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Sex-dependence of anxiety-like behavior in cannabinoid receptor 1 (*Cnr1*) knockout mice

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Abstract

Epidemiological data suggest women are at increased risk for developing anxiety and depression, although the mechanisms for this sex/gender difference remain incompletely understood. Pre-clinical studies have begun to investigate sex-dependent emotional learning and behavior in rodents, particularly as it relates to psychopathology; however, information about how gonadal hormones interact with the central nervous system is limited. We observe greater anxiety-like behavior in male mice with global knockout of the cannabinoid 1 receptor (*Cnr1*) compared to male, wild-type controls as measured by percent open arm entries on an elevated plus maze test. A similar increase in anxiety-like behavior, however, is not observed when comparing female *Cnr1* knockouts to female wild-type subjects. Although, ovariectomy in female mice did not reverse this effect, both male and female adult mice with normative development were sensitive to *Cnr1* antagonist-mediated increases in anxiety-like behavior. Together, these data support an interaction between sex, potentially mediated by gonadal hormones, and the endocannabinoid system at an early stage of development that is critical for establishing adult anxiety-like behavior.

Keywords

Cannabinoid 1 receptor; anxiety; estrogen; development; sex; gender

Introduction

Human epidemiological data consistently suggest that women are at increased risk for major depression and anxiety disorders [1]. Pre-clinical behavioral neuroscience studies, however, have historically modeled emotional behavior and psychiatric disease in exclusively male samples. Given the epidemiological data and the largely exclusive focus on male animal models, it is imperative to include a sex-dependent component of analysis in animal models of psychopathology. Comprehensive sex-based analysis will allow researchers to determine

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how gonadal hormones, which generally circulate at different levels in men and women, interact with central transmitter systems and neural circuits, particularly those that underlie emotion and psychopathology. These types of analyses may clarify the greater prevalence of specific psychiatric disorders among women.

In the pursuit of identifying factors that differentially confer risk or resilience to psychopathology according to sex/gender, investigation of the endogenous cannabinoid system may offer clarity. Across species, the cannabinoid system appears to be critical for emotion and psychopathology [2, 3]. Moreover, the cannabinoid system also seems to be regulated differently in men and women. Sex/gender-differences are observed related to cannabis use, where women appear to more susceptible to dependence [4]. The cannabinoids are also reported to exert sex/gender-specific effects with respect to stress, emotion, and psychopathology, although investigation in this area is still minimal and the direction of these sex/gender-specific effects is not always consistent [3]. These sex/gender-dependent differences may stem from a bidirectional interaction between the cannabinoid system and the estrogen system [5].

We hypothesize that constitutive global knockout of *Cnr1* may have a differential sex-dependent effect on anxiety-like behavior in mice on an elevated plus maze. The elevated plus maze is a valid test of anxiety-like behavior in rodents and has been used to screen pharmacotherapies for affective disorders [6]. In male subjects, global knockout or antagonism of *Cnr1* increases anxiety-like behavior on a number of different paradigms [2]. Here, we demonstrate divergent anxiety-like behaviors of male and female *Cnr1* transgenic mice on an elevated-plus maze test.

Materials and Methods

Animals

Adult male and free-cycling female (estrous cycle was not monitored) C57BL/6J and *Cnr1* transgenic mice were group housed in a temperature-controlled (24° C) animal colony, with *ad libitum* access to food and water, on a 12 hour light-dark cycle. *Cnr1* transgenic mice (C57BL/6J background) were a generous gift from Dr. Carl Lupica at NIDA [7].

Experimental subjects were genotyped by PCR using primers “CB1F” (wild-type forward: 5’ GTA CCA TCA CCA CAG ACC TCC T), “CB1wt” (wild-type reverse: 5’ GGA TTC AGA ATC ATG AAG CAC TC) and “CNK03” (mutant reverse: 5’ AAG AAC GAG ATC AGC AGC CTC T). Wild-type and *Cnr1* knockout amplicons are ~300 base pairs and ~150 base pairs, respectively. Homozygous constitutive *Cnr1* knockout and wild-type littermates from in house heterozygous breeding pairs were weaned at 3 weeks of age and tested at 8-12 weeks of age. All behavioral procedures were performed during the light cycle.

Surgery

Ovariectomy or sham surgeries were performed on 8-week old female *Cnr1* transgenic mice. Anesthesia was induced and maintained using isoflurane. For the ovariectomies, ovaries were gently pulled out and removed via cauterization and subsequent severing of the

fallopian tubes. Sham surgeries were conducted identical to ovariectomy with the exception of ovary removal. Behavioral testing commenced two weeks after surgery.

Behavior

Elevated plus maze—Subjects were handled once per day for the two days prior to testing. Subjects were placed in the center of an elevated plus maze apparatus to explore for 5 minutes in dim lighting (25 lux, measured from the perspective of the subject in the center of the elevated plus maze). The EPM consists of two open arms ($50 \times 6.5 \text{ cm}^2$) and two closed arms with a wall ($50 \times 6.5 \times 15 \text{ cm}^3$) attached to a common central platform ($6.5 \times 6.5 \text{ cm}^2$) to form a cross. The maze was elevated 65 cm above the floor. Behavior was videotaped and hand-scored offline for time on open arms, time on closed arms, open arm entries, and closed arm entries. Observer was not blinded to subject genotype, sex, or drug group assignment. Mice were considered to be on the open or closed arm when all four feet entered an arm. Percent open arm entries (percent open arm entries = (open arm entries / (open arm entries + closed arm entries)) * 100) was used as a measure of anxiety-like behavior. Separate cohorts were used for each behavioral experiment. All subjects tested were included in the analyses, as no subjects fell off the elevated plus maze.

Drugs

The *Cnr1* antagonist, SR141716A (Cayman Chemical 9000484), was dissolved in a vehicle of 2.5% DMSO/0.1% Tween-80 in saline and administered at a dose of 3 mg/kg. SR141716A was administered intraperitoneally (IP) 20 minutes prior to testing on an elevated plus maze.

Statistics

Two-way ANOVA was performed using IBM SPSS Statistics 21 software. The results are presented as mean + SEM. A *post hoc* Bonferroni correction was used where appropriate.

Results

Female *Cnr1* knockout subjects do not exhibit increased anxiety-like behavior compared to male *Cnr1* knockout littermates

Wild-type and *Cnr1* knockout male and female littermates were tested on an elevated plus maze and assessed for 1) time spent on open arms, 2) time spent on closed arms, 3) open arm entries, and 4) closed arm entries (N, wild-type males = 17, *Cnr1* knockout males = 12, wild-type females = 13, *Cnr1* knockout females = 9). We detected a significant main effect of genotype on percent open arm entries ($F_{1,47}=9.32$, $p<0.01$, Fig. 1A). We also found a significant genotype by sex interaction in our analysis of percent open arm entries ($F_{1,47}=4.35$, $p<0.05$). We did not observe a main effect of sex. *Post hoc* Bonferroni tests revealed a significant difference between male wild-type versus male *Cnr1* knockouts ($p<0.01$) and female wild-type subjects versus male *Cnr1* knockouts ($p<0.05$) on percent open arm entries. We did not detect a significant difference between female wild-type versus female *Cnr1* knockout littermates. These data suggest that male *Cnr1* knockout subjects exhibit increased anxiety-like behavior compared to male wild-type subjects, whereas

female *Cnr1* knockout subjects exhibit similar levels of anxiety-like behavior compared to female and male wild-type subjects.

In analyzing the raw values, we detected the same main effect of genotype ($F_{1,47}=5.10$, $p<0.05$) and a genotype x sex interaction on open arm entries ($F_{1,47}=4.53$, $p<0.05$). We did not detect a main effect of sex or genotype, or a genotype x sex interaction on closed arm time, closed arm entries, or open arm time (data not shown). Furthermore, we did not detect a main effect of genotype or sex, or a genotype x sex interaction on total number of entries (closed arm entries + open arm entries), suggesting that all subjects exhibited comparable motor behavior (data not shown).

Ovariectomy does not increase anxiety-like behavior in *Cnr1* knockout females

To test the hypothesis that ovarian hormones mediate a protective effect against increased anxiety-like behavior caused by *Cnr1* knockout, we performed ovariectomy (OVX) and sham surgeries on 8-week old wild-type and *Cnr1* knockout females (N, wild-type sham females = 11, *Cnr1* knockout sham females = 9, wild-type OVX females = 10, *Cnr1* knockout females = 8). Two weeks after surgery, subjects were assessed for anxiety-like behavior, as in the prior experiment (Fig. 1B). We did not detect a significant main effect of surgery or genotype, nor did we observe a surgery by genotype interaction on percent open arm entries. Similarly, we did not detect a significant main effect of surgery or genotype, nor did we observe a surgery x genotype interaction on open arm entries, closed arm time, closed arm entries, open arm time, or total entries (data not shown). These data replicate our previous finding - global knockout of *Cnr1* does not increase anxiety-like behavior in female mice as in male mice. Interestingly, ovariectomy does not increase anxiety-like behavior in female *Cnr1* knockout subjects, suggesting that gonadal hormones might interact with the cannabinoid system early in development to differentially mediate anxiety-like behavior in males and females.

A *Cnr1* antagonist, SR141716A, increases anxiety-like behavior in male and female C57BL/6J mice

Next, we administered the *Cnr1* antagonist SR141716A at a dose of 3 mg/kg to male and female adult C57BL/6J mice to further examine whether the previously observed interaction between sex and *Cnr1* knockout is developmentally-mediated (N, vehicle males = 11, SR141716A males = 13, vehicle females = 12, SR141716A females = 11). We observed a significant main effect of drug on percent open arm entries ($F_{1,43}=7.43$, $p<0.01$, Fig. 1C). We did not detect a significant main effect of sex or a drug by sex interaction on percent open arm entries. *Post hoc* Bonferroni tests revealed a significant difference between males administered vehicle versus males administered SR141716A ($p<0.01$), males administered SR141716A versus females administered vehicle ($p<0.05$), and males administered vehicle versus females administered SR141716A ($p<0.05$). We observed a trend when comparing females administered vehicle versus females administered SR141716A ($p<0.1$).

We also observed a significant main effect of drug on open arm entries ($F_{1,43}=11.49$, $p<0.01$), closed arm time ($F_{1,43}=16.49$, $p<0.001$), closed arm entries ($F_{1,43}=5.62$, $p<0.05$), and open arm time ($F_{1,43}=5.14$, $p<0.05$). We did not detect a significant drug x sex

interaction on any measure of anxiety-like behavior (data not shown). Furthermore, we did not detect a main effect of drug or sex, or a drug x sex interaction on total number of entries.

Discussion

These data demonstrate an interaction between sex and the endocannabinoid system in anxiety-like behavior, where: 1) adult female *Cnr1* knockout subjects do not exhibit increased anxiety-like behavior as in adult male *Cnr1* knockout littermates; 2) ovariectomy in adult females does not increase anxiety-like behavior in *Cnr1* knockouts; however, 3) the *Cnr1* antagonist, SR141716A, increases anxiety-like behavior in both male and female wild-type C57BL/6J mice.

Our results are supported by prior literature. Global knockout of *Cnr1* or administration of a *Cnr1* antagonist increases anxiety-like behavior in male rodents [2]. However, other data demonstrate differential effects of the cannabinoids on anxiety-like behavior, which could be dependent on a balance between GABAergic and glutamatergic neurotransmission or, alternatively, promiscuous activation of receptors beyond *Cnr1* [8, 9]. Furthermore, an effect of the cannabinoids on anxiety-like behavior appears to be contingent on the aversiveness of the test situation [10]. Thus, our observation of a genotype and drug, and, further, genotype x sex interaction effect on anxiety-like behavior may be specific to our experimental conditions.

We did not observe a main effect of sex on anxiety-like behavior in any of our experiments, consistent with some data, but in contrast to other reports [11, 12]. To our knowledge, no study has assessed differences in anxiety-like behavior between male and female *Cnr1* knockout mice, however, one study fails to observe a difference in anxiety-like behavior between male and female mice with conditional knockout of *Cnr1* in either GABAergic or glutamatergic neuronal populations [9]. Furthermore, no study has assessed whether *Cnr1* antagonists have an effect on anxiety-like behavior in female *Cnr1* knockout mice as in male *Cnr1* knockout mice [8].

As acute administration of a *Cnr1* antagonist increases anxiety-like behavior similar to *Cnr1* knockout mice, it is presumed that an anxiogenic effect of *Cnr1* knockout is not developmentally mediated [2]. Our data demonstrating that female *Cnr1* knockout mice exhibit normative anxiety-like behavior, which was not altered by ovariectomy, compared to increased anxiety-like behavior in *Cnr1* antagonist administered C57BL/6J females suggests that there is a developmental component to an effect of *Cnr1* knockout on anxiety-like behavior. This is underlined by data which do not observe a difference in anxiety-like behavior between male and female mice with conditional knockout of *Cnr1* in either GABAergic or glutamatergic neuronal populations, as *Cnr1* could be present during a critical period of development in these strains [9]. Our data indicate that some factor present during early development in females - potentially during the prenatal through the early postnatal developmental critical period - may detect and compensate for aberrant *Cnr1* signaling that contributes to increased anxiety-like behavior in adult males. Alternatively, males, perhaps due to an interaction between *Cnr1* knockout and an early development surge in testosterone, may be more vulnerable to development of the observed anxiety-like

phenotype. Male rat pups have higher levels of anandamide and 2-AG, the two major endogenous cannabinoids, potentially stemming from lower levels of observed endocannabinoid degradative enzymes [13]. Furthermore, the cannabinoid system is differentially dysregulated in male and female rats in response to early maternal deprivation, supporting the hypothesis that sex-specific organization of the cannabinoid system early in development impacts later adolescent and adult sex-specific behaviors [13-15]. Regardless, an exact interpretation of a developmental mechanism by which gonadal hormones interact with the cannabinoid system to mediate differential anxiety phenotypes in male and female mice is limited by the scope of this study and the relative dearth of investigation in this area. Future experiments should investigate the effect of gonadal hormones during early development, which are critical for sexual differentiation, in order to determine the exact nature and timing of a *Cnr1*/gonadal hormone interaction in anxiety-like behavior. The adult male brain phenotype results from exposure of the late gestational/early neonatal brain to estradiol, which is synthesized from testicular testosterone [16]. In this vein, future studies could examine an effect of neonatal testosterone or estradiol injection in female *Cnr1* knockout mice to determine whether early testosterone administration causes an increase in anxiety-like behavior as in male *Cnr1* knockout mice. Moreover, there is evidence that the pubertal surge in hormones, aside from a surge early in development, can also differentially organize the male and female brain [17]. As we performed ovariectomy on 8-week old, adult females, we are not able to exclude the possibility that a pubertal surge in hormones contributed to sex differences in anxiety-like behavior in our subjects.

Comparatively more is known regarding an interaction between the cannabinoid system, behavior, sex/gender, and the gonadal hormones during adulthood relative to early development. Sex/gender-differences are observed related to cannabis use and dependence [4]. Chronic stress differentially affects plasticity of the cannabinoid system in the hippocampus according to sex [18]. The cannabinoid system also appears to modulate prefrontal dendritic complexity and cognitive flexibility disparately in males and females [19]. In rats, an anxiolytic effect of estradiol may be mediated by fatty acid amide hydrolase (FAAH), an endocannabinoid degradative enzyme [20].

Several clinical studies observe interactions between the cannabinoid system, sex/gender, and mental health. Males with a specific *Cnr1* SNP haplotype (rs806377) are at greater risk for ADHD compared to females with the same haplotype [21]. The same SNP is associated with PTSD; however, a sex/gender x SNP x PTSD diagnosis interaction is not reported [21]. Intriguingly, *in vivo* PET data demonstrate sex/gender differences in *Cnr1* receptor regulation, in particular an upregulation of *Cnr1* in women, and especially in women with PTSD [3]. In a study of Major Depression, the *CNR1* rs1049353 G allele is reported to confer resistance to antidepressant treatment, particularly in women [22].

While both the clinical and pre-clinical data suggest interplay between the cannabinoid system and sex that is critical to emotional behavior and psychopathology, the direction of this interaction is not always consistent. Furthermore, our data suggest that females are more resilient with regard to anxiety-like behavior, which is counterintuitive given that the epidemiological data indicates increased prevalence of affective disorders among women [1, 23]. Looking forward, it will be important to address divergence between animal model data

and epidemiological data. Transgenic mice, in particular the four core genotypes (FCG) mice, may be instrumental in this vein, as they can allow assessment of the contribution of sex chromosomes, developmental hormone exposure, and adult circulating hormones to differences in behavior according to sex [24].

Several caveats of our study should be acknowledged. While we did not measure gonadal hormone levels in our female subjects, the ovariectomy data suggest that some sex-specific factor interacts with a *Cnr1* deficiency during early neural development, limiting the information that adult hormone level monitoring might lend to the conclusions of this study. However, data suggest that proestrous (when estradiol levels are higher), compared to diestrous, mice and rats spend more time on the open arms of the EPM [25, 26]. Estrous cycle monitoring may be particularly critical in the future, additionally, as *Cnr1* binding appears to fluctuate according to phase of the estrous cycle [27]. Additional tests of anxiety-like behavior (e.g. open field test, elevated plus maze under aversive light conditions) should be conducted to expand on the findings reported here, particularly as anxiety-like behavior of *Cnr1* knockout mice may depend on the aversiveness of the test situation [28]. Data from these studies suggest that we may observe an alternative outcome when measuring anxiety-like phenotypes of male and female *Cnr1* transgenic mice in more aversive contexts. Additionally, while recent evidence indicates that the EPM appropriately assays anxiety-like behavior in male mice, the EPM may provide a measure of general activity in females [29, 30]. This complicates interpretation of the present study, however, as noted – we did not observe a main effect of sex on total entries made by subjects on the EPM. Future studies that use an anxiety task less dependent on general locomotion, such as the Vogel punished drinking task, could clarify these issues.

Altogether, our data suggests the cannabinoid system may regulate anxiety-like behavior in a sex-dependent manner, potentially via changes to the brain early in development. Pre-clinical and clinical studies should continue to address a potential interaction between the cannabinoid system, sex/gender, and emotion, as identifying an interaction might be explanatory regarding sex/gender differences related to affective disorders.

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Highlights

We compare anxiety-like behavior of male and female *Cnr1* transgenic mice.

Male *Cnr1* KOs make less percent open arm entries on an elevated plus maze compared to male WT.

Female WT and female *Cnr1* KO do not significantly differ on percent open arm entries.

Ovariectomy does not affect percent open arm entries.

Cnr1 antagonist administration decreases percent open arm entries in both sexes.

Fig. 1. Anxiety-like behavior in cannabinoid receptor 1 (*Cnr1*) knockout mice is sex-dependent
(A) We observe a significant genotype by sex interaction in our analysis of percent open arm entries ($p < 0.05$). *Post hoc* tests reveal a significant difference between male wild-type versus male *Cnr1* knockouts ($p < 0.01$) and female wild-type subjects versus male *Cnr1* knockouts ($p < 0.05$). We do not detect a significant difference between female wild-type versus female *Cnr1* knockout littermates. **(B)** Ovariectomy does not increase anxiety-like behavior in *Cnr1* knockout females. We do not detect a significant main effect of surgery or sex, nor do we observe a surgery by sex interaction on percent open arm entries. **(C)** We

observe a significant main effect of 3 mg/kg SR141716A on percent open arm entries ($p < 0.01$). *Post hoc* tests reveal a significant difference between males administered vehicle versus males administered SR141716A ($p < 0.01$), males administered SR141716A versus females administered vehicle ($p < 0.05$), and males administered vehicle versus females administered SR141716A ($p < 0.05$). We observe a trend when comparing females administered vehicle versus females administered SR141716A ($p < 0.1$).

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Table 1

Non-significant elevated plus maze data from wild-type and *Cnr1* knockout male and female mice

Group	Open Arm Time		Percent Open Arm Time		Closed Arm Time		Closed Arm Entries		Total Entries	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
WT/Male	25.71	6.17	8.29	1.99	198.47	9.70	10.41	1.04	14.18	1.24
KO/Male	24.58	13.68	7.93	4.41	234.42	10.64	8.42	1.32	9.58	1.56
WT/Female	36.16	16.88	11.66	5.44	212.46	15.17	9.15	1.04	12.23	1.35
KO/Female	33.44	10.71	10.79	3.46	217.89	15.73	9.33	1.96	12.33	2.51

Non-significant elevated plus maze data from wild-type and *Cnr1* knockout, sham and ovariectomized female mice

Table 2

Group	Open Arm Time		Percent Open Arm Time		Open Arm Entries		Percent Open Arm Entries		Closed Arm Time		Closed Arm Entries		Total Entries	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
WT/Sham	12.86	3.30	5.41	1.47	2.82	0.42	18.43	2.24	235.82	8.21	12.45	1.19	15.27	1.36
WT/OVX	27.60	10.85	10.86	4.40	3.70	1.08	20.69	4.62	232.90	12.85	12.10	0.38	15.80	1.18
KO/Sham	34.33	10.08	12.81	3.64	3.56	0.77	17.88	3.81	229.89	9.72	15.11	1.15	18.67	1.44
KO/OVX	25.38	12.89	9.72	4.92	3.13	1.33	15.17	4.88	233.50	13.15	11.63	1.92	14.75	3.04

Table 3

Non-significant elevated plus maze data from vehicle and 3 mg/kg SR141716A-administered male and female mice

Group	Total Entries	
	Mean	SEM
Vehicle/Male	17.73	0.78
SR141716A/Male	16.85	0.73
Vehicle/Female	17.92	1.04
SR141716A/Female	18.45	0.89