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Sexually-dimorphic alterations in cannabinoid receptor density depend upon prenatal/early postnatal history

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Abstract

Recent research has demonstrated that the endogenous cannabinoid system is central to the brain's response to stress. As part of an ongoing collaboration, we sought to examine the effects of prenatal and early postnatal rearing and housing conditions on developing endocannabinoid systems. We compare brain cannabinoid receptors (CBR) in offspring of either prenatal vehicle intubated or non-treated dams (Experiment 1) or in rats derived from a vendor and shipped at weaning to a collaborating lab (Experiment 2). From postnatal day (PND) 23, all rats were either housed in isolated conditions or enriched conditions with 3 rats/cage and a variety of stimulus objects changed twice a week. All rats underwent 5 days of handling as controls for a behavior study and all rats were sacrificed at approximately PND48-50 within 2 hours of the last behavioral test. All brains were processed together for CB1 receptor binding using ³H CP55,940 in prefrontal cortex, striatum, amygdala and hippocampus. Conditions in the two labs were as similar as possible since the two studies were intentionally designed to be comparable and contemporary. Results show that 1) comparing offspring of non-treated dams to offspring of dams receiving daily vehicle intubations, males show decreased CB1 binding in most brain regions while females only showed alterations in the hippocampus and these were increases in the offspring of the vehicle-intubated dams. 2) When comparing offspring of non-treated dams in NY with those derived from a vendor, shipped and maintained in the collaborating lab, this latter group showed reduced CB1 binding in prefrontal cortex in males and increased binding in all four brain regions in females. Therefore, overall, both prenatal handling (intubations) and being vendor-derived, shipped and maintained in a collaborating facility reduced CB1 receptors in males and increased them in females in key limbic brain regions. Effects of environmental enrichment or isolation were minor with only the prefrontal cortex showing an increase in binding in the isolated animals that were

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Conflict of interest

The authors declare that they have no conflicts of interest

offspring of the vehicle-intubated dams. These results support the ideas that prenatal/early postnatal conditions produce different effects in males and females and override the effects of enrichment/isolation on cannabinoid receptors. Behavioral responses to cannabinoid challenges would therefore be expected to vary depending on sex, prenatal/early postnatal history and postweaning conditions of the rats. Since exogenous cannabinoids act through the CBR, the present data may provide a molecular basis for discrepant behavioral effects reported across various labs in the literature as well as sex differences seen following stress and/or manipulation of the cannabinoid system.

Keywords

Endocannabinoids; Sex differences; Prenatal stress; Postnatal stress

Introduction

The endogenous cannabinoid system, which develops during prenatal and postnatal life in humans and animals, is essential to mount appropriate behavioral and neuroendocrine responses to the environment. Cannabinoid receptors and their endogenous ligands, *N*-arachidonylethanolamide (anandamide/AEA) and 2-arachidonoylglycerol (2-AG), are present in the rat brain as early as gestational days 11–14 (Rodriguez de Fonseca et al, 1993; Berrendero F, et al 1999], while in humans, cannabinoid receptors have been found from week 14 of gestation (Glass et al, 1997; Wang et al, 2003). The endocannabinoid system influences many processes during development through the modulation of neurotransmitter release and action (Viveros et al, 2005; Gaffuri et al; 2012). Additionally, many studies have shown the importance of the endocannabinoid system in modulating hypothalamic-pituitary-adrenal (HPA) axis activity throughout development (for review, Lee and Gorzalka, 2012). CB1 receptor signaling is stress responsive, and repeated exposure to chronic unpredictable stress in adulthood has been shown to reduce CB1 receptor signaling, a change associated with anhedonia and anxiety (for review, Hillard 2014; McEwen et al., 2015). Prenatal stress is widely known to alter behavior and neuroendocrine responses throughout life. While the types of stressors utilized in studies of rodents during the latter third of pregnancy have varied (immobilization, isolation on a platform), alterations in behavior, neuroendocrine responses and gene expression have been described in the offspring and these alterations are frequently sexually dimorphic (Bowman et al., 2004; Mychasiuk et al., 2011, Muhammad et al., 2011; Vallee et al., 1997). Postnatal stress, usually in the form of maternal separation or deprivation, typically induces hyper-reactive HPA axis activity, as well as increases in anxiety and depressive-like measures (Maccari et al., 2014). Maternal separation during the early postnatal period has been shown to have long lasting effects on stress responses and the responses of the cannabinoid system (Llorente-Berzal et al., 2011, Marco et al., 2014;). Numerous types of stressors can alter the subsequent responses to drugs. Although typically not considered as stressors, transport and shipping of mice and rats can increase both behavioral and endocrine measures of stress (Tuli et al., 1995, van Ruiven et al., 1998). Shipping during the peripubertal period produces alterations in response to gonadal hormones that persist into adulthood (Laroche et al., 2009) and shipping at weaning produces sex-specific alterations in behavioral responses to THC (Wiley and Evans, 2009).

Housing conditions of the rats can be varied to examine the effects of enriched or isolated conditions on cognition, emotion and reward (see review by (Simpson and Kelly 2011). While the conditions can vary from one study to the next, enrichment typically occurs from postnatal day (PND) 21/22 (weaning) until the behavioral test is conducted. Isolation is generally believed to be stressful for a naturally social animal like the rat (Fox et al. 2006) while housing with novel objects and with peers is considered less stressful (Belz et al, 2003). Maternal isolation has also been shown to alter offspring play behavior compared to social maternal housing during pregnancy (Honeycutt and Alberts, 2005). Therefore, housing conditions both during prenatal and postnatal life can impact behavior and stress responsivity.

Since both exogenous and endogenous cannabinoids act through the CB1R, alterations in receptor expression suggest changes in the endogenous cannabinoid system, which may produce altered responses to stressors and to drugs. The brain regions that are involved in the response of the endocannabinoid system to the environment include the hippocampus, amygdala and prefrontal cortex (McLaughlin & Gobbi, 2012). In the present study, we measured CB1R densities in these brain regions in rats that either were bred in house and experienced prenatal stress (dams intubated) or were offspring of non-treated dams (Experiment 1) or were vendor-derived, shipped at weaning and raised in a collaborating facility operating with parallel procedures (Experiment 2). In addition we studied the effects of different post-weaning housing conditions in both facilities on CB1R. The findings show that prenatal stress of the mothers and being derived from a vendor and shipped at weaning do alter CB1R densities and that the effects are different in males and females.

Materials & Methods

Experiment 1

Sprague-Dawley rats (VAF strain, Charles River Laboratories, Raleigh, NC) were assigned to treatment groups (see prenatal dosing below). Rats were kept under a 12 h light-dark cycle (lights on at 7:00 h) and temperature of 20–22 °C. Females in proestrus were placed with males of the same strain at 4:00 PM. The next morning, rats were checked for sperm by vaginal smear. If sperm was present, that day was designated as gestational day 1 (G1). Pregnant dams were individually housed in plastic cages with bedding and randomly assigned to receive daily intragastric ((IG) intubations of vehicle or no-treatment. Daily intubations of water (sterile water, Baxter, the vehicle-intubated group) began 1 week after arrival of the rats in the vivarium using a 16 gauge straight feeding needle and continued through mating and up to the day before delivery (G22). The non-treated dams received no handling for the duration of the pregnancy. These dams were controls for a large prenatal dosing study described in Dow-Edwards et al., 2014. On the day of birth (usually G23), designated as postnatal day 1 (PND1), all pups were sexed, toe-clipped, and weighed. Litters were culled to 10 pups (5 males, 5 females) and pups from vehicle-intubated dams were surrogate fostered to non-treated dams on that same day. Cross-fostering was not used and pups from non-treated dams were not fostered due to absence of available dams. At PND 21 animals were weaned, ear punched and separated into same sex cages containing 5 littermates until PND 23. On PND 23, rats were housed in one of two conditions: either one

rat/cage (isolated environment) or with 3 same-sex littermates/cage which also contained stimulus objects (enriched environment) (see details of housing conditions in Dow-Edwards et al, (2014). Rats in both housing conditions had access to food and water ad lib. Subjects were weighed at weekly intervals from PND 8 to PND 42 prior to behavior testing. All procedures were approved by the IACUC in accord with the recommendations of the American Academy of Lab Animal Science. All experiments were carried out in accordance to the Guide for the Care and Use of Laboratory Animals, Eighth Edition, National Research Council, Department of Health and Human Services, 2011.

As part of their postnatal experience on PND 44, pups in the current study served as saline-saline controls for conditioned place preference (CPP) testing. The results have been previously reported (Dow-Edwards et al, 2014; Zakharova et al., 2009a,b) and the 5-day paradigm is presented here for informational purposes only. For this procedure, rats were individually placed in 42 × 42 cm Plexiglas boxes with solid white walls on one side and vertical black/white stripes on the other for a period of 30 min. On the following 3 days, the boxes were partitioned to prevent access to both sides; the rats were injected with saline (1ml/kg body weight) and placed on one side of the box for 30 min in the morning and 3–4 hours later, injected with saline and placed on the opposite side of the box for 30 min. On the 5th day, the partition in the box was removed and the rats were placed in the box for 30 min under observation. Within 2 hours of this final test session, subjects were decapitated, brains removed, frozen and stored at –80°C until shipment to U. Miami for sectioning and processing.

Experiment 2

Sprague-Dawley rats (vendor-derived) (VAF strain, Charles River Laboratories, Raleigh, NC) were from the same facility in Raleigh that supplied the breeders for Experiment 1. The standard operating procedure of this vendor is to house each pregnant female separately in a single cage. Litters are culled to 14 most often retaining only the males and remain housed in these cages until the day of shipping on PND 20. The pups are placed in shipping crates (5–8 same sex per crate) and trucked to Miami overnight in climate controlled vehicles. For this experiment, only non-littermate pups were ordered (that is, in each shipment, rats from different litters were requested). The pups arrived in the Miami lab on PND21 and were housed in same sex cages until PND23. On PND 23, housing conditions were identical as those described above in Experiment 1; isolated or enriched. The objects and rotation cycle of the objects were intentionally designed to be identical to those of the NY lab described above. As in Experiment I, on PND 44, pups underwent CPP training as described above (again these rats were the saline-saline controls) and were sacrificed within 2 hr of the final CPP test. Brain tissue was harvested and handled as described below.

Receptor autoradiography

Tissue processing for Experiments 1 and 2: All brains were processed for receptor densities at the University of Miami. Brains were sectioned coronally at 20 µm, sections taken on plus slides and stored at –80°C. Sections were then processed for CB1 receptor densities using ³H CP 55,940 binding as described in Werling et al, (2009). Frozen sections were thawed to room temperature and incubated for 2 hours with 3nM [³H]CP55,940 in buffer

(50mM Tris-HCl, pH 7.4 with 5%BSA). Sections were washed 4 × 30 min in ice-cold buffer (50mM Tris-HCl with 1% BSA) followed by 5 min at 25°C in buffer containing 50mM Tris-HCl with 0.5% formaldehyde. Slides were then dipped quickly in ice-cold deionized water and dried. Non-specific binding was defined as binding in the presence of 10uM CP55,940. Slides and standards were apposed to Kodak Biomax MR film for 18 days. Films were developed and images were analyzed using NIH Image J software. Binding densities were quantified using curves generated from labeled standards.

Statistical analysis

Experiment 1—Data for each region and associated sub-regions were analyzed using mixed linear models. A separate model was constructed for each region. The fixed effects were maternal treatment (vehicle intubated or nontreated), sex, sub-region and their mutual interactions. Housing (isolated, enriched) was entered as a factor but interactions with this factor were not assessed due to small sample sizes in 2 cells. Litter was introduced as a random effect. The dependent variable was square-root-transformed; the intra-subject covariance matrix was modeled as unstructured. Simple effects analyses were conducted in the presence of significant interactions and bootstrap-simulated adjustments to simple effects *p*-values were applied to deal with multiple testing. Model residuals were inspected for skew and outliers. Model-generated back transformed group means are presented with 95% confidence intervals. *P*-values <0.05 were considered significant. SAS (SAS Institute, Cary, NC) Release 9.4 software was used.

Experiment 2—As for Experiment 1, except that the treatment factor was vendor-derived and shipped vs. vivarium born/non-treated dam. This analysis relied on each vendor-derived animal coming from a different litter as requested.

Housing

In order to examine the effects of housing under the different experimental conditions, a third set of mixed linear models was constructed using the square-root-transformed *averaged* score across sub-regions, with a separate model for each of the 4 brain regions. Fixed effects were condition (NT, veh and VD), housing (isolated, enriched), sex and their respective 2 way interactions. Litter was introduced as a random factor.

Results

Table 1 shows the group sizes for Experiment 1 and 2; each treatment condition, two sexes and two housing conditions.

Experiment 1

Seventeen litters contributed to this experiment. Data for the 4 brain regions were analyzed separately.

For prefrontal cortex, there was a significant treatment x sex x sub-region interaction (*p*<0.001). The sub-regions included: CG3: area 3 of the cingulate cortex, CG1: area 1 of the cingulate cortex, FR2: area 2 of the frontal cortex, AID: agranular insular cortex, IL:

infralimbic area. Simple effects analysis by sex and sub-region resulted in significant decreases in binding in males whose mothers were vehicle intubated compared to the male offspring of the non-treated dams (Figure 1). Females showed no significant effects of the prenatal treatment in prefrontal cortex.

For striatum, there was a significant treatment x sex x sub-region interaction ($p=0.006$) with offspring of vehicle-intubated dams showing lower binding in all striatal subregions in males and no significant alterations in females (Figure 1). Striatal sub-regions were DM: dorsomedial, DL: dorsolateral; VM: ventromedial; VL: ventrolateral; CORE: nucleus accumbens core; SHELL: nucleus accumbens shell.

For hippocampus, the 3 way interaction was not significant but there was a significant treatment by sex interaction ($p<0.001$). Sub-regions of the hippocampus were CA1, CA2, CA3 regions of hippocampus and DG or dentate gyrus. Simple effects comparisons by sex and sub-region showed that male offspring of vehicle-intubated dams had decreased binding in CA1 and CA2 sub-regions while female offspring of vehicle-intubated dams showed significantly increased binding in all sub-regions of the hippocampus (Figure 2).

For the amygdala, there was a significant treatment x sex x sub-region interaction ($p=0.039$). Two sub-regions of the amygdala were analyzed: BLA or basolateral amygdala and BMA or basomedial amygdala. Simple effects analysis by sex and sub-region revealed that only the BLA of male rats showed a significant decrease in offspring of vehicle intubated dams (Figure 2).

Experiment 2

This experiment compared the binding in brains of offspring of non-treated rats from NY with rats derived from a vendor, shipped and maintained in a collaborating facility in an effort to compare the effects of prenatal and postnatal histories on the development of the endocannabinoid system. Data for 4 brain regions; prefrontal cortex, striatum, hippocampus and amygdala were analyzed separately with “treatment” in this case referring to the non-treated NY group versus the vendor-derived Miami group.

For the prefrontal cortex, there was a significant treatment by sex by sub-region interaction [$p<0.001$] (Figure 3). Simple effects analysis indicated that vendor-derived (VD) and non-treated (NT) groups differed in both males and females with CB1R being lower in vendor-derived males than NT males and higher in vendor-derived females than NT females in all sub-regions (Figure 3).

In striatum, there was a significant sex x treatment x sub-region interaction (Figure 3) with lower binding in vendor-derived males in the VL sub-region [$p=0.0129$]. For females, there were higher binding levels in the vendor-derived group in the DL, VM and VL regions [$p's<0.01$].

In hippocampus, there was no significant 3 way interaction but there was a significant treatment by sex interaction [$p<0.001$] (Figure 4). Vendor-derived females showed robust increases in binding in all hippocampal sub-regions compared to the NT females while none of the sub-regions differed across the males. (Figure 4).

For the amygdala, there was no significant 3-way interaction but the interaction of treatment and sex was [$p=0.001$] with vendor-derived females showing increased binding compared to NT females in both the BLA and BMA sub-regions [$p<.001$] and no differences between the male groups (Figure 4).

Housing

For striatum, hippocampus and amygdala, there were no significant interactions or main effects of housing. For prefrontal cortex, however, there was a significant housing x treatment interaction ($F[2,28]=3.58$, $p=0.041$). Only the vehicle group showed a significant effect of housing and this was an increase in isolated animals ($p=0.009$) (Figure 5).

Discussion

Overall, CBR varied by prenatal/postnatal treatments and sex. In Experiment 1, rats born to vehicle-intubated dams showed decreases in CP 55,940 binding in prefrontal cortex(PFC), striatum, hippocampus and amygdala (uniformly or in select sub-regions) in males and increases only in the hippocampus of females. In Experiment 2, being derived from a vendor, shipped and raised in a collaborating lab reduced CBR in PFC and striatum in males (compared to the NT group) and increased binding in components of all brain regions in females. Therefore, compared to NT controls, being exposed to prenatal stress had greater effects in males and being vendor-derived, shipped and raised in a collaborating lab had greater effects in females. Also, importantly, regardless of the stressor, males showed decreases in binding and females showed increases. In general, our results support those of others who examined maternal deprivation (MD) during the early postnatal period where whole litters are separated from the dam for 24 hr beginning on PND9. When examined in adulthood, either decreases in hippocampal CB1R (Suarez et al 2009, Lopez-Gallardo et al 2012) or no change was induced by MD (Zamberletti et al 2012). Other studies show a very different profile of effects. Such as, Marco et al. (2014) reported that MD increased CB1R gene expression in frontal cortex and amygdala while simultaneously decreased CB1R in ventral striatum males while females showed the opposite pattern of change in adolescence (PND46). One factor, in addition to the age of the analysis may be that this study examined CB1R gene expression rather than the protein itself. While all MD studies utilize “in-house” bred subjects, some dams are shipped during pregnancy which is expected to contribute to variability in the results (see prenatal stress below).

Other types of stressors have also been found to produce sex-specific changes in the endocannabinoid system. For example, chronic unpredictable stress during adolescence decreases CBR in male hippocampi and increases CBR in females when examined in adulthood (Reich et al, 2009). After 2 days of repeated stress during adolescence, males showed a decrease in CBR mRNA expression in PFC 7 days later (Xing et al. 2014). Others have reported that 10 days of mild stress during adolescence or adulthood in males increases CBR in PFC acutely but adults receiving stress as adolescence showed decreased CBR (Lee & Hill, 2013). Therefore, several models have shown that CB1R are affected and the direction of change depends on the age at which the stress is experienced, the number of days between the stress and the assessment and the sex of the animal.

Clearly the endocannabinoid system is responsive to stress throughout the lifespan and our data provide evidence that it is sensitive to prenatal stress, being vendor-derived, shipped and reared in one facility or being vivarium derived from non-treated dams and not experiencing overnight shipping. While the overall housing conditions in the 2 facilities (NY and Miami) may be different in ways not detectable to the researchers, the endocannabinoid system is responsive to the combination of influences and this change in basal tone (as suggested by changes in receptor density) likely produces differences in behavioral responses to exogenous cannabinoids and other drugs. In fact, Wiley and Evans in 2009 reported that shipping at weaning increased behavioral responses to THC in several behavioral categories, which differed in males and females. Their two groups of rats were derived from the same vendor and housed in the same vivarium following weaning. The only difference was being vendor-derived and shipped. Presumably the behavioral differences they report reflect underlying differences in the endocannabinoid system providing additional support for our findings reported here. Our results are consistent with the idea that prenatal vehicle intubations or being vendor-derived, etc., disrupts the normal ontogeny of the endocannabinoid system differently in males and females and that this disruption may result in altered behavioral responses to exogenous cannabinoids and dysregulated affective states.

The rodent medial PFC is functionally equivalent with the primate dorsolateral prefrontal cortex and as such maintains balance between the cortex and amygdala to maintain proper executive function in the face of stress (McLaughlin et al., 2014). Cannabinoid receptors in PFC have been shown to play a role in anxiolytic/genic and anti-depressant actions of cannabinoids (see McLaughlin & Gobbi, 2012 for review). Increased cannabinoid concentrations in ventromedial PFC facilitate extinction of fear-potentiated startle while infusions of antagonists reduce potentiated startle (Lin et al, 2009) suggesting that cannabinoid receptors in ventromedial PFC play a role in adaptation to aversive environments. Alterations in CB1R within the medial PFC in VD offspring and offspring exposed to maternal intubations are likely to manifest as abnormal responses to stress, abnormal mood altering effects of cannabinoids, and abnormal adaptation to aversive environments in sex-specific ways.

The hippocampus has been divided into two --dorsal and ventral with wholly different functions. The ventral hippocampus is associated with emotion and affect while the dorsal hippocampus is associated with cognitive functions (Fanselow & Dong, 2010). We analyzed CB1R in dorsal hippocampus and found that males did not show an effect of being vendor-derived but did if their dams had been intubated and these were decreases (Figure 2). Females showed robust increases in CB1R in hippocampus regardless of their stress histories (Figures 2&4). Cannabinoid agonists injected into the CA1 region of dorsal hippocampus result in an anxiogenic response on the elevated plus maze while cannabinoid blockers produce an anxiolytic response (Roohbakhsh et al., 2007). Therefore, we might expect opposite effects of cannabinoid agonists in stressed males and females. Cannabinoid receptors in dorsal hippocampus are also involved in fear memory consolidation (de Oliveira Alvares et al., 2010). Chronic uncontrollable fear has been shown to alter CB1R in DG and CA3 regions (Hill et al., 2009). Therefore, early stress which alters CB1R in dorsal hippocampus in both sexes could contribute to a range of abnormalities from impaired cognitive function to abnormal fear responses with or without exogenous cannabinoids.

McLaughlin and Gobbi (2012) characterize the relationship between the amygdala and PFC as oppositional in that imbalances between these structures impair emotional behavior and that the endocannabinoid system is positioned to regulate this relationship. Acquisition and consolidation of conditioned fear responses, extinction of emotionally aversive memories and alterations in anxiety have all been reported following manipulation of the amygdaloid cannabinoid system. Endocannabinoid signaling in the amygdala appears to be important for behavioral and neuroendocrine habituation to stress (McLaughlin & Gobbi, 2012). Although alterations in CB1R in amygdala in the current study were not as robust as in other brain regions, there was a reduction in BLA in males in Experiment 1 and an increase in both sub-regions in females in Experiment 2. These sex-specific effects of early stress undoubtedly contribute to lasting alterations in fear responses, extinction and anxiety.

Endocannabinoids have been known to play a role in reward circuits for some time. Intra-accumbal injections of cannabinoid agonists can produce conditioned place preference like morphine (Karimi et al., 2013). Neuroplastic changes in nucleus accumbens following training with highly palatable food have been shown to rely on the CB1R (Guegan et al., 2013) and striatal CB1Rs play a role in the emotional consequences of stress and rewarding experiences (DeChiara et al., 2010a) as well as hedonic reward processing (Friemel et al., 2014). In addition, voluntary activity and sucrose consumption enhance CB1R sensitivity in the striatum (DeChiara et al., 2010b). Prenatally stressed males in Experiment 1 showed decreases in CB1R throughout the striatum while in Experiment 2, stressed females showed robust increases throughout the dorsal striatum, not the ventral striatum. There is a clear need to investigate the sparing of the female n. accumbens from the effects of early stress to understand why significant changes in rearing conditions do not produce alterations in the endocannabinoid system, which we can detect with our methods. However, since maternal adrenalectomy results in a dramatic increase in CB1R in caudate putamen (Mailleux & Vanderhaeghen, 1993), our results in males showing a decrease following maternal intubations (which presumably increase corticosterone levels) is consistent with the idea that glucocorticoids regulate CB1R in caudate during fetal development.

Sex Differences

Sex differences in the endocannabinoid system and the behavioral effects of exogenous cannabinoids have been known for many years (see review by Fattore & Fratta, 2010). Generally, males show greater effects of exogenous cannabinoids on food intake, energy homeostasis and decreased sexual behavior than females and females show greater effects on analgesia, motor activity, depression, increased sexual desire, catalepsy and anxiety. The underlying endocannabinoid system also shows sex differences including the distribution of the CB1 receptors, the responses to repeated exogenous cannabinoids, basal activity of the endocannabinoid system, the rate of desensitization of the receptors, effects of stress on receptors and behavior, and differences in the molecular signaling at inhibitory synapses in the hippocampus (Tabatadze et al., 2015; Viveros et al., 2012; Lopez-Gallardo et al., 2012; Burston et al., 2010; Mateos et al., 2011).

Males and females undergo maturation at different ages but at the age of assessment in the current study, females have completed puberty while males have not. Also, it has been

reported that at PND 48–50, receptors in membranes derived from limbic forebrain in males are similar to those in females (Rodriguez de Fonseca et al., 1993). However, recent investigators report that adolescent females have significantly greater expression of CB1R genes, mRNA and protein than males in frontal cortex and hippocampus while expression in amygdala and striatum were similar (Marco et al. 2014, Xing et al. 2014). In adulthood, on the other hand, several authors have reported that males show greater CB1R than females in most brain regions but that receptors in females show greater stimulation following agonist administration (Castelli et al., 2014; Llorente-Berzil et al, 2011; Reich et al, 2009; see van Laere et al 2008 for human study). Since all brain regions examined in the current study demonstrated an interaction between sex and prenatal/postnatal history, main effects of sex were not obtained. Clearly, early stress exposure has sex-specific effects on the endocannabinoid system.

Prenatal stress

Prenatal stress from daily maternal intubations throughout pregnancy reduced CB1R binding in the PFC, striatum and sub-regions within the hippocampus and amygdala in male offspring and increased CB1R binding in hippocampus of female offspring (Experiment 1). For those pups derived from the vendor, we have no information about prenatal conditions but presumably, since the vendor is a commercial operation, they attempt to minimize stress to maximize productivity. Importantly, prenatal stress induces sex-specific changes in gene expression in prefrontal cortex and hippocampus such that the hippocampi of females showed changes in genes that code for growth factors, an effect related to altered depressive-like behavior (Mychasiuk et al, 2011). Others have reported prenatal stress-induced sex-specific alterations in a range of behaviors including stress responses (Bhatnagar et al 2005) (see review by Fernandez-Guasti et al 2012). However, few authors have described alterations in CB1R following prenatal stress. Laviola and coworkers administered glucocorticoids to pregnant mice and found increases in CB1R in striatum and hippocampus (Ceci et al, 2014; Macri et al., 2012). However, oral administration of corticosterone does not mimic the natural stress responses that undoubtedly result in an outpouring of endogenous corticoids, vasoconstriction and an altered maternal physiology. Gastric intubation increases corticosterone levels in pregnant mice verifying that this procedure is stressful (Walker et al, 2012; Vandenberg et al, 2014). While dams may habituate to repeated stressors such as intubation, Experiment 1 shows that this procedure produces robust sex-specific effects on CB1R throughout the brain.

Effects of environment

Although the analysis of the effects of isolation/enrichment was somewhat limited due to the small sample sizes in some groups, we did see that isolation increased CB1R in PFC in the offspring of vehicle-intubated dams. It has been reported that CB1R mRNA is increased throughout the cortex in adult male rats reared in isolation (Robinson et al, 2010) but this was not replicated by Sciolino et al., (2010). While isolation is considered a stressor for a naturally social rat, social housing in adulthood decreases corticosterone levels in females and increases them in males (Brown & Grunberg 1995). Males generally show lower corticosterone levels when housed alone (Brown & Grunberg, 1995). On the other hand,

Belz et al (2003) reported that corticosterone levels were reduced in males and females by the addition of enrichment to the cage. We did not observe interactions between housing condition and sex but again, the sample sizes were relatively small when the groups were divided by sex. Certainly isolation/enrichment can potentially affect the endocannabinoid system and this relationship should be carefully considered in future studies.

Other factors influencing the results

Brake and coworkers (Brake et al. 2004) and Vallee (et al., 1997) have shown that daily handling during the postnatal period results in a dampening of the effects of later stress on a variety of measures and that both maternal separation and non-handling during the early postnatal period result in hyper-reactivity to novelty and increased responses to a mild stressor. All of our non-treated and vehicle-intubated subjects were weighed at weekly intervals and underwent biweekly cage changes, activities which constitute mild handling during the early postnatal period. In spite of this handling, maternal vehicle intubations still produced significant effects in all of the brain regions analyzed (Experiment 1) with males and females showing opposite changes. Others also report that early handling alters the effects of prenatal stress (Wakshlak et al, 1990; Smythe et al, 1996). For our study, rats shipped from the supplier at PND 21 have presumably experienced no handling during the postnatal period. However, all rats in both Experiment 1 and 2 underwent CPP training between PND 44 and 49. This procedure involved daily weighing, 2 saline injections on 3 days and exposure to the Plexiglas chamber for 30 min 8 times across the 5 days of training. While these procedures were identical across both labs, the fact that the rats were handled during mid-adolescence may have influenced the results.

Another factor that is likely to contribute to the results is the variable fostering events to which the animals were exposed. In Experiment 1, offspring of vehicle-intubated dams were fostered on day of birth to non-treated dams, while the offspring of non-treated dams were raised by their natural mothers. It has been reported that fostering alters maternal behavior which in turn, alters pup behavior in inbred mice (Cox et al, 2013). Therefore, it is possible that fostering the offspring of vehicle-intubated dams and not the offspring of non-treated dams contributed to the differences in binding in Experiment 1. In those rats that were vendor-derived, litters were variably fostered and litter composition was also variable. Most often, litters were culled to 14 males with pups from several dams comprising a single litter (pers. communication). All the litters in the NY vivarium were culled to 10 retaining 5 pups of each sex when possible. It has been reported that alterations in maternal behavior with all-male litters result in pups with a range of abnormal behaviors (Moore, 1984; Suvorov & Vandenberg, 2016). Certainly all these factors that produce alterations in behavior could contribute to alterations in CBR as reported in the current study.

One additional factor that could contribute to the findings in Experiment 2 in addition to the effects of shipping is the differences between the NY facility and the Miami facility. We made every effort to control for cage size, housing conditions (enriched and isolated), light cycles etc but certainly room size, noise levels, chow and other factors could be different between the facilities and could potentially contribute to the results. Clearly, additional

studies should be performed to control for the multiple factors, which could influence CB1R and the endocannabinoid system.

Conclusions

In summary, the prenatal/postnatal histories of rats produce sex-specific effects on CB1 receptors. Prenatal stress in Experiment 1 decreased CB1R throughout prefrontal cortex and striatum and selectively decreases in hippocampus and amygdala in males while females showed only increases in hippocampus. Being vendor-derived, shipped and raised in a collaborating facility had relatively greater effects in females since females showed increases throughout the prefrontal cortex, striatum, hippocampus and amygdala while males primarily showed decreases in the prefrontal cortex. The influence of being raised in an enriched or isolated environment was relatively minor compared to the influence of varying prenatal/postnatal conditions. CB1Rs often reflect function of the endocannabinoid system, thus these data suggest that differing prenatal and postnatal conditions exert sex-specific alterations in the endogenous cannabinoid system, which can affect mood and emotionality as well as behavioral responses to subsequent exogenous cannabinoid administration.

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Highlights

Adolescent rat brains were processed for cannabinoid receptors (CB1R) following prenatal or periweaning stressors

Prenatal stress (daily maternal intubations) globally reduced CB1R in males and increased CB1R in females in hippocampus

Being vendor-derived, shipped and raised in a collaborator's lab reduced CB1R in prefrontal cortex of males and globally increased CB1R in females

Since early stress decreased CB1 binding in males and increased it in females, stress can alter responsivity of the endocannabinoid system in sex-specific ways.

Figure 1.

^3H CP 55,940 binding in sub-regions of the prefrontal cortex and striatum of non-treated and vehicle-intubated groups collapsed across housing condition in Experiment 1 (* $p < 0.05$ compared to NT group). Model-generated means + 95% confidence interval of 10 male and 12 female offspring of NT rats; 10 male and 12 female offspring of vehicle-intubated dams. Prefrontal cortex: CG3: area 3 of the cingulate cortex, CG1: area 1 of the cingulate cortex, FR2: area 2 of the frontal cortex, AID: agranular insular cortex, IL: infralimbic area. Striatum: DM: dorsomedial, DL: dorsolateral; VM: ventromedial; VL: ventrolateral; CORE: nucleus accumbens core; SHELL: nucleus accumbens shell).

Figure 2.

^3H CP 55,940 binding in sub-regions of the hippocampus and amygdala of non-treated and vehicle-intubated groups collapsed across housing condition in Experiment 1 (* $p < 0.05$ compared to NT group). Model-generated means + 95% confidence interval of 10 male and 12 female offspring of NT rats; 10 male and 12 female offspring of vehicle-intubated dams. Hippocampus: CA1, CA2, CA3 regions of hippocampus; DG: dentate gyrus; amygdala: BLA: basolateral amygdala; BMA: basomedial amygdala. Note difference in Y axis between hippocampus and amygdala.

Figure 3.

³H CP 55,940 binding in sub-regions of the prefrontal cortex and striatum of non-treated and vendor-derived groups collapsed across housing condition (* $p < 0.05$ compared to NT group). Model-generated means + 95% confidence interval of 12 male and 11 female vendor-derived rats; 10 male and 12 female offspring of non-treated dams.

Figure 4.

³H CP 55,940 binding in sub-regions of the hippocampus and amygdala of non-treated and vendor-derived groups collapsed across housing condition. (* $p < 0.05$ compared to NT group). Model-generated means + 95% confidence interval of 12 male and 11 female vendor-derived rats; 10 male and 12 female offspring of non-treated dams. Note difference in Y axis between hippocampus and amygdala.

Figure 5.

³H CP 55,940 binding in prefrontal cortex in 3 groups of rats showing effects of housing from P23 to day of sacrifice in either enriched (enr) or isolated (iso) cages (see methods for details). Model-generated means + 95% confidence interval. N=12 enr NT, 10 iso NT, 7 enr veh, 7 iso veh, 12 enr VD, 11 iso VD. (* p<0.05 compared to enr group).

Table 1

Numbers of animals in each group

Experiment 1: F1 of non-treated dams (11)		F1 of vehicle intubated dams(6)
Enriched	6 M 6 F	2 M 5 F
Isolated	4 M 6 F	4 M 3 F
Experiment 2: F1 of non-treated dams (11)		Vendor-derived/shipped pups(23 dams)
Enriched	6 M 6 F	6 M 6 F
Isolated	4 M 6 F	6 M 5 F