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The long-term effects of stress and kappa opioid receptor activation on conditioned place aversion in male and female California mice

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Abstract

Psychosocial stress leads to the activation of kappa opioid receptors (KORs), which induce dysphoria and facilitate depression-like behaviors. However, less is known about the long-term effects of stress and KORs in females. We examined the long-term effects of social defeat stress on the aversive properties of KOR activation in male and female California mice *(Peromyscus californicus)* using a conditioned place aversion paradigm. Female California mice naïve to social defeat, formed a place aversion following treatment with 2.5 mg/kg of the KOR agonist U50,488, but females exposed to defeat did not form a place aversion to this dose. This supports the finding by others that social defeat weakens the aversive properties of KOR agonists. In contrast, both control and stressed males formed an aversion to 10 mg/kg of U50,488. We also examined EGR1 immunoreactivity, an indirect marker of neuronal activity, in the nucleus accumbens (NAc) and found that stress and treatment with 10 mg/kg of U50,488 increased EGR1 immunoreactivity in the NAc core in females but reduced activation in males. The effects of stress and U50,488 on EGR1 were specific to the NAc, as we found no differences in the bed nucleus of the stria terminalis. In summary, our data indicate important sex differences in the long-term effects of stress and indicate the need for further study of the molecular mechanisms mediating the behavioral effects of KOR in both males and females.

Keywords

kappa opioid receptor; aversion; sex difference; nucleus accumbens; social defeat stress

1. Introduction

Psychosocial stress induces the activation of kappa opioid receptors (KORs) and is an important risk factor for depression [1]. KORs are considered to be a promising target for

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new therapeutics to treat depression and anxiety because activation of KORs by agonists induces dysphoria [2-4]. The administration of KOR antagonists before psychosocial stress blocks behavioral phenotypes such as social withdrawal [5], behavioral despair [6-8], anxiety [9], and drug seeking behaviors [10-13]. A common theme in studies examining the therapeutic effects of KOR antagonists is that the behavioral experiments are conducted within 24 hours or less after stress. However, psychosocial stress may induce important long-term neuroadaptations that affect KOR signaling.

Previous reports observed that the KOR agonist U50,488 decreases social interaction behavior [14] and that administration of the KOR antagonist norBNI before social defeat prevents decreases in social interaction [5]. However, stress may have different long term effects that alter the role of KORs in social behavior. In male mice that repeatedly lost aggressive interactions, U50,488 increased social interaction, but the same dose of U50,488 decreased social interaction in male mice with no experience in aggressive interactions [15, 16]. In these studies, aggressive contests occurred over a 10-day period, a much longer time frame than studies examining effects of KOR antagonists in the context of stress. Similarly, administration of the long lasting KOR antagonist JDTic before 10 days of social defeat stress did not prevent the development of decreased social interaction or anhedonia in male mice [17]. These data suggest that, over time, neuroadaptations induced by stress may alter the function of KORs.

Place conditioning studies provide a useful measure of the aversive properties of KOR agonists, and these studies suggest that long-term exposure to stress may alter the aversive properties of KORs. In male mice, short-term activation of KORs induces aversion [4, 18] and restores drug-seeking behavior in the reinstatement model of stress-induced drug relapse [11, 19-21]. For example, a conditioned place preference (CPP) for cocaine was induced in male mice and then extinguished [20]. In male mice naïve to stress, treatment with U50,488 reinstated the CPP for cocaine, and a single episode of acute forced swim stress before U50,488 treatment potentiated this effect. However, U50,488 did not reinstate cocaine CPP in mice exposed to either 5 days of social defeat stress (completed the day before reinstatement testing) or 3 weeks of chronic mild stress (completed 10 days before reinstatement testing) [20]. These data suggest that stress has different short- and long-term effects on KOR activation.

Another gap in the literature is that most research on KOR function has been conducted on males. However, depression and anxiety are more common in women than men [22] and sex differences in physiological responses to stress are an important risk factor [23-25]. Shershen et al. [26] showed that the KOR agonist U62,066 potentiated cocaine-induced locomotor activity in female mice during the first 20 minutes of testing, but this difference dissipated over the next hour. However, Wang et al. [27] reported that the KOR agonist U50,488 decreased cocaine-induced locomotor activity in female but not male guinea pigs across 90 minutes. It is possible that KOR agonists affect locomotor activity differently in mice compared to guinea pigs. In contrast, female rats were less sensitive than males to the depressive-like effects of KOR agonist U50,488 in an intracranial self-stimulation paradigm [28]. Finally, no sex differences were observed in the effects of KOR facilitation of selective aggression toward novel conspecifics in male and female prairie voles [29]. To our

knowledge, no previous study has examined the long-term effects of stress on the function of KORs in both males and females.

We studied *Peromyscus californicus* (California mouse), a monogamous species of rodent, in which both the male and the female are aggressive towards conspecifics [30]. This allowed us to observe the effects of social defeat stress in males, as well as females. In California mice, three episodes of social defeat induced social aversion in females but not males [25, 31, 32]. We used a place aversion assay to quantify the aversive properties of the KOR agonist U50,488 and to determine whether social defeat stress has different effects on aversion in males and females. Previously, males formed a place aversion to 10 mg/kg of U50,488, whereas females showed aversion to 2.5 mg/kg [14]. We hypothesized that long-term effects of defeat stress would reduce the aversive properties of U50,488. Here, we tested the effects of social defeat stress and two doses of U50,488 on conditioned place aversion (CPA) in males and females. We then examined how stress and U50,488 affected immediate early gene induction in nucleus accumbens (NAc) core and shell regions. The NAc is an important site of KOR action that is very sensitive to defeat stress [25] and also an important site mediating KOR-induced aversion [18]. Specifically, in male C57Bl6 mice optogenetic stimulation of the ventral NAc shell induced aversion, whereas stimulation of the dorsal NAc shell induced preference, as measured by real time conditioned place preference [18]. We quantified early growth response 1 (EGR1, also known as Zif268), an immediate early gene that, like c-fos, can be used as an indirect marker of neuronal activity. EGR1 protein activation is rapid and transient, peaking between 1-2 hours after neuronal activation [33]. We also examined EGR1 activity in several subregions of the bed nucleus of the stria terminalis (BNST) because of its strong connections with the mesolimbic dopamine system [34] and involvement in reward and aversion [35]. We hypothesized that increased EGR1 expression would be observed in the ventral NAc of animals that formed stronger U50,488-induced aversion.

2. Methods

2.1. Animals and housing conditions

Adult male and female California mice (3-4 months old) were obtained from our breeding colony at UC Davis. They were group housed with 2-3 same-sex animals per cage. Animals were maintained in a temperature-controlled room (68-74°F) on a 16L-8D cycle (lights off at 1400) with *ad libitum* water and food (Harlan Teklad 2016, Madison, WI). Mice were housed in Polycarbonate plastic cages containing Sanichip bedding, nestlets, and envirodri. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) and conformed to NIH guidelines. Social defeat stress was conducted during lights out (1400-1700) under dim red light (3 lux). Conditioned place aversion (CPA) testing was conducted during lights on (800-1400).

2.2. Social Defeat Stress

Male and female California mice were randomly assigned to social defeat stress or control handling for three consecutive days [32, 36]. Mice assigned to social defeat were placed in the home cage of an aggressive, same-sex, sexually-experienced resident mouse. The

experimental mouse remained in the resident's cage for either 7 min or 7 attacks, whichever occurred first. Control mice were introduced to a clean, empty cage for 7 min. Each experimental mouse was exposed to a different resident for each of the three episodes of defeat stress.

2.3. Place conditioning procedure

Two weeks after social defeat stress or control handling, conditioned place aversion (CPA) was conducted as described in Robles et al. [14] (Fig. 1). The apparatus consisted of three visually distinct interconnected standard sized mouse cages (28 × 17.5 × 11 cm). 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 and textured plastic floor). After each test the apparatus was cleaned with Quatricide (1:64, Quatricide PV in water, Pharmacal Research Labs, Inc). Clear polypropylene lids were used to cover the apparatus during experiments to allow for video recording.

Conditioned place aversion was conducted over 4 days during the light cycle. The protocol was designed so that the mice learn to associate vehicle or drug injection with a given chamber. On day 1 (pre-test) mice were placed in the apparatus and allowed to explore freely for 30 min while being tracked in real time with a visual tracking system (Any-maze Stoelting). For each mouse, initial place biases were corrected for by assigning drug conditioning to the preferred side chamber (Robles et al., 2014; McLaughlin et al., 2003).

Mice were randomly assigned to be conditioned with either vehicle (10% Tween 80 in sterile PBS), 2.5 mg/kg, or 10 mg/kg of (±)U50,488 (Tocris, Ellisville, MO, USA) administered i.p. On days 2 and 3, each mouse received two training sessions. In the morning, each mouse received an i.p. injection of vehicle and was placed in the unconditioned chamber for 30 min. Afterwards mice were returned to their home cages. Three hours later, each mouse received an injection of either vehicle, 2.5 mg/kg, or 10 mg/kg of U50,488 and was placed in the conditioned chamber for 30 min. During training sessions, the entrance to the center chamber was closed so mice were confined to a single chamber. On day 4, each mouse was given free access to the entire apparatus for 30 min (post-test) and was tracked with the video tracking system. The time spent in each cage and the total distance traveled were both recorded. On day 5 mice were injected i.p. with their assigned conditioned drug, and, 1 hour later, anesthetized with isoflurane and euthanized by decapitation. Brains were immediately collected and fixed in 5% acrolein in PBS overnight at 4°C. Brains were then transferred to 20% sucrose in PBS overnight at 4°C and then frozen and stored at −40°C.

2.4. Immunohistochemistry

Brains were sectioned on a cryostat at 40μm and stored in cryoprotectant (50% v/v phosphate buffer, 30% w/v sucrose, 1% w/v polyvinylprolidone, 30% v/v ethylene glycol) at −20°C. Every third section was processed for immunohistochemistry. Sections were washed three times in PBS followed by a 10-minute incubation in 1% sodium borohydride in PBS. Sections were then washed twice in PBS and endogenous peroxidases were quenched by submerging sections in H_2O_2 (0.3% in PBS) for 20 min. Sections were washed once in PBS and blocked in 10% normal goat serum (NGS) in PBS on an orbital shaker for 2 hours at

room temperature. Sections were washed once in PBS and then incubated in rabbit anti-EGR1 antibody (Cat. No. 4153S, Cell Signaling, 1:4000) diluted with 2% NGS in PBS with triton × (PBS-TX, 0.5%) for 24 h. Specificity of the EGR1 primary antibody for immunohistochemistry has been demonstrated with a blocking peptide [38]. On day 2 sections were washed three times in PBS and then incubated in biotinylated goat-anti-rabbit antibody (Vector Laboratories, Burlingame, CA, 1:500) in 2% NGS with PBS-TX 0.5% for 2 hours at room temperature. Sections were washed three times in PBS and incubated in avidin-biotin complex (ABC Elite Kit, Vector Laboratories) for 30 min. Sections were then washed three times in PBS and developed in nickel enhanced diaminobenzidine (Vector Laboratories) for 10 min. Sections were washed twice in PBS and mounted onto plus slides (Fisher, Pittsburgh, PA). Slides were dehydrated in ethanol followed by Histoclear (National Diagnostics, Atlanta, GA) and coverslipped with Permount (Fisher).

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 (Campi et al., 2013). The background for each image was normalized by adjusting the exposure time. The number of immunopositive cells in each brain region were counted using Image J (NIH, Bethesda, MD) by an observer unaware of treatment assignments. Different sized frames were used depending on the brain area so cell count data are presented as number of positive cells per mm^2 (Fig. 3).

2.5. Statistical Analysis

Time spent in the conditioned chamber during the pre-test and post-test was analyzed using repeated measures ANOVA testing for effects of test, sex, stress, and dose followed by paired t-tests to compare pre-test and post-test scores. For each animal and brain area, the EGR1 cell counts for the left and right sides of the brain were averaged. Due to heterogeneity of variance, EGR1 cell counts in the NAc were square root transformed. Cell counts were analyzed using a three-way ANOVA (sex, stress, and dose). Significant sex by stress interactions were examined further by analyzing males and females separately using a two-way ANOVA (stress and dose), and then planned comparisons were used to identify group differences when significant two-way interactions were detected.

Spearman correlations were used to correlate EGR1 counts in the NAc with aversion scores (time spent in conditioned chamber during the post-test minus time spent in the conditioned chamber during the pre-test) for females. The p-values for the correlation analyses were adjusted for multiple tests using the Benjamini-Hochberg approach with a false discovery rate (FDR) adjustment of 10%.

3. Results

3.1. Effects of U50,488 on place aversion formation

Repeated measures analysis showed that the effects of U50,488 on place aversion were different in male and female mice and that social defeat had important effects on the development of place aversion (Fig. 2; four-way interaction; $F_{2,110}=2.911$, $p=0.059$). Control females treated with 2.5 mg/kg U50,488 spent significantly less time in the conditioned cage

during the post-test compared to the pre-test (Fig. 2a; paired t-test; t_8=3.16, p=0.01). However, in stressed females, 2.5 mg/kg U50,488 did not induce place aversion in the conditioned cage (Fig. 2b). Neither control nor stressed males formed an aversion to 2.5 mg/kg of U5,488. Similar to our previous study [14], no evidence of place aversion was observed at the 10 mg/kg dose of U50,488 in either control or stressed females. These results suggest that defeat stress may reduce the aversive properties of U50,488 in females. There were no significant differences in distance traveled during either the pre-test or the post-test (all p's > 0.20).

Control males treated with 10 mg/kg U50,488 formed a place aversion in the conditioned chamber (Fig. 2c; paired t-test; t_{12}=2.27, p=0.046). Unlike females, stressed males also showed evidence of place aversion to 10 mg/kg U50,488 (Fig. 2d; paired t-test; t_8=2.30, p=.050). Similar to our previous studies [14], no evidence of place aversion was observed at the 2.5 mg/kg dose of U50,488 in males. For the most part, there were no differences in time spent in the unconditioned or center cages during the pre- and post-tests. One exception was that stressed males treated with 2.5 mg/kg U50,488 spent more time in the unconditioned chamber during the post-test (Table 1; paired t-test; t_8=2.82, p=.023).

As expected in males and control females, vehicle injections had no effect on time spent in the conditioned cage. However, in stressed females, vehicle injections unexpectedly reduced time in the conditioned cage during the post-test (Fig. 2b; paired t-test; t_9=2.84, p=0.019).

3.2. Effects of U50,488 on EGR1 immunoreactivity in the nucleus accumbens and the bed nucleus of the stria terminalis

In the nucleus accumbens (NAc) core, a three-way ANOVA analysis indicated that the effect of U50,488 on EGR1 immunoreactivity depended on sex and stress (Fig. 4; three-way interaction; $F_{1,75}$=7.86, p<.01). This effect was primarily driven by sex differences at the 10 mg/kg U50,488 dose. Stressed females had more EGR1-postive cells than control females at the 10 mg/kg U50,488 dose (Fig. 4A, p<.01), while stressed males had fewer EGR1-postive cells than control males treated with 10 mg/kg U50,488 (Fig. 4D, p<.02). Additionally, 10 mg/kg U50,488 increased EGR1-postive cells in control males compared to vehicle (p<.02). These results suggest that defeat stress has sex-specific effects on the NAc core that alter its sensitivity to U50,488.

In the NAc ventral shell, a three-way ANOVA also indicated that the effect of U50,488 on EGR1 immunoreactivity depended on sex and stress (Fig. 4; three-way interaction; $F1,_{75}$=4.17, p=.045). This interaction was driven primarily by sex differences at the 2.5 mg/kg dose. Control males treated with 2.5 mg/kg U50,488 had more EGR1 positive cells than stressed males treated with this dose (Fig. 4E, p<.01). In females there was a main effect of dose (Fig. 4B; main effect of dose; $F_{2,35}$=4.28, p=.022), which was driven by decreased EGR1 cell counts in females treated with 10 mg/kg U50,488. While there was no evidence for defeat stress to reduce EGR1 responses in females, defeat stress reduced the EGR1 response to the low dose of U50,488 in males.

Similarly, in the NAc dorsal shell, a three-way ANOVA indicated that the effect of U50,488 differed depended on sex and stress (Fig. 4C; three-way interaction; $F1,_{75}$=6.77, p<.01). In

general, EGR1-positive cells in the dorsal shell were more abundant in stressed females than control females (Fig. 4C; main effect of stress; $F_{1,35}$=11.78, p<.01). Significant increases in EGR-1 positive cells were observed in females treated with vehicle (p<.05) and 2.5 mg/kg U50,488 (p<.05), but not at the 10 mg/kg dose. No significant differences were observed in males. Overall, defeat stress appears to increase EGR1 responses in the dorsal shell of females but not males.

In the posterior ventral lateral BNST, females had higher EGR1 positive counts compared to males (Table 2; main effect of sex; $F1,_{76}$=4.25, p=.043). In the posterior dorsal lateral BNST, overall, stressed mice had higher EGR1 positive counts compared to controls (Table 2, main effect of stress; $F1,_{74}$=5.89, p=.018). We found no significant differences in the anterior BNST, posterior ventral medial BNST, or the posterior dorsal medial BNST. These results suggest that EGR1 responses to U50,488 are stronger in females than males in the ventral lateral BNST, while in the dorsal lateral BNST, defeat stress has more important effects on EGR1 than sex.

3.3. Correlations between place aversion behavior and EGR1 immunoreactivity

We correlated aversion score during CPA (time spent in conditioned chamber during post-test minus time spent in conditioned chamber during pre-test) with EGR1-positive cell counts. In the NAc ventral shell, control females treated with 2.5 mg/kg U50,488 showed a negative correlation between EGR1 and CPA score (Table 3, Fig. 5B, Spearman's ρ= -.943, FDR adjusted p=.045), whereas this correlation was not significant in stressed females. The only other significant correlation between CPA score and EGR1 cell counts was in the NAc core of vehicle treated control females (Table 3, Fig. 5A, Spearman's ρ= .964, FDR adjusted p<.001).

4. Discussion

Accumulating evidence suggests that stressors induce long-term changes in brain function that alter the aversive properties of KOR. Here we showed that a low dose of U50,488 that induced place aversion in unstressed females failed to induce aversion in females exposed to defeat stress. Unexpectedly, U50,488 maintained its ability to induce aversion in males exposed to defeat stress, albeit at a higher dose. These results suggest that there are important sex differences in how defeat stress impacts KOR function. We also showed that in females, stress increased EGR1 immunoreactivity in the NAc core but reduced activation in males and, that overall, stress increased activation in the dorsal shell of the NAc in females but not in males. In general, EGR-1 immunoreactivity did not closely track place aversion data, suggesting that different approaches are needed to identify neural circuits mediating KOR-induced aversion.

4.1. Effects of stress and U50,488 on CPA

An important finding of the place aversion study was that defeat stress blocked the ability of the 2.5 mg/kg dose of U50,488 to induce place aversion in female California mice. This aligns with previous studies showing that over the long-term, stress can alter KOR-mediated behaviors. In male mice that repeatedly lost aggressive interactions over a 10-day period,

U50,488 increased social interaction, but the same dose of U50,488 decreased social interaction in male mice with no experience in aggressive interactions [15, 16]. Similarly, Al-Hasani *et al.,* [20] found that male mice exposed to either three weeks of chronic mild stress or five days of social defeat stress did not exhibit U50,488-induced reinstatement for cocaine CPP. One possible mechanism for reduced sensitivity to KOR agonists following stress is desensitization, which can occur after repeated activation of KOR, either by stress or a KOR agonist. For example, repeated treatment with U50,488 leads to prolonged KOR phosphorylation, as well as analgesic tolerance in male mice [39]. Recovery of KOR function following desensitization can take several weeks [39, 40]. Currently it is unclear whether defeat or chronic mild stress induces KOR desensitization.

Our study identified sex differences in dose-response curves for U50,488, replicating results from a previous place aversion study on California mice [14]. In both studies, unstressed female California mice formed an aversion to 2.5 mg/kg but not 10 mg/kg U50,488, while unstressed males formed a place aversion to 10 mg/kg but not 2.5 mg/kg U50,488. Russell *et al.* [28] also reported that male rats were more sensitive than females to 10 mg/kg of U50,488 in an intracranial self-stimulation (ICSS) paradigm. However, males were also more sensitive to lower doses of U50,488 such as 2.5 mg/kg. It's not clear whether species differences (California mouse vs. rat) or different behavioral assays (CPA vs. ICSS) drive the different dose-response curves. Thus, while differences in the sensitivity of males and females to KOR agonists have been consistently reported, the direction of these differences is context dependent. One possible mechanism for context-dependent effects of defeat on KOR is allosteric activation of receptors. For the MOR, different signaling pathways can be activated by different exogenous or endogenous ligands [41]. Furthermore, allosteric modulators can change the affinity and/or efficacy of MOR ligands [42], as well as modify the second messenger systems used by MOR [43]. Interestingly, social defeat increased prodynorphin mRNA has stronger effects on mu opioid receptor (MOR) expression in male California mice than female California mice [44]. It is possible that context, experience, or sex may engage similar mechanisms to lead to sex differences in effects of KOR on behavior.

An unexpected finding in this study was that stressed females treated with vehicle spent less time in the conditioned chamber in the post-test compared to the pre-test. We hypothesize that vehicle injections led to release of endogenous dynorphin in stressed females, as exposure to repeated stress induces KOR activation [7], but further studies are needed to test this

4.2. Effects of stress and U50,488 on EGR1 activation

Stress and U50,488 affected EGR1 counts in the NAc but not the BNST. Although the BNST may be an important source of dynorphin and KOR [45], our results indicate that EGR1 is more responsive to KOR stimulation in the NAc than the BNST. We originally predicted that mice that exhibited a CPA to U50,488 would show increased EGR1 immunoreactivity in the ventral shell of the NAc and that mice that did not show aversion would show no change in EGR1 immunoreactivity [18]. In contrast, stressed females treated with vehicle or 2.5 mg/kg U50,488 had more EGR1 positive cells in the dorsal shell of the

NAc than controls. One factor that may have prevented EGR1 from closely tracking with aversion is that mice were in their home cages at the time the brains were collected. The context may be an important variable affecting how the NAc and BNST respond to U50,488.

One question raised by our results is how social defeat stress alters endogenous KOR ligands or KOR activity. In California mice, real-time PCR analysis of prodynorphin mRNA in the NAc showed no effects of social defeat, although mean levels were lower in stressed males and females [46]. In male C57Bl6 mice, a single episode of defeat increased prodynorphin mRNA in the NAc but 10 days of defeat reduced prodynorphin mRNA [17]. Interestingly, social defeat also decreased in prodynorphin mRNA in the medial preoptic area of sexually experienced male California mice (Kowalcyzk and Trainor, in prep.). These results suggest that effects of defeat on prodynorphin transcription are anatomically specific. It's also possible that effects of defeat on dynorphin protein expression could differ from mRNA. The translation of prodynorphin is complex; several splice variations allow for anatomically specific expression of the full-length peptide [47]. Visualization of dynorphin protein in nuclei such as the NAc is a challenge (L.M. Coolen personal communication), and typically requires the use of colchicine treatment to reduce axon transport of dynorphin from the cell body [48]. In contrast, sex differences in KOR binding have been reported in guinea pigs. Quantitative autoradiography for KORs showed that males and females differed in KOR binding in several brain regions; males exhibited increased binding in areas of the cortex, caudate putamen, claustrum, medial geniculate nucleus, and the cerebellum, while females exhibited increased binding in the dentate gyrus and hypothalamus [27]. However, there were no sex differences found in the NAc or the BNST [27]. Similarly, autoradiography data in male and female prairie voles indicated no sex differences in KOR levels in the core or shell of the NAc [49]. Interestingly, infusion of norBNI into the NAc core of prairie voles increased aggression in females but not males [29], which could indicate sex differences in the downstream effects of KORs in the NAc core. Going forward, autoradiography analyses of KOR binding in males and females exposed to defeat stress could provide insights into changes in receptor expression. Based on our behavioral results, we expect social defeat to reduce KOR binding to a greater extent in females compared to males.

Correlational analyses in the NAc suggested that defeat induced subtle changes in KOR function. In control females, the 2.5 mg/kg dose of U50,488 induced aversion and higher EGR1 cell counts in the ventral shell were correlated with stronger aversion scores. This relationship was absent in stressed females that did not form aversion to U50,488. This aligns with previous data indicating the ventral shell of the NAc is important in regulating aversion [18] and suggests the possibility that KORs in ventral shell may be desensitized following defeat. However, both control and stressed males formed an aversion to 10 mg/kg U50,488, but we did not see a correlation between aversion score and EGR1 counts in any subregion of the NAc. Overall, it is difficult to draw definitive conclusions relating EGR1 counts to aversion during CPA testing because we did not examine activation in the NAc during CPA testing. One possible way to address this question would be to tag neurons responding to U50,488 during CPA with H2B-GFP, a long lasting fluorescent protein [50]. For example, H2B-GFP driven by the c-fos promoter was used to tag hippocampal neurons during fear conditioning, enabling analysis and manipulation of these cells after learning

occurred [50]. Tagging neurons responding to U50,488 during CPA training with H2B-GFP would allow for quantification of aversion before histological analysis.

Our behavioral results in females are consistent with previous studies indicating that the aversive effects of KORs are weakened in animals exposed to stressors such as social defeat. Overall, these results indicate that there are important sex differences in the relationship between stress and KOR activation, which is important in light of evidence showing antidepressant effects of KOR antagonists in males. Continued studies on females and better tools for quantifying dynorphin, such as dynorphin reporter mice [18], are needed for future studies investigating the impact of stress on KOR function.

5. Conclusion

In summary, we observed that stress blocked the aversive effect of U50,488 in females but not males. These results highlight the importance of studying the long-term effects of stress in both males and females. Further investigation of sex differences in response to KOR activation is needed due to growing evidence that males and females respond differently to stress and KOR activation [14, 27, 28]. Effects of stress on the circuits mediating the behavioral effects of KORs [20, 51] could also impact the relationship between stress and the development of disorders such as depression [1]. A deeper understanding of the relationship between KORs, stress, and sex could aid in the development of new treatments for depression, addiction, and pain.

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Highlights

- Low dose of kappa opioid receptor agonist induced aversion in control, but not stressed, females
- High dose of kappa opioid receptor agonist induced aversion in control and stressed males
- Stress and kappa agonist increased EGR1 activity in the nucleus accumbens in females

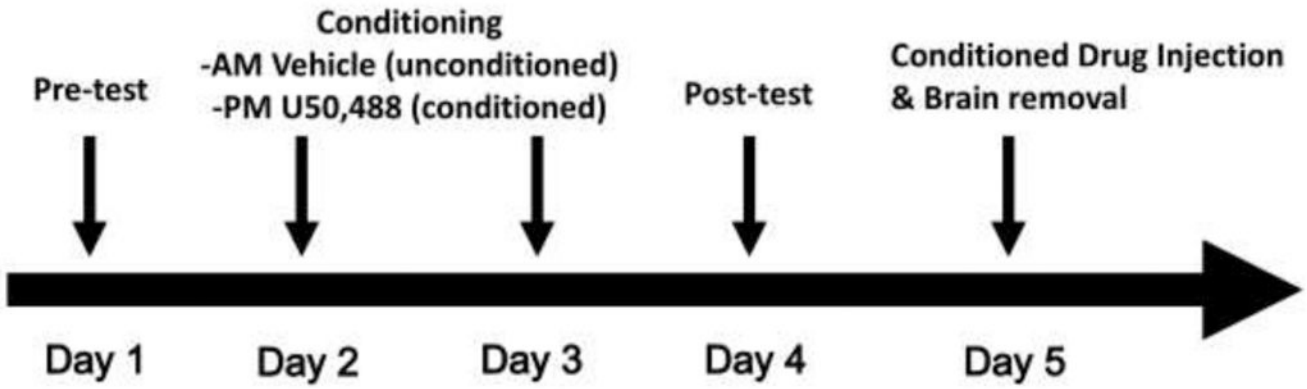

Figure 1.
Timeline for conditioned place aversion experiments.

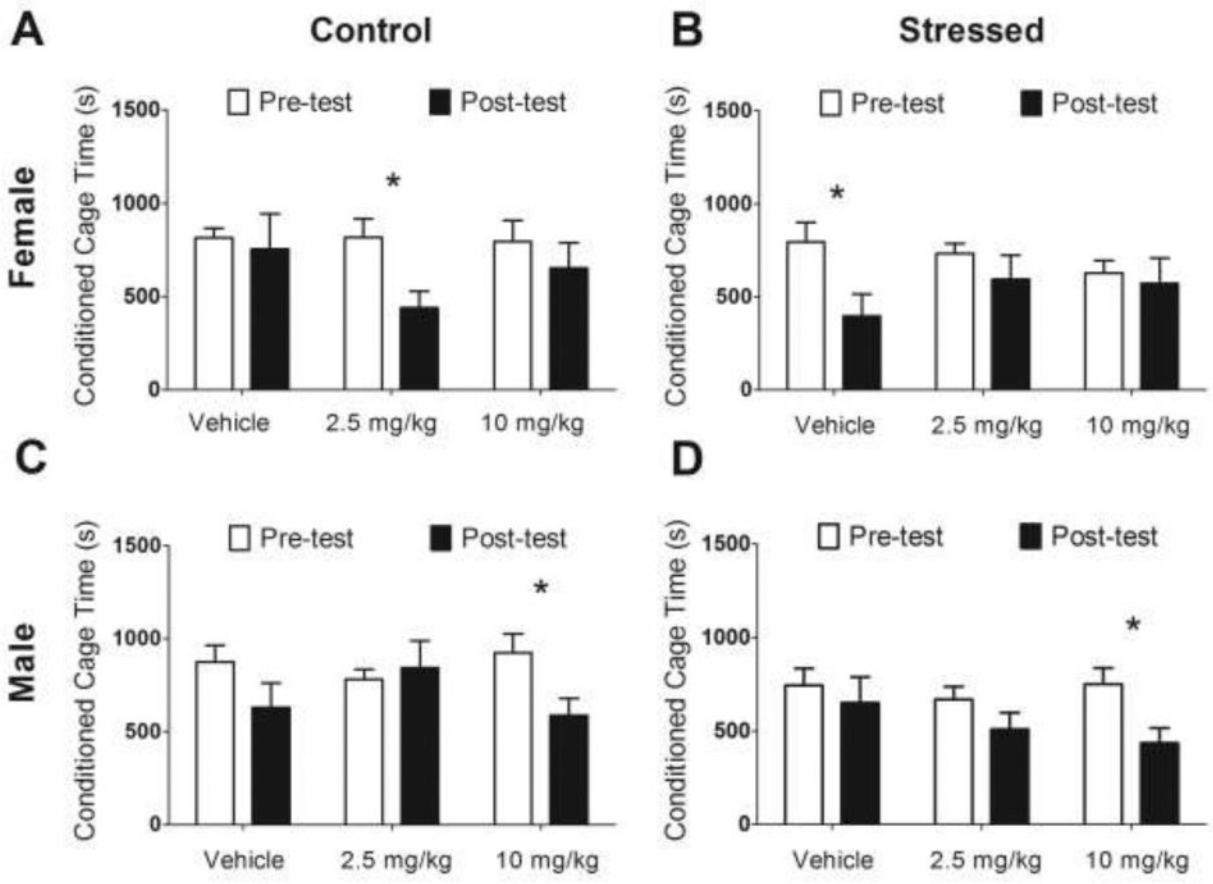

Figure 2.

Time spent in conditioned chamber during pre-test and post-test for (A) control females, (B) stressed females, (C) control males, and (D) stressed males. * $p < 0.05$, n=9-15 per group.

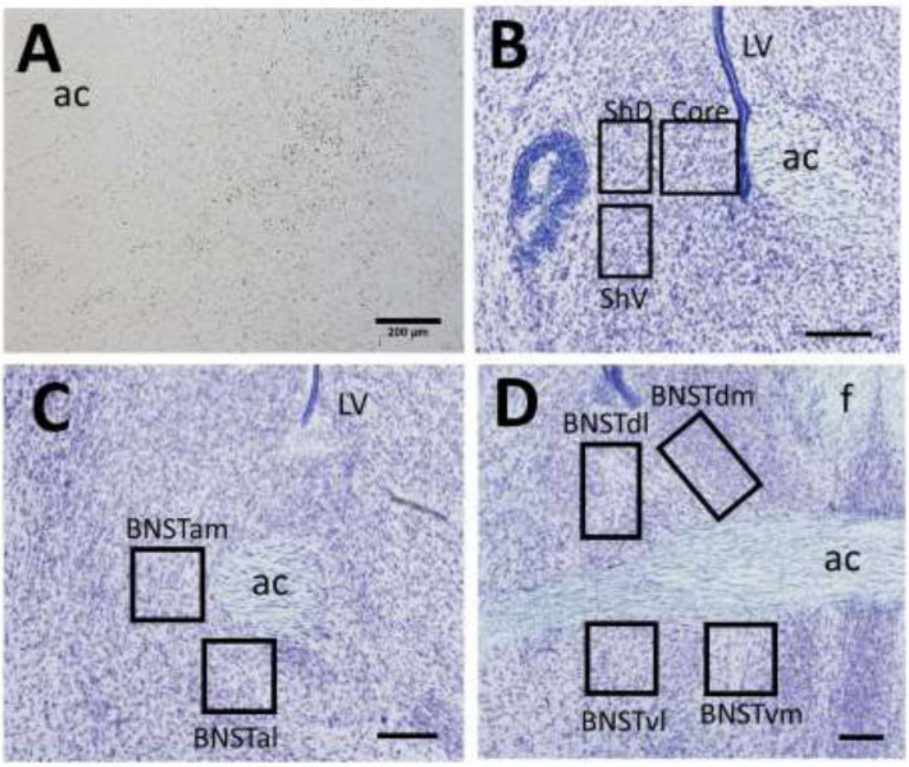

Figure 3.

(A) Representative photomicrograph of EGR1 staining in the NAc, (B) areas quantified in the NAc, (C) areas quantified in the anterior BNST, and (D) areas quantified in the posterior BNST. LV = lateral ventricle, ac = anterior commissure, core = NAc core, ShD = dorsal shell of NAc, ShV = ventral shell of NAc, BNSTam = anterior medial BNST, BNSTal = anterior lateral BNST, BNSTdl = dorsal lateral BNST, BNSTdm = dorsal medial BNST, BNSTvl = ventral lateral BNST, BNSTvm = ventral medial BNST, f = fornix. Scale bars = 200 μm.

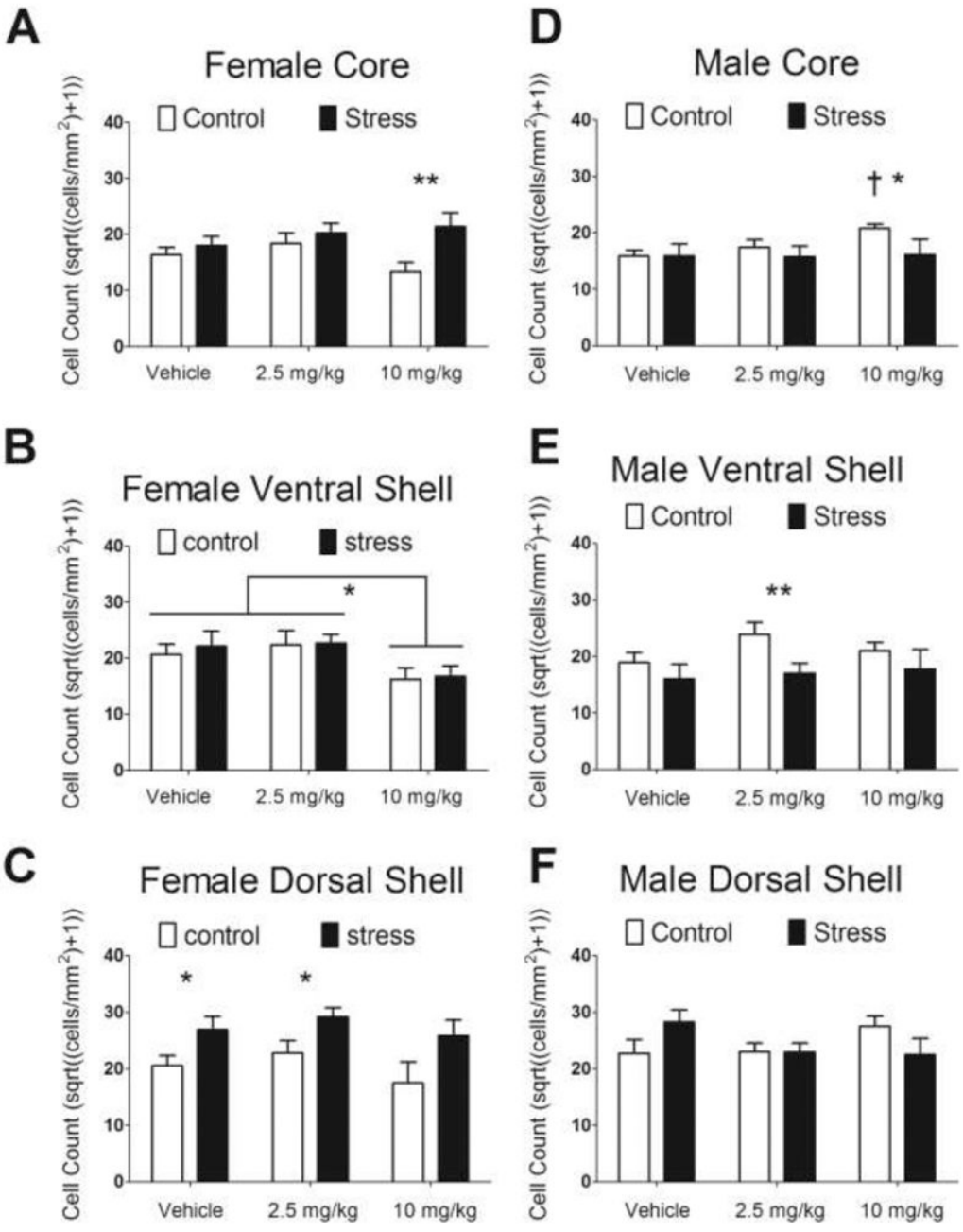

Figure 4.

Cell counts for EGR1 in the NAc. * $p < 0.05$, ** $p < 0.01$, † $p < 0.05$ vs. vehicle treated animals of same stress group, n=5-9 per group.

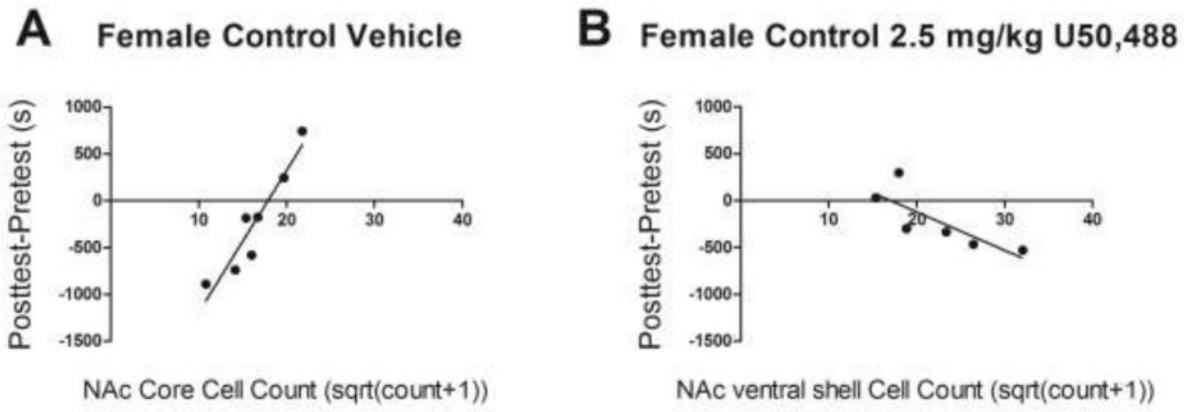

Figure 5.

Correlations between aversion score (post-test minus pre-test) and EGR1 counts in the NAc for (A) control females treated with vehicle (Spearman's ρ= .964, FDR adjusted p<.001), (B) control females treated with 2.5 mg/kg U50,488 (Spearman's ρ= -.943, FDR adjusted p=. 045), n=6-7 per group.

Table 1

		Female					
		Unconditioned			Center		
		Vehicle	2.5 mg/kg	10mg/kg	Vehicle	2.5 mg/kg	10mg/kg
Control	pre-test	500 ± 31	440 ± 72	456 ± 33	489 ± 63	545 ± 84	588 ± 53
	post-test	445 ± 141	592 ± 122	509 ± 91	602 ± 122	749 ± 115	640 ± 148
Stressed	pre-test	417 ± 59	422 ± 27	349 ± 48	587 ± 58	640 ± 66	815 ± 99
	post-test	763 ± 176	358 ± 50	396 ± 71	638 ± 123	842 ± 98	815 ± 153
		Male					
		Unconditioned			Center		
		Vehicle	2.5 mg/kg	10mg/kg	Vehicle	2.5 mg/kg	10mg/kg
Control	pre-test	432 ± 32	436 ± 43	372 ± 49	493 ± 75	583 ± 51	504 ± 66
	post-test	469 ± 107	293 ± 61	512 ± 133	700 ± 128	664 ± 111	700 ± 109
Stressed	pre-test	369 ± 67	402 ± 40	423 ± 33	694 ± 91	729 ± 84	627 ± 72
	post-test	331 ± 48	566 ± 75*	527 ± 153	813 ± 141	725 ± 80	838 ± 117

Time spent in unconditioned and center chambers during pre-test and post-test for control and stressed males and females. Data are shown as mean ± SEM.

* $p < 0.05$ vs. pre-test, n=9-15 per group.

Table 2

			Anterior BNST								
			Vehicle			2.5 mg/kg U50,488			10 mg/kg U50,488		
			Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
Anterior Medial	Male	Control	562.7	90.1	9	703.4	70.6	7	536.0	48.8	7
		Stress	500.8	66.7	8	532.7	48.4	9	736.0	170.8	7
	Female	Control	547.9	95.6	7	664.8	92.8	7	629.4	158.9	5
		Stress	707.8	92.0	6	651.5	79.4	5	581.0	95.3	10
Anterior Ventral	Male	Control	655.3	50.9	9	755.6	94.8	7	780.6	69.4	7
		Stress	721.1	64.5	8	766.5	59.6	9	669.2	57.6	7
	Female	Control	724.6	87.2	7	758.9	84.3	7	823.4	170.8	5
		Stress	827.0	45.4	6	672.8	34.1	5	842.1	72.7	10
			Posterior BNST								
			Vehicle			2.5 mg/kg U50,488			10 mg/kg U50,488		
			Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
Ventral lateral	Male	Control	659.1	73.7	9	799.1	83.3	7	677.3	104.7	7
		Stress	750.6	83.9	8	734.4	87.3	9	802.9	113.1	7
	Female	Control	721.9	57.7	7	837.8	79.3	6	901.1	155.1	5
		Stress	914.9	77.6	7	902.5	76.1	6	792.2	78.4	10
Ventral Medial	Male	Control	652.4	88.9	9	683.8	49.4	7	720.3	58.4	7
		Stress	734.4	92.1	8	684.9	64.7	9	652.9	79.3	7
	Female	Control	659.9	56.5	7	600.6	78.6	6	816.6	114.4	5
		Stress	645.2	87.6	7	713.5	45.4	6	734.4	72.6	10
Dorsal Lateral	Male	Control	666.7	74.8	9	653.5	65.0	7	626.3	73.2	6
		Stress	830.1	143.1	8	762.2	73.5	9	673.6	129.9	6
	Female	Control	663.9	75.4	7	552.2	87.2	6	493.1	136.5	5
		Stress	786.6	101.1	7	738.1	61.8	6	698.6	97.0	10
Dorsal Medial	Male	Control	783.8	96.1	9	703.9	81.0	7	738.7	100.9	6
		Stress	1000.2	170.8	8	781.8	145.8	9	578.1	123.3	6
	Female	Control	615.5	62.3	7	640.5	104.6	6	578.1	123.3	5

			Anterior BNST								
			Vehicle			2.5 mg/kg U50,488			10 mg/kg U50,488		
			Mean	SEM	n	Mean	SEM	n	Mean	SEM	n
		Stress	747.2	85.3	7	853.3	83.6	6	801.9	94.9	10

EGR1 counts in the anterior and posterior subregions of the BNST.

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Table 3

Sex	Stress	Dose U50,488	NAc Subregion	Spearman's correlation	p-value	Benjamini-Hochberg P-value
Female	Control	Vehicle	Core	0.964***	<.001	<.001
Female	Stressed	Vehicle	Core	-0.029	0.957	1
Female	Control	Vehicle	Ventral Shell	0.679	0.094	0.309
Female	Stressed	Vehicle	Ventral Shell	0.029	0.957	1.000
Female	Control	Vehicle	Dorsal Shell	0.643	0.119	0.309
Female	Stressed	Vehicle	Dorsal Shell	-0.086	0.872	1
Female	Control	2.5 mg/kg	Core	-0.886	0.019	0.114
Female	Stressed	2.5 mg/kg	Core	0	1	1
Female	Control	2.5 mg/kg	Ventral Shell	-0.943*	0.005	0.045
Female	Stressed	2.5 mg/kg	Ventral Shell	-0.536	0.215	0.479
Female	Control	2.5 mg/kg	Dorsal Shell	-0.829	0.042	0.189
Female	Stressed	2.5 mg/kg	Dorsal Shell	-0.107	0.819	1
Male	Control	10 mg/kg	Core	-0.452	0.260	0.479
Male	Stressed	10 mg/kg	Core	0.543	0.266	0.479
Male	Control	10 mg/kg	Ventral Shell	0.310	0.456	0.631
Male	Stressed	10 mg/kg	Ventral Shell	0.486	0.329	0.538
Male	Control	10 mg/kg	Dorsal Shell	0.595	0.120	0.309
Male	Stressed	10 mg/kg	Dorsal Shell	0.429	0.397	0.596

Correlations between aversion score (post-test minus pre-test) and EGR1 counts in the NAc.

*$p < 0.05$,

***$p < 0.001$, n=6-8 per group.