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## Activation of Kappa Opioid Receptors in the Dorsal Raphe Have Sex Dependent Effects on Social Behavior in California Mice

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### Abstract

Kappa opioid receptor activation has been linked to stress and anxiety behavior, thus leading to kappa antagonists being popularized in research as potential anxiolytics. However, while these findings may hold true in standard models, the neuromodulatory effects of social defeat may change the behavioral outcome of kappa opioid receptor activation. Previous research has shown that social defeat can lead to hyperactivity of serotonergic neurons in the dorsal raphe nucleus, and that inhibition of this increase blocks the social deficits caused by defeat. Kappa opioid receptor activation in the dorsal raphe nucleus works to decrease serotonergic activity. We injected the kappa opioid receptor U50,488 directly into the dorsal raphe nucleus of male and female, defeat and control adult California mice. Here we show evidence that U50,488 induces anxiety behavior in control male California mice, but helps relieve it in defeated males. Consistent with previous literature, we find little effect in females adding evidence that there are marked and important sex differences in the kappa opioid system.

### Keywords

Kappa opioid receptor; dorsal raphe nucleus; social defeat; U50; 488; California mouse

### 1. Introduction

Psychological stress is a major risk factor of depression. The kappa opioid receptor (KOR) is a class of opioid receptor that is activated during stress by the endogenous opioid dynorphin [1]. Acute KOR activation has been found to contribute depression- and anxiety-like behaviors induced by stress [2], and has also been found to contribute to pro-addictive behavior [3]. Social stressors in particular appear to induce robust activation of the kappa

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opioid system [4, 5]. There is growing evidence that activation of KOR during stress contributes to long term increases in depression- and anxiety-like behaviors that persist long after stressors themselves have ended. For example, KOR antagonists administered immediately before episodes of social defeat block stress-induced social avoidance (Williams et al., in review) whereas KOR antagonists administered after social defeat are ineffective at reducing social avoidance [6]. It also appears that social stress has different short and long term effects on the KOR system. While acute social defeat increases expression of dynorphin in the nucleus accumbens, chronic social defeat decreases dynorphin expression [7]. This finding is consistent with studies showing that over the long term, stress exposure reduces the aversive properties of KOR [8–11]. Together, these results suggest that stress induces neuroadaptations that alter how KOR modulate behavior. The dorsal raphe nucleus may be one region showing important changes [12].

Evidence from several sources indicates that the DRN is highly responsive to social stress [13]. In male hamsters social defeat increased c-fos mRNA in the DRN [14]. Immunohistochemistry approaches show that either a single or repeated episodes of social defeat can induce c-fos immunoreactivity specifically within serotonergic neurons of the DRN of male rats [15, 16]. In male hamsters this effect was limited to more rostral and ventral subdivisions of the DRN [17]. Consistent with these data, electrical recordings of DRN neurons of male tree shrews showed very large increases in activity during exposure to an aggressive opponent [18]. Increased activity of serotonergic neurons during episodes of defeat is associated with increased concentrations of extracellular serotonin in both the DRN [19] and hippocampus [20]. Multiple episodes of defeat have been linked with increased serotonin in amygdala and hypothalamus [21] and decreased expression of inhibitory 5-HT<sub>1A</sub> autoreceptors in DRN [22], PFC [23], and desensitizes receptors in the cortex [24]; all of which may reflect hyperactivity of DRN serotonin neurons. Overall, these results suggest that defeat stress induces both acute and chronic activation of DRN serotonergic neurons, with chronic activation leading to greater neuromodulation throughout the brain.

However, studies examining the role of KOR have reported a different connection between stress and serotonergic function. Inhibition of KOR in the DRN blocked defeat-induced reinstatement of cocaine place-preference [25], while deletion of p38 $\alpha$  MAP kinase (the putative mechanism of KOR-induced aversion) in DRN serotonin neurons blocked social avoidance immediately after a single episode of social defeat [26]. However, when the KOR agonist U69,493 was applied to DRN slices from stress naïve male mice, the excitability of serotonergic neurons was reduced [27]. This is somewhat surprising because most studies indicate that defeat increases the activity of serotonin neurons. Interestingly, KOR-induced inhibition of excitability in serotonergic neurons was reduced in slices from male mice exposed to repeated swim stress. This finding is in agreement with the hypothesis that repeated stress exposure alters the effects of KOR on neural circuits and behavior.

There is a major gap in our understanding of KOR modulation of behavior because the vast majority of studies have been conducted using male rodents, and there is growing evidence for important sex differences in KOR function [28, 29]. Here, we used the California mouse (*Peromyscus californicus*) model of social defeat to test whether social defeat modulates the behavioral effects of KOR within the DRN in males and females. The California mouse is a

monogamous species in which both sexes are aggressive, which allows for the study of social defeat in males and females [30]. In a novel environment, three episodes of social defeat induces social avoidance in females [31, 32] and in males that receive an acute injection immediately before behavior testing [33, 34]. When behavior is assessed in a familiar home cage, social defeat reduces investigation of social odors [35], decreases sucrose consumption [36], and increases freezing in response to an intruder [37] in both males and females. Sex differences in behavioral responses to KOR ligands have been observed in California mice. In a place aversion assay, females form place aversions to a lower dose of the KOR agonist U50,488 than males [38, 39]. However, in a forced swim test norBNI reduced immobility in males but not females [40], consistent with other studies suggesting that males may be more sensitive KOR than females [41, 42]. Previous work showed that activation of KOR within the DRN induced anxiety- and depression-like behavior, so we expected that similar responses in stress naïve mice. However, in light of evidence that defeat increases baseline activity of DRN serotonin neurons, we hypothesized that activation of KOR in stressed mice could yield very different behavioral effects.

## 2. Methods and Materials

### 2.1 Animals and housing

Male and female California mice were from the UC Davis breeding colony and began testing between 60–180 days old. Originally, mice were housed 2 or 3 to a cage with same-sex cage mates of comparative age. After the cannula placement surgery, animals were singly housed and remained thus for the duration of the experiment. All housing cages were clear polypropylene cages with Sanichip bedding and were enriched with nestlets and enviro-dri. Room temperature was maintained between 68–74 °F, with a 16L:8D light:dark cycle (lights off at 1400). A longer photoperiod was chosen as short ones have been linked with increased aggression [43]. Water and food (Harlan Teklad 2016; Harlan, Hayward, CA, USA) were provided *ad libitum*. All behavior testing were conducted during lights out (1430–1730) under dim red light (3 lux). All procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

### 2.2 DRN Coordinate Determination

To determine stereotaxic coordinates for the DRN in California mice (Fig. 1A), we used immunohistochemistry to create a map tryptophan hydroxylase (TPH) staining in the brainstem (Fig. 1B). Brains from two male mice were immersion fixed with acrolein and cut on a cryostat at 40  $\mu$ m and stored in cryoprotectant (50% v/v phosphate buffer, 30% w/v sucrose, 1% w/v polyvinylpyrrolidone, 30% v/v ethylene glycol) at 20 °C. Sections were then washed three times in phosphate buffered saline (PBS) and incubated in 1% sodium borohydride in PBS for 10 min. Sections were then blocked in 10% normal rabbit serum and 0.3% hydrogen peroxide in PBS for 20 min. Sections were then incubated in primary anti-TPH antibody (Sigma T8575-1VL) dissolved in 2% normal rabbit serum and 0.5% triton X (TX) in PBS overnight at 4 °C on an orbital shaker. The sections were then washed three times in PBS before transferring to biotinylated rabbit anti-sheep antibody in 2% normal rabbit serum in PBS TX (Vector Laboratories, Burlingame, CA, USA, 1:500) for 2 h.

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 2 min. Sections were then rinsed in PBS and mounted onto plus slides (Fisher, Pittsburgh, PA, USA). Slides were dehydrated in ethanol followed by HistoClear (National Diagnostics, Atlanta, GA, USA) and coverslipped with Permount (Fisher). Images were taken via microscope with a color camera at 4x magnification.

Next, we made fluoro-ruby injections via Hamilton syringe under 3–5% isoflurane anesthesia to determine the correct stereotaxic coordinates to hit the region. To avoid the central sinus, the mouse head was raised from a flat position at –4mm to +1.5mm via the mouth grip. The Hamilton syringe was inserted at a 10-degree angle perpendicular to the midline to allow for an approach from a more lateral direction and still reach medial DRN. Based on these injections we determined the target coordinates of lambda +1.3mm, –1.2mm lateral to midline, angled 10-degree to reach the DRN coordinates of bregma –5.04mm, ventral –4.3mm.

### 2.3 Social Defeat

Male and female California mice were randomly assigned to either social defeat stress or to remain naïve to defeat. All social defeat was done during the dark cycle when mice are naturally active. Each mouse assigned to social defeat was placed in the cage of an aggressive same-sex mouse resident mouse. Each episode lasted 7 min or until the resident attacked the focal mouse 7 times (whichever occurred first), and this was repeated on 3 consecutive days with three different residents. Naïve mice were introduced into a clean cage for 7 min, again for 3 consecutive days. Immediately after defeat/control conditions mice were returned to their home cage.

### 2.4 Cannula Placement Surgery

Animals were anesthetized with 3–5% isoflurane and implanted with a stainless steel cannula guide. A single cannula guide (Plastics One; C315G/SPC, Length 4.2mm) was lowered into a burr hole (#105 dremel bit, 1/16' tip) at a 10-degree angle at +1.3mm rostral to lambda, and –1.2mm lateral to the midline. The guide was attached to the skull with skull screws (plastics one, 00-96 X 1/16) and acrylic dental cement. A dummy cap (Plastics One; C315DC/1/SPC, Length 4.2mm) was inserted into the guide and kept there except during treatment administration. Mice received subcutaneous injections of Carprofen (5mg/kg) immediately before surgery and for three days following. Mice were given five days of recovery during which they were monitored and handled daily.

### 2.5 Drug Administration

Mice were randomly assigned to receive infusions of either aCSF, 0.25µg of U50,488 (Tocris), or 0.50µg of U50,488 were made into the DRN were made using internal cannula (Plastics One, C315I/SPC, Length 5.2mm). Injections were made 30 minutes after the start of the dark cycle using Hamilton syringes attached to an automatic micropump (PHD 2000, Harvard Apparatus, Cambridge, MA) at a flow rate of 0.1 µL/minute, for a volume of 200nL. The internal cannula was kept in place for an additional 30 seconds before it was

removed and the dummy cap replaced. Test animals were then returned to their home cage for 30 min before testing. We used an angled injection approach to avoid the adjacent cerebral aqueduct. However, it's still possible that some drug may have diffused in to the aqueduct. We believe the possibility of off-target effects of U50,488 is low because the doses of U50,488 we used are relatively low [44] and we did not see evidence for behavioral effects of U50,488 in mice with misplaced injection sites. A previous study examining behavioral effects of corticotropin releasing hormone in the DRN showed that a 20-fold higher dose infused in to the ventricle was required to see behavioral effects similar to those of local DRN infusion [45].

## 2.6 Social Interaction Test

Social interaction testing occurred in a large (89 × 63 × 60 cm) arena and all behaviors were recorded via animal tracking software (Anymaze, Stoelting). The first phase of the social interaction test was a three-minute open field test. During this portion, distance traveled and time spent in the center of the apparatus were recorded to measure the extent to which the test animal exhibited anxiety-like behavior. After the completion of the open field phase, an empty mesh wire cage was placed at one end of the arena. This marked the beginning of the three-minute acclimation phase. During the time, time spent within 8cm of the cage (interaction zone) and times spent in corners opposite to the cage were recorded. Finally, a novel intact same-sex conspecific was placed in an identical wire-mesh cage in the same location as the previous one had been during the acclimation phase. This marked the start of a three-minute interaction phase. During this time, the number of seconds the test animal spent in the interaction zone and time spent in opposite corners was also recorded. After the completion of the interaction phase, the test animal was promptly returned to the home cage in the usual home setting.

## 2.7 Resident Intruder Test

Resident-intruder testing was performed and scored as previously described [46, 47]. Before testing mice were moved to the testing room to habituate for 10 minutes. Next an unfamiliar same-sex intruder was placed inside the cage with the test mouse. Interactions were recorded for 7 minutes and later scored for behavior. Behavior was scored by an observer without knowledge of treatment group. Two anxiety behaviors freezing and escaping were recorded. Freezing was coded when the resident stood still on four paws and didn't move for >2 seconds. Escaping was recorded when the resident kept to the side of the cage and jumped side to side. Chasing, an aggressive behavior, was recorded and defined as the resident mouse chasing the intruder. Finally, anogenital sniffing, a social investigatory behavior, was coded when the resident's nose was in proximity to the anogenital region of the intruder.

## 2.8 Perfusions and Histology

20 hours after the completion the resident intruder test animals were perfused with 4% paraformaldehyde and brains removed. Histology we performed to determine whether injections were correctly placed in to the DRN (Fig. 1C). Behavioral data from mice that did not have injection sites within the dorsal raphe were omitted from statistical analysis.

## 2.9 Data Analysis

Social interaction and resident intruder data were analyzed with two-way ANOVA followed by planned comparisons testing for the effect of treatment or defeat using R. Homogeneity of variance was confirmed with a Fligner-Killeen test. This test indicated that freezing and chasing data had heterogeneous variance, so these data were log transformed. Planned comparisons (independent contrasts) were used to compare U50,488 treated mice with aCSF controls as well as the effect of defeat stress within each dose [48].

## 3. Results

### 3.1 Resident Intruder

In males, there was a significant interaction between treatment and defeat on freezing behavior (Fig. 2B,  $F_{2,25} = 3.7$ ,  $p < 0.05$ ). Planned comparisons showed that social defeat increased freezing behavior in mice treated with aCSF compared to controls ( $p < 0.01$ ,  $d = 1.92$ ). In contrast, stressed males treated with 0.50 $\mu$ g U50,488 had lower levels of freezing than stressed males treated with aCSF ( $p < 0.01$ ,  $d = 2.00$ ). Interestingly, U50,488 appeared to have opposite effects on freezing in control and stressed males at the 0.25 $\mu$ g dose. Although differences were nonsignificant, effect sizes were large. In control males treated with 0.25 $\mu$ g U50,488 mean freezing was increased ( $p = 0.07$ ,  $d = 1.24$ ) but in stressed males this same dose was associated with decreased freezing ( $p = 0.05$ ,  $d = 1.19$ ) relative to aCSF treated mice. Effects of U50,488 were weaker on escaping behavior (Fig. 2C, dose x stress  $F_{2,24} = 2.7$ ,  $p = 0.08$ ). Planned comparisons showed a nonsignificant increase (with large effect size) in escape behavior in control males treated with 0.50 $\mu$ g U50,488 ( $p = 0.07$ ,  $d = 1.23$ ). There was also evidence that effects of U50,488 on anogenital sniffing were different in control and stressed males (Fig 2E, dose x stress,  $F_{2,24} = 2.7$ ,  $p = 0.08$ ). Planned comparisons showed that stressed males treated with aCSF engaged in significantly less anogenital sniffing than controls ( $p < 0.03$ ,  $d = 1.16$ ). Interestingly, stressed males treated with 0.5 $\mu$ g of U50,488 showed a non-significant increase in anogenital sniffing (with large effect size) compared to stressed males treated with aCSF ( $p = 0.08$ ,  $d = 1.14$ ). There were no significant differences in chasing, boxing or biting.

In females, there were no significant differences in freezing, escaping, chasing, or boxing. There was a nonsignificant main effect of defeat on biting across all three drug groups (Table 1,  $F_{1,30} = 3.6$ ,  $p = 0.06$ ), but planned comparisons yielded no differences. There was a weak effect of U50,488 on anogenital sniffing treatment (Fig 3E,  $F_{2,29} = 2.6$ ,  $p = 0.09$ ). Planned comparisons showed that 0.5 $\mu$ g of U50,488 reduced anogenital sniffing in control females ( $p < 0.05$ ,  $d = 1.28$ ).

### 3.2 Social Interaction

In males, there was a significant main effect of defeat on time spent in the interaction zone during the interaction phase of the test (Fig. 4B,  $F_{1,29} = 4.2$ ,  $p < 0.05$ ). Although there was no significant dose x defeat interaction ( $p > 0.5$ ), planned comparisons revealed a decrease in social interaction significant only at the 0.25 $\mu$ g dose ( $p < 0.05$ ,  $d = 1.33$ ). There were no differences in the acclimation phase (Fig. 4C). In males, there was evidence for an effect of defeat on total distance traveled during the open field phase (Fig. 4E  $F_{1,30} = 4.3$ ,  $p < 0.05$ ).

Planned comparisons showed that at the 0.50 $\mu$ g U50,488, stressed mice traveled less than control mice ( $p < 0.02$ ,  $d = 0.45$ ) but this difference was not significant for the 0.25 $\mu$ g dose or aCSF. All other behaviors showed no significant differences.

In females, surprisingly, there were no differences during the interaction phase in time spent in the interaction zone or in the corners opposite the interaction zone. There were also no differences in behavior during the acclimation phase. During the open field phase, there was a main effect of dose on time spent in the open field section (Fig. 5C,  $F_{2, 32} = 3.8$ ,  $p < 0.02$ ). Planned comparisons showed that control females treated with 0.50 $\mu$ g U50,488 spent less time in the center than control females treated with aCSF ( $p < 0.05$ ,  $d = 2.15$ ).

#### 4. Discussion

Here we found that KOR activation induced anxiety-like behaviors in control males, while having the opposite effect in stressed males. In stressed males, a higher dose of U50,488 was required to affect behavior compared to control males, suggesting that stress may have desensitized KOR. Finally, effects of U50,488 were weaker in females, consistent with previous reports that acute effects of KOR are stronger in males than females. Together, these results indicate that within the DRN, the effects of KOR on behavior and sex- and experience-dependent.

Social defeat increased freezing and reduced levels of anogenital sniffing in aCSF treated males. The low dose (0.25 $\mu$ g) of U50,488 also increased freezing behavior in the resident intruder test and decreased anogenital sniffing (a social investigatory behavior) in control males while escape behavior was increased by the high dose (0.50 $\mu$ g). These data are consistent with previous studies indicating that KOR activation mediates defeat-induced freezing behavior [4] and acute social avoidance [49]. Freezing behavior in control males was not affected by 0.5  $\mu$ g of U50,488, consistent with previous reports of U-shaped curves in place aversion studies [38, 50]. For stressed males treated with the high dose of U50,488, freezing was reduced and anogenital sniffing increased. Thus, in the resident-intruder test, U50,488 had largely opposite effects on behavior in control and stressed males. U50,488 reduces the excitability of DRN serotonin neuron [5, 51], which may contribute to the anxiolytic effects we observed in stressed males. The activation of KOR receptors in stressed males may restore the activity of DRN serotonin neurons to a baseline rate. Male rats exposed to uncontrollable stress had more reactive DRN serotonergic neurons when confronted with an acute challenge [52], an effect that is likely mediated by a stress-induced reductions in inhibitory 5HT<sub>1A</sub> autoreceptors [22, 23]. Stress induced changes in the distribution of CRH receptors in the DRN may also facilitate increased reactivity of DRN serotonin neurons. Some male rats take a more proactive strategy when faced with aggressive resident [53], resisting social defeat with defensive postures. This proactive phenotype is associated with increased membrane expression of the type 2 CRH receptor [54], which enhances the excitability of DRN serotonin neurons [55]. Previous work in mice and other species have linked proactive coping strategies with increased aggression and perseverative behavior [56], both of which are observed in male California mice exposed to social defeat [37, 57]. The behavioral effects of CRH receptor activation in the amygdala



have been found to be KOR-dependent, but less clear how these signaling pathways interact in the DNR [58].

While effects of U50,488 were relatively strong in males, females showed a decreased sensitivity to U50,488 infusions. Previous studies have also found that the acute effects of KOR agonists are stronger in males than females [28, 29, 59]. This suggests that there could be important sex differences in neural circuits mediating KOR action. In guinea pigs U50,488 induced freezing behavior to a greater extent in males than females [60]. Sex differences in freezing and escape behavior have been observed in the learned helplessness model, in which rats are first exposed to inescapable shocks and later given an opportunity to escape from shocks. In male rodents, prior exposure to inescapable shock reduces the probability of avoiding escapable shock, and this effect is mediated in part by activation of the dorsal raphe [61]. Interestingly, female rats are much less likely to develop this learned helplessness [62]. This is consistent with other evidence showing greater reactivity of the DRN to stress in males than females [63]. These results are thus consistent with our finding of stronger effects of KOR activation in the DRN of males versus females. A somewhat surprising result was that we observed no effect of defeat on social interaction behavior in females treated with aCSF. Defeat-induced social avoidance in females has been one of the most consistent phenotypes in the California mouse model of defeat [35, 64, 65]. Although stressed male California mice typically do not exhibit social avoidance, in two studies stressed males that receive injections administered 30 minutes before testing did exhibit social avoidance [33, 34]. Here, stressed females treated with aCSF showed high levels of social interaction, suggesting that the infusion itself was not overly stressful. In addition, 0.50 $\mu$ g of U50,488 induced an anxiety-like response in the open-field test and reduced anogenital sniffing, which can both be interpreted as anxiogenic responses. However, the lack of the expected social withdrawal after defeat does shed some caution as to the ability to generalize defeated female data. The stark contrast between male and female response to KOR manipulation in this study highlights the sex dependent differences in the kappa opioid system and emphasizes the importance of investigating both sexes.

Another interesting facet of the results was that U50,488 had stronger effects in the resident-intruder test versus the social interaction test. A major difference between these tests is the familiarity of the environment with the resident-intruder test occurring in familiar home cage versus the social interaction test occurring in a novel environment. Previous work in California mice demonstrates that behavior can be remarkably different in familiar versus novel environments [66, 67]. The DRN is also highly sensitive to the environment. For example, male rats that received DRN lesions reduced social behavior in a resident-intruder test but not in the novel context of a social interaction test [68]. Our results suggest that KOR modulation of the DRN has stronger effects on social behavior in familiar environments versus novel environments, at least in males.

The serotonergic system is well known for its modulation of aggressive behavior. Lesions of the DRN and inhibition of serotonin synthesis have both been found to increase instigation of aggression by residents in resident-intruder tests but not increase the instigation of aggression by intruders [69, 70]. Overall, interventions that decrease serotonin output are usually associated with increased aggression [71]. While activation of KOR has been linked

to increased aggression, this effect was observed in the nucleus accumbens and in a specific context of pair bonding [72]. We did not see changes in offensive aggression levels (biting and upright posture) in either males or females treated with U50,488 in the DRN. This suggests that the effects of U50,488 were not driven by functional changes in aggression circuits. Finally, defeat may have altered KOR expression. Social defeat has been found to decrease KOR mRNA within the nucleus accumbens of male C57B16/J [73] and increase KOR mRNA within the medial preoptic area of male California mice [74]. To our knowledge, the effects of social defeat on KOR expression have not been examined.

## 5. Conclusions

In conclusion, we found that the effects of KOR activation in the DRN were context- and sex-specific. In males, lower doses of U50,488 induced anxiety-like responses in control males while in stressed males given higher doses of U50,488 had anxiolytic responses. These results fit with data showing that social defeat decreases 5HT<sub>1A</sub> receptor negative feedback on serotonergic neurons. It may be the case that engaging KOR in stressed males generates an alternative source of negative feedback independent of 5HT<sub>1A</sub>. Overall, effects of U50,488 infusions were weaker in females, consistent with previous reports of greater sensitivity to KOR in males. Interestingly, the strongest behavioral effects of U50,488 infusions were found when behavior testing took place in a familiar environment. Overall the effects of KOR activation were experience-, sex-, and context-specific. This complexity of regulation needs to be considered when evaluating the therapeutic potential of KOR dependent pharmaceuticals.

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## Abbreviations

<b>aCSF</b>	artificial cerebrospinal fluid
<b>DRN</b>	dorsal raphe nucleus
<b>KOR</b>	kappa opioid receptor
<b>TPH</b>	tryptophan hydroxylase
<b>PBS</b>	phosphate buffered saline

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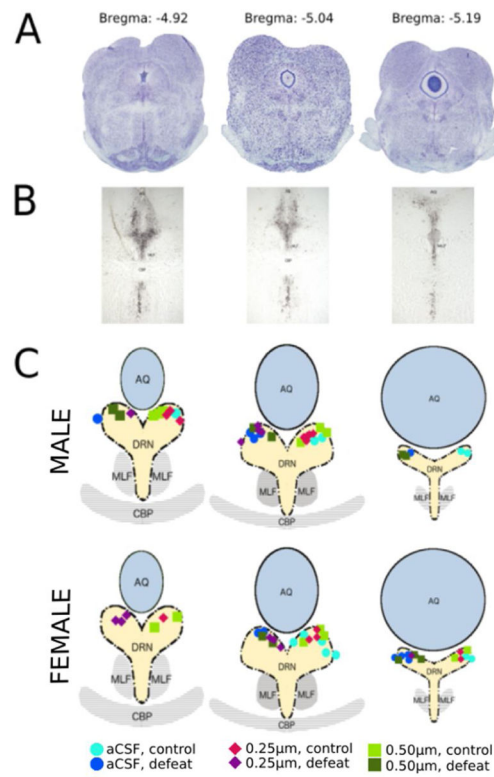
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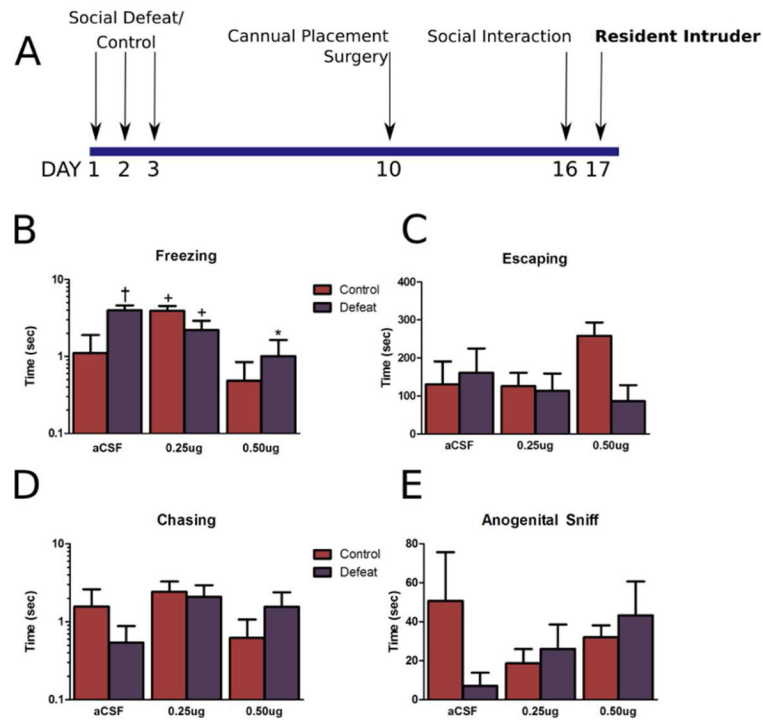
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### Figure 1. Cannula Placements

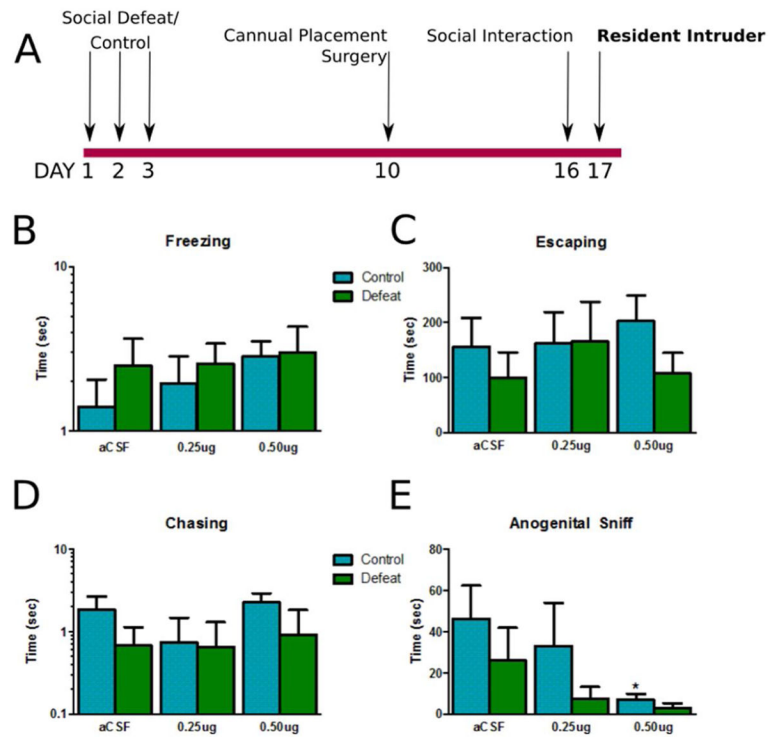
Nissl stain of coronal sliced California mouse brain tissue containing DRN (Fig. 1A), courtesy of brainmaps.org. Tryptophan hydroxylase DAB stain of serotonergic neurons (Fig. 2A) corresponding to the regions represented in Fig. 1A. All cannula placements in DRN for both males and females (Fig. 1C). AQ= Cerebral aqueduct, CBP= Cerebellar peduncles, DRN = dorsal raphe nucleus, MLF = Medial longitudinal fascicle





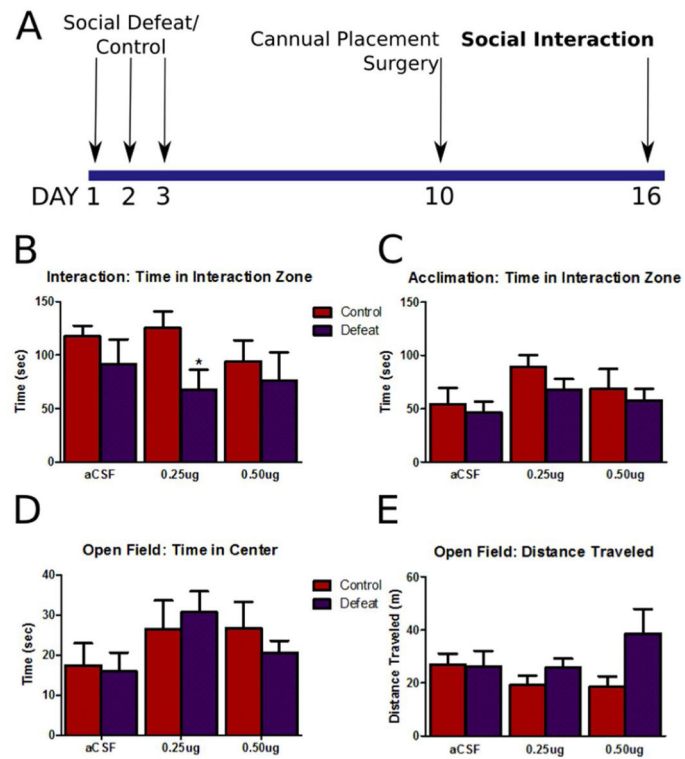
**Figure 2. Male Resident Intruder Data**

Experimental timeline (A). Behaviors measured in resident test animal: freezing (B), escaping (C), chasing (D), and anogenital sniffing (E).  $*$  =  $p < 0.05$  vs aCSF,  $+$  =  $p = 0.07$  vs aCSF,  $†$  =  $p < 0.05$  vs control.  $n = 4-6$  per group.



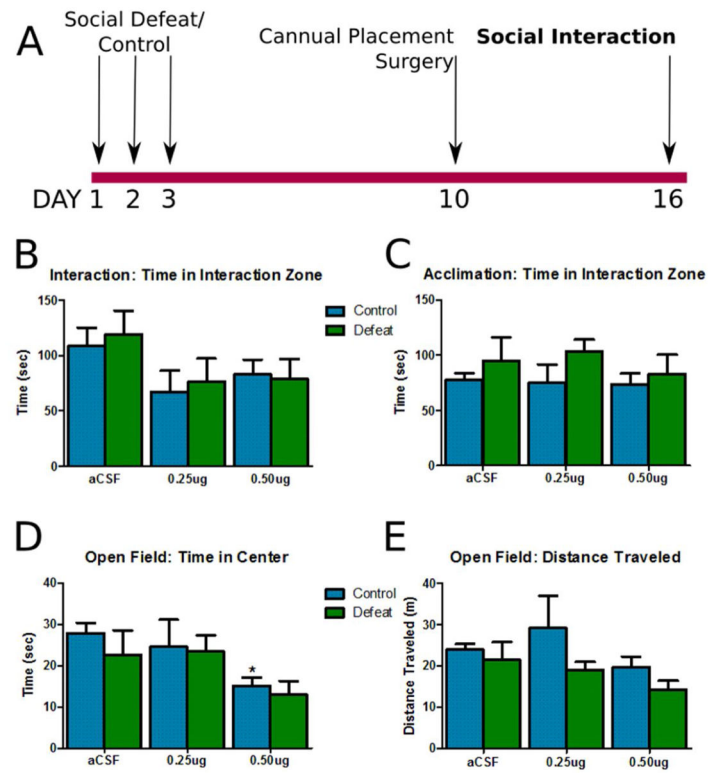
**Figure 3. Female Resident Intruder Data**

Experimental timeline (A). Behaviors measured in resident test animal: freezing (B), escaping (C), chasing (D), and anogenital sniffing (E). \*  $p < 0.05$  vs aCSF.  $n = 5-7$  per group.



#### Figure 4. Male Social Interaction Data

Experimental timeline (A). Time spent in interaction zone during interaction phase of the social interaction test (B). Time spent in interaction zone during the acclimation phase (C). Time spent in center of arena (D) and distance traveled (E) during the first phase. \* =  $p < 0.05$  vs control,  $n = 5-8$  per group.



**Figure 5. Female Social Interaction Data**

Experimental timeline (A). Time spent in interaction zone during interaction phase of the social interaction test (B). Time spent in interaction zone during the acclimation phase (C). Time spent in center of arena (D) and distance traveled (E) during the first phase. \* =  $p < 0.05$  vs aCSF,  $n = 6-7$ .

**Table 1**Resident Intruder offensive aggression (mean  $\pm$  s.e.).

	Bites (freq)		Upright Posture (freq)	
	Male	Female	Male	Female
aCSF, control	0.8 $\pm$ 0.5	3.1 $\pm$ 1.6	15.8 $\pm$ 6.5	11.4 $\pm$ 4.5
aCSF, defeat	0.6 $\pm$ 0.3	0.7 $\pm$ 0.6 <sup>†</sup>	6.4 $\pm$ 2.3	5.2 $\pm$ 3.0
0.25ug, control	2.25 $\pm$ 1.6	3.3 $\pm$ 2.9	7.5 $\pm$ 2.8	12.0 $\pm$ 3.6
0.25ug, defeat	1.6 $\pm$ 1.6	0.2 $\pm$ 0.1 <sup>†</sup>	7.0 $\pm$ 2.9	7.7 $\pm$ 4.5
0.50ug, control	0.0 $\pm$ 0.0	4.0 $\pm$ 1.5	5.3 $\pm$ 2.6	3.0 $\pm$ 1.7
0.50ug, defeat	2.5 $\pm$ 1.5	1.0 $\pm$ 0.7 <sup>†</sup>	2.5 $\pm$ 1.2	4.0 $\pm$ 1.2

<sup>†</sup> main effect of defeat p=0.06