/kg, s.c.) 10 min after drug injection, $n = 6$. (f) Females exhibit a rightward shift in the EKC dose–response curve, $n = 6$. Data expressed as means ± *SEM*

3.3 | X versus Y chromosome contribution to sex difference

To determine whether this sex difference is due to genes on the X chromosome or the Y chromosome, the XY* model was used. Baseline tail flick latencies were not significantly different based on number of X chromosomes or the presence or absence of a Y chromosome (Figure 3a; $F_Y(1, 34) = 0.25$, $p = 0.62$; $F_X(1, 34) = 1.03$, $p = 0.32$, $F_{Interaction}(1, 34) = 3.40$, $p = 0.07$, 6–11). There was no significant difference in U50,488H antinociception (10 mg/kg, s.c.) between mice with or without a Y chromosome (one X females versus one X males; two X females versus two X males), but there was a significantly lower U50,488H antinociception in mice with two X chromosomes compared to mice with one X chromosome (one X females versus two X females; Figure 3b; $F_{X\ dose}(1, 47) = 6.23$, $p = 0.02$, $F_{Y\ dose}(1, 47) = 0.57$, $p = 0.40$, $F_{Interaction}(1, 47) = 0.5$, $p = 0.38$, $n = 6$–11). This effect remained when data were transformed to %MPE ($F_{X\ dose}(1, 31) = 4.43$, $p = 0.044$, $F_{Y\ dose}(1, 31) = 0.61$, $p = 0.44$, $F_{Interaction}(1, 31) = 1.13$, $p = 0.30$, $n = 6$–11).

4 | DISCUSSION

Using genetic manipulation of the X and Y chromosomes, we assessed the contribution of sex chromosomes and gonadal hormones on KOR analgesia. We found that in FCG mice, XX animals exhibited significantly less KOR analgesia than XY animals. This chromosomal effect occurred in mice with testes or ovaries. The difference was replicated in the XY* model, which revealed that the XX–XY difference was caused by differences in the number of X chromosomes, not by the presence of absence of the Y chromosome.

Sex hormones did not significantly influence KOR antinociception in either gonad-intact or gonadectomized animals (Figure 2). This suggests that the activational and organizational effects of gonadal hormones play a minor role in this observed sex difference. While this seemingly contradicts published literature describing activational effects of female gonadal hormones on KOR analgesia (Abraham et al., 2018; Clemente-Napimoga et al., 2009), we did observe a significant interaction between gonadal hormones and sex chromosomes in mediating baseline withdrawal thresholds, which indicate gonadal hormones may augment or inhibit the sex

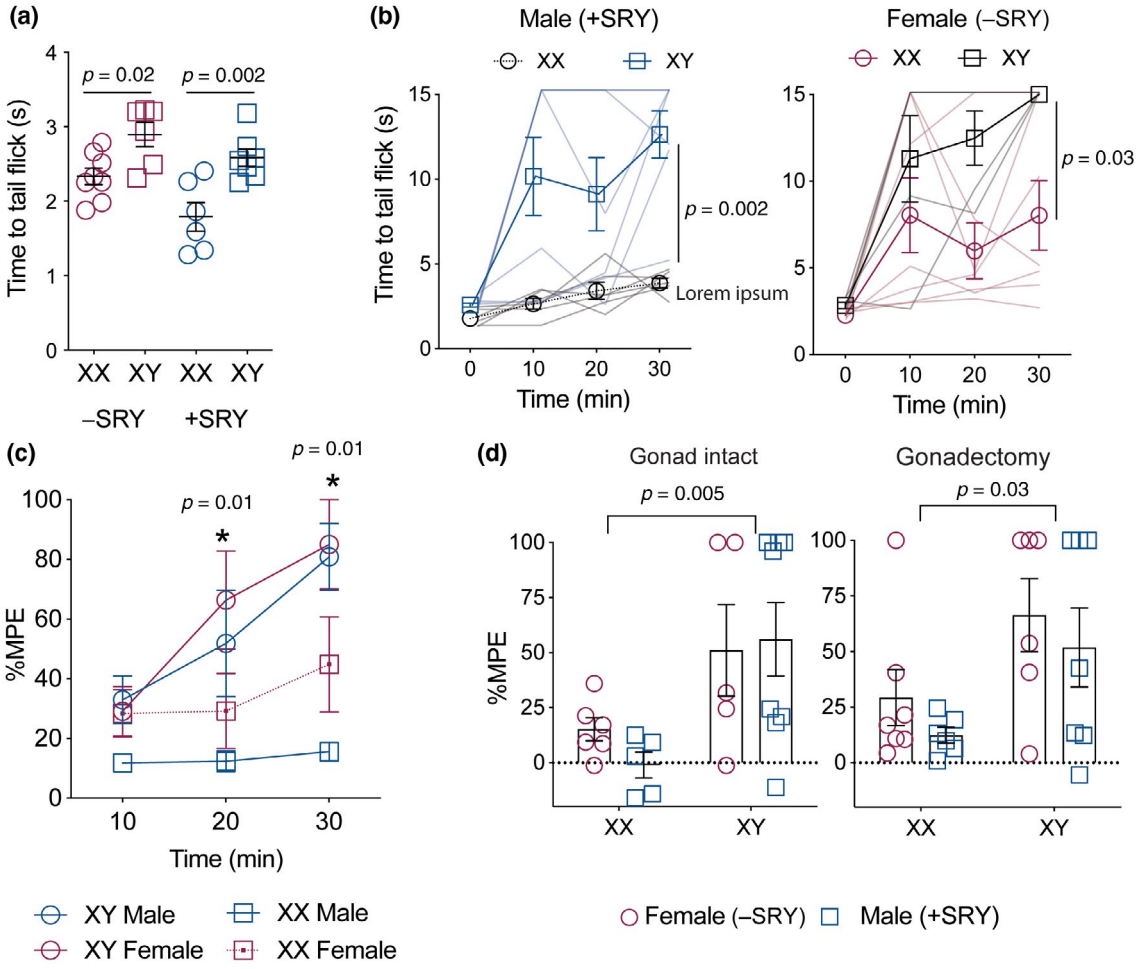

FIGURE 2 KOR antinociception varies with sex chromosome, not gonadal hormones. (a) Baseline tail withdrawal thresholds vary by sex chromosome and gonadal hormone, with animals with a Y chromosome displaying higher baseline pain withdrawal thresholds than animals with two X chromosomes, $n = 6–8$. (b) U50 antinociception (10 mg/kg, s.c.) is significantly higher in XY than XX mice, regardless of type of gonad. Data were compared with three-way ANOVA with time, sex chromosome, and gonadal hormone as independent factors. Males and females are plotted separately for clarity, $n = 6–8$ (c) Tail withdrawal thresholds were transformed to percent maximum potential effect (%MPE), $n = 6–8$. (d) Removal of gonads does not affect U50 sex differences in tail withdrawal, $n = 5–8$. Data presented as mean ± *SEM*

chromosome effect. Moreover, given that previous reports indicate that the magnitude of KOR sex difference depends on species, strain, and pain modality tested (Barrett et al., 2002; Craft & Bernal, 2001; Liu, Schnell, Wessendorf, & Gintzler, 2013), the contribution of gonadal hormones to KOR sex differences may well depend upon such factors. Finally, it should be emphasized that this study does not directly assess whether fluctuations in gonadal hormones contribute to observed sex differences. We did not assess female reproductive cycle nor did we measure gonadal hormone levels in individual animals. Therefore, it is possible gonadal hormones influence KOR analgesia within the context of the sex chromosome complement, and more research is warranted to investigate these processes. KOR agonists also effectively activate the hypothalamic–pituitary–adrenal axis, and we have shown previously that a significant portion of KOR analgesia is driven by stress-induced analgesia (Taylor et al., 2015). Future studies are needed to determine whether the sex chromosome complement also contributes to sex differences in KOR activity in stress pathways. It is unlikely that differences in KOR analgesia between males and females are due to pharmacokinetic differences, given that U50,488H metabolism and brain concentrations have been shown to be the same between the sexes (Laman-Maharg, 2018; Russell et al., 2014).

Our results are the first to indicate that sex chromosomes contribute an important portion of the sex differences in KOR analgesia. We found that genes located on the X chromosome contribute to lowered KOR antinociception in females (Figure 3). Our results also indicate a significant interaction between X chromosomes genes and gonadal hormones (Figure 2a), which suggests this X chromosome effect is more robust in gonadal males over females. While this study does not identify the specific genes responsible, there are some intriguing possibilities. The mouse X chromosome contains over a thousand genes. However, due to the process of X inactivation, only a minority of genes on the X chromosome are bi-allelically expressed in females (X escapees). In the rodent, 13 X chromosome genes are potential escapees

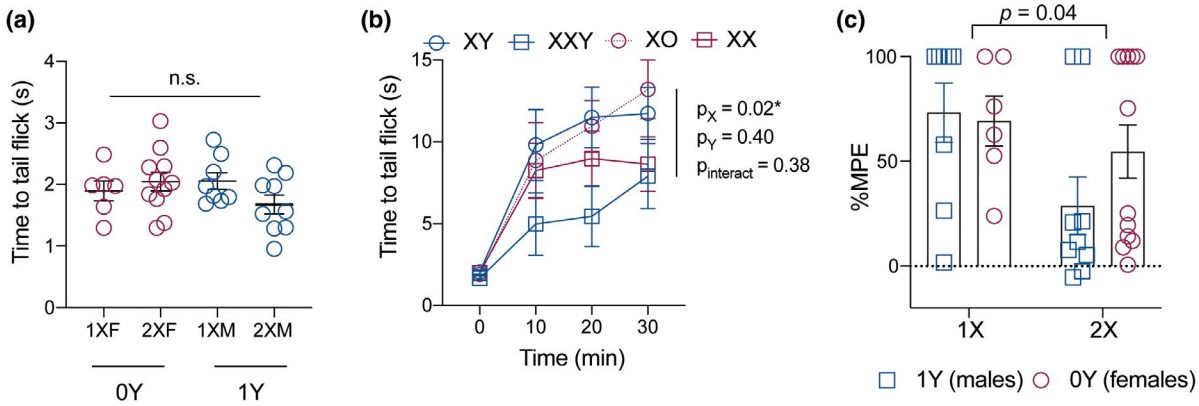

FIGURE 3 KOR antinociception varies with the X chromosome, not with Y chromosome. (a) In the XY*model, baseline tail withdrawal latencies were not significantly different between any of the genotypes, $n = 6–11$. (b) U50 antinociception (10 mg/kg, s.c.) tended to be lower in animals with two X chromosomes compared to one X chromosome. The presence or absence of the Y chromosome (SRY) did not affect withdrawal thresholds, $n = 6–11$. (c) Tail withdrawal thresholds 20 min following U50 administration were transformed to percent maximum effect (%MPE), $n = 6–11$. Data presented as mean ± *SEM*. Data were compared with two- or three-way ANOVA, with time, X chromosome, and Y chromosome as independent factors

(Berletch, Yang, Xu, Carrel, & Disteche, 2011). Of these 13, six express mRNA at higher levels in the XX than XY brain (Xu, Burgoyne, & Arnold, 2002). Most of these genes are involved in transcriptional regulation, and are widely expressed across tissues, and thus may have wide ranging influence on many other genes and proteins. Particularly interesting are two X escapee genes, *Kdm6a* and *Kdm5c*, which are expressed higher in XX than XY cells from the brain, immune system, adipose tissues, liver, and heart (Chen et al., 2012; Itoh et al., 2019; Li et al., 2014; Xu, Deng, & Disteche, 2008). These genes are histone demethylases, and thus have broad epigenetic effects across the genome. Recently sex differences in expression of *Kdm6a* have been implicated in causing sex differences in mouse models of bladder cancer (Kaneko & Li, 2018) and autoimmune diseases (Itoh et al., 2019). These and other X escapees are top candidate for regulation of KOR analgesia (Arnold, 2017; Golden et al., 2019).

One intriguing possibility is that X chromosome genes control the expression or function of KOR. Indeed, sex differences in KOR agonist binding and activity have been noted. For example, PET imaging using radiolabeled KOR ligand ([^{11}C]LY2795050) indicate men had higher KOR availability than women in multiple brain regions, including cerebellum, frontal cortex, and parietal cortex (Vijay et al., 2016). In animal studies, autoradiography with tritiated KOR agonist U69,593, showed greater binding in males throughout the brain, including somatosensory and insular cortices (Wang et al., 2011). KOR have also been shown to be differentially regulated in males and females in chronic pain (Liu et al., 2019). Finally, immunocytochemical analysis of downstream signaling events (p44/p42 MAPK) indicate reduced KOR receptor activation throughout the brain, but particularly in the amygdala (Rasakham, McGillivray, & Liu-Chen, 2012). This is of interest given the importance of the amygdala in modulating pain and stress responses (Kang-Park, Kieffer, Roberts, Siggins, & Moor, 2015; Nation et al., 2018).

In conclusion, our study indicates that genes on the X chromosome contribute to observed sex difference in KOR analgesia between males and females. These results indicate fundamental differences KOR signaling, driven in part, by genes located on the X chromosome.

Declaration of Transparency

The authors, reviewers and editors affirm that in accordance to the policies set by the *Journal of Neuroscience Research*, this manuscript presents an accurate and transparent account of the study being reported and that all critical details describing the methods and results are present.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

Conceptualization, A.M.W.T, A.P.A. and C.J.E.; *Methodology*, H.H. and A.P.A.; *Investigation*, A.M.W.T., S.M. and H.H.; *Formal Analysis*, A.M.W.T. and S.M.; *Resources*, A.P.A.; *Writing – Original Draft*, A.M.W.T., C.I.C., A.P.A. and C.J.E.; *Writing – Review & Editing*, A.M.W.T., A.P.A. and C.J.E.; *Visualization*, A.M.W.T. and C.I.C.; *Supervision*, A.M.W.T., A.P.A. and C.J.E.; *Funding Acquisition*, A.P.A. and C.J.E.; *Project Administration*, A.M.W.T.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article.

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