G-protein coupled estradiol receptor 1 in dorsolateral striatum modulates cocaine preference in male rats

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Running Head (50-character limit): GPER1 activation attenuates cocaine CPP in males

Keywords (3-6 words or phrases): addiction, sex differences, GPER1

Funding Support (sponsor and number): R01DA039952 to JBB

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Abstract

There are sex differences in susceptibility to addiction. Cultural influences play a role in drug seeking behaviors; however, sex differences are found in rodent models of addiction as well, suggesting that there are also neurobiological factors involved. Extensive research has shown that estradiol facilitates enhanced motivation and drug seeking in female but not male rodents. Estradiol treatment also potentiates cocaine induced dopamine overflow in the dorsolateral striatum (DLS) of females. Together, these findings suggest that estradiol potentiates the rewarding effects of cocaine in females only. However, testosterone is aromatized to estradiol in the male brain but thus far, it's action in males is not well understood. These studies used pharmacological treatments to manipulate ERα, ERβ and GPER1 in the DLS to investigate how estradiol receptors regulate preference for cocaine in both sexes. Antagonism of ERα or ERβ did not alter cocaine CPP in males, but GPER1 activation blocked cocaine preference and inhibition of GPER1 enhanced cocaine CPP in males. Surprisingly, there was no effect of GPER1 activation in females. There were no sex differences in relative mRNA expression of ERα, ERβ and GPER1 in the dorsal striatum. Together, these results indicate that estradiol is playing differential roles in males and females such that it may be protective against drug seeking in male rats while enhancing it in females.

Significance Statement

Drug addiction effects men and women differently but the way we treat addiction is not tailored by sex like it should be. Apart from cultural influence, one reason for sex differences in drug taking could be due to differences in neurobiological function and actions of gonadal hormones. This research article identifies a novel, protective, role for estradiol receptor, GPER1, in male rats only. This discovery could lead to sex-specific therapeutic targets for addiction treatment.

Introduction

More men report abusing drugs but interestingly, more women are seeking treatment for addiction (Kosten et al., 1993). At the time of entering treatment, women report taking higher amount of drug and report having enhanced cravings, compared to their male counterparts (Kosten et al., 1993; Back et al., 2011). In part, this could be due to the fact that women escalate through the stages of addiction more rapidly than males and because women have shorter periods of abstinence between bouts of drug use (Kosten et al., 1993; Lynch et al., 2002). Historically, we have attributed sex-specific behaviors related to addiction as being mediated by cultural influences combined with differences in neurobiological function between males and females, including a sex-specific role of the gonadal hormone estradiol (E2) that enhances drug taking in females (Carroll et al., 2004).

Thus far, both clinical and pre-clinical research models into the biological bases for sex differences in addiction have focused on how E2 alters the rewarding properties psychostimulants in females. Previous research has highlighted that when E2 levels are high, women report an enhanced euphoria or “high” when abusing smoked cocaine (Sofuoglu et al., 1999). Similarly addicted female rats show enhanced cocaine craving when E2 and progesterone levels are high, during estrus, compared to when hormone levels are low during diestrus (Nicolas et al., 2019). Female rodents are also more motivated to work for cocaine when E2 levels are high (Roberts et al., 1989; Hu et al., 2004; Becker and Hu, 2008). Together, these data suggest that E2 plays a major role in facilitating addiction-like behaviors in females.

The rewarding effects of cocaine are understood to be mediated by the drug’s direct effects on dopamine (DA) reuptake within the dorsal and ventral striatum (Pettit and Justice, 1989; Kiyatkin, 1993). Cocaine administration increases synaptic DA levels and repetitive administration leads to enhancement of D1 DA receptor sensitivity (Henry and White, 1991). Each of the aforementioned effects of psychostimulants are present in both males and females. However, there are apparent sex differences in the effects of cocaine on DA release and

receptor binding that are driven by circulating gonadal hormones. In females, E2 acts on GABAergic neurons and disinhibits DA terminals, which increases DA release in striatal tissue but this mechanism hasn't been reported in males (Yoest et al., 2018b). Prior research utilizing in-vivo microdialysis has measured higher levels of amphetamine or cocaine induced increase in DA in dialysate from the dorsal striatum of ovariectomized (OVX) females treated with E2 compared to females without E2 treatment (Cummings et al., 2014). However, there was no direct effect of E2 treatment on DA levels upon cocaine administration for intact or castrated male rats (Cummings et al., 2014). In the ventral striatum, E2 treatment acutely increases the effect of cocaine on stimulated DA release in female but not male rats (Yoest et al., 2018a). Chronic intermittent administration of cocaine leads to sensitization of drug-induced behavioral effects, such as locomotor and rotational behavior (Robinson and Becker, 1986; Becker, 1990; Peris et al., 1991). E2 increases the magnitude of sensitization to psychostimulant drugs in females only (Robinson et al., 1982; Hu and Becker, 2003).

The role of E2 in males is important to understand but is significantly understudied. In males, testosterone is the major gonadal hormone produced by the testis, however, it is aromatized to E2 in the brain and in the peripheral system. Smaller amounts of E2 are also released by the adrenal gland and gonads of males. In both sexes, E2 action is complicated in that it is highly dependent on developmental age, sex, and brain region (Gillies and McArthur, 2010). From rodent studies, we know that during development, E2 masculinizes the male brain including the neural pathways that facilitate aggressive behaviors (Wu et al., 2009). In females, E2 is important for sexual maturation and is elevated in a cyclic pattern to cause sexual receptivity in mature females.

Estradiol binds to estradiol receptors alpha (ERα), beta (ERβ) and G-protein coupled estradiol receptor 1 (GPER1). Our results indicate that all three receptors are present in the dorsal striatum (dSTR). The cellular location of traditional estradiol receptors (ER)s, α and β are dependent on their association with caveolin proteins and are either in the cell nucleus or

membrane (Razandi et al., 1999; Luoma et al., 2008). The actions of GPER1 are quite independent and mainly localized in the endoplasmic reticulum (Otto et al., 2008). In females, ERα and GPER1 in the dSTR are localized to GABAergic medium spiny neurons as well as cholinergic inter-neurons (Almey et al., 2012, 2016).

To date, few studies have identified what role E2 plays in mediating drug abuse liability in males, if any. One reason for this could be that we historically hypothesized an increase in behaviors after E2 treatment, thus, studies were designed to detect either an increase in DA-release or an increase in motivation, rather than a decrease. The current study was able to avoid this confound by utilizing a Conditioned Place Preference (CPP) paradigm, which measures both increases and decreases in the amount of time spent in a drug-paired chamber. These studies detect that in addition to the effects of E2 to enhance drug taking in females, sex differences in motivation for cocaine may also be mediated by effects of E2 to decrease preference for cocaine in males. In the experiments reported here we examine the contribution of E2 acting at GPER1 on cocaine preference in male and female rats.

Methods

Animals

Male and female Sprague-Dawley rats (Charles River Breeding Laboratory, Portage, MI, USA, RRID:RGD734476) approximately 75 days old on arrival were maintained on a 14:10 light/dark cycle (lights on at 08:00 h) in standard laboratory cages. Animals were initially housed in same-sex pairs, until undergoing surgery, when they were subsequently housed individually. Rats had ad libitum access to water and phytoestrogen-free rat chow (2017 Teklad Global, 14% protein rodent maintenance diet, Harlan rat chow; Harlan Teklad, Madison, WI, USA). Both males and females were weighed daily to determine good health. All animal care and experimental procedures were carried out in accordance with the National Institutes of Health guidelines on

laboratory animal use and care, using a protocol approved by University of Michigan Institutional Animal Care and Use Committee.

Conditioned Place Preference (CPP)

Stereotaxic Surgery

One week after arriving in the laboratory, rats underwent surgery for the implantation of bi-lateral guide cannula aimed at the DLS (AP: +0.4 ML: +/-3.6 DV: -4.0). On the day of surgery, rats were injected with carprofen (5mg/kg s.c.) and 30 minutes later anesthetized with ketamine (50mg/kg i.p.) and dexmedetomidine (0.25mg/kg i.p.), then prepared in a stereotaxic frame. At the conclusion of the surgery, rats were given atipamezole hydrochloride (0.5mg/kg i.p.) and 3ml 0.9% saline (s.c.). Every 24 hours for three days post-surgery, rats were given carprofen (5mg/kg s.c.) prophylactically for post-operative pain. No animal underwent behavioral testing for at least 7 days after surgery.

During surgery, solid stylets were inserted into the hollow guide cannula that were fixed on animal's heads. These stylets were flush with the bottom of the guide cannula and did not protrude into the brain. At the conclusion of the first day of behavioral testing (pre-test), the solid stylets were removed and replaced with hollow stylets, which protruded the guide cannula by 1mm and delivered treatment directly into the DLS.

Drug Preparation

Hollow stylets were prepared as previously described (Becker et al., 1987). Briefly, a powder mixture of ICI182,780:cholesterol (1:10) was dissolved in absolute ethanol and then poured into a petri dish for 48 hours, until all of the ethanol had evaporated. Under a dissection microscope, stylets were tapped into the powder so that the area of the stylet was covered. Sides of the guide cannula were cleaned using 100% ethanol. The same method was used for stylets filled with G1:cholesterol (1:10), G15:cholesterol (1:10), and exemestane:cholesterol (1:10).

Drugs were obtained from the following sources: ICI182,780 (Santa Cruz Biotechnology, purity ≥ 98%); G1 (Cayman Chemical, purity ≥ 98%); G15 (Cayman Chemical, purity ≥ 95%);

exemestane (Tocris Bioscience, purity ≥ 99%); cholesterol (Santa Cruz Biotechnology, purity ≥ 92%)

Behavioral Testing Paradigm

Animals were tested on a CPP paradigm that took place over 10 consecutive days. On day 1, rats were placed in a novel three-compartment chamber and were allowed to move freely between all compartments for 30 minutes. For eight days thereafter, animals were trained to associate each of the three chambers with another stimulus (drug paired; neutral; vehicle paired). Each conditioning session began with a 10-minute confinement in the center neutral chamber of the apparatus so that animals would habituate to the neutral chamber. Then, animals were removed from the chamber and received an intra-peritoneal (i.p.) injection of either cocaine or vehicle and immediately placed in one of the side chambers (drug paired; vehicle paired) for 30 minutes. Animals were conditioned with either 5mg/kg cocaine or 10mg/kg cocaine depending on the experiment. Every other day animals were conditioned to the other stimulus such that drug and vehicle were conditioned 4 times each. A biased design was utilized so that each individual animal's initially preferred chamber was paired with vehicle, and their initially un-preferred chamber was paired with cocaine. ANYMAZE tracking software (Stoelting Co., Wood Dale, IL 60191) was utilized to track animal behavior within the chamber (how much time the animal spent in each of the compartments of the chamber).

Euthanasia and Tissue Preparation

Animals received 0.5 ml of FatalPlus (i.p.source?). Once the animal was fully sedated, they were perfused transcardinally with 0.1M phosphate buffered saline followed by 4% paraformdehyde. After perfusion, the brain of each rat was dissected and post-fixed in 4% paraformdelyde for 24 hours and afterwards stored in 10% sucrose. Brains were sliced on either a microtome or cryostat in 60-micron sections then were mounted on slides, stained with cresyl violate and cover slipped. Sections were analyzed for accurate probe placement by a blink

observer. Only animals which had accurate guide cannula placement were included in the final analysis.

Quantitative Polymerase Chain Reaction (qPCR)

Euthanasia and tissue preparation

Naïve male and female (n = 8/sex) rats were given 0.5 ml of FatalPlus (i.p.). Once the animal was fully anesthetized, animals were rapidly decapitated and their brain was dissected, then immediately placed into ice-cold saline. The DLS from both hemispheres was micro-dissected from each animal and stored at -80°C for later processing.

Tissue was extracted using a phenol-chloroform reaction using Trizol (Cat. No. 97064-950, Amresco) as the lysis reagent. Next, a QuantiTect® Reverse Transcription Kit was used for genomic DNA wipeout and cDNA synthesis (Cat. No. 205314, Qiagen). Relative gene expression was measured using a RealMasterMix™ Fast SYBR Kit (Cat. No. A25742, Applied Biosystems) for the following genes of interest ERα (Cat. No. 249900, Qiagen Primer), ERβ (Cat. No. 249900, Qiagen Primer), and GPER1 (Cat. No. 249900, Qiagen Primer). Samples were run in triplicates at the Biomedical Research Core Facilities at the University of Michigan.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism v8.0 (GraphPad, San Diego, CA). CPP data were analyzed using repeated measures two-way ANOVAs. A Bonferroni correction was used for multiple comparisons when appropriate. qPCR data were analyzed using non-parametric Mann-Whitney comparisons. The threshold for significance in all experiments was set to $p<0.05$ and data are presented as mean ± SEM. Power analyses were calculated

Results

In the first experiment, we determined whether there is a sex difference in the ability to form a CPP for cocaine at 5mg/kg (n = 9 males; n = 10 females) or 10mg/kg doses (n = 9 males; n = 10 females). All animals received cholesterol (vehicle) treatment intra-DLS. Not surprisingly,

females exhibit a CPP at the lower dose and males did not (Fig. 1A). A two-way repeated measures ANOVA that compared test session (pre-test vs test) by sex revealed a significant interaction ($F_{(1,17)} = 9.301$; $p = 0.0072$). Following, a Bonferroni post hoc test was used for multiple comparisons and showed that females spent significantly more time in the drug paired chamber on pre-test compared to test, and males did not (female, 3.65 ± 1.01, $p = .0043$; male, 0.8259 ± 1.065). These results are in line with previous research that indicates females exhibit CPP for cocaine at lower doses than males do. At the 10mg/kg dose however, both sexes acquire a CPP for cocaine. A two-way ANOVA was used to compare test session by sex revealed a main effect of test session (Fig. 1B), ($F_{(1,17)} = 30.22$; $p < 0.0001$). These animals were grouped after being run as separate controls throughout the following listed studies and are represented as the cholesterol treated animals in the following statistical analyses.

We first determined the effect of ICI treatment intra-DLS on CPP in both sexes (n = 8/sex). In males (Fig. 2A), a two-way repeated measures ANOVA revealed a treatment x test session interaction ($F_{(1,15)} = 6.655$; $p = 0.0209$). A Bonferroni multiple comparisons test revealed that cholesterol treated animals increased time spent in the drug paired chamber (4.403 ± 1.043, $p = 0.0015$) but ICI treated males did not (0.04821 ± 1.106). For females (Fig. 2B), there was a main effect of test session ($F_{(1,16)} = 13.99$; $p = 0.018$) but no effect of treatment and no interaction. Together, these results suggest an effect of ICI on CPP formation for male, but not female rats. Still unclear is whether this effect on CPP from ICI in males is due to the antagonistic effect on ER alpha and beta, or the agonistic effect of GPER1.

The goal of our follow up study was to differentiate the effects of inactivation of ER alpha and beta verse activation of GPER1, using G1, a selective GPER1 agonist. Fig. 3A represents males treated with G1 (n=8) compared to cholesterol. A two-way repeated measures ANOVA revealed a similar effect of G1 as treatment with ICI did. There was a significant interaction between test session and treatment ($F_{(1,15)} = 5.111$; $p = 0.0391$) and multiple comparisons revealed G1 treated males did not acquire a CPP for cocaine (1.272 ± 1.008), but control

animals did (4.403 ± 0.95). These results support that GPER1 activation in the DLS is sufficient for blocking CPP for 10mg/kg cocaine in males. Fig. 3B represents females treated with G1 (n = 8) or cholesterol and again reveals a main effect of test session ($F_{(1,16)} = 13.48$; $p = 0.0021$) only, indicating that regardless of treatment, females acquired a CPP.

It is clear that activating GPER1 intra DLS decreases CPP for cocaine in males but we wondered whether we could reverse this effect by administering G15, a selective GPER1 antagonist. Shown in Fig. 4, we delivered G15 intra-DLS (n = 8) and conditioned for 5mg/kg cocaine, a dose that does not produce a CPP in control males. However, a two-way repeated measures ANOVA revealed a significant interaction between test session and treatment ($F_{(1,16)} = 8.194$; $p = 0.0119$). A Bonferroni post hoc test revealed that males treated with G15 acquired a CPP while cholesterol males did not (1.591 ± 0.6143, $p = 0.0406$; 0.8259 ± 0.5791; respectively).

We then examined whether altering endogenous production of estradiol in the DLS would affect CPP by administering an aromatase inhibitor intra-DLS. CPP was not affected by intra-DLS exemestane when compared to control in either sex (n = 10 females; n = 6 males). A two-way repeated measures ANOVA did not reveal any main effect of test session or treatment and did not reveal a significant interaction in males (Fig. 5A). In females (Fig. 5B), a main effect of test session was found, as expected ($F_{(1,18)} = 23.91$; $p = 0.0001$).

Quantitative analyses of estradiol receptor expression in the dorsal striatum

Using qPCR, we investigated potential sex differences in the quantity of mRNA expression of ERs in the DLS. All samples (n = 8/sex) were analyzed relative to the housekeeping gene, Hprt1. Data were analyzed using an unpaired, nonparametric (Mann-Whitney) test. There were not sex differences found in relative gene expression of ERα ($p = 0.1949$), ERβ ($p = 0.6454$), or GPER1 ($p = 0.5737$) (Fig. 6A-C, respectively). Each graph represents male gene expression, relative to females. These data suggest that the sex specific behaviors outcomes of GPER1

manipulation are likely due to differences in the downstream effects of receptor activation, rather than sex differences in overall expression.

Discussion

Our study is the first to report that ER activation in the DLS modulates preference formation for cocaine in male rats and this effect is mediated by GPER1. We have shown that activation of GPER1 in the DLS blocks cocaine CPP while inhibition of the receptors produces a significant preference for cocaine at a dose that generally does not induce CPP in males. We also identified the relative RNA expression of ERs in the dSTR and revealed no sex differences, indicating that the sex specific effect of GPER1 activation/inhibition is not due to an overall sex difference in the synthesis of receptors within this brain region.

We initially hypothesized that the pharmacological manipulation of ER in the DLS of females would alter their cocaine CPP, based on previous findings that E2 regulates the rewarding properties of cocaine in females. We predicted that administering ICI, an ERα and ERβ antagonist would inhibit CPP formation but our results do not support this. We postulate that this could be due to the fact that ICI is also an agonist for GPER1 and that we would have needed to give an antagonist all three receptors in order to inhibit preference formation. In both sexes we administered 10% drug:vehicle and although this was a sufficient dose in males, it could also be that females need a higher or lower dose in order to see a behavioral effect.

Previous findings have implicated the ventral striatum as an important brain region for the rewarding effects of cocaine in females. Treatment of E2 acutely enhanced stimulated DA release after cocaine administration in female but not male rats (Yoest et al., 2018a). Our pilot studies did investigate the effect of ICI in the nucleus accumbens but did not see an effect on cocaine CPP. However, it is possible that ICI administration in both the dorsal and ventral striatum simultaneous could alter CPP formation in females.

It is also possible that GPER1 does not regulate the rewarding effects of drugs of abuse in females. Females show enhanced rotational behavior and sensitization after cocaine exposure compared to males and this effect is mediated by ERα (Hu and Becker, 2003; Schultz et al., 2009). Work in dissociated medium spiny neurons from dorsal striatum has shown E2 decreases L-type calcium current, which implicates ERß (Mermelstein et al., 1996; Boulware and Mermelstein, 2009). Finally, E2 treatment reduces GABA release, and overexpression of ERα in the dSTR also enhances the inhibitory effects of E2 on GABA release (Hu et al., 2006; Schultz et al., 2009). Thus, ERα and ERβ are implicated in the effects of E2 on striatal function in females.

Our study used qPCR to explore relative RNA expression of ERα, ERβ and GPER1 and we did not find any sex differences. An obvious limitation is that we did not determine overall protein expression in this brain region and other techniques should be used to solidify whether or not expression of ERs differs between males and females. We speculate that the localization of these receptors could also be different between males and females. We know that in the dSTR, membrane receptors mediate the rapid effects of E2 but this work has primarily used female animals (Yoest et al., 2018b).

Finally, the failure to find an effect of exemestane on cocaine CPP could indicate that a higher concentration was needed to completely eliminate endogenous E2 synthesis. Alternatively, it could be that there is a balance among the three ERs in the DLS and blocking GPER-1 only, while ERα and ERβ are active, it what mediates the effect reported here.

In conclusion, we report that we have identified a novel role for GPER1 in males. To our knowledge, this is the first set of studies to show that activation or inhibition of GPER1 in the dSTR is sufficient to alter cocaine preference formation in males. Given these results, we postulate that GPER1 is a potential target for decreasing motivation to attain cocaine in males, which is currently under investigation in our laboratory. Together, these findings present a unique pharmacological target for addiction in males. We recognize that treating men with

estrogenic compounds might create side effects that would be poorly tolerated. However, there are selective ER modulators that are agonists in brain, while antagonists in the periphery. The discovery of a ligand that binds to GPER1 receptors in the brain selectively could be of important therapeutic value.

Other Acknowledgements

In alphabetical order, we thank Kendra Beaudoin, Hannah Epstein, Brianna Graham, Lahin Lalani and Benjamin Lipkin for assisting in craniotomies, day-to-day animal handling as well as behavioral testing. We thank Brandon Luma for the construction of the chambers used in the CPP behavioral Paradigm. We would also like to acknowledge Caitlin Posillico for her integral role in tissue processing and qPCR data collection.

Conflict of Interest Disclosure

The authors have no conflicts of interest to disclose.

Author Contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. *Conceptualization:* Quigley, J.A. and Becker, J.B.; *Methodology:* Quigley, J.A. and Becker, J.B.; *Investigation:* Quigley, J.A.; *Formal Analysis:* Quigley, J.A.; *Resources:* Becker, J.B.; *Writing – Original Draft:* Quigley, J.A.; *Writing – Revision and Editing:* Quigley, J.A. and Becker, J.B.; *Visualization:* Quigley, J.A.; *Supervision:* Becker, J.B.; *Funding:* Becker, J.B.

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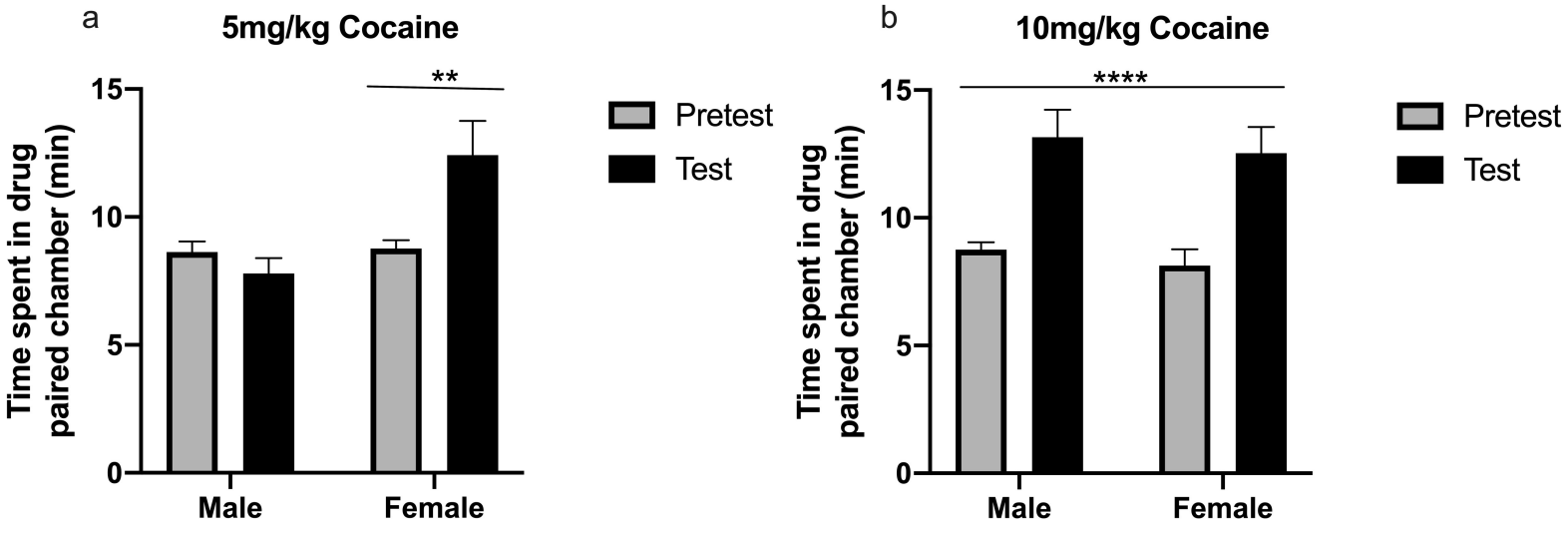

Figure 1

There are sex differences in cocaine CPP at 5mg/kg but not 10mg/kg cocaine. (a) At 5mg/kg dose, only female rats spend more time in the drug paired chamber on Test, compared to Pretest. (b) Both female and male rats spend more time in the drug paired chamber after conditioning with a 10mg/kg cocaine dose. Bars represent group mean (+SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

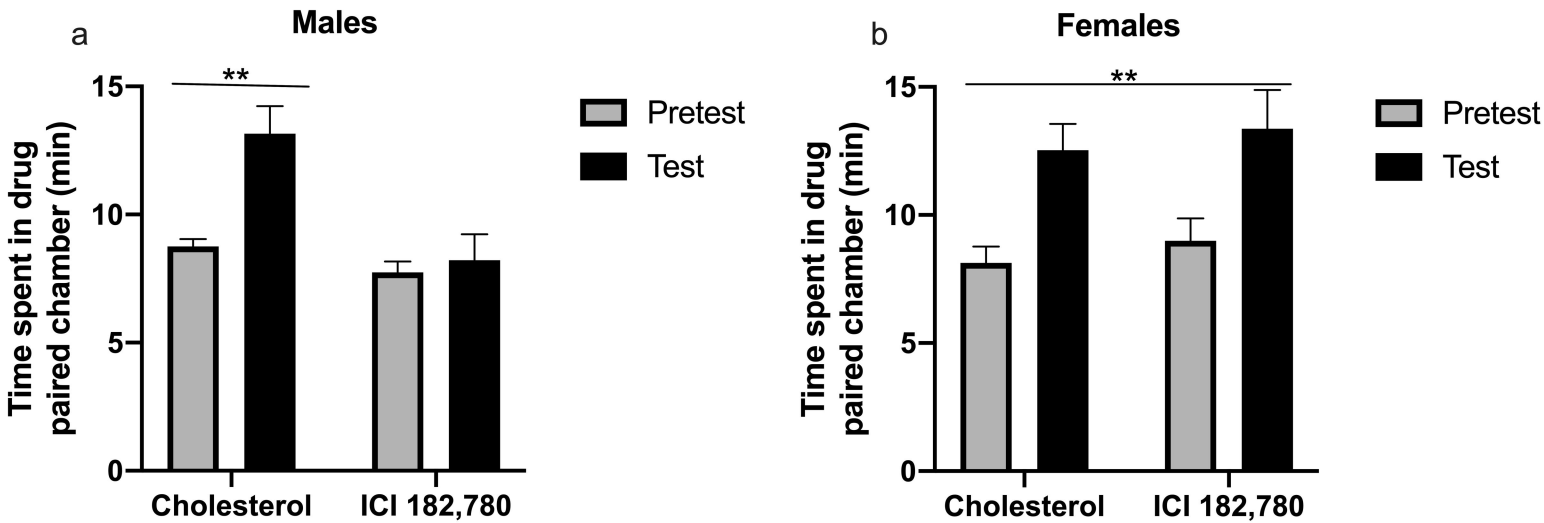

Figure 2

Treatment of ICI intra-DLS inhibits 10mg/kg cocaine CPP in males only. (a) Control males spend more time in the drug paired chamber on Pretest verse Test, but ICI treated males do not. (b) Both vehicle and ICI females acquire a CPP for cocaine. Bars represent group mean (+SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

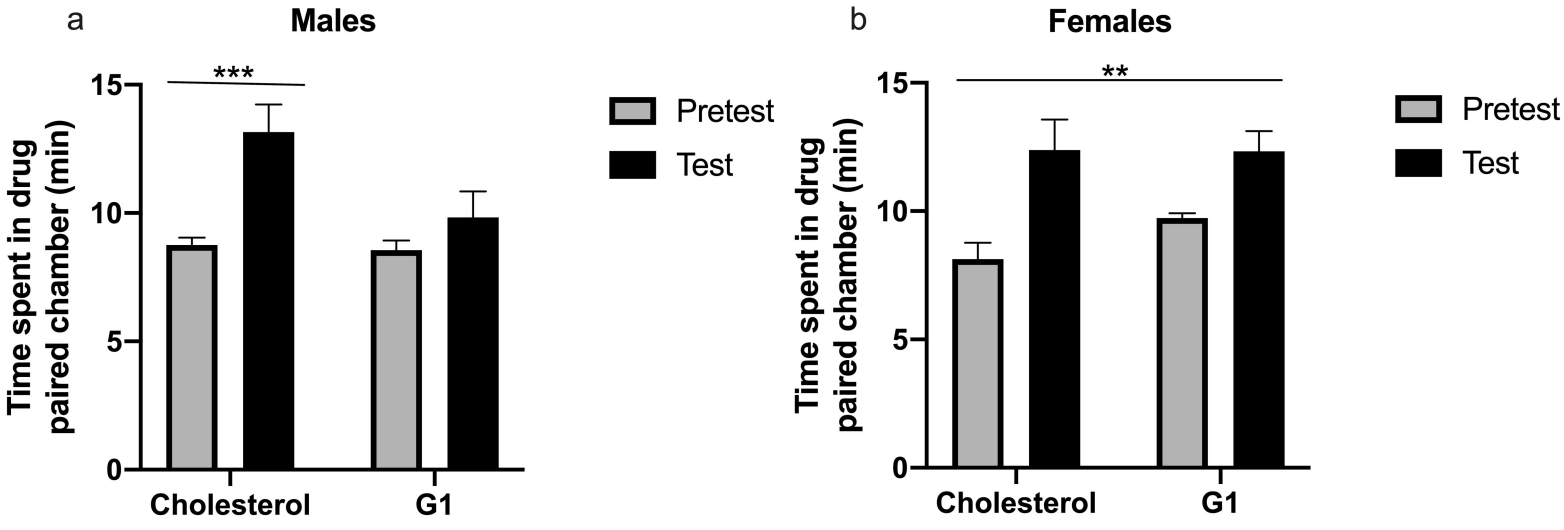

Figure 3

Treatment of G1 intra-DLS inhibits 10mg/kg cocaine CPP in males only. (a) Control males spend more time in the drug paired chamber on Pretest verse Test, but G1 I treated males do not. (b) Both vehicle and G1 females acquire a CPP for cocaine. Bars represent group mean (+SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

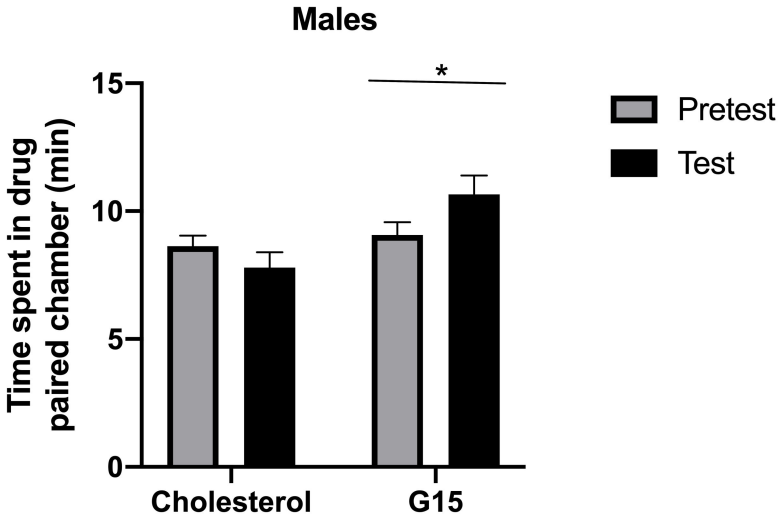

Figure 4

At 5mg/kg cocaine, control males do not increase time spent in the drug paired chamber on Pretest verse Test but G15 treated males do not. Bars represent group mean (+SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

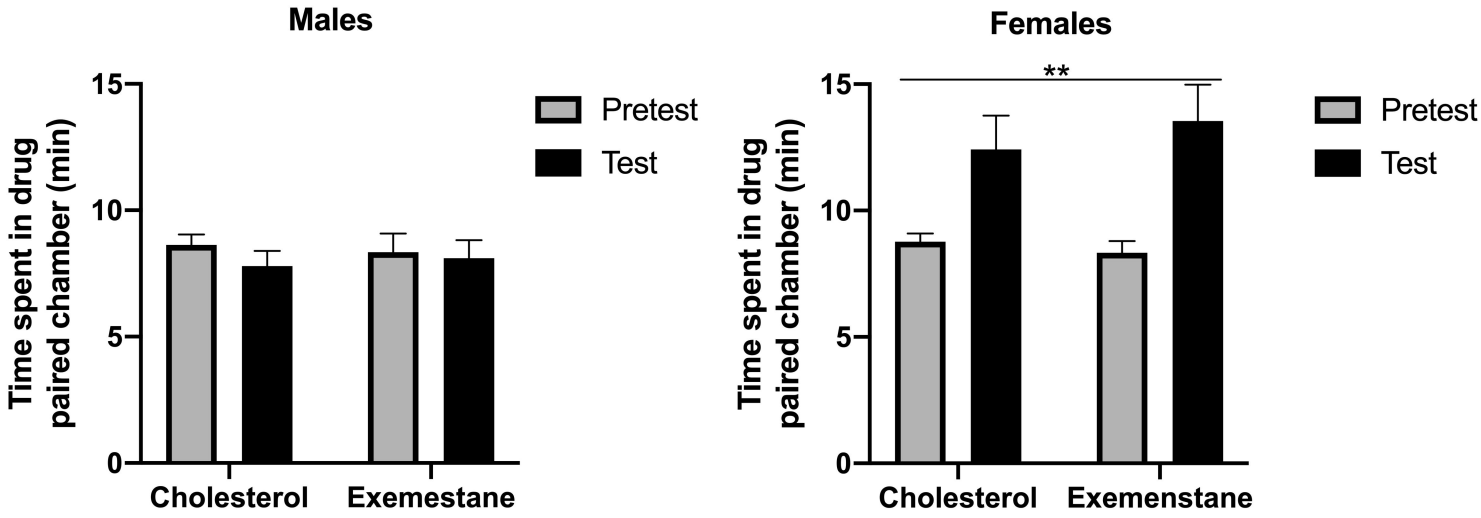

Figure 5

Treatment of an aromatase inhibitor intra-DLS does not change CPP formation for 5mg/kg cocaine in either sex. (a) Neither control nor exemestane males spent significantly more time in the drug paired chamber on Pretest verse Test sessions. (b) Both vehicle and Exemestane females acquired a CPP for cocaine at 5mg/kg. Bars represent group mean (+SEM). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

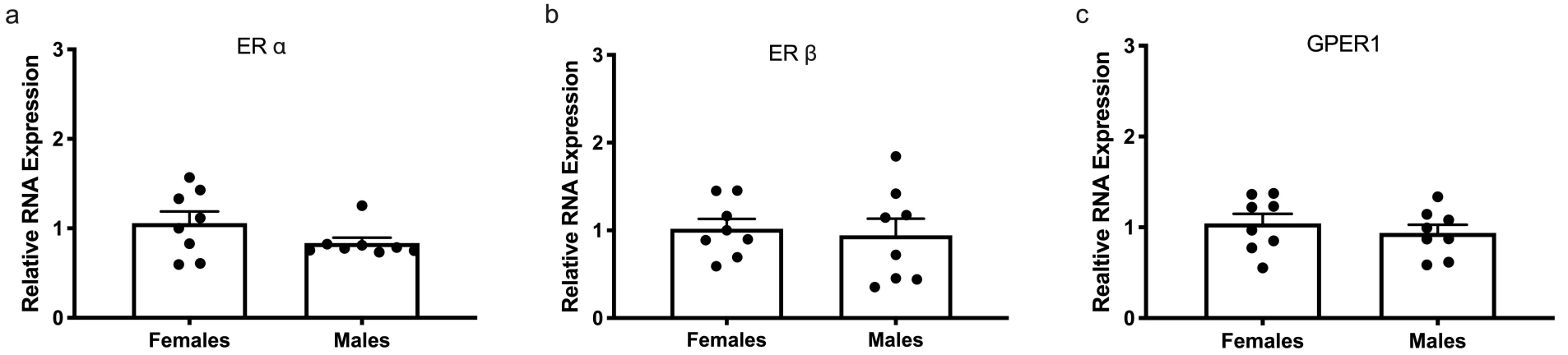

Figure 6

There are no sex differences in relative RNA expression of estradiol receptors within the DLS.