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Research report

Effects of kappa opioid receptors on conditioned place aversion and social interaction in males and females

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HIGHLIGHTS

- Low doses of kappa opioid receptor agonist induced place aversion in females but not males.
- Acute effects of kappa agonist on social interaction behavior are similar in males and females.
- Kappa agonist induces activation of p38 map kinase in microglia in the brain.

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ABSTRACT

The effects of kappa opioid receptors (KOR) on motivated behavior are well established based on studies in male rodents, but relatively little is known about the effects of KOR in females. We examined the effects of KOR activation on conditioned place aversion and social interaction in the California mouse (*Peromyscus californicus*). Important differences were observed in long-term (place aversion) and short-term (social interaction) effects. Females but not males treated with a 2.5 mg/kg dose of U50,488 formed a place aversion, whereas males but not females formed a place aversion at the 10 mg/kg dose. In contrast the short term effects of different doses of U50,488 on social interaction behavior were similar in males and females. Acute injection with 10 mg/kg of U50,488 (but not lower doses) reduced social interaction behavior in both males and females. The effects of U50,488 on phosphorylated extracellular signal regulated kinase (pERK) and p38 MAP kinase were cell type and region specific. Higher doses of U50,488 increased the number of pERK neurons in the ventrolateral bed nucleus of the stria terminals in males but not females, a nucleus implicated in male aggressive behavior. In contrast, both males and females treated with U50,488 had more activated p38 cells in the nucleus accumbens shell. Unexpectedly, cells expressing activated p38 co-expressed Iba-1, a widely used microglia marker. In summary we found strong sex differences in the effects of U50,488 on place aversion whereas the acute effects on U50,488 induced similar behavioral effects in males and females.

1. Introduction

Activation of KOR induces an aversive state, producing dysphoric like behaviors [38]. Initial work suggested that females might be more sensitive to KOR activation, as clinical studies showed that the analgesic effects of KOR agonists following dental surgery were stronger in women versus men [27,28]. Further study has demonstrated that sex differences in the analgesic effects of KOR are dependent on the pain modality assessed [33,54,64,46]. Much less is known about whether the behavioral effects of KOR differ in males and females, although recent reports also suggest that sex differences are context-dependent. Injections of the KOR-specific agonist U50,488 had stronger effects on posture and locomotor behavior in male Guinea pigs, but were more effective at blocking cocaine-induced hyperactivity in females [81]. Activation of KOR by U50,488 also was more effective at inhibiting intrancranial self-stimulation (which stimulates brain reward systems) in males compared to females [66]. Suppression of reward-related circuits is thought to contribute to dysphoria. Activation of KOR has been reported to induce dysphoria in humans [60,80] and dysphoric-like states in rodents [42]. In rodents,

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dysphoric-like states frequently lead to the formation of a conditioned place aversion [11,67,20]. The ability of KOR activation to induce place aversion has not been previously reported in females.

Kappa opioid receptors have also been reported to modulate social behaviors, particularly in the context of social conflict. The KOR antagonist nor-binaltorphimine (nor-BNI) reduced submissive behaviors in male C57Bl6 mice exposed to social defeat stress [48] and increased social interaction behavior immediately after exposure to defeat stress [13]. One of the only studies to examine the effects of KOR on social behaviors in females demonstrated that infusions of nor-BNI into the nucleus accumbens (NAc) shell reduced resident-intruder aggression in both male and female prairie voles [65]. These effects of KOR on aggressive and submissive behaviors were induced by relatively short term manipulations of KOR function. Other studies have suggested that certain experiences, such as defeat stress, may induce long term neuroplastic changes in the effects of KOR on social behavior. While U50,488 decreased social approach behavior in C57Bl6 mice that had won aggressive encounters, the same treatment increased social interaction in mice exposed to defeat stress for three weeks [41].

We examined the effects of the selective KOR agonist U50,488 on behavior in female and male California mice (*Peromyscus californicus*). Unlike other rodents, both male and female California mice are aggressive [69], which has allowed for the study of social defeat stress in females [74]. We recently observed that the inhibitory effects of dopamine D1 receptors in the NAc shell on social interaction behavior were stronger in females than males [15]. However, there were no sex differences in D1 receptor mRNA expression. Medium spiny neurons in the NAc that express D1 receptor also express dynorphin [31], the primary endogenous ligand for KOR [19]. Based on these data, we hypothesized that female California mice would be more sensitive than males to KOR stimulation. We tested this hypothesis with a dose–response study examining the effects of U50,488 on conditioned place preferences. Although other specific KOR agonists are available, U50,488 is one of the best characterized KOR ligands in studies of place preference. We also examined the effects of U50,488 on social interaction behavior. Finally, we examined the effects of U50,488 on the activation of extracellular signal regulated kinase (ERK) and p38 MAP kinase, as these pathways have been found to mediate the effects of KOR [4,12,61]. We examined core and shell regions of the NAc as previous reports have shown that the NAc is an important site of KOR action. We also examined the bed nucleus of the stria terminalis, which acts as a link between the mesolimbic dopamine and social behavior circuits [57,58].

2. Methods

2.1. Animals

California mice were bred in the UC Davis laboratory colony. All animals in the study were three month old sexually inexperienced adults. Females were tested at different stages of the estrous cycle as each mouse was tested over the course of one week. Estrous cycle was not continuously monitored because we previously determined that vaginal lavage has strong effects on behavior in California mice [69]. Mice were individually marked with ear punches and housed 2–3 per cage in same sex groups. Animals were housed in clear polypropylene cages with Sani-chips bedding, environ-dri (Shepherd, Milford, NJ), and cotton nestlets. Harlan Tekland 2016 food and water were provided *ad libitum*. Mice were maintained on a 16 h light/8 h dark cycle (lights off 15:00 PST). All experiments were approved by the UC Davis Institutional Animal Care and Use Committee. The mice were maintained in accordance with the recommendations of the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*.

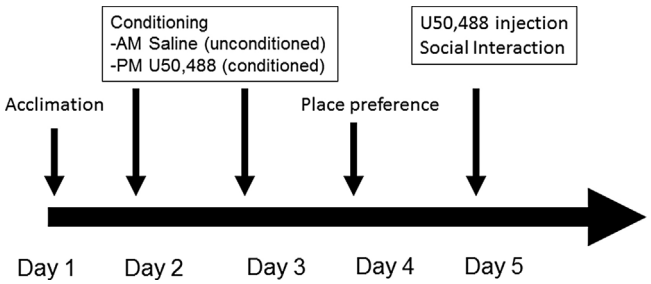

Fig. 1. Timeline for place preference experiments and social interaction testing.

2.2. Conditioned place aversion

The conditioning apparatus consisted of three interconnected standard sized mouse cages (28 × 17.5 × 11 cm). All three of the cages were visually distinct. The center cage (black and white horizontal stripe background) was connected to a left cage (black and white vertical stripe background) and a right cage (black dots on a white background). Each compartment contained no bedding and the apparatus was cleaned with Quatricide (1:64, Quatricide PV in water, Pharmacal Research Labs, Inc) after each conditioning or testing session. Clear polypropylene lids were used to cover the apparatus during experiments to facilitate video recording.

Place preference testing was conducted over four days (Fig. 1). On day 1 each mouse was given free access to the entire arena for a 30 min acclimation test (between 0800 and 1200). Mice were tracked in real time with a visual tracking system (Any-maze, Stoelting). Repeated measures ANOVA showed that on day 1 of testing mice spent significantly more time in the center chamber compared to the side chambers (Table 1, $F_{2,61} = 15.23$, $p < 0.001$). There was also a slight but significant preference for the left chamber compared to the right ($p = 0.03$), but there were no sex or treatment differences (all p's > 0.8). For each mouse, initial place biases were corrected for by assigning drug conditioning to the preferred side chamber [49,63]. Although more mice were conditioned in the left chamber (Table 1), there were no systematic differences between males and females or between the different drug treatments. Importantly, conditioning procedures did not change mean chamber preferences (Table 1). This suggests that the effects of conditioning are based on the aversive or rewarding aspects of U50,488 and not due to nonspecific effects on activity or anxiety-like behavior [77]. Mice were randomly assigned to be conditioned with either 0, 2.5, 5, or 10 mg/kg of the KOR selective agonist U50,488 (Tocris, Ellisville, MO, USA) dissolved in vehicle consisting of sterile phosphate buffered saline (PBS) with 10% Tween 80 (Fisher Scientific, Fair Lawn, NJ, USA).

Conditioning sessions took place on Days 2 and 3 based on previous methods [8]. Each day consisted of one training session in the unconditioned chamber (AM) and one training session in the conditioned chamber (PM). First, between 0800 and 1100 h each mouse was injected i.p. with vehicle and confined to the unconditioned chamber for 30 min. Subjects were confined to the unconditioned chamber by blocking off the entry way to the center chamber with heavy tape. Between 1200 and 1500 h mice

Table 1
Cage preferences before and after conditioning. $^{**}p < 0.01$ vs center, † vs. left side $p < 0.05$.

	Cage time (s)		
	Center cage	Left side	Right side
Pre-test	813 ± 43	544 ± 35**	438 ± 32**,†
Post-test	812 ± 72	580 ± 48**	408 ± 44**,†
Number conditioned	23	46	

Table 2
Abnormal/splaying posture rating scale (based on [81]).

Score	Description
0	No change in normal body condition
1	One leg is splayed forward or sideways, moderately elongated
2	Two legs are splayed, moderately elongated
3	More than 2 legs are splayed, elongated completely, animal does not move, except head
4	More than 2 legs are splayed, elongated completely, animal does not move
5	More than 2 legs are splayed, elongated completely, animal does not move, hind legs splayed backward

were injected i.p. with the assigned dose of U50,488 and confined to the conditioned chamber for 30 min. During all conditioning sessions, mice were observed for abnormal postures and immobility. Normal posture in California mice consists of a huddled position with the tail in the air. California mice treated with i.p. injections of U50,488 demonstrated consistent changes in posture such as extension of the hindlimbs and/or forelimbs, resting the tail on the cage flooring, or lying flat on the cage. These changes in posture were quantified using a Likert rating scale of 0–5, previously used to quantify the effects of KOR agonists on posture in Guinea pigs [9,81]. Scores rate the extent to which the forelimbs and/or hind limbs are extended outward (splaying), and whether their bodies are elongated (Table 2). Mice were assigned a score every 5 min during each conditioning trial. On Day 4, each mouse was given free access to the entire apparatus for 30 min and was tracked with the video tracking system. The time spent in each cage and the total distance traveled were both recorded.

2.3. Social interaction

On Day 5 (Fig. 1) each mouse was tested in the social interaction test in the dark phase (1500–1700) as previously described [75,30]. Each mouse was injected (i.p.) with the same dose of U50,488 used in the afternoon conditioning sessions on Days 2 and 3. Forty-five minutes later subjects were tested during the dark phase of the light cycle under dim red illumination.

The apparatus for social interaction testing consisted of a large open field ($89 \times 63 \times 60$ cm) and a wire cage. There were three phases of the test, 3 min each. First, during the open field phase, the mouse was habituated to the empty arena. Next, during the acclimation phase, an empty wire cage ($14 \times 17 \times 14.5$ cm) was placed into the arena at one end. During the social interaction phase an unfamiliar, same-sex sexually inexperienced mouse was placed into the novel cage. Time spent within 8 cm of the wire cage and in two corner zones (8×8 cm) opposite the cage was recorded using a video tracking system (Any-maze, Stoelting). Time spent in the center of the arena during the open field phase was recorded as a measure of anxiety-like behavior and distance traveled during the open field phase was used as an estimate of locomotor activity. Mice were anesthetized with isoflurane and euthanized by decapitation 40 min after the testing. Brains were immediately collected and were fixed in 5% acrolein in PBS overnight at 4 °C. Brains were then transferred to 20% sucrose overnight at 4 °C and then frozen and stored at −40 °C.

2.4. Immunohistochemistry and quantification

Brains were sectioned at 40 μm on a cryostat and stored in cryoprotectant (50% v/v phosphate buffer, 30% w/v sucrose, 1% w/v sucrose, 1% w/v polyvinylpyrrolidone, 30% v/v ethylene glycol) at −20 °C. Two different sets of sections were used for immunohistochemical staining for p-ERK and p-p38 based on previously published protocols [72]. Sections were washed three times in phosphate buffered saline (PBS) for 5 min, followed by a 10 min wash in 1% sodium borohydride in PBS. Two PBS washes for 5 min were done before blocking in 10% normal goat serum and 0.3% hydrogen peroxide in PBS for 30 min. Sections were washed with PBS twice before being incubated overnight at 4 °C in primary pERK (Cat. No. 4376S, Cell Signaling, 1:300) or phosphorylated p38 (p-p38, Cat. No. 4511s, Cell Signaling, 1:500) in 2% normal goat serum and 0.5% Triton X (TX) in PBS. Day 2, sections were washed three times in PBS for 5 min, followed by incubation in secondary biotinylated goat anti-rabbit (Vector Laboratories, Burlingame, CA, 1:500) in 2% normal goat serum and 0.5% TX in PBS for 2 h. Sections were washed three times in PBS and incubated in avidin-biotin complex (ABC Elite Kit, Vector Laboratories) for 30 min. Three PBS washes followed and then sections were developed in nickel enhanced diaminobenzidine (Vector Laboratories) for 90 s. Sections were then washed in PBS two times and mounted onto Superfrost Plus microscope slides (Fisher Scientific). Once dry, slides were dehydrated for 1 min in 100% ethanol followed by 1 min in Histoclear (National Diagnostics, Atlanta, GA) and coverslipped with Permount (Fisher Scientific).

Specificity for the p-ERK primary antibody in California mice has been previously described [72]. To determine specificity of the p-p38 antibody we confirmed that omission of primary antibody resulted in no positive staining (Fig. 2B). We also demonstrated that pre-incubation with the immunizing peptide for the p-p38 antibody prevented positive staining (Fig. 2C). To identify the cell types that expressed p-ERK or p-38, we conducted additional double labeling experiments using neuN (to label neurons), GFAP (to label astrocytes), and Iba-1 (to label microglia). Sections were incubated in sodium borohydride and then blocked in 10% normal donkey serum for 30 min. Sections were then double labeled for p-p38 (Cat. No. 4511s, Cell Signaling, 1:500) and mouse antibodies directed at either NeuN (Cat. No. MAB377, Millipore, 1:500) or GFAP (Cat. No. N206A, Neuromab, 1:500). For Iba-1 we used a rabbit antibody Iba-1 (Cat. No. 019-19741, Wako, 1:100). Sections were incubated in primary antibodies overnight at 4 °C, washed in PBS, and then incubated in secondary antibodies for 2 h. NeuN and GFAP were visualized using donkey anti-mouse Alexa Fluor 488 (Abcam ab 150105, 1:500). To visualize Iba-1 we first incubated these sections in in goat anti-rabbit fab fragment (Jackson immune Cat No. 111-007-003, 40 mcg/ml) for 1 h. After washing in PBS, these sections were incubated with donkey anti-goat Alexa Fluor 488 (Abcam ab 150129, 1:500) for 2 h. We visualized p-p38 using donkey anti-rabbit Alexa Fluor 555 antibodies (Abcam ab 150074, 1:500) for 2 h. Sections were then washed in PBS, mounted on slides and coverslipped with VectaShield (Vector).

Images of the left and right side of each brain area were imaged with a Zeiss AxioImager based on a mouse brain atlas and previous descriptions of the California mouse NAc and BNST [16]. The background for each image was normalized by adjusting the exposure time. The number of immunopositive cells in each brain region was counted in frame of uniform size (0.3×0.3 mm) using Image J (NIH, Bethesda, MD) by an observer unaware of treatment assignments. Cell count data are presented as number of positive cells per mm^2.

2.5. Statistical analyses

For each afternoon conditioning session, we used splaying ratings to calculate an area under the curve score (Prism Graphpad, La Jolla, CA) to summarize the effect of the injection on posture. Scores for the two conditioning sessions were averaged. Due to the non-normal distribution of these scores, splaying data were

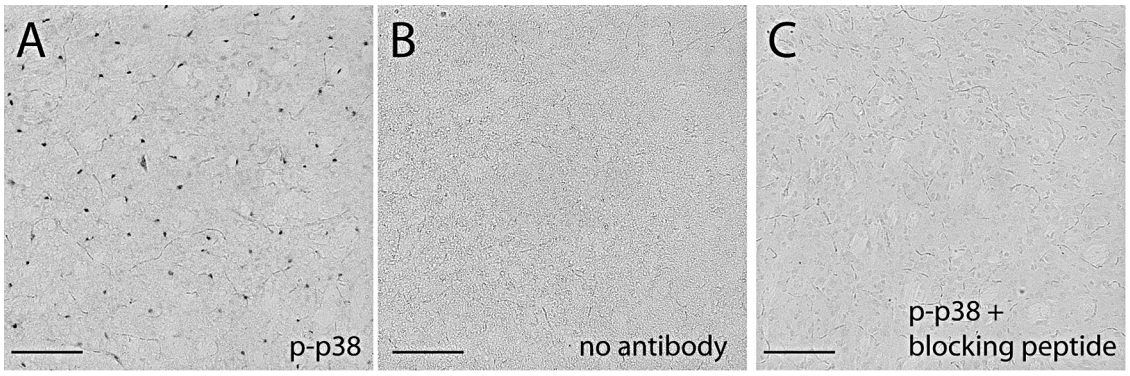

Fig. 2. Immunostaining of phosphorylated p38 MAP kinase in California mouse nucleus accumbens (A). No staining was observed when the primary antibody was omitted (B), or if the primary antibody was preabsorbed with the immunizing peptide (C). Scale bar = 100 μm.

analyzed using nonparametric statistics. For place preference tests, we calculated difference scores for the conditioned chamber, unconditioned chamber, and center chamber by subtracting pretest scores (day 1) from posttest scores (day 4). Difference scores were analyzed with two-way ANOVA testing for effects of dose and sex. To test whether place preferences were affected by the location of conditioning, we conducted an additional 3-way ANOVA on these data including sex, dose, and conditioning site (left or right).

Social interaction data were analyzed with two-way ANOVA (sex and dose). For each brain area, we averaged cell counts from the left and right side and analyzed these scores with two-way ANOVA (sex and dose). Spearman rank correlations were used to correlate splaying with place preference or social interaction behavior.

3. Results

3.1. Effects of U50,488 on posture during conditioning

The effects of U50,488 on behavior during conditioned place aversion studies suggest that the short term effects of KOR in males and females are similar, but that there are significant sex differences in the long term effects of KOR. Splaying was never observed during morning conditioning sessions with vehicle (data not shown). During afternoon conditioning sessions, U50,488 induced splaying behavior in both males (Fig. 3, Kruskal-Wallis $p < 0.01$), and females (Fig. 3, Kruskal-Wallis $p < 0.01$), and there were no significant sex differences. All three doses of U50,488 induced significant increases in splaying compared to vehicle in both males and females. There was a nonsignificant trend for females to show a greater degree of splaying than males (Mann-Whitney $p = 0.05$). There were no significant differences in locomotor activity in morning or afternoon conditioning sessions.

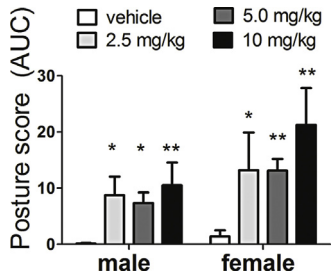

Fig. 3. Effects of U50,488 on splaying behavior averaged across the two afternoon conditioning sessions. Posture ratings were taken every 5 min during conditioning and used to calculate an area under the curve (AUC) score. $^{*}p < 0.05$, $^{**}p < 0.01$ Mann-Whitney U test vs. saline. $n = 7$–12 per group.

3.2. Effects of U50,488 on place preference formation

On day 4, U50,488 induced conditioned place aversions at different doses in males and females (Fig. 4A, $F_{3,61} = 5.14$, $p < 0.01$). Males treated with 10 mg/kg of U50,488 spent significantly less time in the conditioned chamber compared to saline treated males (Fig. 4A). Males treated with 5 mg/kg or 2 mg/kg were not significantly different from saline. In females, a conditioned place aversion was induced by the lowest dose in females (Fig. 4A, $p < 0.01$ vs. saline). Intriguingly, the highest dose of U50,488 induced a conditioned place preference in females ($p < 0.05$ vs. saline). On average, females showed a greater increase in time spent in the unconditioned chamber compared to males (Fig. 4B, $F_{3,61} = 9.1$, $p < 0.01$). In the center chamber, there was a nonsignifcant trend for a sex × stress interaction (Fig. 4C, $F_{3,61} = 2.59$, p 0.06). Females treated with 10 mg/kg spent significantly less time in the center cage compared to saline treated females ($p < 0.001$). We also ran additional 3-way ANOVA analyses including conditioning location (left or right) as a factor. These analyses confirmed that conditioning location was not a confounding variable. Conditioning location did not explain a significant portion of variance in place preference for the conditioned, unconditioned, or center chamber (all p's > 0.36). More importantly, the location of conditioning did not alter the effects of sex, dose, sex × dose for any variable we examined.

3.3. Effects of U50,488 on social interaction behavior

During social interaction testing on day 5, the effects of U50,488 were stronger in the presence of social stimuli. During the social interaction phase, the highest dose of U50,488 reduced social interaction behavior (Fig. 5A, $F_{3,61} = 6.24$, $p < 0.001$) and increased time spent in the corners of the arena opposite the social stimulus (Fig. 5B, $F_{3,61} = 3.29$, $p = 0.03$). There were no significant sex differences in these variables. There were no significant differences in behavior during the acclimation phase in the absence of a social stimulus (Table 3). There were also no significant differences in locomotor behavior in the open field phase and there were no differences in time spent in the center of the arena (Table 3).

3.4. Correlations between posture, place preferences, and social interaction behavior

Correlational analyses suggest that U50,488 might have different short and long term effects on behavior. Posture scores and social interaction behavior were quantified within 30 min of an i.p. injection, and these variables were negatively correlated with each other (Fig. 6A, Spearman rank correlation $\rho = -0.50$, $p < 0.001$). In contrast, place preference behavior was assessed in a drug free state

Table 3
Behavior during the acclimation and open field phases of testing immediately before social interaction testing.

		Acclimation phase		Open field phase	
		Interaction zone	Corner zone	Center zone	Distance (m)
Females	Vehicle (n = 9)	108.5 ± 9.8	9.2 ± 3.4	37.3 ± 4.7	22.7 ± 2.8
	2.5 mg/kg (n = 7)	118.1 ± 6.6	7.1 ± 0.9	26.2 ± 4.1	21.3 ± 2.9
	5 mg/kg (n = 12)	122.5 ± 7.0	5.5 ± 1.2	32.6 ± 5.9	26.5 ± 1.7
	10 mg/kg (n = 6)	94.6 ± 17.9	6.0 ± 2.2	33.9 ± 4.4	23.3 ± 3.3
Males	Vehicle (n = 10)	102.6 ± 8.3	3.8 ± 1.0	37.0 ± 7.0	27.2 ± 2.6
	2.5 mg/kg (n = 8)	106.8 ± 11.5	6.0 ± 1.6	35.7 ± 7.4	27.4 ± 4.2
	5 mg/kg (n = 9)	97.6 ± 7.1	6.1 ± 1.9	25.7 ± 3.7	22.4 ± 2.4
	10 mg/kg (n = 7)	94.8 ± 12.1	5.6 ± 1.3	25.0 ± 2.5	27.4 ± 5.8

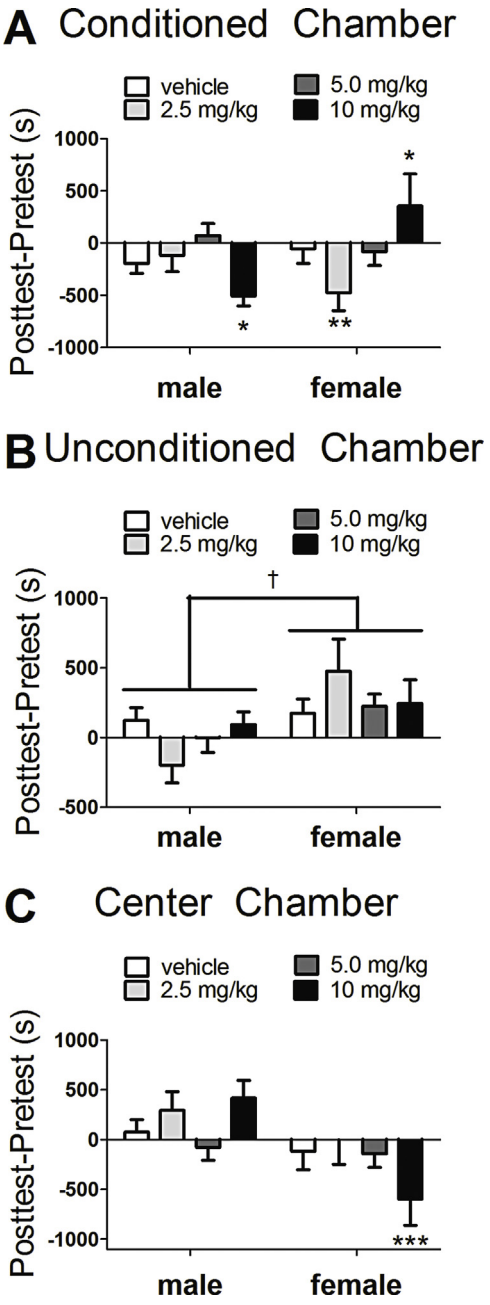

Fig. 4. Changes in place preferences in the conditioned chamber (A), unconditioned chamber (B) and center chamber (C). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus saline; †sex difference $p < 0.01$. n = 7–12 per group.

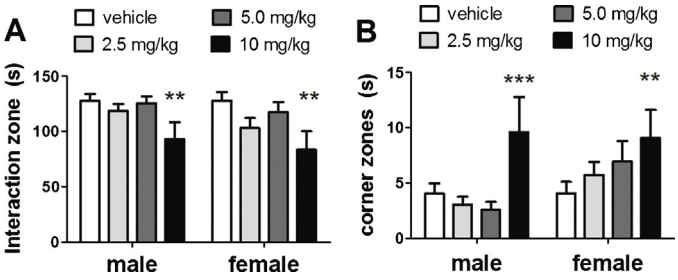

Fig. 5. Effects of U50,488 on time spent in the interaction (A) and corner (B) zones during the social interaction test. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle.

approximately 20 h after the last treatment of U50,488. There was no significant correlation between splaying and place preference in the conditioned chamber (Fig. 6B, Spearman rank correlation $\rho = -0.04$, $p = 0.74$).

3.5. Effects of U50,488 on p-ERK and p-p38 immunoreactivity in the nucleus accumbens and bed nucleus of the stria terminalis

Unexpectedly, p-p38 immunoreactivity was restricted exclusively to cells co-expressing the microglia marker Iba-1 (Fig. 7). In the NAc shell, U50,488 treatment increased the number of p-p38 positive cells (Fig. 8A, $F_{3,39} = 3.44$, $p < 0.05$). There were no sex differences or interactions. There were no significant differences in p-p38 positive cells in the NAc core (Fig. 8B). There were no significant differences in the number of p-ERK positive cells in either

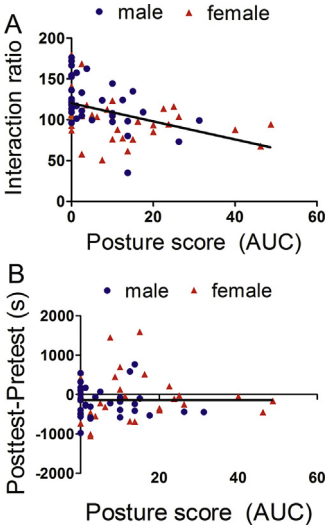

Fig. 6. Correlations between splaying during afternoon conditioning sessions (Days 2 and 3) and social interaction ratio (A, Spearman $\rho = -0.5$, $p < 0.001$) and place preference in the conditioned chamber (B, Spearman $\rho = -0.04$, $p = 0.74$).

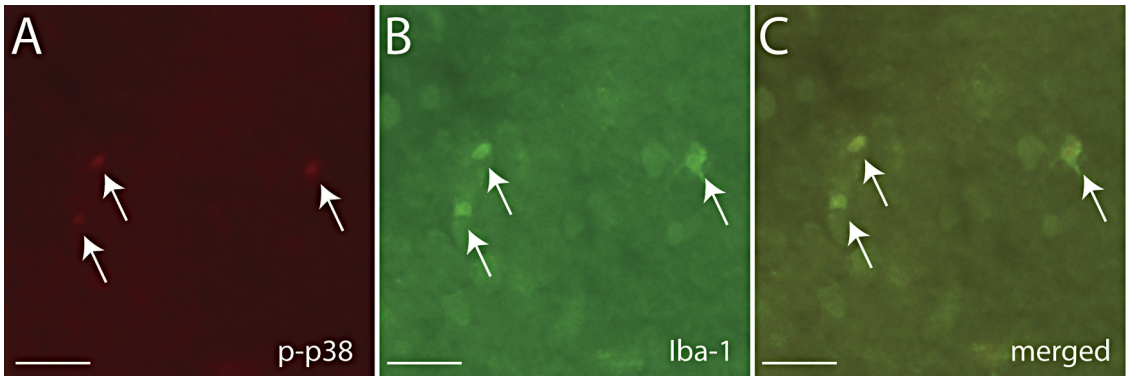

Fig. 7. Immunostaining of p-p38 (A) and Iba-1 (B) in California mouse NAc. The merged image (C) demonstrates co-localization of p-p38 and Iba-1 in the same cells. Black indicates double-labeled cells. Scale bar = 100 μm.

NAc core or shell (Fig. 8D, E). There were no significant correlations between cell counts and behavior in the social interaction test.

In the BNSTam the high dose of U50,488 increased the number of p-ERK positive cells (Fig. 9D, $F_{3,39} = 4.52$, $p < 0.01$). In the BNSTvl, males had more p-ERK positive cells than females (Fig. 9E, $F_{3,39} = 29.95$, $p < 0.01$). Animals treated with U50,488 also had more positive p-ERK cells ($F_{1,39} = 2.90$, $p < 0.05$), but this effect was driven primarily by changes in males. Males but not females treated with the 5 or 10 mg/kg dose had significantly more p-ERK positive cells compared to saline. Males had more p-p38 positive cells than females in both the BNSTam (Fig. 9A, $F_{1,39} = 6.09$, $p < 0.05$) and the BNSTvl (Fig. 9B, $F_{1,39} = 7.76$, $p < 0.1$) but there were no effects of U50,488 or interactions. There were no significant correlations between cell counts and behavior in the social interaction test.

4. Discussion

This study assessed both long term and short term effects of KOR activation in both males and females. In place preference tests, which measured long term effects of KOR, males and females had different dose–response curves. Females but not males formed place aversions at low level KOR stimulation. In contrast at the highest dose of U50,488 males formed place aversions but females formed place preferences. When the short term effects of KOR stimulation were quantified in the social interaction test, males and females showed more similar behavioral responses. Social stress activates KOR signaling pathways, and in previous studies we observed that the effect of defeat stress on social interaction behavior is stronger in female California mice versus males [74,75,30]. Social withdrawal in females is a long lasting behavioral change, which is consistent with our observation that females were more sensitive to the long term effects of U50,488 in the place preference tests. In contrast, when U50,488 was administered immediately before social interaction tests, KOR activation had similar effects in males and females. Previous studies have demonstrated that KOR agonists can induce rapid activation of ERK via G protein-dependent mechanisms but that long-term effects are more dependent on β-arrestins [10,68,85]. Together these results suggest that KOR mediated place aversion may be mediated via β-arrestin signaling whereas acute induction of splaying or social withdrawal by KOR activation may more dependent on G protein mediated signaling.

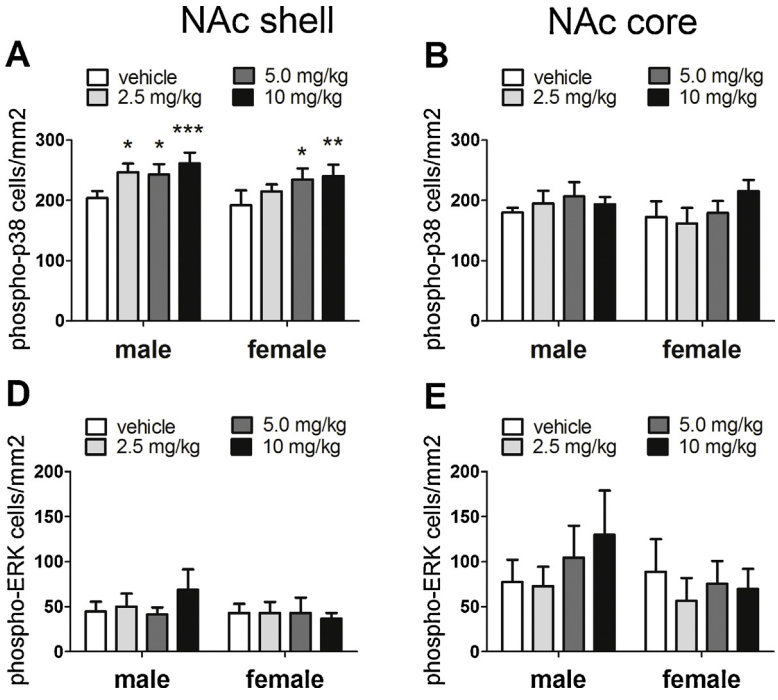

Fig. 8. Cell counts for p38 (A, B) and ERK (D, E) in the NAc shell and core. Representative photomicrographs for phosphorylated p38 (C) and ERK (D) immunostaining. $^{*}p < 0.05$ vs saline, $^{**}p < 0.01$ vs saline, $^{***}p < 0.001$ vs saline. $n = 5$–6 per group.

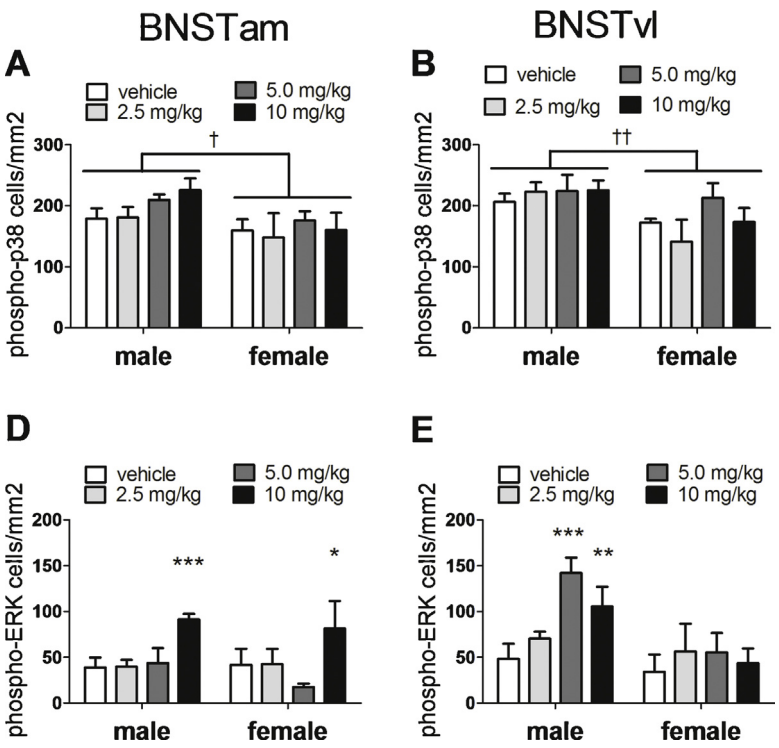

Fig. 9. Cell counts for p38 (A, B) and ERK (D, E) in the BNSTam and BNSTvl. Representative photomicrographs for phosphorylated p38 (C) and ERK (D) immunostaining. $^{*}p < 0.05$ vs saline, $^{**}p < 0.01$ vs saline, $^{***}p < 0.001$ vs saline. †sex difference $p < 0.05$, ††sex difference $p < 0.01$. $n = 5$–6 per group.

4.1. Effects of U50,488 on behavior

The most striking sex difference in the behavioral effects of U50,488 was observed in a test that examined a long-term behavioral change: conditioned place aversion. Similar to a recent report in male rats [82], only the 10 mg/kg dose of U50,488 induced place aversion in male California mice. In contrast, female California mice developed place aversion to 2.5 mg/kg of U50,488 but formed a place preference at 10 mg/kg. Similar biphasic dose–response curves for place aversion have been observed in male rats using either peripheral [55,3] or central [2,1] administration of U50,488. Our results suggest that the dose–response curve to the aversive properties of U50,488 is shifted to the left in female California mice. The observation that females treated with 10 mg/kg of U50,488 developed a place preference raises the possibility of nonspecific activation of μ opioid receptors (MOR), which mediates the formation of place preferences by morphine [70]. However, U50,488 has poor affinity for MOR [21] and we observed increased splaying behavior during conditioning at all doses of U50,488 in both males and females. Intriguingly, several forms of KOR-mediated analgesia in rodents are dependent on NMDA receptors in males but not females [34,35]. Activation of NMDA receptors has important modulatory effects on the formation of mu-opioid dependent place preferences [78,22], suggesting that sex differences in KOR-NMDA interactions could impact sex differences in sensitivity to KOR mediated place aversion. Currently, little is known about KOR-NMDA interactions in the context of place preferences.

Splaying was observed shortly after U50,488 administration, and was correlated with behavior in the social interaction test. Indeed, only the highest dose of U50,488 reduced time spent in the interaction zone with a social stimulus and increased time spent in the corner zones opposite the social stimulus. We observed no effects of any dose of U50,488 on distance traveled during the open field phase (conducted 3 min before the social interaction phase), which indicates that reduced social interaction was not due to sedation. Thus, while we observed sex differences in the long term effects of U50,488 in the place preferences tests, we observed no sex differences in the short term effects of U50,488. We originally predicted that U50,488 would have stronger effects on social interaction behavior in females, because KOR is activated by stress [42] and defeat stress has stronger effects on female social interaction behavior than males [74,75,30]. Thus, the lack of strong sex differences in the social interaction test was unexpected. A key factor may be the time course of KOR activation. In female California mice, social withdrawal behavior is stronger four weeks after defeat compared to 24 hours [74]. If KOR mediates effects of defeat stress on social interaction behavior, long term effects of KOR mediated by β-arrestins may be more important.

We observed little evidence for anxiogenic effects of U50,488 during open field testing. Previous studies in male rodents have recorded anxiogenic effects of KOR activation [62,39,84], but all of these studies were conducted during the inactive phase (lights on). Our results are more consistent with a previous study conducted during the active phase (lights off), which reported no effect of prodynorphin deletion on behavior in the zero-maze test of anxiety-like behavior [7]. The behavioral effects of opioids can depend on whether testing is conducted during the active phase or inactive phase [36]. Overall, these results suggest that the anxiogenic effects of KOR signaling may be stronger during the inactive phase. Splaying was not observed during social interaction testing. The lack of splaying might be due to increased arousal as the mice were tested during the active phase in a novel environment.

Open field and social interaction testing were conducted after a third treatment of U50,488, raising the possibility that animals may have developed tolerance with repeated U50,488 treatment [6]. However, previous reports of KOR tolerance require more frequent and higher doses of U50,488 over a longer time frame compared to our study [50]. For example, in a study of intracranial self-stimulation, multiple doses of 10 mg/kg of U50,488 did not induced tolerance in male or female rats [66]. Finally, activation of KOR might have different effects on male–female behavioral interactions (as opposed to the same-sex interactions observed in our

study). While activation of KOR by U50,488 reduces sexual interest in male rats [44], it enhances copulatory behavior in estradiol-primed female rats [59]. This suggests that there may be important differences in KOR-sensitive circuits mediating social interaction in same sex individuals versus sexual behavior.

4.2. Effects of U50,488 on phosphorylated p38 in the nucleus accumbens

Previous studies have implicated activation of p38 within neurons as a key mechanism mediating the aversive effects of KOR [11,13,43]. Although U50,488 increased activation of p38 in the NAc and reduced social interaction behavior, these two variables were not significantly correlated. This may be because increases in p-p38 immunoreactivity we observed occurred in microglia not neurons. The p-p38 antibody we used has been used in immunoblot analyses in previous studies, and we confirmed its specificity in California mice. Kappa opioid receptor-microglia interactions can downregulate inflammatory signaling in the brain [23], but so far the behavioral implications of these interactions have received little attention.

Microglia are best known for their role in mediating immune responses [25], and are often studied in the context of brain injury or ischemia. However, microglia have broader effects on brain function by altering synaptic structure. Microglia can align with apical dendrites, and may even eliminate inactive synapses [79,29]. This suggests that microglia may play a role in facilitating neuroadaptive changes that are induced by psychosocial stress [51,18]. Restraint stress increases microglia proliferation in the brain, a process facilitated by corticosterone [56]. Restraint stress also increases the number of microglia in the NAc shell, NAc core, dorsal BNST, infralimbic cortex, and hippocampus but not amygdala or paraventricular nucleus [76]. Defeat stress increases microglia activity in frontal cortex [71]. Increased cytokine signaling by microglia has been hypothesized to be an important risk factor for mood disorders such as major depression [52]. Certain sub-populations of patients with depression have increased levels of inflammatory cytokines [86], which in animal models are closely linked to depression-like behaviors such as social withdrawal [24]. Our results suggest that these pathways could be regulated by KOR signaling via p38.

We also found that U50,488 increased p38 activation in an anatomically specific fashion. In a previous study we observed that dopamine D1 receptor antagonists infused into the NAc shell increased social interaction behavior in stressed female California mice [15]. Based on this result we predicted that U50,488 would increase the number of p-p38 cells, because D1 neurons in the NAc coexpress dynorphin [31], the primary endogenous ligand for KOR [19]. To some degree our prediction was correct, as U50,488 indeed increased the number of p-p38 positive cells in the NAc shell. However, this effect was not stronger in female than males and we had not expected to see this effect to be mediated by microglia. Our current results indicate that it will be important for future studies to consider whether stress induced activation of KOR signaling in the NAc affects neurons or microglia. Given the role of microglia in synaptic remodeling, an intriguing possibility is that microglia may be more important for mediating the long term effects of KOR signaling.

4.3. Effects of U50,488 on phosphorylated ERK in the BNST

Higher doses of U50,488 also increased pERK immunostaining in ventral BNST in males but not females. This raises an interesting question regarding the nature of social interaction and aggressive behavior. In male CD-1 mice both estrogen receptor α (ERα) and c-fos immunostaining in ventral BNST show strong positive correlations with resident-intruder aggression [73]. In male California mice, pERK immunostaining in ventral BNST is also positively correlated with male aggressive behaviors (Villalon Landeros and Trainor unpublished data). Furthermore, treatment with KOR antagonists decrease male resident-intruder aggression in prairie voles [65]. Together these data suggest that activation of KOR increases activity of the ventral BNST (either directly or indirectly), possibly facilitating aggressive behavior. However, this hypothesis appears to be at odds with our observation that higher doses of U50,488 reduced social approach; a critical first step to engage in aggression. Different neuroendocrine mechanisms govern aggressive behavior in familiar and unfamiliar environments [26]. This suggests the possibility that the effects of KOR activation may differ in familiar versus unfamiliar environments. Indeed, the behavioral effects of the KOR agonist salvanorin A depend on whether the individual is in a familiar or novel environment [17]. It will be important to test whether there are sex differences in the effects of KOR in familiar versus unfamiliar environments.

Forced swim stress induces KOR-dependent activation of ERK [14], which has interesting implications for the relationship between stress and aggressive behavior. Interestingly, while defeat stress reliably reduces aggressive behavior [40,47], other forms of stress such as chronic mild stress can increase aggressive behavior [53]. This suggests that different forms of stress would have differential effects on KOR signaling networks and that defeat stress would suppress ERK phosphorylation in the BNSTvm. Dynorphin neurons in the CEA have been shown to control GABAergic signaling in the dorsolateral BNST via ERK [45]. It is not clear whether a similar pathway regulates GABAergic transmission in the ventral BNST where we observed U50,488 upregulation of pERK.

5. Conclusions

Our main finding was that sex differences in the behavioral effects of KOR were more substantial when long term outcomes were assessed while short term behavioral effects of KOR were more similar in males and females. This suggests that there may be stronger sex differences in β-arrestin mediated KOR signaling, which is considered to be the primary mechanism mediating long term effects of KOR. Recent studies in rodents have reported stronger effects of KOR agonists in males versus females, although these studies have focused primarily on short term behavioral effects. In addition to considering short term and long term effects of KOR signaling, it will be important to examine the behavioral effects of KOR agonists in females of different rodent species. Male and female California mice exhibit sex differences in brain anatomy [16], immune responses [37], and aggressive behavior [74] that are similar to domestic rats and mice. However sex differences in body size [83], parental behavior [5] and glucocorticoid levels [32] that are commonly observed in domestic rodents are less pronounced or absent in California mice. Observing the effects of KOR in males and females of different species will be necessary to determine whether social organization exerts a consistent effect on KOR action.

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